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The association between viral infection and human cancers

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The association between viral infection and human cancers

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Editorial: The association between viral infection and human cancers

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Editorial on the Research Topic
The association between viral infection and human cancers

The World Health Organization estimates that 15.4% of all cancers are attributable to infections and 9.9% are linked to viruses (Plummer et al., 2016). Cancers that are attributable to infections have a greater incidence than any individual type of cancer worldwide. Eleven pathogens have been classified as carcinogenic agents in humans by the International Agency for Research on Cancer (IARC; Bouvard et al., 2009). After *Helicobacter pylori*, the four most prominent infection-related causes of cancer are estimated to be viral: human papilloma virus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), and Epstein–Barr virus (EBV; Zapatka et al., 2020). They are all DNA viruses, except for HCV. Viral infection can lead to uncontrolled cell proliferation and transformation through interfering with cellular regulatory proteins, inactivating tumor suppressor genes, evading host immune responses, inducing persistent inflammatory reactions, causing epigenetic changes, stimulating angiogenesis and activating telomerase (Chu et al.).

The persistent high-risk HPV (HR-HPV) infection has been strongly associated with several types of cancer, such as cervical cancer (99.7%), head and neck squamous cell carcinomas (60%), anal cancer (93%), vulvar cancer (69%), vaginal cancer (75%), and penile cancers (47%) (Brakebill et al., 2023; Liu and Wallace, 2023; Pisani and Cenci, 2024). Lin et al. reported a significantly higher risk of breast cancer in HPV patients than in non-HPV patients, with an adjusted hazard ratio of 2.271 in Taiwan. The mechanism by which HPV causes cervical cancer is relatively well-defined. The rodent and human cells have been shown to undergo immortalization and transformation upon sustained expression of E6 and E7 proteins translated from HPV early genes in cell lines (Lou et al., 2022). An article by Chu et al. reports the successful construction of a recombinant virus that expresses HPV16 E7 protein in cervical cancer cells and induces the up regulation of CD36 gene, which is involved in HPV-related oncogenic pathways. This also provides a potential platform for developing replicative HPV recombinant vaccines. On the correlation between the prognosis of HPV infection and lesion recurrence, Lu et al. conducted a retrospective study after cervical conization in 300 patients. They found that the HPV-negative rates increased over time after surgery. Also, patients with HPV type 16 infection had the highest risk of cervical squamous intraepithelial lesions.

Another group of DNA viruses closely associated with tumors are members of the family *Herpesviridae*. Lee et al. have found that Kaposi's sarcoma-associated human herpes virus (KSHV) infection can induce high-mobility group box 1 (HMGB1) to transfer from the nucleus of endothelial cells to the extracellular space and secrete into the culture medium. HMGB1 plays a key role in the pathogenesis of Kaposi's sarcoma (KS) by regulating the secretion of cytokines and growth factors, affecting the tumor microenvironment. Although HCMV is not a clearly defined oncogenic virus, numerous reports in recent years have shown its correlation with various types of tumors (Hu et al., 2021, 2022). In tumor cells, HCMV may hijack the mRNA nuclear export machinery, thereby changing the translation of cellular genes and promoting tumor progression (Li et al.).

An oncolytic virus can be either a naturally occurring DNA virus, or a genetically engineered virus that selectively infects and kills cancer cells, while sparing normal cells. It can also stimulate the immune system to attack the tumor. Hao et al. provides insights into the molecular mechanisms of EV-A71 oncolysis and its potential anti-tumor efficacy in glioma. They identified *PTBP1*, a gene that is downregulated by EV-A71 infection in glioma cells, as a potential prognostic biomarker and therapeutic target for glioma1.

The research articles included in this Research Topic investigated the cellular transformation mechanisms employed by DNA tumor viruses and also shed light on novel therapeutic targets, diagnostic tools, and treatment strategies that can be implemented in clinical settings to effectively treat tumors caused by oncogenic viruses. In conclusion, these articles clearly develop a better understanding of the underlying molecular processes involved in viral-induced tumorigenesis.

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MH: Conceptualization, Writing—original draft, Writing—review & editing. BW: Conceptualization, Writing—review & editing. JL: Conceptualization, Writing—review & editing. CW: Conceptualization, Writing—review & editing.

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References

Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., et al. (2009). A review of human carcinogens—part B: biological agents. *Lancet Oncol* 10, 321–322. doi: 10.1016/s1470-2045(09)70096-8

Brakebill, A., Morgan, A., and Lieberman, R. W. (2023). Primary HPV screening vs. cotesting for cervical cancer. *J. Am. Med. Assoc.* 330:2121. doi: 10.1001/jama.2023.20370

Hu, M., Wang, B., and Wu, C. (2022). The role of DNA viruses in human cancers. *Front. Cell Infect. Microbiol.* 12:1103505. doi: 10.1177/11769351231154186

Hu, M., Yu, B., Zhang, B., Wang, B., Qian, D., Li, H., et al. (2021). Human cytomegalovirus infection activates glioma activating transcription factor 5 via microRNA in a stress-induced manner. *ACS Chem. Neurosci.* 12, 3947–3956. doi: 10.1021/acschemneuro.1c00576

Liu, X., and Wallace, N. (2023). HPV and HPV-associated cancer. *J. Med. Virol.* 95:e29177. doi: 10.1002/jmv.29177

Lou, H., Boland, J. F., Li, H., Burk, R., Yeager, M., Anderson, S. K., et al. (2022). HPV16 E7 nucleotide variants found in cancer-free subjects affect E7 protein expression and transformation. *Cancers* 14:4895. doi: 10.3390/cancers 14194895

Pisani, T., and Cenci, M. (2024). Prevalence of multiple high risk human papilloma virus (HR-HPV) infections in cervical cancer screening in Lazio Region, Italy. *Cancer Diagn. Progn.* 4, 42–45. doi: 10.21873/cdp.10283

Plummer, M., de Martel, C., Vignat, J., Ferlay, J., Bray, F., and Franceschi, S. (2016). Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob. Health* 4, 609–616. doi: 10.1016/S2214-109X(16)3 0143-7

Zapatka, M., Borozan, I., Brewer, D. S., Iskar, M., Grundhoff, A., Alawi, M., et al. (2020). The landscape of viral associations in human cancers. *Nat. Genet.* 52, 320–330. doi: 10.1038/s41588-019-0558-9

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Implications of viral infections and oncogenesis in uterine cervical carcinoma etiology and pathogenesis

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Background: Uterine Cervical Carcinoma (UCC) is the most prevalent gynecological malignancy globally, with a rising incidence in recent years. Accumulating evidence indicates that specific viral infections, including human papillomavirus (HPV), Epstein-Barr virus (EBV), Hepatitis B and C viruses (HBV and HCV), and human herpesvirus (HHV), may contribute to UCC development and progression. Understanding the complex interplay between viral infections and UCC risk is crucial for developing novel preventative and therapeutic interventions.

Methods: This comprehensive review investigates the association between viral infections and UCC risk by examining the roles of various viral pathogens in UCC etiology and pathogenesis, and possible molecular mechanisms. Additionally, we evaluate current diagnostic methods and potential therapeutic strategies targeting viral infections for UCC prevention or treatment.

Results: The prevention of UCC has been significantly advanced by the emergence of self-sampling for HPV testing as a crucial tool, allowing for early detection and intervention. However, an essential challenge in UCC prevention lies in understanding how HPV and other viral coinfections, including EBV, HBV, HCV, HHV, HIV, or their concurrent presence, may potentially contribute to UCC development. The molecular mechanisms implicated in the association between viral infections and cervical cancer development include: (1) interference of viral oncogenes with cellular regulatory proteins, resulting in uncontrolled cell proliferation and malignant transformation; (2) inactivation of tumor suppressor genes by viral proteins; (3) evasion of host immune responses by viruses; (4) induction of a persistent inflammatory response, contributing to a tumor-promoting microenvironment; (5) epigenetic modifications that lead to aberrant gene expression; (6) stimulation of angiogenesis by viruses; and (7) activation of telomerase by viral proteins, leading to cellular immortalization. Additionally, viral coinfections can also enhance oncogenic potential through synergistic interactions between viral oncoproteins, employ immune evasion strategies, contribute to chronic inflammation, modulate host cellular signaling pathways, and induce epigenetic alterations, ultimately leading to cervical carcinogenesis.

Conclusion: Recognizing the implications of viral oncogenes in UCC etiology and pathogenesis is vital for addressing the escalating burden of UCC. Developing innovative preventative and therapeutic interventions requires a thorough understanding of the intricate relationship between viral infections and UCC risk.

KEYWORDS

human papillomavirus (HPV), Epstein-Barr virus (EBV), human herpesvirus (HHV), uterine cervical carcinoma (UCC), molecular mechanisms, hepatitis B and C viruses (HBV and HCV)

Introduction

Uterine cervical carcinoma (UCC) remains a major global health concern, accounting for significant morbidity and mortality in women worldwide. Despite considerable research efforts and advances in screening and prevention strategies, UCC continues to pose a substantial public health challenge, particularly in low- and middle-income countries (Srinath et al., 2022; Ton et al., 2022; Dau et al., 2023; Tin et al., 2023). A comprehensive understanding of the etiological factors underlying the development of UCC is crucial for devising improved prevention, early detection, and treatment strategies. Although extensive research has been conducted on the etiology and pathogenesis of UCC, the precise mechanisms underlying its development are not yet fully understood. Various factors, such as age (Basoya and Anjankar, 2022; Yuan et al., 2023), obesity (Frumovitz et al., 2014; Coffey et al., 2016; Sassenou et al., 2021; Bohn et al., 2022), hormonal imbalances (Zidi et al., 2020; Iversen et al., 2021; Lasche et al., 2022), genetic predisposition (Chandra et al., 2022; Liu et al., 2022b; Yadav et al., 2023a), and environmental exposures (Kycler et al., 2017; Korsakov et al., 2022), have been implicated in UCC development. Recently, a growing body of evidence has highlighted viral infections, particularly the Human Papillomavirus (HPV), as a key factor in UCC pathogenesis (Figueiredo et al., 2023; Gilham et al., 2023; Smith et al., 2023).

Viruses, as obligate intracellular parasites, are well-recognized for their ability to manipulate host cellular processes, potentially leading to malignant transformation. Several viruses, such as HPV, Epstein-Barr virus (EBV) (Abudoukadeer et al., 2015; Luo et al., 2019), and hepatitis B and C viruses (HBV and HCV) (Mahmood et al., 2002; Ferber et al., 2003), have been identified as oncogenic, playing crucial roles in the pathogenesis of various human cancers. Nevertheless, the association between viral infections and UCC remains an active area of investigation, with inconsistent findings reported in the literature.

This review aims to provide a comprehensive overview of the current state of knowledge regarding the relationship between viral infections and UCC risk. We will discuss the evidence supporting the involvement of various viruses in the development of UCC, focusing on their potential roles in oncogenesis, molecular mechanisms, and clinical implications. Furthermore, we will explore the challenges and future directions in the study of viral infections and UCC, emphasizing the need for well-designed epidemiological and molecular studies to better understand the intricate interplay between viruses and UCC malignancy. Ultimately, elucidating the role of viral infections in UCC may lead to novel preventive and therapeutic strategies for this prevalent and life-threatening disease.

Human papillomavirus (HPV) infection and etiology of UCC

There are over 100 different types of HPV, which are categorized into three risk groups: high-risk, intermediate-risk, and low-risk, based on their association with UCC (**Table 1**; Muñoz et al., 2003). High-risk HPV types are strongly associated with UCC, as they can cause persistent infections and lead to the development of precancerous lesions, which may eventually progress to cancer. The high-risk types include HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (**Table 1**). Notably, HPV 16 and 18 are the most common high-risk strains, responsible for approximately 70% of UCC cases worldwide. Two of these species, α9 (also known as HPV16-like) and α7 (also known as HPV18-like), are of particular importance in the context of UCC diagnosis (**Table 1**; Evans et al., 2022). HPV-16 is a high-risk HPV type belonging to the Alphapapillomavirus genus. It is responsible for nearly 50–60% of all cervical cancer cases, making it the most prevalent and oncogenic HPV type (Youn et al., 2020). The carcinogenic potential of HPV-16 is primarily attributed to the expression of two viral oncoproteins, E6 and E7. These oncoproteins play a crucial role in HPV-16-mediated cervical carcinogenesis by disrupting the normal cellular regulatory pathways. The E6 oncoprotein of HPV-16 targets the tumor suppressor protein p53, promoting its ubiquitin-mediated degradation and thus impairing its ability to regulate cell cycle progression, apoptosis, and DNA repair (Zhang et al., 2022). The E7 oncoprotein, on the other hand, binds to and inactivates the retinoblastoma protein (pRb), a key cell cycle regulator, leading to uncontrolled cell proliferation and genomic instability. Furthermore, E6 and E7 can cooperate to induce chromosomal aberrations, telomerase activation, and immortalization of infected cervical epithelial cells, eventually resulting in malignant transformation (**Figure 1**; Liu et al., 2019). The strong association between HPV-16 and UCC underscores the importance of HPV vaccination and screening programs to prevent infection and early detection of cervical precancerous lesions. Currently, available prophylactic HPV vaccines, such as the bivalent, quadrivalent, and nonavalent vaccines, provide protection against HPV-16 and other high-risk HPV types. These vaccination

TABLE 1 Human papillomavirus (HPV) and uterine cancer risk.

Country and date	HPV strains	Main findings	Applications	References
Arbyn et al. (2022a)–United States	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.	Higher agreement for target amplification-based DNA assays compared to signal amplification-based DNA assays or RNA assays	HPV test agreement/concordance targets may provide criteria to extend existing validations toward alternative sampling approaches.	Arbyn et al., 2022a
Arbyn et al. (2022b)–Belgium	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.	Compared with validated DNA assays, APTIMA HPV Test was similarly sensitive and slightly more specific for CIN2 + .	APTIMA HPV assay is a target amplification nucleic acid probe test for the *in vitro* qualitative detection of E6/E7 viral mRNA from 14 high-risk types of human papillomavirus	Arbyn et al., 2022b
Serrano et al. (2022)–Spain	HPV strains 16, 18, 31, 59, 66, 53, 33, 58, 45, 56, 52, 35, 68, 51, 39, 82, 26, 73, 6, 11, 81.	Of countries with screening programs, 12% recommend self-sampling: nine as the primary method and eight for underscreened populations.	The information can be beneficial for decision-making in both new and existing programs.	Serrano et al., 2022
Muñoz-Bello et al. (2022)–Mexico	HPV strains 16 and 18	The epidemiology of HPV-16 and HPV-18 intratype variants in the Mexican population, as well as their association with UCC.	Investigating intratype HPV variants linked to cancer may facilitate the development of targeted UCC prevention and patient outcome prediction strategies.	Muñoz-Bello et al., 2022
Evans et al. (2022)–Canada	HPV α9 species (HPV16-like), HPV α7 species (HPV18-like)	The immune landscape of HPV-positive and HPV-negative UCC displays significant disparities, with subtle differences observed between HPV α9 and α7 UCC.	Altered patient outcomes between HPV-negative and HPV-positive UCC and potentially between UCC associated with different HPV types.	Evans et al., 2022
Costa et al. (2023)–Belgium	HPV strains 16, 18, 31, 59, 66, 53, 33, 58, 45, 56, 52, 35, 68, 51, 39, 82, 26, 73, 6, 11, 81.	Opt-in strategies were less effective than send-to-all strategies.	Self-samples represent a great opportunity to increase UCC screening.	Costa et al., 2023
Schiffman et al. (2011)–United States	HPV strains 16, 18, 31, 33, 45, 52, and 58.	Discusses the role of HPV testing in UCC prevention and screening strategies	Incorporating HPV testing in UCC prevention programs could enhance screening efficacy and inform public health policy.	Schiffman et al., 2011
Walboomers et al. (1999)–Netherlands	HPV strains 16, 18, 45, 31, 33, 52, 35, and 58.	Establishes that HPV infection is a necessary cause of UCC worldwide.	The causal link between HPV infection and UCC justifies the creation and deployment of HPV vaccines to prevent UCC.	Walboomers et al., 1999
Bosch et al. (2002)–Spain	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59.	Reviews the causal relationship between HPV infection and UCC.	The causal link between HPV infection and UCC justifies the creation and deployment of HPV vaccines to prevent UCC.	Bosch et al., 2002
Muñoz et al. (2003)–France, Spain	High-risk types include HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Intermediate-risk types include HPV strains 68, 73, and 82. Low-risk types include HPV strains 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108.	Classifies the various HPV genotypes and their association with UCC.	HPV types associated with UCC provided a basis for the development of vaccines targeting specific high-risk HPV types.	Muñoz et al., 2003
zur Hausen (2002)–Germany	HPV strains 16, 18, 31, 33, 35, 45, 52, and 58.	Reviews the molecular mechanisms of papillomavirus in cancer development and its clinical applications.	Fundamental and clinical HPV research in cancer has contributed to the innovation of diagnostic tools, therapies, and preventive approaches.	zur Hausen, 2002
Castellsagu et al. (2001)–Spain, Mozambique	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66.	Investigates the HPV genotypes present in a rural population in Mozambique.	HPV genotypes in rural Mozambique informed regional vaccination programs and public health strategies tailored to specific populations.	Castellsagu et al., 2001

(Continued)

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TABLE 1 (Continued)

Country and date	HPV strains	Main findings	Applications	References
de Sanjose et al. (2010)–Spain, multiple international collaborators	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66	Analyzes the attribution of different HPV genotypes in invasive UCC cases worldwide.	HPV genotype attribution in invasive UCC helps guide the development of vaccines targeting the most common and high-risk HPV types.	de Sanjose et al., 2010
Brown et al. (2009)–United States	HPV strains 6, 11, 16, and 18	Studies the impact of the quadrivalent HPV vaccine on the infection and disease caused by non-vaccine oncogenic HPV types.	The impact of the quadrivalent HPV vaccine could be used to inform vaccination strategies and guide public health recommendations.	Brown et al., 2009
Kreimer et al. (2015)–United States, multiple international collaborators	HPV strains 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59.	Evaluates the efficacy of fewer than three doses of an HPV-16/18 AS04 adjuvanted vaccine.	The efficacy of fewer than three doses of an HPV-16/18 vaccine, potentially leading to more cost-effective and accessible vaccination programs.	Kreimer et al., 2015
Joura et al. (2015)–Austria, multiple international collaborators	HPV strains 6, 11, 16, 18, 31, 33, 45, 52, and 58.	Assesses the efficacy of a valent HPV vaccine against infection and intraepithelial neoplasia in women.	The efficacy of a 9-valent HPV vaccine, potentially improving protection against a broader range of high-risk HPV types.	Joura et al., 2015
Plummer et al. (2016)–France, United Kingdom	HPV strains 16 and 18	Estimates the global burden of cancers attributable to infections, including HPV associated UCC.	The global burden of cancers attributable to infections, including HPV, informing public health policies and strategies for cancer prevention.	Plummer et al., 2016
Insinga et al. (2003)–United States	HPV strains 6 and 11	Estimates the health and economic burden of genital warts in a set of private health plans in the United States.	The health and economic burden of genital warts in the United States, highlighting the need for prevention and control strategies.	Insinga et al., 2003
Chen et al. (2018)–China		Provides an overview of cancer incidence and mortality in China, including UCC.	UCC incidence and mortality in China, informing cancer control policies and strategies, including HPV vaccination programs.	Chen et al., 2018
Tota et al. (2013)–Canada		Discusses epidemiologic approaches to evaluate the potential for HPV type replacement post vaccination.	Potential HPV type replacement post vaccination, which may guide the development and monitoring of future vaccination programs.	Tota et al., 2013
Huh et al. (2017)–United States	HPV strains 6, 11, 16, 18, 31, 33, 45, 52, and 58.	Presents the final efficacy, immunogenicity, and safety analyses of a nine-valent HPV vaccine in women aged 16-26 years.	The efficacy, immunogenicity, and safety analyses of a 9-valent HPV vaccine informed vaccination guidelines and policies.	Huh et al., 2017
Ackermann et al. (2005) Germany		UCC development because of HPV infection.	Prevention of HPV infection.	Ackermann et al., 2005
Brewster et al. (1999) United States		HPV is unrelated to the neoplastic transformation process of UCC.		Brewster et al., 1999
Montoya-Fuentes et al. (2001) Mexico	HPV strains 16, 18, 35, and 58	Viral presence in UCC and advanced squamous intraepithelial lesions is inconclusive for confirming or dismissing HPV infection involvement in HGSILs and UCC.		Montoya-Fuentes et al., 2001
Nakagawa et al. (2010) Brazil	HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.	A robust association exists between HPV infection and UCC neoplasia, which may progress to UC.	These include getting vaccinated against HPV, practicing safe sex, getting regular Pap tests, and avoiding smoking.	Nakagawa et al., 2010
Maskey et al. (2019)–Nepal		High-grade lesions exhibit greater infiltrating T cell density than low-grade lesions or normal tissue, with differences in T cell distribution between HPV-negative and HPV-positive samples.	Identifying T lymphocyte subpopulations related to cervical neoplasia grades may enable the development of targeted therapies.	Maskey et al., 2019

(Continued)

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TABLE 1 (Continued)

Country and date	HPV strains	Main findings	Applications	References
Sankaranarayanan et al. (2009)–India	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.	Research should prioritize high-risk HPV types prevalent in rural India, with HPV16 and HPV18 demonstrating the strongest UCC risk association.	HPV testing was associated with a significant reduction in the numbers of advanced UCC and deaths from UCC.	Sankaranarayanan et al., 2009
Bhatla and Singhal (2020)–India	HPV strains 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	HPV, a necessary cause of UCC, is detected through primary HPV screening for pre-neoplastic lesions or cytology-based screening involving cell examination.	Primary HPV screening offers superior pre-neoplastic lesion detection sensitivity, improved negative test reassurance, and safely extended screening intervals.	Bhatla and Singhal, 2020
Hildesheim et al. (2001)–United States	44 HPV types (2, 6, 11, 13, 16, 18, 26, 31, 32, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 72, 73, 83, AE2, AE4, AE5, AE6, AE7, AE8, W13B, AP155)	Among HPV-positive women, multiparity and smoking are risk factors for UCC. Oral contraceptive use may be associated with UCC too.	Preventing smoking and oral contraceptive use.	Hildesheim et al., 2001
Malevolti et al. (2022)–Italy		A significant association was found between cigarette smoking and the risk of UCC. The risk of CC increased with pack-years and smoking duration and decreased linearly with time since quitting, reaching that of never smokers about 15 years after quitting.	By highlighting the role of smoking in increasing UCC risk, this research supports the need for smoking cessation programs and public health policies that discourage smoking.	Malevolti et al., 2022
Marlow et al. (2007)–United Kingdom		UCC development because of HPV infection.	HPV vaccine and testing	Marlow et al., 2007
Lee et al. (2003)–Cancer letters Republic of Korea	High-risk HPV type (16/18/31/33/35/39/45/51 /52/56/58/59/66/68/69) and low-risk HPV types (6/11/34/40/42/43/44).	Subjects infected with multiple HPV types had a 31.8-fold higher risk of UCC, while the single HPV type had a 19.9-fold increased risk	The detection and typing of HPV infection by HPV DNA Chip.	Lee et al., 2003
Labani et al. (2014)–India		Cervical HPV detection exhibits high sensitivity (85%) for CINIII + lesions and moderate sensitivity (53%) for CINII + lesions	HPV testing is superior to VIA and Pap test for the detection of high-grade UCC.	Labani et al., 2014
Giuliano et al. (2002)–United States	HPV strain 16 and 18	In the ≤23 years group, approximately 76% of high-risk HPV-infected individuals are potentially at risk for UCC development.	HPV testing as the sole primary screening test	Giuliano et al., 2002
Cuzick et al. (2006) United Kingdom		Among 60,000 females, HPV testing demonstrated higher CIN2 + detection sensitivity than cytology (96.1 vs. 53.0%) but lower specificity (90.7 vs. 96.3%).	HPV testing as the sole primary screening test, with cytology reserved for women who test HPV positive	Cuzick et al., 2006

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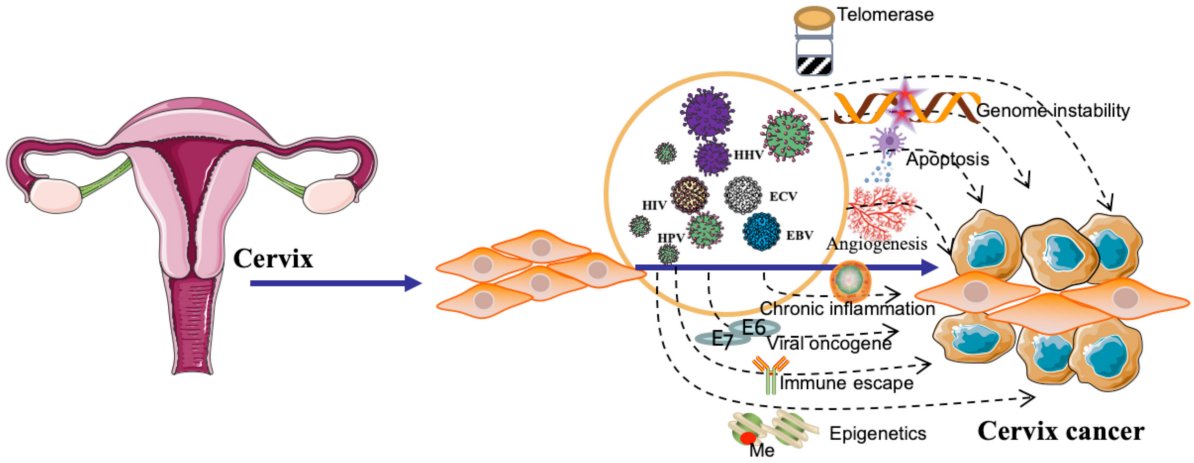

FIGURE 1
Viral pathogenesis of cervical cancer. The figure highlights the role of viral oncogenes in deregulating cellular processes, leading to uncontrolled cell proliferation and malignant transformation. Additionally, the figure emphasizes the involvement of viral infections in promoting immune escape, chronic inflammation, angiogenesis, and epigenetic modifications. Furthermore, the illustration demonstrates the influence of viruses on the activation of telomerase and the induction of genome instability, contributing to the development of cervical cancer.

programs have been shown to significantly reduce the incidence of HPV-16-associated cervical intraepithelial neoplasia and invasive UCC (Pham et al., 2020). Moreover, persistent HPV-16 infection serves as a valuable biomarker for the early identification of women at high risk for cervical cancer development. Molecular testing for HPV-16 and other high-risk HPV types in cervical cancer screening programs can enhance the sensitivity and specificity of detecting cervical precancerous lesions, thereby improving the overall effectiveness of cervical cancer prevention strategies (Kim et al., 2020).

Human papillomavirus α9 species includes HPV types 16, 31, 33, 35, 52, and 58. HPV α7 species includes HPV types 18, 39, 45, 59, and 68. These species comprise several high-risk HPV types, which are strongly associated with the development of UCC. UCC diagnosis typically involves a combination of screening and testing methods. Regular UCC screening, such as the Papanicolaou (Pap) test or liquid-based cytology, can identify abnormal cell changes in the cervix. If abnormal cells are detected, additional testing, such as HPV DNA testing or colposcopy, may be recommended. HPV DNA testing helps to identify high-risk HPV types, including those within the α9 and α7 species, that are associated with an increased risk of UCC. HPV α9 and α7 species, which encompass several high-risk HPV types, play a crucial role in the development of UCC. Regular screening, early detection, and appropriate follow-up care are vital for preventing the progression of precancerous lesions into invasive UCC. Additionally, HPV vaccination can help protect against the most common high-risk HPV types, including those in the α9 and α7 species, thereby reducing the risk of UCC. Intermediate-risk HPV types have a weaker association with UCC compared to high-risk types. They include HPV strains 68, 73, and 82 (**Table 1**). These types may contribute to the development of cancer in combination with other risk factors, but they are not as aggressive as high-risk types. Low-risk HPV types are not typically associated with UCC but can cause benign lesions, such as genital warts or mild dysplasia. These include HPV strains 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 (**Table 1**). Although low-risk types do not generally lead to cancer, it is still essential to monitor and treat any HPV infection to maintain overall health. The relationship between UCC and HPV types is based on the risk categories. High-risk types are the primary cause of UCC, while intermediate-risk types have a weaker association, and low-risk types are generally not linked to the development of UCC. Over time, the accumulation of genetic mutations and chromosomal abnormalities in infected cells contributes to the progression from low-grade to high-grade precancerous lesions (cervical intraepithelial neoplasia, CIN) and, eventually, to invasive UCC.

There are several proposed mechanisms by which HPV might contribute to the development of UCC, such as by promoting genomic instability (Porter and Marra, 2022), deregulating cell cycle control (Singh et al., 2022), and inhibiting apoptosis (**Figure 1**; Yadav et al., 2023b). HPV may downregulate the expression of major histocompatibility complex (MHC) molecules, hindering antigen presentation and recognition by cytotoxic T cells (Ferrall et al., 2021). Viral infections can induce a persistent inflammatory response, characterized by the infiltration of immune cells and the release of pro-inflammatory cytokines, chemokines, and reactive oxygen species. This chronic inflammation may contribute to the formation of a tumor-promoting microenvironment (Hemmat and Bannazadeh Baghi, 2019); HPV infection may cause epigenetic modifications, such as DNA methylation (van den Helder et al., 2022), histone modifications (Lourenço de Freitas et al., 2021), and non-coding RNA regulation (Liu et al., 2022a), which can lead to the aberrant expression of genes involved in cell cycle regulation, apoptosis, and DNA repair, thereby facilitating carcinogenesis (**Figure 1**). HPV can disrupt the apoptotic machinery, thereby promoting the survival and proliferation of UCC (Liu et al., 2020). HPV can cause genome instability by integrating its viral DNA into the host genome. This integration disrupts the normal function of cellular genes and regulatory elements, leading to the dysregulation of cell cycle control and DNA repair mechanisms (**Figure 1**; Kamal et al., 2021).

Understanding the molecular biology of HPV infection and UCC development has been crucial in developing prevention and screening strategies, such as HPV vaccines and HPV-based UCC screening tests, to reduce the incidence and mortality of UCC worldwide (**Table 1**). HPV vaccines, such as Gardasil (Choi et al., 2023) and Cervarix (Roy et al., 2023), protect against the most common high-risk HPV types (particularly HPV 16 and 18). By preventing infection with these types, the vaccines can effectively reduce the likelihood of developing precancerous cervical lesions and, ultimately, UCC. Widespread vaccination has the potential to substantially decrease the overall incidence of UCC. Studies have already shown a decline in the prevalence of high-risk HPV infections and precancerous cervical lesions in vaccinated populations. When a significant portion of a population is vaccinated against HPV, it can create herd immunity, which means that even unvaccinated individuals will be indirectly protected due to reduced virus circulation in the population. Herd immunity, also known as community immunity, occurs when a substantial proportion of a population is immunized against a contagious disease, subsequently reducing the overall circulation of the pathogen and indirectly protecting unvaccinated individuals (Fine et al., 2011). The impact of herd immunity in UCC prevention is particularly relevant given the etiological role of HPV in the development of this malignancy. Persistent high-risk HPV infection is responsible for virtually all cases of UCC (Bosch et al., 2002). Therefore, achieving herd immunity through widespread HPV vaccination has the potential to considerably decrease the incidence of UCC. Several factors contribute to the development of herd immunity in the context of HPV vaccination. Firstly, the widespread vaccination of adolescents, both male and female, can significantly reduce the prevalence of high-risk HPV strains in the population. This reduced prevalence can lead to a decline in the transmission of HPV to unvaccinated individuals, thereby lowering their risk of developing UCC (Markowitz et al., 2012). Secondly, herd immunity can benefit specific population groups that may be at higher risk for HPV infection or cervical cancer but have lower vaccination rates. For example, certain minority or socioeconomically disadvantaged populations might face barriers to accessing HPV vaccination. Herd immunity can provide a measure of protection to these groups by reducing the overall circulation of high-risk HPV strains (Harper and DeMars, 2017). Recent studies have provided evidence of the positive impact of herd immunity on cervical cancer prevention. A study by Drolet et al. found that in countries with high HPV vaccination coverage, the prevalence of vaccine-targeted HPV types decreased by 83% among 13–19-year-old females and 66% among 20–24-year-old females, indicating a substantial reduction in the circulation of high-risk HPV strains (Drolet et al., 2019). Furthermore, the study observed a decrease in HPV prevalence among unvaccinated females, suggesting the presence of herd immunity.

However, it is important to note that HPV vaccines do not protect against all HPV types that can cause UCC, nor do they eliminate the risk entirely. Therefore, even vaccinated individuals should continue to undergo regular UCC screenings as recommended by healthcare professionals. With a reduction in the prevalence of high-risk HPV infections and UCC, there may be a reduced need for frequent UCC screenings (e.g., Pap smears or HPV tests) and associated treatments. This can lead to lower healthcare costs and improved quality of life for women. Self-sampling for HPV testing has emerged as a valuable tool in UCC prevention, offering several benefits that can help improve the overall effectiveness of screening programs and facilitate early detection of HPV infections, particularly among high-risk groups (Racey et al., 2013; Arbyn et al., 2018). Some key advantages of self-sampling for HPV testing during UCC prevention include: Self-sampling allows women to collect their samples in the privacy of their homes, without the need for a clinical appointment. This can lead to higher participation rates, especially among women who may be reluctant or unable to attend traditional clinic-based screenings due to cultural, logistical, or financial barriers (Sancho-Garnier et al., 2013; Gupta et al., 2018). Self-sampling provides a more comfortable and less invasive alternative to clinician-collected samples. Many women find self-sampling less intimidating and more acceptable, which can encourage them to undergo regular HPV testing as part of their UCC prevention routine (Waller et al., 2009; Arrossi et al., 2016). Self-sampling can help reduce costs associated with clinic visits, clinician time, and resources (Abuelo et al., 2014). By facilitating increased participation in HPV testing, self-sampling can contribute to more cost-effective UCC screening programs (Elfström et al., 2014). Self-sampling can improve access to UCC screening among hard-to-reach populations, such as women living in remote areas or those with limited access to healthcare services (Safaeian et al., 2007). By making HPV testing more accessible, self-sampling can help reduce health disparities and improve UCC prevention efforts in underserved communities (Bennett et al., 2018). With increased participation in HPV testing through self-sampling, more women can be screened for high-risk HPV infections. Early detection of these infections allows for timely intervention, such as close monitoring or treatment, to prevent the development of precancerous lesions and UCC (Ronco et al., 2014). It is important to note that the accuracy of self-sampling depends on the quality of the sample collected and the type of HPV test used (Arbyn et al., 2014). High-quality self-sampling kits and sensitive HPV tests are essential for reliable results (Petignat et al., 2007). In conclusion, self-sampling for HPV testing is an important tool in UCC prevention, as it can increase participation rates, improve access to screening, and facilitate early detection and intervention, ultimately contributing to a reduction in the incidence of UCC (Ogilvie et al., 2007; Nelson et al., 2017). It is important to consider other risk factors for UCC, such as obesity (Urbute et al., 2022), smoking (**Table 1**; Hildesheim et al., 2001; Malevolti et al., 2022), hormone replacement therapy, tamoxifen use, and a family history of Lynch syndrome (Kwolek et al., 2023), as these factors have more consistent associations with UCC risk.

The role of EBV in UCC risk

Epstein-Barr virus, also known as human herpesvirus 4 (HHV-4), is a virus that has been associated with various types of cancers, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and some types of stomach cancer (gastric carcinoma). However, the association between EBV and UCC is not well-established. Some studies have detected EBV DNA or viral proteins in UCC tissues (Blanco et al., 2020; Castro et al., 2020; Feng M. et al., 2021; Macleod and Reynolds, 2021), while others have not found any significant association between EBV

infection and UCC (**Table 2**; De Oliveira et al., 1999; Noel et al., 2001).

Several mechanisms have been proposed to elucidate the role of EBV in the development of UCC. Two crucial viral proteins, Epstein-Barr nuclear antigen 1 (EBNA-1) and latent membrane protein 1 (LMP-1), have been implicated in the oncogenic process during EBV infection. EBNA-1 is a multifunctional protein involved in the replication, maintenance, and segregation of the EBV episome in latently infected cells. It is expressed in all EBV-associated malignancies and plays a pivotal role in the persistence of viral episomes within host cells. Additionally, EBNA-1 contributes to the immortalization of infected cells by altering cellular gene expression and promoting genomic instability. During clinical diagnosis, the detection of EBNA-1 expression may serve as a marker of latent EBV infection and its associated cervical malignancies (Hoseini Tabatabaie et al., 2023). LMP-1, on the other hand, is a transmembrane protein that functions as a constitutively active mimic of the tumor necrosis factor receptor (TNFR) family, stimulating multiple signaling pathways that promote cell survival, proliferation, and differentiation. LMP-1 exerts its oncogenic effects by activating the nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. Detection of LMP-1 expression during clinical diagnosis can indicate the presence of an active EBV infection and suggest a more aggressive disease course, as LMP-1 is implicated in immune evasion, angiogenesis, and metastasis (Awasthi et al., 2023). A well-executed retrospective cohort study contributes to our understanding of the prevalence of EBV by targeting EBNA-1 and LMP-1 in cervical cancer specimens, with the authors reporting a 22.2% prevalence (n = 22) in their cohort of 99 patients. This is a valuable addition to the existing body of knowledge on EBV and cervical cancer. The authors provide evidence for the prognostic value of EBV status in cervical cancer patients, demonstrating that the 1-year and 5-year OS rates were higher in the EBV-positive group compared to the EBV-negative group. This finding highlights the potential clinical utility of EBV status as a prognostic biomarker (**Table 2**; Castro et al., 2020). EBV infection has been associated with epigenetic modifications in host cells, including DNA methylation (Fujii et al., 2022), histone modifications (Fujii et al., 2022), and non-coding RNA regulation (Kolesnik et al., 2021). EBV has developed various strategies to evade the host immune response, allowing the virus to persist in infected cells. This immune evasion can lead to chronic inflammation and contribute to the development of a favorable microenvironment for UCC initiation and progression (**Figure 1**; Pandey et al., 2021).

There have been some studies suggesting that coinfection with EBV and HPV may increase the risk of UCC development. The hypothesis is that the two viruses could act synergistically, with EBV potentially promoting HPV-induced cervical carcinogenesis (**Table 2**; da Carvalho and de Melo, 2019; Blanco et al., 2020). Co-infection with EBV, HPV, and human immunodeficiency virus (HIV) could potentially increase the risk of UCC development (Denny et al., 2008; Kelly et al., 2018). Infection with these high-risk types can lead to the formation of abnormal cervical cells, which may progress to UCC if not detected and treated early. HIV infection impairs the immune system, making it more difficult for the body to fight off infections, including HPV. Women with HIV are more likely to have persistent HPV infections, which can increase the risk of developing UCC (Clifford et al., 2017). Additionally, HIV-infected women are more likely to have faster progression from pre-cancerous lesions to invasive UCC compared to women without HIV. Although EBV is not directly linked to UCC, co-infection with HIV could further increase the risk due to the impaired immune response. Co-infections can complicate the clinical picture, and it is essential for healthcare providers (**Table 2**; Feng M. et al., 2021). HPV, EBV, and KSHV coinfections can stimulate angiogenesis (**Figure 1**), the formation of new blood vessels, by upregulating pro-angiogenic factors (e.g., VEGF and IL-8), which may promote tumor growth and metastasis (Dai et al., 2018; Hemmat and Bannazadeh Baghi, 2019). Upon coinfection, EBV and HPV oncoproteins have been shown to cooperatively activate several critical signaling pathways, including PI3K/AKT, MAPK/ERK, JAK/STAT, β-catenin, and p53 (Vranic et al., 2018). These pathways are known to regulate various cellular processes, such as cell survival, proliferation, differentiation, and migration, which are often dysregulated in cancer. Consequently, the simultaneous activation of these pathways by EBV and HPV oncoproteins can contribute to enhanced UCC development and progression.

Given the current state of research, the direct relationship between EBV and UCC risk remains unclear. Further studies are needed to investigate the potential association between EBV and uterine cancer, as well as the underlying mechanisms involved if such a link exists.

HBV and HCV infection increases the development of UCC risk indirectly

Hepatitis B viruses is a DNA virus that primarily infects the liver and can cause both acute and chronic hepatitis. HBV is a well-established risk factor for liver cancer, specifically hepatocellular carcinoma (HCC). However, the association between HBV infection and uterine cancer, particularly UCC, is not well-established. There is limited research on the potential relationship between HBV and uterine cancer, and the available studies do not provide sufficient evidence to establish a clear link between HBV infection and the risk of developing uterine cancer (**Table 3**). It is important to note that HBsAg (hepatitis B surface antigen) is associated with hepatitis B infection, which primarily affects the liver, while HPV is the main causative agent for UCC. Both HBV and HPV infection can potentially contribute to a weakened immune system, which may make it more difficult for the body to fight off other infections, including HPV. A weakened immune system could theoretically increase the risk of developing UCC in HPV positive individuals, but the primary risk factor remains the HPV infection itself (**Table 3**; Ferber et al., 2003; Luo et al., 2022). Although there is not a direct causal link between HBV infection and squamous cell UCC, some studies have suggested a potential association between the two. Serological markers of HBV infection, such as HBsAg, hepatitis B e antigen (HBeAg), and hepatitis B core antibody (anti-HBc), can provide information about the presence and stage of HBV infection in an individual. These markers may have prognostic value in certain cancer types (**Table 3**; Feng X. et al., 2021). The presence of HBsAg in serum indicates an ongoing

TABLE 2 Association of Epstein-Barr virus (EBV) with UCC risk.

Country and date	EBV strains or coinfection	Main findings	Applications	References
Blanco et al. (2020)–Chile	EBV and HPV	The study explores the epidemiological and molecular aspects of EBV and HPV coinfection, suggesting that EBV and HPV coinfection could increase the risk of UCC development by affecting multiple signaling pathways.	Investigates the role of EBV and HPV coinfection in UCC, discussing epidemiology, molecular mechanisms, and potential therapeutic targets.	Blanco et al., 2020
Castro et al. (2020)–Peru	EBV	EBNA-1 and LMP-1 in cervical cancer specimens, with the authors reporting a 22.2% prevalence in UCC patients. Tthe 1-year and 5-year OS rates were higher in the EBV-positive group compared to the EBV-negative group.	This finding highlights the potential clinical utility of EBV status as a prognostic biomarker of UCC.	Castro et al., 2020
Feng M. et al. (2021)–China	EBV, HPV, and HIV	The study reports that EBV and HPV coinfection in Chinese women living with HIV was significantly associated with high-grade cervical intraepithelial neoplasia, supporting the role of EBV as a potential cofactor in the development of cervical lesions.	Studies the role of EBV and HPV coinfection in cervical intraepithelial neoplasia in Chinese women living with HIV.	Feng M. et al., 2021
Macleod and Reynolds (2021)–Eastern and Southern Africa	EBV	This scoping review highlights the high prevalence of HPV infection and UCC among women who sell sex in Eastern and Southern Africa, emphasizing the need for targeted interventions to reduce the burden of HPV-related disease.	Reviews the prevalence of HPV infection and UCC among women who sell sex in Eastern and Southern Africa.	Macleod and Reynolds, 2021
De Oliveira et al. (1999)–Brazil	EBV	The study found no evidence of EBV infection in cervical carcinomas, suggesting that EBV may not play a significant role in UCC.	Reports the lack of EBV infection in cervical carcinomas.	De Oliveira et al., 1999
Noel et al. (2001)–Belgium	EBV	The investigation of lymphoepithelioma-like carcinoma of the uterine cervix revealed evidence of HPV infection but not EBV, indicating that HPV may be the primary causative agent.	Presents evidence of HPV infection but not EBV in lymphoepithelioma-like carcinoma of the uterine cervix.	Noel et al., 2001
Yordanov et al. (2020)–Bulgaria	EBV	The single-center study found no correlation between EBV and lymphoepithelioma-like carcinoma of the uterine cervix, while a strong association with high-risk HPV types was observed.	Examines the correlation between EBV and HPV infection in lymphoepithelioma-like carcinoma of the uterine cervix.	Yordanov et al., 2020
Kienka et al. (2019)–United States	EBV	The study reports that EBV, but not human cytomegalovirus, is associated with high-grade HPV-associated cervical lesions in women from North Carolina.	Investigates the association of EBV with high-grade HPV-associated cervical lesions in women in North Carolina.	Kienka et al., 2019
Cameron et al. (2020)–United States	EBV	HIV-infected women with genital tract specimens positive for both HPV and EBV were found to be at a higher risk for abnormal cervical cytology.	Assesses the risk of abnormal cervical cytology in HIV-infected women testing positive for both HPV and EBV.	Cameron et al., 2020
Vranic et al. (2018)–Qatar	EBV	This brief update discusses the association between EBV and UCC, highlighting the potential role of EBV as a cofactor in the development of HPV-associated cervical lesions.	Provides an update on the role of EBV in UCC.	Vranic et al., 2018
de Lima et al. (2018)–Brazil	EBV	A meta-analysis found a significant association between EBV and cervical carcinoma, supporting the role of EBV as a potential cofactor in cervical carcinogenesis.	Conducts a meta-analysis to assess the association between EBV and cervical carcinoma.	de Lima et al., 2018

(Continued)

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TABLE 2 (Continued)

Country and date	EBV strains or coinfection	Main findings	Applications	References
Sasagawa et al. (2000)–Japan	EBV	The study found that EBV gene expression was more frequently observed in invasive UCC than in cervical intraepithelial neoplasia, suggesting a potential role for EBV in UCC progression.	Compares EBV gene expression in cervical intraepithelial neoplasia and invasive UCC, in relation to HPV infection.	Sasagawa et al., 2000
Khashman et al. (2020a)–Iraq	EBV	The study investigated the expression of EBV latent membrane protein 1 (LMP1) in Iraqi women with cervical carcinoma, although the main findings were not provided in the citation.	Examines the expression of EBV latent membrane protein 1 (LMP1) in Iraqi women with cervical carcinoma.	Khashman et al., 2020a
Yordanov et al. (2019)–Bulgaria	EBV	The immunohistochemical study showed HPV and EBV infection in patients with lymphoepithelioma-like carcinoma of the uterine cervix, supporting a possible role for both viruses in the development of this rare subtype of UCC.	Studies the immunohistochemical expression of HPV and EBV in patients with lymphoepithelioma-like carcinoma of the uterine cervix.	Yordanov et al., 2019
da Carvalho and de Melo (2019)–Brazil	HPV and EBV	The study highlights the association between HPV and EBV infections in UCC, discussing the potential interactions and molecular mechanisms involved in cancer development.	Reviews the association between HPV and EBV infections and cancer of the uterine cervix.	da Carvalho and de Melo, 2019
Lau et al. (2021)–Taiwan	HPV and EBV	The case report describes a rare occurrence of an Epstein-Barr Virus-associated smooth muscle tumor (EBV-SMT) of the cranio-cervical junction in an immunocompetent patient, emphasizing the need for further research on EBV-SMT in this population.	Presents a case history of an EBV-associated smooth muscle tumor (EBV-SMT) of the cranio-cervical junction in an immunocompetent patient.	Lau et al., 2021
Okoye et al. (2023)–Southern Nigeria	EBV-LMP1	The prevalence of HPV/EBV-LMP1 copresence was high in invasive UCC compared with non-invasive UCC cases	EBV DNA should equally be investigated during HPV testing of suspected UCC cases to identify individuals with poorer prognoses.	Okoye et al., 2023
Sosse et al. (2022)–France		The role of EBV as potential cofactors in cervical carcinogenesis.	The study could lead us to develop new therapeutics and preventive vaccines	Sosse et al., 2022

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infection, either acute or chronic (Lok and McMahon, 2009). HBeAg is a secreted viral protein that reflects active viral replication and is associated with high infectivity (Hadziyannis, 1995). Anti-HBc is an antibody produced in response to the HBcAg, which is a component of the viral nucleocapsid. The presence of anti-HBc indicates previous exposure to HBV, either resolved or ongoing (Chu and Liaw, 2010). In a study involving 277 cervical cancer patients, the seropositivity rates for HBsAg, HBeAg, and anti-HBc were found to be 4.33, 0.72, and 13.00%, respectively, indicating a potential relationship between HBV infection and cervical cancer (Wu et al., 2021). The detection of HBsAg and HBcAg in a subset of cervical cancer cases with seropositive HBsAg, as well as the increased risk of cervical cancer in individuals with both HBsAg and HPV positive, underscores the potential interplay between HBV infection and HPV in cervical cancer development. This observation highlights the importance of considering co-infections when examining UCC risk factors (Luo et al., 2022).

Hepatitis C viruses is a well-known risk factor for liver cancer, particularly hepatocellular carcinoma. However, its association with uterine cancer, particularly UCC, is unclear and not well-established (**Table 3**; Mahmood et al., 2002; Chhetri, 2010). The prevalence of HBV and HCV infections was found among UCC patients compared to the general population. The association between chronic HBV infection and cervical cancer disappears among HPV-positive patients but remains significant for patients younger than 50 years after adjusting for HPV infection and parity. Co-infection with sexually transmitted infections (STIs), such as HIV, HBV, HCV, can also increase the risk of developing UCC. The seroprevalence of STIs among cervical cancer suspected women in Ethiopia, highlights a significant public health concern. The authors report an overall STI prevalence of 16.6% (67/403) in the study population, with the prevalence of HIV, HBV, HCV, and syphilis being 8.9, 2.5, 1, and 7.2%, respectively. This information helps to bridge the existing knowledge gap concerning the burden of coinfection in this UCC population (Abebe et al., 2021).

The clinical importance of EBV, HBV, and HCV in the etiology of cervical cancer has become an area of increasing interest among researchers and clinicians. The exact mechanisms by which EBV may contribute to cervical cancer development remain unclear, but possible explanations include the induction of genomic instability, inhibition of apoptosis, and promotion of cell proliferation (**Figure 1**). The possible mechanisms through which HBV may contribute to chronic inflammation, immune suppression, or molecular mimicry. Further investigation is warranted to determine the clinical relevance of HBV in the context of cervical cancer and to assess whether prevention and control of HBV infection may have implications for cervical cancer risk reduction. Similar to HBV, HCV is primarily implicated in liver diseases but has also been suggested as a possible co-factor in cervical cancer development. Some studies have reported an increased prevalence of HCV infection among cervical cancer patients, raising the possibility of an association between the two conditions. The potential mechanisms linking HCV to cervical cancer remain speculative and may involve chronic inflammation or immune dysregulation. In conclusion, the clinical importance of EBV, HBV, and HCV in cervical cancer etiology remains an emerging area of investigation. Although these viruses are not considered primary causative agents of cervical cancer, their potential role in the development of this malignancy warrants further study. Understanding the mechanisms through which these viruses may contribute to cervical carcinogenesis could have important implications for the prevention, diagnosis, and treatment of cervical cancer, ultimately improving patient outcomes and public health strategies.

Other virus strains and UCC risk

Human cytomegalovirus (HCMV) has been associated with various malignancies, including glioblastoma and prostate cancer. It has been detected in some UCC tissues (Kienka et al., 2019; Khashman et al., 2020b; Ghadicolaee et al., 2021), but the relationship is not well-established. Human T-cell lymphotropic virus type 1 (HTLV-1) is a retrovirus known to be associated with adult T-cell leukemia/lymphoma (ATL) and a neurological disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although HTLV-1 has not been directly implicated in UCC, some studies have suggested a potential link between HTLV-1 infection and an increased risk of developing UCC (Schierhout et al., 2020). The connection between HTLV-1 and UCC is not as well-established as that of HPV, which is the primary cause of UCC. However, there is some evidence to suggest that HTLV-1 infection might influence the persistence or progression of HPV infection, potentially increasing the risk of UCC in HTLV-1-infected women (Ibrahim Jaber and Qasim Dhumad, 2022; Rosadas and Taylor, 2022). Additionally, it has been suggested that HTLV-1 might indirectly contribute to UCC development by impairing the immune system and reducing the body's ability to control HPV infection (Ibrahim Jaber and Qasim Dhumad, 2022).

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a virus primarily associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. While KSHV has been extensively studied in relation to these conditions, its direct association with UCC is not well-established. Although KSHV and HPV are both viruses that can cause cancer, their roles in the development of UCC are distinct. HPV is considered the primary etiological agent for UCC, while KSHV has not been definitively linked to this malignancy. There have been some studies investigating the potential role of KSHV in UCC (Mukerebe, 2022), but the evidence is not yet strong enough to establish a direct association. Further research is needed to determine if KSHV plays a role in the development or progression of UCC or if it has any interaction with HPV in cervical carcinogenesis.

Human herpesvirus (HHV), particularly HHV-2, also known as herpes simplex virus type 2 (HSV-2), has been suggested to play a role in the development of UCC (Mukerebe, 2022). HSV-2 is primarily responsible for genital herpes infections, which can cause genital ulcers and increase the risk of acquiring other sexually transmitted infections (STIs), including HPV (Moharreri and Sohrabi, 2021). While HSV-2 itself is not considered a direct cause of UCC, its association with genital ulcers and the increased risk of acquiring HPV could potentially contribute to the development of UCC. Furthermore, HSV-2 infection might cause local inflammation and immunosuppression in the cervical area, which could facilitate the persistence and progression of HPV infection, ultimately leading to UCC (Vitali et al., 2020).

TABLE 3 The indirect association between hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and the increased risk of developing uterine cervical Cancer (UCC).

Country and date	Virus strains	Main findings	Applications	References
Luo et al. (2022)–China	HBC and HCV	HBsAg and HPV positive had an increased risk of UCC	HBV infection should be avoided.	Luo et al., 2022
Feng X. et al. (2021)–China	HBV	HBV infection is associated with poor prognosis in patients with primary UCC.	The main application is investigating the prognostic impact of hepatitis B virus (HBV) infection in patients with primary UCC.	Feng X. et al., 2021
Chhetri (2010)–India	HCV	A case report suggests an association between HCV infection and carcinoma cervix, highlighting the need for further studies.	This article focuses on a case report and brief review of the literature on the association between chronic hepatitis C virus (HCV) infection and carcinoma cervix.	Chhetri, 2010
Ferber et al. (2003)–United States	HBV	Integrations of HBV and HPV into the human telomerase reverse transcriptase (hTERT) gene were found in liver and UCC, suggesting a possible oncogenic mechanism.	The study examines the integrations of HBV and human papillomavirus (HPV) into the human telomerase reverse transcriptase (hTERT) gene in liver and UCC.	Ferber et al., 2003
Mahmood et al. (2002)–United Kingdom	HCV	HCV RNA was detected in normal cervical smears of HCV-seropositive patients, indicating a possible role for HCV in cervical pathogenesis.	This research aims to detect HCV RNA in normal cervical smears of HCV-seropositive patients.	Mahmood et al., 2002
Shiels et al. (2013)–United States	None mentioned	The non-Hodgkin lymphoma epidemic in the United States was analyzed, with results suggesting factors other than HIV playing a significant role in its development.	The article analyzes the epidemic of non-Hodgkin lymphoma in the United States, disentangling the effect of HIV from 1992 to 2009.	Shiels et al., 2013
Engels et al. (2010)–Republic of Korea	HBV	HBV infection was found to be associated with an increased risk of non-Hodgkin lymphoma in a South Korean cohort study.	This cohort study investigates the association between HBV infection and risk of non-Hodgkin lymphoma in Republic of Korea.	Engels et al., 2010
Kumar et al. (2018)–India	HBV	Multiple idiopathic cervical root resorptions were reported in patients with HBV infection, suggesting a potential association.	The research examines the occurrence of multiple idiopathic cervical root resorptions in patients with HBV infection.	Kumar et al., 2018
Heffernan et al. (2010)–Australia	None mentioned	The global reduction of UCC through HPV vaccines is discussed, with insights drawn from HBV vaccine experience.	The main application is exploring global reduction of UCC with HPV vaccines, drawing insights from the hepatitis B virus vaccine experience.	Heffernan et al., 2010
Wu et al. (2021)–China	HBV	Serological markers of HBV infection have prognostic value in squamous cell UCC.	The study investigates the prognostic value of serological markers of HBV infection in squamous cell UCC.	Wu et al., 2021
Siu et al. (2007)–Hong Kong SAR, China	HBV	Patients with malignant or pre-malignant cervical lesions were found to have an increased risk of becoming hepatitis B carriers.	This research explores the increased risk of becoming an HBV carrier in patients with malignant or pre-malignant cervical lesions.	Siu et al., 2007
Li et al. (2015)–China	HBV	Up-regulation of human UCC proto-oncogene contributes to HBV-induced malignant transformation of hepatocytes by down-regulating E-cadherin.	The article examines the role of human UCC proto-oncogene in contributing to HBV-induced malignant transformation of hepatocytes by down-regulating E-cadherin.	Li et al., 2015

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TABLE 3 (Continued)

Country and date	Virus strains	Main findings	Applications	References
Leroux-Roels et al. (2011)–Belgium	HBV	The immunogenicity and safety of hepatitis B and human papillomavirus type 16/18 AS04-adjuvanted UCC vaccines were found to be satisfactory when coadministered in an accelerated schedule.	The study evaluates the immunogenicity and safety of the hepatitis B vaccine given in an accelerated schedule coadministered with the HPV type 16/18 AS04-adjuvanted UCC vaccine.	Leroux-Roels et al., 2011
Dimond et al. (2021)–United States	HBV	A fatal hepatitis B reactivation case was reported in a patient receiving chemoradiation for UCC, highlighting the need for careful monitoring and management of HBV during treatment.	This case report discusses fatal hepatitis B reactivation in a patient receiving chemoradiation for UCC.	Dimond et al., 2021

Human immunodeficiency virus is a virus that attacks the immune system, specifically the CD4 + T cells, which play a crucial role in immune response. HIV infection can progress to acquired immunodeficiency syndrome (AIDS) when the immune system is severely damaged, leaving the individual vulnerable to various opportunistic infections and cancers. There is a strong association between HIV and UCC. HIV infection weakens the immune system, making it difficult for the body to fight off HPV infections and prevent the progression of pre-cancerous lesions to UCC (Marima et al., 2021; Stelzle et al., 2021). HIV-infected women have a higher prevalence of HPV infection and are more likely to be infected with multiple high-risk HPV types. They are also more likely to have persistent HPV infections, which increases the risk of developing UCC (D'andrea et al., 2019). HIV-infected women tend to have a faster progression from HPV infection to the development of precancerous lesions and invasive UCC compared to women without HIV infection (Moscicki et al., 2019; Tawe et al., 2020). HIV-infected women may have a poorer response to UCC treatments, such as surgery, radiation, and chemotherapy, due to their compromised immune systems. To reduce the risk of UCC in HIV-infected women, regular UCC screening (e.g., Pap smears or HPV tests) is recommended. Additionally, the administration of the HPV vaccine can help protect against the high-risk HPV types responsible for UCC. Antiretroviral therapy (ART) can also help to improve the immune system in HIV-infected individuals, potentially reducing the risk of developing UCC (Shin et al., 2019). It is important to note that the link between many types of viruses and UCC is still not fully understood, and more research is needed to establish a clear relationship between the two. HPV remains the primary risk factor for UCC, and the prevention and control of HPV infection through vaccination and screening are the most effective strategies for reducing the incidence of UCC. More research is needed to determine the exact relationship between different virus types and UCC, as the current evidence is limited and inconclusive.

The future challenge for the viral etiology of UCC

The future challenges for the viral etiology of UCC, particularly in relation to HPV, include (1) Improving vaccination coverage, although the HPV vaccine has proven effective in reducing the prevalence of high-risk HPV strains associated with UCC, improving vaccination coverage globally remains a challenge, especially in low- and middle-income countries where the burden of UCC is high; (2) Understanding HPV and other viral coinfections, such as EBV, may potentially play a role in UCC development. Further research is needed to elucidate the mechanisms and significance of these coinfections; (3) expanding screening programs: screening programs using HPV DNA testing have shown promise in detecting precancerous lesions, but implementing and expanding these programs worldwide, particularly in resource-limited settings, remains a challenge; (4) addressing disparities in access to care, disparities in access to preventive care, such as vaccination and screening, contribute to the high burden of UCC in certain populations. Efforts should be made to address socioeconomic, cultural, and logistical barriers

to care; (5) developing novel therapeutic approaches, although current treatments for UCC, such as surgery, radiation, and chemotherapy, can be effective, there is a need for novel therapies that target the viral etiology of the disease, such as antiviral drugs and immunotherapies; (6) enhancing public awareness and education, public awareness and understanding of the role of HPV in UCC and the importance of vaccination and screening are essential to reduce the burden of the disease. Educational campaigns targeting various populations can help increase vaccine uptake and participation in screening programs; (7) investigating the role of viral genetic variation, the role of genetic variation within HPV types and its impact on UCC risk and vaccine efficacy needs further investigation; (8) Studying the impact of the HPV vaccine on non-cervical HPV-associated cancers, the HPV vaccine has the potential to prevent other HPV-associated cancers, such as oropharyngeal, anal, and penile cancers. Further research is needed to understand the long-term impact of vaccination on these cancers; (9) Addressing vaccine hesitancy: Vaccine hesitancy remains a challenge in certain populations, and efforts should be made to address misconceptions and promote confidence in the HPV vaccine, and (10) Long-term monitoring and surveillance, continued monitoring and surveillance of HPV prevalence, vaccine efficacy, and UCC incidence are crucial to assess the long-term impact of vaccination and screening programs and inform public health policy.

Author contributions

DC and TL: review conception, design, data collection, and quality analysis. TL and YY: the data extraction of the included studies, analysis, and interpretation of results. DC and YY: draft manuscript preparation and the critical revision of the manuscript. All authors reviewed the results and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abebe, M., Eshetie, S., and Tessema, B. (2021). Prevalence of sexually transmitted infections among cervical cancer suspected women at University of Gondar Comprehensive Specialized Hospital, North-west Ethiopia. *BMC Infect. Dis.* 21:378. doi: 10.1186/s12879-021-06074-y

Abudoukadeer, A., Niyazi, M., Aikula, A., Kamilijian, M., Sulaiman, X., Mutailipu, A., et al. (2015). Association of EBV and HPV co-infection with the development of cervical cancer in ethnic Uyghur women. *Eur. J. Gynaecol. Oncol.* 36, 546–550.

Abuelo, C., Levinson, K., Salmeron, J., Sologuren, C., Fernandez, M., and Belinson, J. (2014). The Peru cervical cancer screening study (PERCAPS): The design and implementation of a mother/daughter screen, treat, and vaccinate program in the Peruvian jungle. *J. Commun. Health* 39, 409–415. doi: 10.1007/s10900-013-9786-6

Ackermann, S., Renner, S., Fasching, P., Poehls, U., Bender, H., and Beckmann, M. (2005). Awareness of general and personal risk factors for uterine cancer among healthy women. *Eur. J. Cancer Prev.* 14, 519–524.

Arbyn, M., Castle, P., Schiffman, M., Wentzensen, N., Heckman-Stoddard, B., and Sahasrabuddhe, V. (2022a). Meta-analysis of agreement/concordance statistics in studies comparing self-vs clinician-collected samples for HPV testing in cervical cancer screening. *Int. J. Cancer* 151, 308–312.

Arbyn, M., Simon, M., de Sanjosé, S., Clarke, M., Poljak, P., Rezhake, R., et al. (2022b). Accuracy and effectiveness of HPV mRNA testing in cervical cancer screening: A systematic review and meta-analysis. *Lancet Oncol.* 23, 950–960.

Arbyn, M., Smith, S., Temin, S., Sultana, F., and Castle, P. (2018). Detecting cervical precancer and reaching underscreened women by using HPV testing on self samples: Updated meta-analyses. *BMJ* 363:k4823. doi: 10.1136/bmj.k4823

Arbyn, M., Verdoodt, F., Snijders, P., Verhoef, V., Suonio, E., Dillner, L., et al. (2014). Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: A meta-analysis. *Lancet Oncol.* 15, 172–183.

Arrossi, S., Ramos, S., Straw, C., Thouyaret, L., and Orellana, L. (2016). HPV testing: A mixed-method approach to understand why women prefer self-collection in a middle-income country. *BMC Public Health* 16:832. doi: 10.1186/s12889-016-3474-2

Awasthi, P., Dwivedi, M., Kumar, D., and Hasan, S. (2023). Insights into intricacies of the latent membrane protein-1 (LMP-1) in EBV-associated cancers. *Life Sci.* 313:121261. doi: 10.1016/j.lfs.2022.121261

Basoya, S., and Anjankar, A. (2022). Cervical cancer: Early detection and prevention in reproductive age group. *Cureus* 14:e31312. doi: 10.7759/cureus.31312

Bennett, K., Waller, J., Chorley, A., Ferrer, R., Haddrell, J., and Marlow, L. (2018). Barriers to cervical screening and interest in self-sampling among women who actively decline screening. *J. Med. Screen.* 25, 211–217. doi: 10.1177/0969141318767471

Bhatla, N., and Singhal, S. (2020). Primary HPV screening for cervical cancer. *Best Pract. Res. Clin. Obstet. Gynaecol.* 65, 98–108.

Blanco, R., Carrillo-Beltrán, D., Osorio, J., Calaf, G., and Aguayo, F. (2020). Role of Epstein-Barr virus and human papillomavirus coinfection in cervical cancer: Epidemiology, mechanisms and perspectives. *Pathogens* 9:685. doi: 10.3390/pathogens9090685

Bohn, J., Hernandez-Zepeda, M., Hersh, A., Munro, E., Kahn, J., Caughey, A., et al. (2022). Does obesity influence the preferred treatment approach for early-stage cervical cancer? A cost-effectiveness analysis. *Int. J. Gynecol. Cancer* 32, 133–140. doi: 10.1136/ijgc-2021-003004

Bosch, F., Lorincz, A., Muñoz, N., Meijer, C., and Shah, K. (2002). The causal relation between human papillomavirus and cervical cancer. *J. Clin. Pathol.* 55, 244–265.

Brewster, W., Monk, B., Burger, R., Bergen, S., and Wilczynski, S. (1999). Does human papillomavirus have a role in cancers of the uterine corpus? *Gynecol. Oncol.* 75, 51–54.

Brown, D., Kjaer, S., Sigurdsson, K., Iversen, O., Hernandez-Avila, M., Wheeler, C., et al. (2009). The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16–26 years. *J. Infect. Dis.* 199, 926–935. doi: 10.1086/597307

Cameron, J., Dennis, D., Herrel, N., Chapple, A., and Hagensee, M. (2020). Risk of abnormal cervical cytology in HIV-infected women testing positive for both human papillomavirus and Epstein-Barr virus in genital tract specimens. *Cancer Causes Control* 31, 365–375. doi: 10.1007/s10552-020-01287-z

Castellsagué, X., Menéndez, C., Loscertales, M. P., Kornegay, J. R., dos Santos, F., Gómez-Olivé, F. X., et al., (2001). Human papillomavirus genotypes in rural Mozambique. *Lancet* 358, 1429–1430. doi: 10.1016/S0140-6736(01)06523-0

Castro, D., Vera, J., Soto-Becerra, P., López-Ilasaca, M., Yabar, A., Cámara, A., et al. (2020). Epstein-Barr virus and its prognostic value in a cohort of Peruvian women with cervical cancer. *medRxiv [Preprint]* doi: 10.1101/2020.08.04.20167841

Chandra, S., Sarkar, S., and Mandal, P. (2022). Identification of novel genetic and epigenetic regulators of different tissue types of cervical cancer. *J. Obstet. Gynaecol. Res.* 48, 3179–3190. doi: 10.1111/jog.15454

Chen, W., Sun, K., Zheng, R., Zeng, H., Zhang, S., Xia, C., et al. (2018). Cancer incidence and mortality in China, 2014. *Chin. J. Cancer Res.* 30, 1–12.

Chhetri, M. (2010). Chronic hepatitis C virus infection and carcinoma cervix–report of a case and brief review of literature. *Apollo Med.* 7, 61–63.

Choi, S., Ismail, A., Pappas-Gogos, G., and Boussios, S. (2023). HPV and cervical cancer: A review of epidemiology and screening uptake in the UK. *Pathogens* 12:298.

Chu, C., and Liaw, Y. (2010). Hepatitis B surface antigen seroclearance during chronic HBV infection. *Antivir. Ther.* 15, 133–143.

Clifford, G., Tully, S., and Franceschi, S. (2017). Carcinogenicity of human papillomavirus (HPV) types in HIV-positive women: A meta-analysis from HPV infection to cervical cancer. *Clin. Infect. Dis.* 64, 1228–1235. doi: 10.1093/cid/cix135

Coffey, K., Gaitskell, K., Beral, V., Canfell, K., Green, J., Reeves, G., et al. (2016). Past cervical intraepithelial neoplasia grade 3, obesity, and earlier menopause are associated with an increased risk of vulval cancer in postmenopausal women. *Br. J. Cancer* 115, 599–606. doi: 10.1038/bjc.2016.165

Costa, S., Verberckmoes, B., Castle, P., and Arbyn, M. (2023). Offering HPV self-sampling kits: An updated meta-analysis of the effectiveness of strategies to increase participation in cervical cancer screening. *Br. J. Cancer* 128, 805–813. doi: 10.1038/s41416-022-02094-w

Cuzick, J., Clavel, C., Petry, K., Meijer, C., Hoyer, H., Ratnam, S., et al. (2006). Overview of the European and North American studies on HPV testing in primary cervical cancer screening. *Int. J. Cancer* 119, 1095–1101. doi: 10.1002/ijc.21955

da Carvalho, M., and de Melo, Y. (2019). Association between human papillomavirus and Epstein-Barr virus infections and cancer of the uterine cervix. *Crit. Rev. Oncog.* 24, 379–383.

Dai, L., Zhao, M., Jiang, W., Lin, Z., Del Valle, L., and Qin, Z. (2018). KSHV co-infection, a new co-factor for HPV-related cervical carcinogenesis? *Am. J. Cancer Res.* 8, 2176–2184.

D'andrea, F., Pellicanò, G., Venanzi Rullo, E., D'ALEO, F., Facciolà, A., Mical, C., et al. (2019). Cervical cancer in women living with HIV: A review of the literature. *World Cancer Res. J.* 6:e1224.

Dau, H., Trawin, J., Nakisige, C., Payne, B., Vidler, M., Singer, J., et al. (2023). The social and economic impacts of cervical cancer on women and children in low- and middle-income countries: A systematic review. *Int. J. Gynaecol. Obstet.* 160, 751–761. doi: 10.1002/ijgo.14395

de Lima, M., Neto, P., Lima, L., Gonçalves Júnior, J., Teixeira Junior, A., Teodoro, I., et al. (2018). Association between Epstein-Barr virus (EBV) and cervical carcinoma: A meta-analysis. *Gynecol. Oncol.* 148, 317–328.

De Oliveira, D., Monteiro, T., De Melo, W., Moreira, M., Alvarenga, M., and Bacchi, C. (1999). Lack of Epstein-Barr virus infection in cervical carcinomas. *Arch. Pathol. Lab. Med.* 123, 1098–1100.

de Sanjose, S., Quint, W., Alemany, L., Geraets, D., Klaustermeier, J., Lloveras, B., et al. (2010). Human papillomavirus genotype attribution in invasive cervical cancer: A retrospective cross-sectional worldwide study. *Lancet Oncol.* 11, 1048–1056.

Denny, L., Boa, R., Williamson, A., Allan, B., Hardie, D., Stan, R., et al. (2008). Human papillomavirus infection and cervical disease in human immunodeficiency virus-1-infected women. *Obstet. Gynecol.* 111, 1380–1387. doi: 10.1097/AOG.0b013e3181743327

Dimond, C., Negroiu, A., Hughes, D., and Patel, J. (2021). Fatal hepatitis B reactivation in a patient receiving chemoradiation for cervical cancer. *J. Oncol. Pharm. Pract.* 27, 1296–1301. doi: 10.1177/1078155220964256

Drolet, M., Bénard, É, Pérez, N., Brisson, M., and Hpv Vaccination Impact Study Group. (2019). Population-level impact and herd effects following the introduction of human papillomavirus vaccination programmes: Updated systematic review and meta-analysis. *Lancet* 394, 497–509. doi: 10.1016/S0140-6736(19)30298-3

Elfström, K., Smelov, V., Johansson, A., Eklund, C., Nauclér, P., Arnheim-Dahlström, L., et al. (2014). Long term duration of protective effect for HPV negative women: Follow-up of primary HPV screening randomised controlled trial. *BMJ* 348:g130. doi: 10.1136/bmj.g130

Engels, E., Cho, E., and Jee, S. (2010). Hepatitis B virus infection and risk of non-Hodgkin lymphoma in South Korea: A cohort study. *Lancet Oncol.* 11, 827–834.

Evans, A., Salnikov, M., Gameiro, S., Maleki Vareki, S., and Mymryk, J. (2022). HPV-positive and-negative cervical cancers are immunologically distinct. *J. Clin. Med.* 11:4825. doi: 10.3390/jcm11164825

Feng, M., Duan, R., Gao, Y., Zhang, H., Qiao, Y., Li, Q., et al. (2021). Role of Epstein-Barr virus and human papillomavirus coinfection in cervical intraepithelial neoplasia in Chinese women living with HIV. *Front. Cell. Infect. Microbiol.* 11:703259. doi: 10.3389/fcimb.2021.703259

Feng, X., Lu, H., Wei, Y., Guan, M., Wang, J., Liu, C., et al. (2021). Prognostic impact of hepatitis B virus infection in patients with primary cervical cancer. *Cancer Med.* 10, 8310–8319. doi: 10.1002/cam4.4358

Ferber, M. J., Montoya, D. P., Yu, C., Aderca, I., McGee, A., Thorland, E. C., et al. (2003). Integrations of the hepatitis B virus (HBV) and human papillomavirus (HPV) into the human telomerase reverse transcriptase (hTERT) gene in liver and cervical cancers. *Oncogene* 22, 3813–3820. doi: 10.1038/sj.onc.1206528

Ferrall, L., Lin, K., Roden, R., Hung, C., and Wu, T. (2021). Cervical cancer immunotherapy: Facts and hopes. *Clin. Cancer Res.* 27, 4953–4973. doi: 10.1158/1078-0432.CCR-20-2833

Figueiredo, D., Ribeiro, I., Penedones, A., Mendes, D., Alves, C., Batel-Marques, F., et al. (2023). Performance of Aptima-HPV in the cervical cancer screening program of Portugal: A cost-analysis. *BMC Womens Health* 23:96. doi: 10.1186/s12905-023-02219-0

Fine, P., Eames, K., and Heymann, D. (2011). "Herd immunity": A rough guide. *Clin. Infect. Dis.* 52, 911–916. doi: 10.1093/cid/cir007

Frumovitz, M., Jhingran, A., Soliman, P., Klopp, A., Schmeler, K., and Eifel, P. (2014). Morbid obesity as an independent risk factor for disease-specific mortality in women with cervical cancer. *Obstet. Gynecol.* 124, 1098–1104. doi: 10.1097/AOG.0000000000000558

Fujii, T., Okabe, A., and Kaneda, A. (2022). Epigenetic contribution to tumorigenesis of host cells by Epstein-Barr virus infection. *Chiba Med. J.* 98, 1–7.

Ghadicolaee, S., Pazhoohan, M., Hasanzadeh, A., Nematolahi, M., Yahyapour, Y., Ranaee, M., et al. (2021). Low frequency of human cytomegalovirus in cancerous and precancerous cervical samples of Iranian women. *Future Virol.* 16, 399–405.

Gilham, C., Sargent, A., Crosbie, E., and Peto, J. (2023). Long-term risks of invasive cervical cancer following HPV infection: Follow-up of two screening cohorts in Manchester. *Br. J. Cancer* 128, 1933–1940. doi: 10.1038/s41416-023-02227-9

Giuliano, A., Sedjo, R., Roe, D., Harri, R., Baldwi, S., Papenfuss, M., et al. (2002). Clearance of oncogenic human papillomavirus (HPV) infection: Effect of smoking (United States). *Cancer Causes Control* 13, 839–846.

Gupta, S., Palmer, C., Bik, E., Cardenas, J., Nuñez, H., Kraal, L., et al. (2018). Self-sampling for human papillomavirus testing: Increased cervical cancer screening participation and incorporation in international screening programs. *Front. Public Health* 6:77. doi: 10.3389/fpubh.2018.00077

Hadziyannis, S. (1995). Hepatitis B e antigen negative chronic hepatitis B: From clinical recognition to pathogenesis and treatment. *Viral Hepat Rev.* 1, 7–36.

Harper, D., and DeMars, L. (2017). HPV vaccines–a review of the first decade. *Gynecol. Oncol.* 146, 196–204.

Heffernan, M., Garland, S., and Kane, M. (2010). Global reduction of cervical cancer with human papillomavirus vaccines: Insights from the hepatitis B virus vaccine experience. *Sex. Health* 7, 383–390. doi: 10.1071/SH09134

Hemmat, N., and Bannazadeh Baghi, H. (2019). Association of human papillomavirus infection and inflammation in cervical cancer. *Pathog. Dis.* 77:ftz048.

Hildesheim, A., Herrero, R., Castle, P. E., Wacholder, S., Bratti, M. C., Sherman, M. E., et al. (2001). HPV co-factors related to the development of cervical cancer: Results from a population-based study in Costa Rica. *Br. J. Cancer* 84, 1219–1226. doi: 10.1054/bjoc.2001.1779

Hoseini Tabatabaie, F., Hosseini, S., Hashemi, S., Safaie, A., and Sarvari, J. (2023). A preliminary sequence analysis of the Epstein-Barr virus nuclear antigen 1 (EBNA1) carboxy-terminal region in cervical and ovarian cancers. *Iran. J. Pathol.* 18, 24–32.

Huh, W., Joura, E., Giuliano, A., Iversen, O., de Andrade, R., Ault, K., et al. (2017). Final efficacy, immunogenicity, and safety analyses of a nine-valent human papillomavirus vaccine in women aged 16–26 years: A randomised, double-blind trial. *Lancet* 390, 2143–2159. doi: 10.1016/S0140-6736(17)31821-4

Ibrahim Jaber, S., and Qasim Dhumad, B. (2022). Expression of HPV-16 L1 gene human papillomavirus (HTLV-1) identified by pap smear. *Arch. Razi Inst.* 77, 2125–2130.

Insinga, R., Dasbach, E., and Myers, E. (2003). The health and economic burden of genital warts in a set of private health plans in the United States. *Clin. Infect. Dis.* 36, 1397–1403. doi: 10.1086/375074

Iversen, L., Fielding, S., Lidegaard, O., and Hannaford, P. (2021). Contemporary hormonal contraception and cervical cancer in women of reproductive age. *Int. J. Cancer* doi: 10.1002/ijc.33585 [Epub ahead of print].

Joura, E., Giuliano, A., Iversen, O., Bouchard, C., Mao, C., Mehlsen, J., et al. (2015). A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. *N. Engl. J. Med.* 372, 711–723.

Kamal, M., Lameiras, S., Deloger, M., Morel, A., Vacher, S., Lecerf, C., et al. (2021). Human papilloma virus (HPV) integration signature in cervical cancer: Identification of MACROD2 gene as HPV hot spot integration site. *Br. J. Cancer* 124, 777–785.

Kelly, H., Weiss, H., Benavente, Y., de Sanjose, S., and Mayaud, P. (2018). Association of antiretroviral therapy with high-risk human papillomavirus, cervical intraepithelial neoplasia, and invasive cervical cancer in women living with HIV: A systematic review and meta-analysis. *Lancet HIV* 5, e45–e58. doi: 10.1016/s2352-3018(17)30149-2

Khashman, B., Al-Zahawi, F., Alwandawi, T., and Ali, M. J. (2020a). Expression of EBV latent membrane protein 1 (LMP1) in Iraqi women with cervical carcinoma. *Biochem. Cell. Arch.* 20:3263.

Khashman, B., Karim, S., Alhilli, H., and Ali, M. (2020b). Possible role of HCMV infection on the development of HPV positive cervical carcinoma in a group of Iraqi women. *Biochem. Cell. Arch.* 20, 1549–1552.

Kienka, T., Varga, M., Caves, J., Smith, J., and Sivaraman, V. (2019). Epstein-Barr virus, but not human cytomegalovirus, is associated with a high-grade human papillomavirus–associated cervical lesions among women in North Carolina. *J. Med. Virol.* 91, 450–456.

Kim, J., Kim, B., Jeon, D., Lee, C., Roh, J., Kim, J., et al. (2020). Type-specific viral load and physical state of HPV type 16, 18, and 58 as diagnostic biomarkers for high-grade squamous intraepithelial lesions or cervical Cancer. *Cancer Res. Treat.* 52, 396–405.

Kolesnik, M., Stepien, E., and Polz-Dacewicz, M. (2021). The role of microRNA (miRNA) as a biomarker in HPV and EBV-related cancers. *J. Preclin. Clin. Res.* 15, 104–110.

Korsakov, A., Kryukova, A., Troshin, V., Milushkina, O., and Lagerev, D. (2022). Cervical and endometrial cancer incidence in the female population from the bryansk region living in conditions of chemical, radioactive and combined environmental contamination (2000-2020). *Life* 12:1488. doi: 10.3390/life12101488

Kreimer, A., Struyf, F., Del Rosario-Raymundo, M., Hildesheim, A., Skinner, S., Wacholder, S., et al. (2015). Efficacy of fewer than three doses of an HPV-16/18 AS04-adjuvanted vaccine: Combined analysis of data from the Costa Rica vaccine and PATRICIA trials. *Lancet Oncol.* 16, 775–786. doi: 10.1016/S1470-2045(15)00047-9

Kumar, V., Chawla, A., and Kaur, A. (2018). Multiple idiopathic cervical root resorptions in patients with hepatitis B virus infection. *J. Endod.* 44, 1575–1577. doi: 10.1016/j.joen.2018.06.017

Kwolek, D., Gerstberger, S., Tait, S., and Qiu, J. (2023). Ovarian, uterine, and vulvovaginal cancers: Screening, treatment overview, and prognosis. *Med. Clin.* 107, 329–355. doi: 10.1016/j.mcna.2022.10.016

Kycler, W., Kubiak, A., Rzymski, P., Wilczak, M., Trojanowski, M., Roszak, M., et al. (2017). Impact of selected environmental factors on attendance in the breast and cervical cancer early detection programme in the Wielkopolska province of Poland during 2007-2012. *Ann. Agric. Environ. Med.* 24, 467–471. doi: 10.26444/aaem/74481

Labani, S., Asthana, S., Sodhani, P., Gupta, S., Bhambhani, S., Pooja, B., et al. (2014). CareHPV cervical cancer screening demonstration in a rural population of north India. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 176, 75–79. doi: 10.1016/j.ejogrb.2014.03.006

Lasche, M., Gallwas, J., and Grundker, C. (2022). Like brothers in arms: How hormonal stimuli and changes in the metabolism signaling cooperate, leading HPV infection to drive the onset of cervical cancer. *Int. J. Mol. Sci.* 23:5050. doi: 10.3390/ijms23095050

Lau, K., Hsu, Y., Lin, Y., Yeap, M., Lee, C., and Chen, K. (2021). Case history on Epstein-Barr Virus-associated smooth muscle tumor (EBV-SMT) of cranio-cervical junction in an immunocompetent patient. *Br. J. Neurosurg.* doi: 10.1080/02688697.2021.1932745 [Epub ahead of print].

Lee, S., Kang, D., Seo, S., Jeong, J., Yoo, K., Jeon, Y., et al. (2003). Multiple HPV infection in cervical cancer screened by HPVDNAChip^{TM}. *Cancer Lett.* 198, 187–192. doi: 10.1016/s0304-3835(03)00312-4

Leroux-Roels, G., Haelterman, E., Maes, C., Levy, J., De Boever, F., Licini, L., et al. (2011). Randomized trial of the immunogenicity and safety of the Hepatitis B vaccine given in an accelerated schedule coadministered with the human papillomavirus type 16/18 AS04-adjuvanted cervical cancer vaccine. *Clin. Vaccine Immunol.* 18, 1510–1518. doi: 10.1128/CVI.00539-10

Li, J., Dai, X., Zhang, H., Zhang, W., Sun, S., Gao, T., et al. (2015). Up-regulation of human cervical cancer proto-oncogene contributes to hepatitis B virus-induced malignant transformation of hepatocyte by down-regulating E-cadherin. *Oncotarget* 6, 29196–29209. doi: 10.18632/oncotarget.5039

Liu, X., Ma, H., Fei, L., Jiang, M., Xia, M., Bai, L., et al. (2020). HPV-mediated down-regulation of NOD1 inhibits apoptosis in cervical cancer. *Infect. Agents Cancer* 15:6. doi: 10.1186/s13027-020-0272-3

Liu, Y., Fan, P., Yang, Y., Xu, C., Huang, Y., Li, D., et al. (2019). Human papillomavirus and human telomerase RNA component gene in cervical cancer progression. *Sci. Rep.* 9:15926.

Liu, Y., Liu, H., Sheng, B., Pan, S., Wang, Z., and Zhu, X. (2022a). The functions of lncRNAs in the HPV-negative cervical cancer compared with HPV-positive cervical cancer. *Apoptosis* 27, 685–696.

Liu, Y., Zhang, Q., and Ni, R. (2022b). Association between genetic variants (rs920778, rs4759314, and rs217727) in LncRNAs and cervical cancer susceptibility in Chinese population: A systematic review and meta-analysis. *Front. Genet.* 13:988207. doi: 10.3389/fgene.2022.988207

Lok, A., and McMahon, B. (2009). Chronic hepatitis B: Update 2009. *Hepatology* 50, 661–662.

Lourenço de Freitas, N., Deberaldini, M., Gomes, D., Pavan, A., Sousa, Â, Dos Santos, J., et al. (2021). Histone deacetylase inhibitors as therapeutic interventions on cervical cancer induced by human papillomavirus. *Front. Cell. Dev. Biol.* 8:592868. doi: 10.3389/fcell.2020.592868

Luo, C., Yu, S., Zhang, J., Wu, X., Dou, Z., Li, Z., et al. (2022). Hepatitis B or C viral infection and the risk of cervical cancer. *Infect. Agents Cancer* 17:54.

Luo, W., Feng, Y., Guo, R., Tang, L., Chen, L., Zhou, G., et al. (2019). Patterns of EBV-positive cervical lymph node involvement in head and neck cancer and implications for the management of nasopharyngeal carcinoma T0 classification. *Oral Oncol.* 91, 7–12. doi: 10.1016/j.oraloncology.2019.01.012

Macleod, C., and Reynolds, J. (2021). Human Papilloma Virus infection and cervical cancer among women who sell sex in Eastern and Southern Africa: A scoping review. *Womens Health* 17:17455065211058349. doi: 10.1177/17455065211058349

Mahmood, M., Baghestanian, M., Thomas, W. R., Battistutti, W., Pischinger, K., Schatten, C., et al. (2002). Detection of hepatitis C virus (HCV) RNA in normal cervical smears of HCV-seropositive patients. *Clin. Infect. Dis.* 35, 966–973. doi: 10.1086/342909

Malevolti, M., Lugo, A., Scala, M., Gallus, S., Gorini, G., Lachi, A., et al. (2022). Dose-risk relationships between cigarette smoking and cervical cancer: A systematic review and meta-analysis. *Eur. J. Cancer Prev.* 32, 171–183. doi: 10.1097/CEJ.0000000000000773

Marima, R., Hull, R., Lolas, G., Syrigos, K., Kgoebane-Maseko, M., Kaufmann, A., et al. (2021). The catastrophic HPV/HIV dual viral oncogenomics in concert with dysregulated alternative splicing in cervical cancer. *Int. J. Mol. Sci.* 22:10115. doi: 10.3390/ijms221810115

Markowitz, L., Tsu, V., Deeks, S., Cubie, H., Wang, S., Vicari, A., et al. (2012). Human papillomavirus vaccine introduction–the first five years. *Vaccine* 30, F139–F148.

Marlow, L., Waller, J., and Wardle, J. (2007). Public awareness that HPV is a risk factor for cervical cancer. *Br. J. Cancer* 97, 691–694.

Maskey, N., Thapa, N., Maharjan, M., Shrestha, G., Maharjan, N., Cai, H., et al. (2019). Infiltrating CD4 and CD8 lymphocytes in HPV infected uterine cervical milieu. *Cancer Manag. Res.* 11, 7647–7655.

Moharreri, M., and Sohrabi, A. (2021). Characteristics of hsv-2, *M. genitalium* and *C. trachomatis* in HPV genotypes associated with cervical intraepithelial neoplasia and genital infections. *Infect. Disord. Drug Targets* 21, 112–118. doi: 10.2174/1871526520666191231142317

Montoya-Fuentes, H., Suárez Rincón, A., Ramírez-Muñoz, M., Arévalo-Lagunas, I., Morán Moguel, M., Gallegos Arreola, M., et al. (2001). The detection of human papillomavirus 16, 18, 35 and 58 in cervical-uterine cancer and advanced degree of squamous intraepithelial lesions in Western Mexico: Clinical-molecular correlation. *Ginecol. Obstet. Mexico* 69, 137–142.

Moscicki, A., Flowers, L., Huchko, M., Long, M., MacLaughlin, K., Murphy, J., et al. (2019). Guidelines for cervical cancer screening in immunosuppressed women without HIV infection. *J. Low. Genit. Tract Dis.* 23, 87–101.

Mukerebe, C. (2022). Kaposi's sarcoma-associated herpesvirus shedding in saliva and cervical secretions in Tanzanian women. *Tanzania J. Health Res.* 23, 157–158.

Muñoz, N., Bosch, F., de Sanjosé, S., Herrero, R., Castellsagué, X., Shah, K., et al. (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* 348, 518–527.

Muñoz-Bello, J., Carrillo-García, A., and Lizano, M. (2022). Epidemiology and molecular biology of HPV variants in cervical cancer: The state of the art in Mexico. *Int. J. Mol. Sci.* 23:8566. doi: 10.3390/ijms23158566

Nakagawa, J., Schirmer, J., and Barbieri, M. (2010). Human papillomavirus (HPV) and uterine cervical cancer. *Rev. Bras. Enferm.* 63, 307–311.

Nelson, E., Maynard, B., Loux, T., Fatla, J., Gordon, R., and Arnold, L. (2017). The acceptability of self-sampled screening for HPV DNA: A systematic review and meta-analysis. *Sex. Transm. Infect.* 93, 56–61. doi: 10.1136/sextrans-2016-052609

Noel, J., Lespagnard, L., Fayt, I., Verhest, A., and Dargent, J. (2001). Evidence of human papilloma virus infection but lack of Epstein-Barr virus in lymphoepithelioma-like carcinoma of uterine cervix: Report of two cases and review of the literature. *Hum. Pathol.* 32, 135–138. doi: 10.1053/hupa.2001.20901

Ogilvie, G., Krajden, M., Maginley, J., Isaac-Renton, J., Hislop, G., Elwood-Martin, R., et al. (2007). Feasibility of self-collection of specimens for human papillomavirus testing in hard-to-reach women. *CMAJ* 177, 480–483.

Okoye, J., Ngokere, A., Onyenekwe, C., Eze, U., and Obioma, O. (2023). Abstract C029: The pattern of mutant p53 protein expression in cervical cancer-associated single and co-infection with human papillomavirus and Epstein-Barr virus. *Cancer Epidemiol. Biomarkers Prev.* 32:C029.

Pandey, N., Chauhan, A., Raithatha, N., Patel, P., Khandelwal, R., Desai, A., et al. (2021). Influence of TLR4 and TLR9 polymorphisms and haplotypes on multiple hrHPV infections and HPV16 copy number in cervical cancer and cervicitis. *Microb. Pathog.* 159:105149. doi: 10.1016/j.micpath.2021.105149

Petignat, P., Faltin, D., Bruchim, I., Tramèr, M., Franco, E., and Coutlée, F. (2007). Are self-collected samples comparable to physician-collected cervical specimens for human papillomavirus DNA testing? A systematic review and meta-analysis. *Gynecol. Oncol.* 105, 530–535.

Pham, C., Juhasz, M., Sung, C., and Mesinkovska, N. (2020). The human papillomavirus vaccine as a treatment for human papillomavirus–related

dysplastic and neoplastic conditions: A literature review. *J. Am. Acad. Dermatol.* 82, 202–212.

Plummer, M., de Martel, C., Vignat, J., Ferlay, J., Bray, F., and Franceschi, S. (2016). Global burden of cancers attributable to infections in 2012: A synthetic analysis. *Lancet Glob. Health* 4, e609–e616.

Porter, V., and Marra, M. (2022). The drivers, mechanisms, and consequences of genome instability in HPV-driven cancers. *Cancers* 14:4623. doi: 10.3390/cancers14194623

Racey, C., Withrow, D., and Gesink, D. (2013). Self-collected HPV testing improves participation in cervical cancer screening: A systematic review and meta-analysis. *Can. J. Public Health* 104, e159–e166. doi: 10.1007/bf03405681

Ronco, G., Dillner, J., Elfström, K., Tunesi, S., Snijders, P., Arbyn, M., et al. (2014). Efficacy of HPV-based screening for prevention of invasive cervical cancer: Follow-up of four European randomised controlled trials. *Lancet* 383, 524–532. doi: 10.1016/S0140-6736(13)62218-7

Rosadas, C., and Taylor, G. (2022). HTLV-1 and co-infections. *Front. Med.* 9:812016. doi: 10.3389/fmed.2022.812016

Roy, V., Jung, W., Linde, C., Coates, E., Ledgerwood, J., Costner, P., et al. (2023). Differences in HPV-specific antibody Fc-effector functions following Gardasil® and Cervarix® vaccination. *Npj Vaccines* 8:39. doi: 10.1038/s41541-023-00628-8

Safaeian, M., Solomon, D., and Castle, P. (2007). Cervical cancer prevention—cervical screening: Science in evolution. *Obstet. Gynecol. Clin. North Am.* 34, 739–760. doi: 10.1016/j.ogc.2007.09.004

Sancho-Garnier, H., Tamalet, C., Halfon, P., Leandri, F., Le Retraite, L., Djoufelkit, K., et al. (2013). HPV self-sampling or the Pap-smear: A randomized study among cervical screening nonattenders from lower socioeconomic groups in France. *Int. J. Cancer* 133, 2681–2687. doi: 10.1002/ijc.28283

Sankaranarayanan, R., Nene, B., Shastri, S., Jayant, K., Muwonge, R., Budukh, A., et al. (2009). HPV screening for cervical cancer in rural India. *N. Engl. J. Med.* 360, 1385–1394.

Sasagawa, T., Shimakage, M., Nakamura, M., Sakaike, J., Ishikawa, H., and Inoue, M. (2000). Epstein-Barr virus (EBV) genes expression in cervical intraepithelial neoplasia and invasive cervical cancer: A comparative study with human papillomavirus (HPV) infection. *Hum. Pathol.* 31, 318–326. doi: 10.1016/s0046-8177(00)80245-2

Sassenou, J., Ringa, V., Zins, M., Ozguler, A., Paquet, S., Panjo, H., et al. (2021). Women with obesity in cervical cancer screening. The double penalty: Underscreening and income inequalities. *Obes. Res. Clin. Pract.* 15, 212–215. doi: 10.1016/j.orcp.2021.03.003

Schierhout, G., McGregor, S., Gessain, A., Einsiedel, L., Martinello, M., and Kaldor, J. (2020). Association between HTLV-1 infection and adverse health outcomes: A systematic review and meta-analysis of epidemiological studies. *Lancet Infect. Dis.* 20, 133–143. doi: 10.1016/S1473-3099(19)30402-5

Schiffman, M., Wentzensen, N., Wacholder, S., Kinney, W., Gage, J., and Castle, P. (2011). Human papillomavirus testing in the prevention of cervical cancer. *J. Natl. Cancer Inst.* 103, 368–383.

Serrano, B., Ibáñez, R., Robles, C., Peremiquel-Trillas, P., de Sanjosé, S., and Bruni, L. (2022). Worldwide use of HPV self-sampling for cervical cancer screening. *Prev. Med.* 154:106900.

Shiels, M., Engels, E., Linet, M., Clarke, C., Li, J., Hall, H., et al. (2013). The epidemic of non–hodgkin lymphoma in the United States: Disentangling the effect of HIV, 1992–2009U. S. NHL trends excluding HIV-infected cases, 1992–2009. *Cancer Epidemiol. Biomarkers Prev.* 22, 1069–1078.

Shin, S., Carpenter, C., Ekstrand, M., Wang, Q., Grover, S., Zetola, N., et al. (2019). Cervical cancer awareness and presence of abnormal cytology among HIV-infected women on antiretroviral therapy in rural Andhra Pradesh, India. *Int. J. STD AIDS* 30, 586–595. doi: 10.1177/0956462419825950

Singh, G., Sharma, S., and Singh, S. (2022). miR-34a negatively regulates cell cycle factor Cdt2/DTL in HPV infected cervical cancer cells. *BMC Cancer* 22:777. doi: 10.1186/s12885-022-09879-5

Siu, S., Cheung, T., Chan, P., Lin, C., and Lo, K. (2007). Patients with malignant or pre-malignant cervical lesion have increased risk of becoming hepatitis B carrier. *J. Exp. Clin. Cancer Res.* 26, 77–81.

Smith, J., Vaz, O., Gaber, C., Des Marais, A., Chirumamilla, B., Hendrickson, L., et al. (2023). Recruitment strategies and HPV self-collection return rates for under-screened women for cervical cancer prevention. *PLoS One* 18:e0280638. doi: 10.1371/journal.pone.0280638

Sosse, S., Tadlaoui, K., Benhassou, M., Elkarroumi, M., Elmzibri, M., and Ennaji, M. (2022). Viral co-infection of oncogenic human papillomavirus with Epstein–Barr Virus, human herpesvirus 8 and Herpes Simplex Virus type 2 in malignant cervical cancer. *Int. Med. J.* 30.

Srinath, A., van Merode, F., Rao, S., and Pavlova, M. (2022). Barriers to cervical cancer and breast cancer screening uptake in low-and-middle-income countries: A systematic review. *Health Policy Plan* 38, 509–527. doi: 10.1093/heapol/czac104

Stelzle, D., Tanaka, L., Lee, K., Ibrahim Khalil, A., Baussano, I., Shah, A., et al. (2021). Estimates of the global burden of cervical cancer associated with HIV. *Lancet Glob. Health* 9, e161–e169.

Tawe, L., MacDuffie, E., Narasimhamurthy, M., Wang, Q., Gaseitsiwe, S., Moyo, S., et al. (2020). Human papillomavirus genotypes in women with invasive cervical cancer with and without human immunodeficiency virus infection in Botswana. *Int. J. Cancer* 146, 1667–1673.

Tin, K., Ngamjarus, C., Rattanakanokchai, S., Sothornwit, J., Aue-Aungkul, A., Paing, A., et al. (2023). Interventions to increase the uptake of cervical cancer screening in low- and middle-income countries: A systematic review and meta-analysis. *BMC Womens Health* 23:120. doi: 10.1186/s12905-023-02265-8

Ton, M., Swami, N., Germar, M., and Dee, E. (2022). HPV mRNA testing in cervical cancer screening: Implications for low- and middle-income countries. *Int. J. Gynecol. Cancer* 32, 1632–1633. doi: 10.1136/ijgc-2022-003959

Tota, J., Ramanakumar, A., Jiang, M., Dillner, J., Walter, S., Kaufman, J., et al. (2013). Epidemiologic approaches to evaluating the potential for human papillomavirus type replacement postvaccination. *Am. J. Epidemiol.* 178, 625–634. doi: 10.1093/aje/kwt018

Urbute, A., Kjaer, S., Kesmodel, U., Frederiksen, K., and Thomsen, L. (2022). Women with obesity participate less in cervical cancer screening and are more likely to have unsatisfactory smears: Results from a nationwide Danish cohort study. *Prev. Med.* 159:107072. doi: 10.1016/j.ypmed.2022.107072

van den Helder, R., Steenbergen, R., van Splunter, A., Mom, C., Tjiong, M., Martin, I., et al. (2022). HPV and DNA methylation testing in urine for cervical intraepithelial neoplasia and cervical cancer detection. *Clin. Cancer Res.* 28, 2061–2068.

Vitali, D., Bagri, P., Wessels, J., Arora, M., Ganugula, R., Parikh, A., et al. (2020). Curcumin can decrease tissue inflammation and the severity of HSV-2 infection in the female reproductive mucosa. *Int. J. Mol. Sci.* 21:337. doi: 10.3390/ijms21010337

Vranic, S., Cyprian, F., Akhtar, S., and Al Moustafa, A. (2018). The role of Epstein-Barr Virus in cervical cancer: A brief update. *Front. Oncol.* 8:113. doi: 10.3389/fonc.2018.00113

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., et al. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189, 12–19.

Waller, J., Bartoszek, M., Marlow, L., and Wardle, J. (2009). Barriers to cervical cancer screening attendance in England: A population-based survey. *J. Med. Screen.* 16, 199–204. doi: 10.1258/jms.2009.009073

Wu, X., Li, L., Li, Y., Jiang, M., Li, K., Li, Z., et al. (2021). Prognostic value of serological markers of hepatitis B virus infection in squamous cell cervical cancer. *J. Cancer* 12, 6620–6628. doi: 10.7150/jca.61249

Yadav, C., Yadav, R., Chhabra, R., Nanda, S., Ranga, S., Kadian, L., et al. (2023a). Correction to: Overview of genetic and epigenetic regulation of human papillomavirus and apoptosis in cervical cancer. *Apoptosis* doi: 10.1007/s10495-023-01822-8 [Epub ahead of print].

Yadav, C., Yadav, R., Chabbra, R., Nanda, S., Ranga, S., Kadian, L., et al. (2023b). Overview of genetic and epigenetic regulation of human papillomavirus and apoptosis in cervical cancer. *Apoptosis* doi: 10.1007/s10495-023-01812-w [Epub ahead of print].

Yordanov, A., Ivanov, I., Dineva, T., Popovska, S., Karcheva, M., Strashilov, S., et al. (2020). Lymphoepithelioma-like carcinoma of the uterine cervix: Correlation with Epstein-Barr virus and human papillomavirus infection. A single-center experience. *Eur. J. Gynaecol. Oncol.* 41, 913–918. doi: 10.31083/j.ejgo.2020.06.2107

Yordanov, A., Karcheva, M., Betova, T., Ivanov, I., Dineva, T., and Strashilov, S. (2019). Immunohistochemical study of human papilloma virus and Epstein–Barr virus in patients with lymphoepithelioma-like carcinoma of the uterine cervix. *Arch. Balk Med. Union* 53, 680–684.

Youn, J., Hur, S., Woo, J., Kim, Y., Lim, M., Park, S., et al. (2020). Pembrolizumab plus GX-188E therapeutic DNA vaccine in patients with HPV-16-positive or HPV-18-positive advanced cervical cancer: Interim results of a single-arm, phase 2 trial. *Lancet Oncol.* 21, 1653–1660. doi: 10.1016/S1470-2045(20)30486-1

Yuan, M., Zhao, X., Wang, H., Hu, S., and Zhao, F. (2023). Trend in cervical cancer incidence and mortality rates in China, 2006-2030: A Bayesian age-period-cohort modeling study. *Cancer Epidemiol. Biomarkers Prev.* doi: 10.1158/1055-9965.EPI-22-0674 [Epub ahead of print].

Zhang, J., Yu, G., Yang, Y., Wang, Y., Guo, M., Yin, Q., et al. (2022). A small-molecule inhibitor of MDMX suppresses cervical cancer cells via the inhibition of E6-E6AP-p53 axis. *Pharmacol. Res.* 177:106128. doi: 10.1016/j.phrs.2022.106128

Zidi, S., Sahli, M., Mezlini, A., and Yacoubli-Loueslati, B. (2020). Association of combined tobacco smoking, hormonal contraceptive use and status matrimonial with cervical cancer evolution in Tunisian women. *Pathol. Oncol. Res.* 26, 217–222. doi: 10.1007/s12253-018-0442-4

zur Hausen, H. (2002). Papillomaviruses and cancer: From basic studies to clinical application. *Nat. Rev. Cancer* 2, 342–350.

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HMGB1, a potential regulator of tumor microenvironment in KSHV-infected endothelial cells

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High-mobility group box 1 (HMGB1) is a protein that binds to DNA and participates in various cellular processes, including DNA repair, transcription, and inflammation. It is also associated with cancer progression and therapeutic resistance. Despite its known role in promoting tumor growth and immune evasion in the tumor microenvironment, the contribution of HMGB1 to the development of Kaposi's sarcoma (KS) is not well understood. We investigated the effect of HMGB1 on KS pathogenesis using immortalized human endothelial cells infected with Kaposi's sarcoma-associated human herpes virus (KSHV). Our results showed that a higher amount of HMGB1 was detected in the supernatant of KSHV-infected cells compared to that of mock-infected cells, indicating that KSHV infection induced the secretion of HMGB1 in human endothelial cells. By generating HMGB1 knockout clones from immortalized human endothelial cells using CRISPR/Cas9, we elucidated the role of HMGB1 in KSHV-infected endothelial cells. Our findings indicate that the absence of HMGB1 did not induce lytic replication in KSHV-infected cells, but the cell viability of KSHV-infected cells was decreased in both 2D and 3D cultures. Through the antibody array for cytokines and growth factors, CXCL5, PDGF-AA, G-CSF, Emmprin, IL-17A, and VEGF were found to be suppressed in HMGB1 KO KSHV-infected cells compared to the KSHV-infected wild-type control. Mechanistically, phosphorylation of p38 would be associated with transcriptional regulation of CXCL5, PDGF-A and VEGF. These observations suggest that HMGB1 may play a critical role in KS pathogenesis by regulating cytokine and growth factor secretion and emphasize its potential as a therapeutic target for KS by modulating the tumor microenvironment.

KEYWORDS

HMGB1, KSHV, herpesvirus, cell proliferation, CRISPR/Cas9 system, cytokine array

1. Introduction

High-mobility group box 1 (HMGB1) is a versatile protein that plays essential roles in normal cellular processes and pathological conditions, particularly inflammation and cancer (Martinotti et al., 2015; Wang and Zhang, 2020). As an immune protein released during tissue damage, infection, or inflammation, HMGB1 plays a crucial role in regulating innate and adaptive immune responses (Yang et al., 2020). In innate immunity, HMGB1 acts as a pro-inflammatory cytokine, stimulating the production of TNF-alpha and IL-1β, thereby promoting inflammation, immune cell recruitment, and activation for threat elimination (Lee

S. A. et al., 2014). In adaptive immunity, HMGB1 enhances antigen presentation through dendritic cell receptor binding, facilitating antigen capture, processing, and presentation to T cells. This interaction drives T cell activation, proliferation, and differentiation into effector cells, shaping the adaptive immune response (Li et al., 2013). Inflammation is crucial for the development of tumors, and HMGB1 is a vital mediator of the inflammatory response. HMGB1 functions as an extracellular signaling molecule and stimulates the production of cytokines and chemokines, contributing to the pro-inflammatory response (Xu et al., 2016). Additionally, HMGB1 can bind to receptors on immune cells, promoting tumor growth and immune evasion (Zhang et al., 2019; Hubert et al., 2021). In cancer, HMGB1 is frequently overexpressed and is associated with poor patient prognosis (Zhang et al., 2015; Xu et al., 2016). HMGB1 influences multiple aspects of cancer progression, including DNA repair, transcription, angiogenesis, and metastasis (Tripathi et al., 2019; Wang and Zhang, 2020).

Kaposi's sarcoma (KS) is a type of cancer that affects the endothelial cells lining blood vessels and is caused by the Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8) (Boshoff et al., 1995; Ganem, 2010). KSHV promotes the development of KS by modulating various signaling pathways that regulate cell proliferation, survival, and angiogenesis (Watanabe et al., 2018). The virus encodes several proteins that activate or inhibit these pathways, leading to the uncontrolled growth of infected cells and the formation of KS lesions (Ganem, 2010). Furthermore, KSHV exploits multiple host genes to create a pro-inflammatory and pro-tumorigenic microenvironment that facilitates tumor growth and immune evasion (Lee M. S. et al., 2014; Jeon et al., 2019; Lee et al., 2023).

HMGB1 enhances KSHV replication and transcription activator (RTA) binding to RTA-responsive elements of KSHV target genes (Song et al., 2004). Furthermore, HMGB1 binds and synergistically upregulates the KSHV *ORF50* promoter in conjunction with RTA (Harrison and Whitehouse, 2008). In a previous study, we demonstrated that intracellular HMGB1 forms complexes with various proteins and that the levels of HMGB1-interacting proteins are altered during latent and lytic replication (Kang et al., 2021a). Furthermore, our findings indicated that extracellular HMGB1 enhances lytic replication, which correlates with viral production.

Our previous study demonstrated that HMGB1 has a crucial role in the replication of KSHV in KSHV-producing cancer cell line. However, the role of HMGB1 in the development of KS remains unclear. Although human endothelial cells are widely regarded as a source of KS spindle cells, the lack of primary human endothelial cells for gene depletion studies has hindered efforts to ascertain the specific functions of cellular proteins in KS pathogenesis. To address the limitations of the study exploring the involvement of HMGB1 in KS development using primary endothelial cells, we employed the CRISPR-Cas9 system to knock out (KO) HMGB1 in immortalized human endothelial cells known as HuARLT cells. Fortunately, we successfully isolated an HMGB1 KO clone from the HuARLT cells, as well as HMGB1 KO HuARLT cells that were infected with KSHV. Using these cell model, our findings revealed that HMGB1 was not essential for the proliferation of uninfected cells. However, in KSHV-infected cells, the absence of HMGB1 significantly inhibited cell proliferation in 2D culture and impaired sphere formation in 3D culture. Further analysis revealed differential expression of various cytokines and growth factors, including those involved in sphere formation and maintenance, in KSHV-infected cells lacking HMGB1. These results suggest that HMGB1 plays a critical role in KS development and that its absence can impair tumor growth and maintenance.

2. Materials and methods

2.1. Cell culture and reagents

iSLK BAC16 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/high glucose (Welgene, Gyeongsan, South Korea) containing 10% fetal bovine serum (FBS; GenDEPOT, Katy, TX, United States) and 1% antibiotic-antimycotic solution (Invitrogen, Waltham, MA, United States). HygromycinB (1.2 mg/mL; Invitrogen), geneticin (250 μg/mL; Invitrogen), and puromycin (1 μg/mL; Invitrogen) were added and the cells were cultured to maintain the latent infection of iSLK BAC16. HuARLT cells (May et al., 2010) were cultured in endothelial cell growth medium 2 (EGM-2, PromoCell, Heidelberg, Germany) supplemented with 2 μg/mL doxycycline (Sigma-Aldrich, St. Louis, MO, United States). All cells were cultured at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2. Virus isolation and infection

The iSLK BAC16 cells harboring recombinant KSHV BAC16 (Brulois et al., 2012) were used to produce virions. iSLK BAC16 cells were treated with 1.2 mM sodium butyrate (Sigma, Burlington, MA, United States) and 50 μg/mL doxycycline (Sigma) for 48 h to induce lytic replication. Upon the induction of lytic replication, DMSO was added to the culture media together with sodium butyrate and doxycycline at 0.1, 0.5%, or 1% of the total volume (Kang et al., 2021b). For virus isolation, the culture medium was collected and centrifuged at 300 × *g* for 10 min at 4°C to remove cell debris from the culture supernatant. The supernatant was centrifuged again at 2,000 × *g* for 10 min at 4°C, and the supernatant was collected. The supernatant was collected and centrifuged at 100,000 × *g* for 1 h at 4°C. The virus pellet was resuspended in cold phosphate-buffered saline (PBS) and stored at −80°C until the viral stock was used. KSHV infection was performed as previously described (Yoo et al., 2008). Briefly, the prepared KSHV stock was added to Gibco Opti-MEM (Invitrogen) containing 5 μg/mL polybrene (Santa Cruz Biotechnology, Santa Cruz, CA, United States). HuARLT cells were seeded onto 6-well culture plates the day before KSHV infection. KSHV infection was performed by centrifugation at 2,600 rpm for 1 h at 25°C. After centrifugation, the medium was changed to endothelial cell growth medium 2 (PromoCell), and the cells were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The KSHV-infected cells were selected with 50 ~ 100 μg/mL of hygromycin B for over 2 weeks.

2.3. Establishment of HMGB1 KO cells using CRISPR/Cas9 system

HMGB-1 KO was performed according to a previously published method that targeted HMGB-1 (Kang et al., 2021a). To establish the

HMGB-1 KO cell line, CRISPR RNA (crRNA) targeting HMGB-1 (catalog number A35509, chr13:30463559–30463537) and tracrRNA were purchased from Thermo Fisher Scientific and annealed according to the manufacturer's instructions. Lipofectamine CRISPRMAX Cas9 transfection reagent (Thermo Fisher Scientific) was used to transfect HuARLT cells with CRISPR RNA and TrueCut Cas9 protein V2. After 3 days, the cells were detached with trypsin–EDTA and counted at the desired concentration. The cells were then seeded into a 96-well culture dish with 0.8 cells per well and 100 μL of medium for cloning. Single clones were analyzed by western blotting and sequencing to confirm HMGB-1 KO. The HMGB-1 KO clone was cultured in a large culture dish and used in subsequent experiments.

2.4. Sequencing to validate KO of HMGB1

To identify the KO cell clones and gene alterations using the CRISPR/Cas9 KO system, sequencing was performed as previously described (Kang et al., 2021a; Lee et al., 2023). DNA was extracted from HMGB-1 KO cells using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, United States), and PCR was performed using a PCR premix (Solgent, Daejeon, South Korea) targeting HMGB-1. The following primers were used: F 5′-GAAAAATAACTAA ACATGGGCAA-3′, and R 5′-GGAGGCCTCTTGGGTGCA-3′. The PCR products were validated by agarose gel electrophoresis and inserted into a vector using a TOPcloner TA kit (Enzynomics, Daejeon, South Korea). The resulting vector was transformed into *Escherichia coli* DH5α (included in the TA kit). Bacteria were cultured on LB plates containing 50 μg/mL ampicillin, and colonies were obtained. Ten colonies were selected, and plasmids were extracted using a Plasmid Mini kit (MGmed, Seoul, South Korea). The plasmids were sequenced by Bionics (Seoul, South Korea) using M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAA CAGCTATGAC-3′) primers.

2.5. Western blotting

Western blot analysis was performed as previously described protocol (Jeon et al., 2021). The primary antibodies used were anti-HMGB-1 (Abcam, Cambridge, MA, United States), anti-β-actin (Sigma-Aldrich), anti-KSHV ORF 50 (Bioss, Woburn, MA, United States), anti-HHV ORF 45 (Thermo Fisher Scientific), and anti-KSHV K8.1 antibodies (Santa Cruz, Santa Cruz, CA, United States). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bethyl; Montgomery, TX, United States) and HRP-conjugated goat anti-rabbit IgG (Bethyl) were used as secondary antibodies. The antibody-reacted membranes were visualized using Amersham ImageQuant 800.

2.6. RNA isolation, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

RNA was extracted using a TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Shiga, Japan) and adjusted to the same concentration per microliter. Reverse transcription was performed using TaKaRa RT Master Mix II (Takara) following the manufacturer's instructions. The resulting complementary DNA was quantified using real-time PCR with TaKaRa SYBR FAST qPCR mix (Takara). qPCR was performed using the following primers: HMGB-1 F, 5′- GAA AAA TAA CTA AAC ATG GGC AA-3′ HMGB-1 R, 5′- CTA AGA AGT GCT CAG AGA-3′, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F, 5′-GGT ATC GTG GAA GGA CTC-3′, GAPDH R, 5′-GTA GAG GCA GGG ATG ATG-3′, ENA-78 F, 5′-CTG CAA GTG TTC GCC ATA GG-3′, ENA-78 R, 5′-GAG GCT ACC ACT TCC ACC TT-3′, PDGFA F, 5′-GTC ATT TAC GAG ATT CCT-3′, PDGFA R, 5′-TAA TTT TGG CTT CTT CCT-3′, G-CSF F, 5′-CAG AGC TTC CTG CTC AAG TG-3′, G-CSF R, 5′-TAG GTG GCA CAC TCA CTC AC-3′, Emmpirin F, 5′-AGG CTG TGA AGT CGT CAG AA-3′, Emmpirin R, 5′-GCC TCC TCA GAG TCA GT-3′, IL-17A F, 5′-TGT GAT CTG GGA GGC AAA GT-3′, IL-17A R, 5′-CCC ACG GAC ACC AGT ATC TT-3′, VEGF F, 5′-ATT ATG CGG ATC AAA CCT-3′, VEGF R, 5′-TTC TTG TCT TGC TCT ATC TT-3′, VEFGA F, 5′-AGG ATG GCT TGA AGA TGT-3′, VEGFA R, 5′-CAC GAAGTG GTG GTG AAG TTC-3′, VEGFC F, 5′-TGT GTC CAG TGT AGA TGA A-3′, VEGFC R, 5′-TCT TCT GTC CTT GAG TTG A-3′ KSHV ORF50 F, 5′-AGA AGG TGA CGG TAT ATC C-3′, KSHV ORF50 R, 5′-CGC TGT TGT CCA GTA TTC-3′, KSHV K8.1 F, 5′-AAC TGA CCG ATG CCT TAA-3′, KSHV K8.1 R, 5′-GCG TCT CTT CCT CTA GTC-3′. The primers used in this study were synthesized by Genotech (Daejeon, South Korea).

2.7. Immunofluorescence assay

Immunofluorescence assay (IFA) was performed as previously described (Lee M. S. et al., 2014; Kang et al., 2021a). The primary antibodies used were anti-HMGB-1 (Abcam), anti-KSHV ORF50 (Bioss), anti-KSHV K8.1 (Santa Cruz), and anti-LNA (Abcam) antibodies. The secondary antibodies used were Alexa Fluor 568-conjugated anti-mouse IgG (Thermo Fisher Scientific) and Alexa Fluor 568-conjugated anti-rabbit IgG (Thermo Fisher Scientific) antibodies. A concentration of 500 ng/mL of 49,6-diamidino-2-phenylindole (DAPI) was used to stain nucleic acids. The stained samples were observed under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under the same conditions.

2.8. Proliferation and cell death assay

To assess cell proliferation, 5000 cells were seeded in each well of a 96-well plate and incubated for 1 day (under specified culture conditions). After incubation, the cells were treated with the WST-1 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. The plate was then incubated for 1 h (under specified culture conditions), after which absorbance was measured at 450 nm using a microplate reader. Three independent experiments were performed, and the data were statistically analyzed. To assess cellular death, 5000 cells were seeded in each well of a 96-well plate and incubated for 1 day (under specified culture conditions). The lactate dehydrogenase (LDH) assay kit (Roche) was used according to the manufacturer's instructions. After the assay, absorbance was measured at 490 nm using a microplate reader. Three independent assays were

performed, and the supernatant LDH levels were normalized to the cellular LDH levels. The data were statistically analyzed.

2.9. Three-dimensional culture of endothelial cell spheroid

Endothelial cells were cultured in a 3-dimensional culture system following a previously published method (Dubich et al., 2021; Lee et al., 2023). Briefly, 4000 endothelial cells were seeded in a 96-well plate coated with 1% agarose (Bio-Rad Laboratories, Hercules, CA, United States) in PBS. The cells were then cultured under adjusted growth conditions with doxycycline and hygromycin B for 2–3 days. The resulting spheroids were harvested from the wells and resuspended in 50 μL of endothelial cell growth media containing 2 μg/mL doxycycline, 0.7 mg/mL of human fibrinogen (Merck, Rahway, NJ, United States), 0.4% methylcellulose (Sigma-Aldrich), and 0.5 U/mL human plasma thrombin (Merck). The resuspended cells were mixed with 50 μL of Matrigel® (BD Biosciences, Franklin Lakes, NJ, United States) and seeded in a 96-well plate. After polymerization, the plate was incubated under optimized cell culture conditions to allow further spheroid growth. To prevent drying, the plate was supplied with a culture medium containing doxycycline.

2.10. Cytokine antibody array

To assess cytokine production, 1×10^6 endothelial cells were seeded onto 100 mm diameter dishes in an endothelial cell growth medium containing 2 μg/mL of doxycycline. After 2 days of culture, the supernatant was harvested and centrifuged at 2000 rpm at 4°C for 10 min to remove cellular debris. The supernatant was analyzed using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, United States), following the manufacturer's instructions. The final membrane was detected using Amersham™ ImageQuant™ 800.

2.11. Statistical analysis

All experiments were performed independently at least three times, and the results are representative data. The mean ± standard deviation values are presented in the graphs. A two-tailed Student's *t*-test was used to compare the data between two different groups. Significant differences are indicated by an asterisk at a p value of less than 0.05 ($^*p < 0.05$; $^{**}p < 0.01$).

3. Results

3.1. Expression of HMGB1 in KSHV-infected human endothelial cells

We investigated whether KSHV infection affected the expression and secretion of HMGB1 in immortalized human endothelial HuARLT cells (Lipps et al., 2017). After infection with recombinant KSHV and KSHV BAC16 (Brulois et al., 2012), we selected KSHV-infected cells and found that HMGB1 expression in the cellular fraction was not significantly altered by KSHV infection (Figure 1A). However, we observed a significant increase in the protein level of HMGB1 in the supernatant of KSHV-infected cells compared with that in mock-infected cells (Figure 1A). This increase was not accompanied by an increase in HMGB1 mRNA expression (Figure 1B). Our results suggest that KSHV infection induces the translocation of HMGB1 from the nucleus into the cytoplasm of infected cells, and that this released HMGB1 may be secreted into the extracellular space. These findings were supported by immunofluorescence assays (IFA), which demonstrated HMGB1 expression in both the nucleus and cytoplasm of KSHV-infected cells but only in the nucleus of mock-infected cells (Figure 1C).

3.2. Establishment of an HMGB1 KO clone in human endothelial cells

To isolate a KO clone of *HMGB1* in HuARLT cells, we utilized a ribonucleoprotein complex composed of the Cas9 protein and *HMGB1*-targeting gRNA. To perform CRISPR/Cas9-mediated knockout, it is necessary to isolate a single cell clone by using limiting dilutions. However, this process can create cellular heterogeneity, which can make it difficult to interpret the results. To avoid this problem, a single clone was isolated from the HuARLT cells before the CRISPR/Cas9-mediated knockout process, and this clone was used for subsequent experiments. After transfection of gRNA and Cas9 protein to HuARLT cells, each clone was separated using a limiting dilution technique. To confirm HMGB1 KO, we employed a PCR-based genotyping method to detect genetic mutations. Subsequently, we cloned the PCR products, including the gRNA sequence, into a T-cloning vector to generate single-copy mutant DNA fragments for sequencing (Figure 2A). We analyzed 10 colonies using conventional sequencing analysis and found that all colonies exhibited the same mutation with an additional 'A' insertion in the Cas9 targeting site, indicating that both alleles might harbor the same mutation. We cannot exclude the possibility that only one allele was detected in the analysis using only 10 colonies. Intriguingly, the mutation induced by the gRNA sequence was the same as that in a previous application of iSLK BAC16 (ref), suggesting that the gRNA sequence might preferentially induce a mutation at a specific site. Furthermore, we analyzed HMGB1 expression in wild tyep (WT) and KO HuARLT clones using western blotting and IFA (Figures 2B,C). The cellular morphology and proliferation of KO clones were not significantly affected compared to those of WT cells, despite the HMGB1 ablation (Figures 2D,E).

3.3. KSHV gene expressions in HMGB1 KO human endothelial cells

We infected an HMGB1 KO HuARLT clone with KSHV and investigated the expression of KSHV viral genes, including ORF50, K8.1, and ORF73 (Figure 3A). In the immunofluorescence assay (IFA), we found that while the latent gene ORF73 was detected in all KSHV-infected WT cells, the early lytic gene (ORF50) and late lytic gene (K8.1) were not detected, which is consistent with previous studies (Lee et al., 2023). In KSHV-infected HMGB1 KO cells, we did not observe any alteration in the viral gene expression of KSHV compared to that

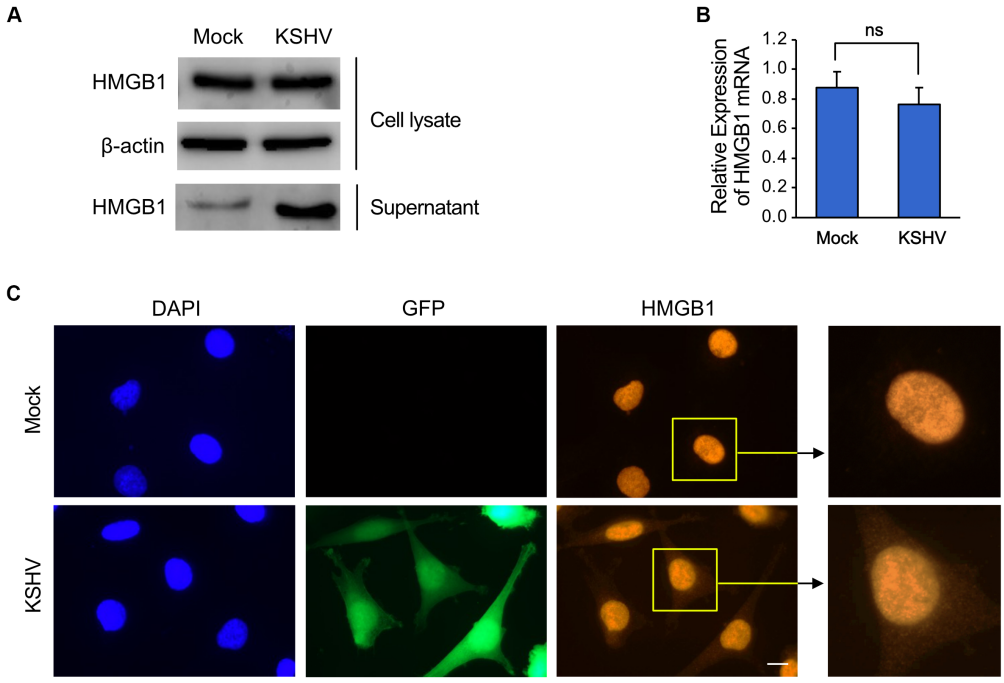

FIGURE 1
The expression of HMGB1 in KSHV-infected human endothelial cells. The immortalized human endothelial HuARLT cells were infected with KSHV, and the infected cells were isolated using hygromycin. The expression of HMGB1 in the KSHV-infected cells was compared with that of the mock-infected cells. **(A)** Western blot analysis for HMGB1 in the cell lysates and supernatant from the KSHV-and mock-infected cells. A representative blot is presented. β-actin was used as a house-keeping protein for normalization. **(B)** Densitometry analysis for the western blot results of **(A)**. Data are shown as the mean ± SD, *n* = 3, ns, not significant. **(C)** Immunofluorescence assay for HMGB1 in mock-and KSHV-infected HuARLT cells. KSHV BAC16 induces green fluorescence protein (GFP) in virus-infected cells. Scale bar, 10 μm.

in KSHV-infected WT cells, indicating that both WT and HMGB1 KO cells showed latent infection in KSHV-infected cells. We obtained consistent results from western blotting and mRNA expression analyses by RT-qPCR, which supported the results of IFA (Figures 3B,C).

3.4. Cell viability of KSHV-infected WT and HMGB1 KO HuARLT cells

The viability of KSHV-infected HMGB1 KO HuARLT cells was compared to that of KSHV-infected WT cells using the WST-1 cell viability assay (Figure 4A). While there was no significant difference in viability between mock-infected WT and HMGB1 KO cells, HMGB1 KO significantly affected the viability of KSHV-infected cells. HMGB1 KO resulted in lower viability of KSHV-infected cells than that of WT cells. The LDH assay was used to determine whether decreased cell viability was caused by increased cell death in HMGB1 KO cells compared to WT cells (Figure 4B). However, cell death was not significantly increased in HMGB1 KO cells following KSHV infection, indicating that the lower cell viability of KSHV-infected HMGB1 KO cells might be the result of a decrease in the cell proliferation rate. KSHV-infected HuARLT cells are known to induce sphere formation in 3D culture (ref). To investigate the effect of HMGB1 KO on sphere formation and maintenance, we induced the formation of spheres with mock-infected WT, mock-infected HMGB1 KO, KSHV-infected WT, and KSHV-infected HMGB1 KO cells (Figure 4C). KSHV-infected WT cells formed larger and more compact spheres than the mock-infected WT cells. Although HMGB1 KO did not significantly affect sphere formation in mock-infected cells, KSHV-infected HMGB1 KO cells showed smaller and less compact spheres than KSHV-infected WT cells. These results indicate that HMGB1 plays a vital role in sphere formation in 3D cultures and may be associated with tumorigenesis.

3.5. Cytokine, chemokine, and growth factor expression profile in WT and HMGB1 KO HuARLT cells infected with KSHV

To investigate the changes in cytokine, chemokine, and growth factor expression in KSHV-infected HuARLT cells with HMGB1 KO, conditioned media were collected from each cell group and applied to a human cytokine antibody array. The array showed that some cytokines, chemokines, and growth factors were upregulated, whereas others were downregulated in HMGB1 KO cells (Figure 5A). This study focused on the downregulation of cytokines, chemokines, and growth factors by HMGB1 KO because cell proliferation was suppressed by HMGB1 KO (Figure 4). The cytokine array showed that CXCL5, PDGF-AA, G-CSF, Emmprin, IL-17A, and VEGF levels were significantly decreased in HMGB1 KO cells compared to those in WT cells (Figure 5B). For these poorly expressed factors in HMBG1 KO cells, mRNA expression analysis confirmed that CXCL5, PDGF-A, and VEGF levels were significantly decreased in HMGB1 KO cells compared to those in WT cells (Figure 5C). However, G-CSF, Emmprin, and IL-17A showed increased mRNA expression or no significant differences between KSHV-infected WT and HMGB1 KO cells. To examine the potential connection between certain proteins and a signaling pathway, HMGB1-related

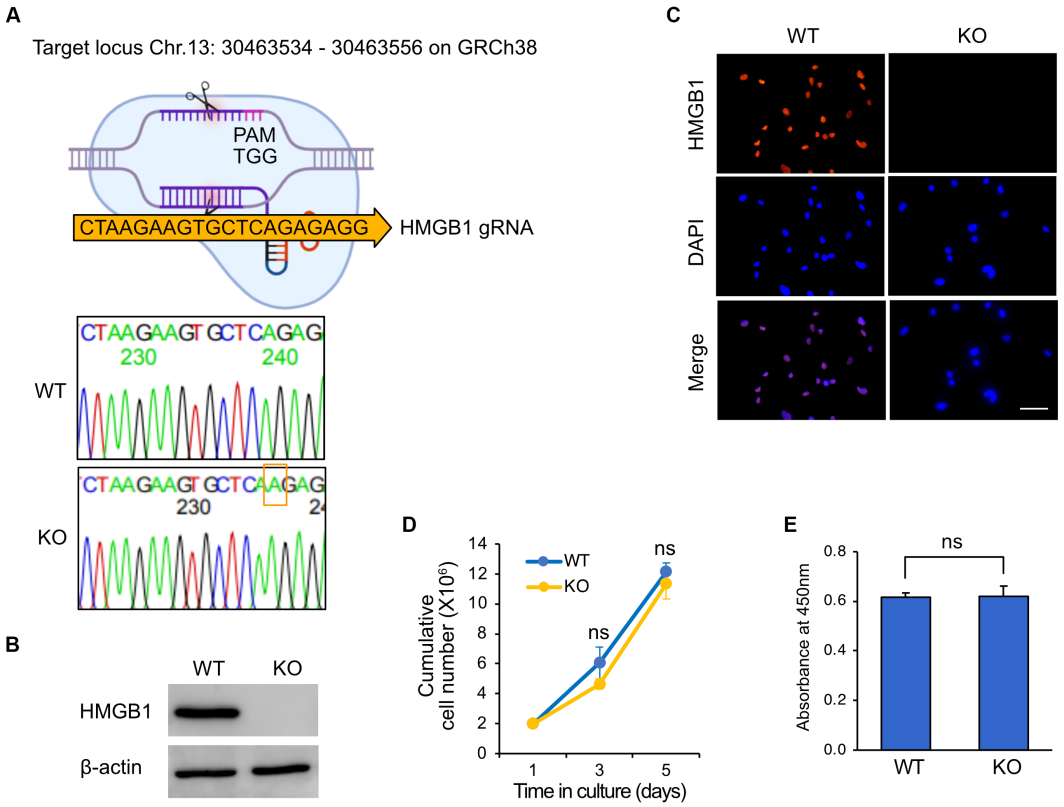

FIGURE 2
Knockout of HMGB1 in HuARLT cells. HuARLT cells were treated against HMGB1 using the CRISPR/Cas 9 system and gRNA against HMGB1. The isolated clone with a knockout (KO) of HMGB1 sequence, HMGB1 protein expression, and cell proliferation. **(A)** Schematic diagram showing CRISPR/Cas9 system with Cas9 targeting site of HMGB1. The PCR products containing gRNA targeting sequences from wild-type (WT) and KO clones were cloned into a T-vector, and the sequences were analyzed by conventional sequencing from 10 colonies. The nucleotide inserted into the CRISPR/Cas9 target site of HMGB1 in the KO clone is indicated by a red box. **(B)** Western blotting for HMGB1 expression in WT and HMGB1 KO cells. **(C)** Immunofluorescence assay for HMGB1 expression. Scale bar, 50 μm. **(D,E)** Proliferation of WT and HMGB1 KO iSLK BAC16 cells. At each time point, live cells were analyzed by trypan blue exclusion **(D)** and WST-1 viability testing **(E)**. Statistical significance of differences is indicated. ns, not significant, Student's *t*-test.

signaling pathways were analyzed by western blotting (Figures 6A,B). When HMGB1 was knocked out, we observed a significant reduction in p38 phosphorylation, suggesting a possible association between p38 and HMGB1 in KSHV-infected endothelial cells. In a previous study using iSLK BAC16 cells, we showed that the phosphorylation of JNK plays a critical role in KSHV replication regulated by HMGB1 (Kang et al., 2021a). However, in endothelial cells, although HMGB1 influenced the overall expression of JNK, we did not observe a suppression of the JNK phosphorylation by knocking out HMGB1. To investigate further, we employed chemical inhibitor targeting the p38 pathway (Figure 6C). By using SB203500, a p38 inhibitor, we examined the relationship between CXCL5, PDGF-A, VEGF-A, and this specific pathway in KSHV-infected cells (Figure 6C), and we found that it affected the mRNA expression of CXCL5, PDGF-A, and VEGF-A.

3.6. Glycyrrhizin inhibited the proliferation and maintenance of the 3D culture sphere in KSHV-infected endothelial cells mediated by HMGB1

Glycyrrhizin is an extracellular HMGB1 inhibitor. When tested on mock-infected cells, no significant difference in cell viability was observed after the addition of glycyrrhizin. However, glycyrrhizin treatment significantly reduced the viability of the KSHV-infected HuARLT cells (Figure 7A). The LDH assay did not show any significant effects of glycyrrhizin on either mock-or KSHV-infected cells (Figure 7B), indicating that the decreased cell proliferation caused by glycyrrhizin was responsible for the suppressed viability of KSHV-infected cells. In 3D cultures, sphere formation was not significantly affected by glycyrrhizin in either mock-or KSHV-infected cells. However, treatment with glycyrrhizin led to an increase in dead cells and debris around KSHV-infected cells that did not express green fluorescence protein (GFP) (Figure 7C). These results suggested that HMGB1 may have different functions in 2D and 3D cultures.

4. Discussion

In our previous study, we established HMGB1 KO in the KSHV-producing cell line iSLK BAC16, in which HMGB1 KO decreased virion production by decreasing the expression of viral genes (Kang et al., 2021a). Several cellular viral proteins interacted with intracellular HMGB1 in the nucleosomal complex, and extracellular HMGB1 induced JNK phosphorylation to enhance the lytic replication of KSHV. Therefore, we demonstrated that HMGB1

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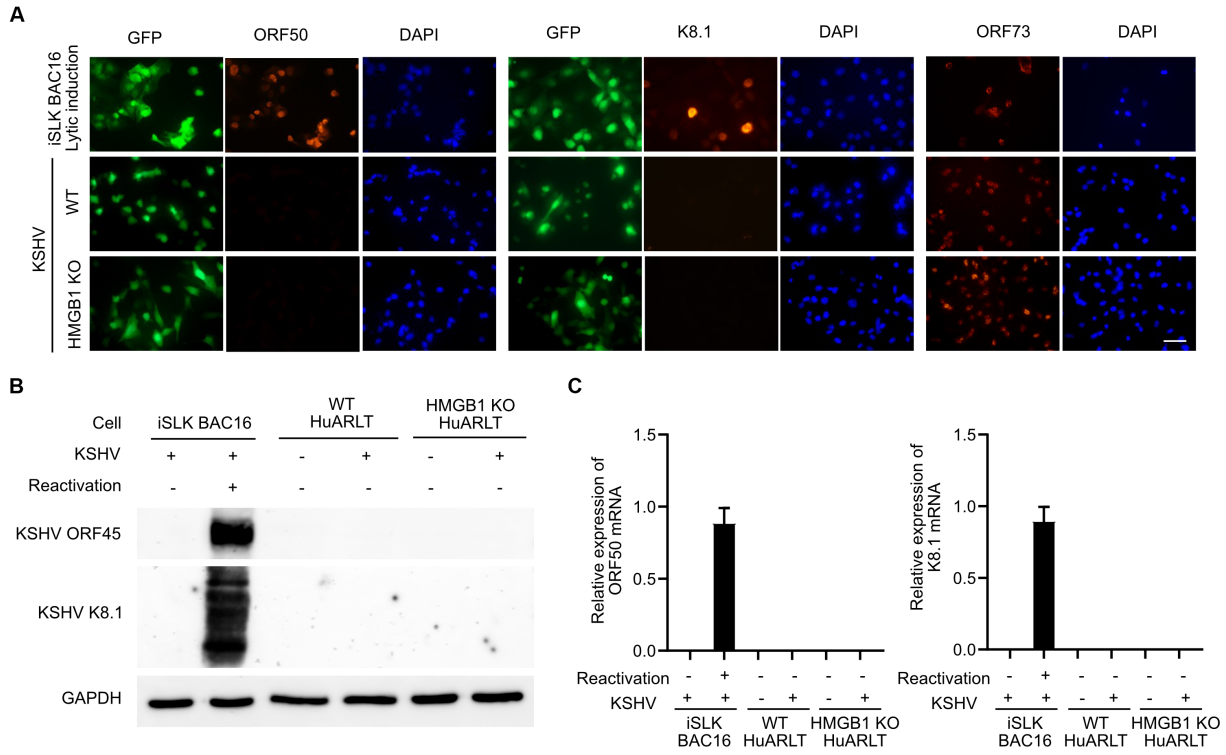

FIGURE 3
The expression of KSHV genes in WT and HMGB1 KO HuARLT cells. **(A)** Immunofluorescence assay (IFA) for KSHV ORF50, K8.1, and ORF73. iSLK BAC16 cells with reactivation were used as a positive control. Scale bar, 50 μm. **(B)** Western blot analysis for KSHV viral proteins. **(C)** Quantitative RT-PCR for KSHV mRNA expression. Data are shown as the mean ± SD, $n = 3$.

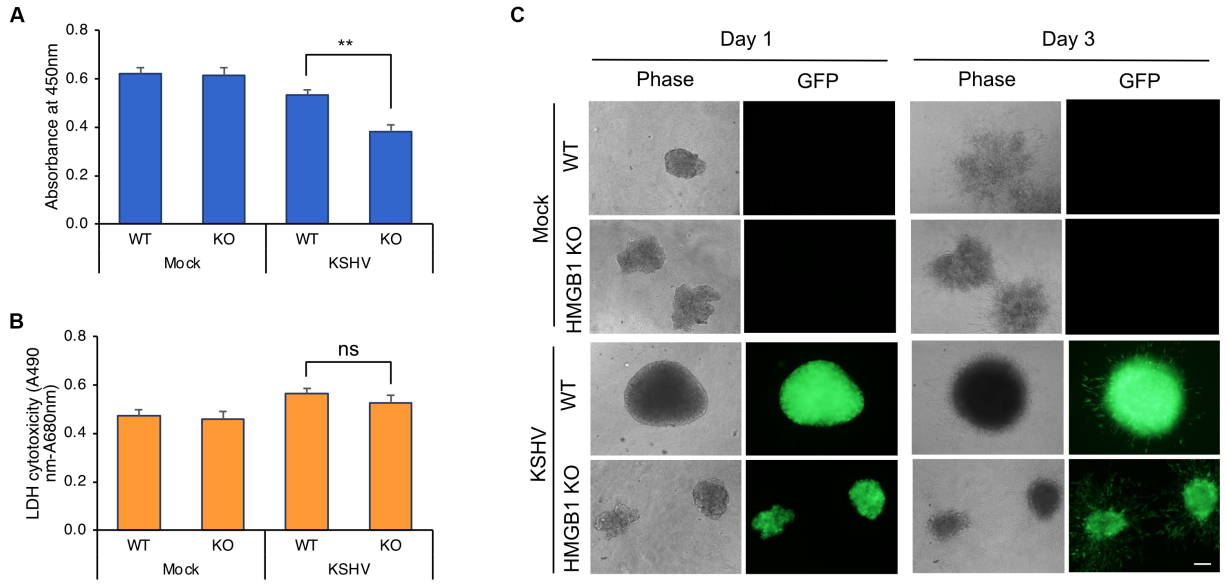

FIGURE 4
Cell viability and 3D culture of KSHV-infected HMGB1 KO HuARLT cells. **(A)** WST-1 cell viability assay. WT, wild-type HuARLT cells; KO, HMGB1 KO HuARLT cells; Mock, mock-infected cells; KSHV, KSHV-infected cells. $^{**}p < 0.01$. **(B)** LDH assay. ns, not significant. **(C)** Sphere formation of cells in 3D culture. Day 1 and 3: the observation time after seeding the sphere on 3D culture. Scale bar, 100 μm.

plays a crucial role in the generation of infectious KSHV progeny during lytic replication. Since HMGB1 is closely associated with cancer development, we investigated the role of HMGB1 in the pathogenesis of Kaposi's sarcoma (KS). While iSLK BAC16 is useful for producing recombinant KSHV, this cell line is not a suitable model for investigating KS development. Therefore, we established

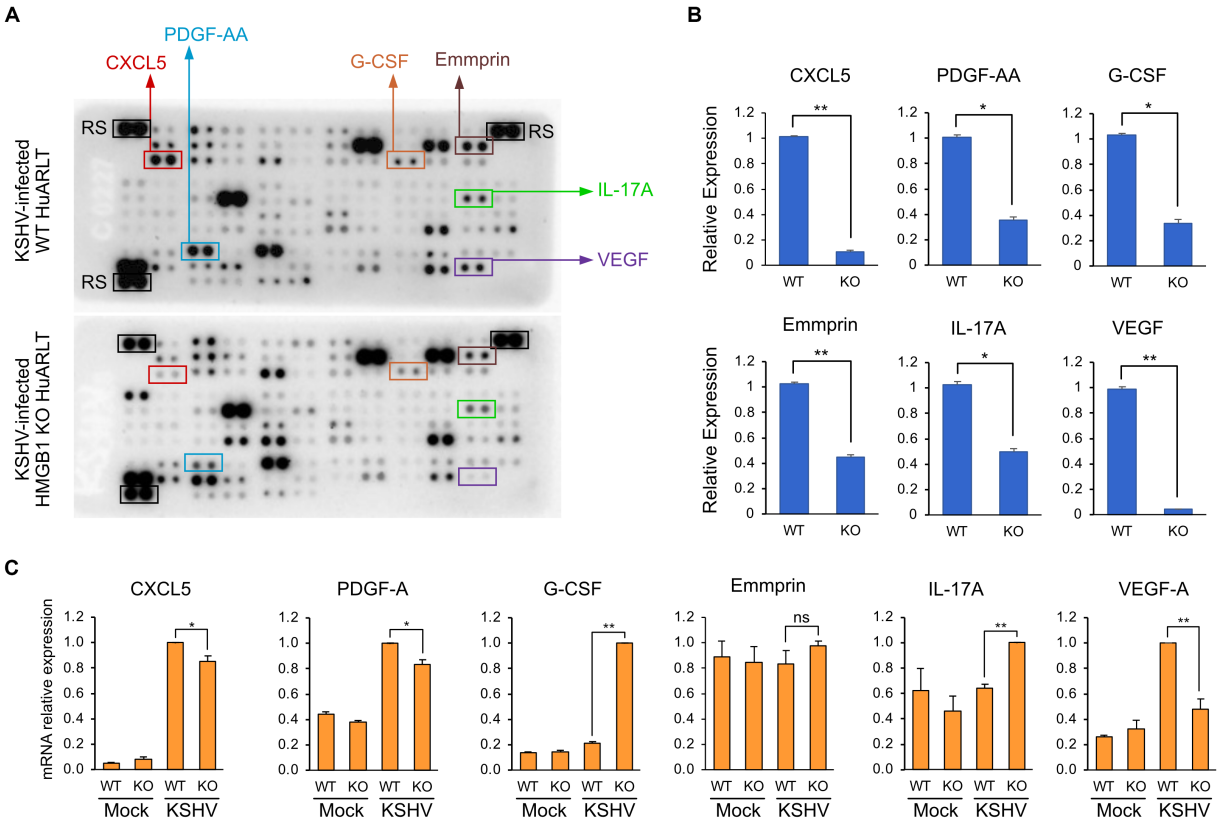

FIGURE 5
Cytokine expression levels in KSHV-infected HuARLT cells with HMGB1 KO. **(A)** Cytokine array. The conditioned media from KSHV-infected WT or HMGB1 KO cells were used for the cytokine array. The decreased proteins in HMGB1 KO cells were indicated as boxes. RS, reference spots as the experimental control. **(B)** Densitometry analysis for the selected proteins in cytokine array of **(A)**. Data are shown as the mean ± SD, $n = 2$, $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** Quantitative RT-PCR for the selected cytokines or growth factors. Data are shown as the mean ± SD, $n = 3$, $^{**}p < 0.01$.

an HMGB1 KO cell line using immortalized human endothelial HuARLT cells.

HuARLT cells derived from HUVEC show tightly controlled proliferation with relevant phenotypic and molecular characteristics of endothelial cells via doxycycline-dependent regulation of two independent immortalizing genes (May et al., 2010). Because this cell line showed a KS-like phenotype with KSHV, it is a suitable model to investigate the pathogenesis of KSHV and a novel drug for the treatment of KS. Furthermore, we demonstrated that this cell line is useful for studying KSHV-host cell interactions by knocking out a target gene using the CRISPR/Cas9 system (Lee et al., 2023).

Many studies have been conducted on the role of HMGB1 in the regulation of cytokine, chemokine, and growth factor production. A previous study demonstrated that the inhibition of HMGB1 using glycyrrhizin led to a reduction in the expression of CCL2 and CXCL5, along with their respective receptors, CCR2 and CXCR2 (Wang et al., 2022). An association between HMGB1 and G-CSF has also been reported in previous studies (Yuan et al., 2020). HMGB1 administration restores blood flow recovery and capillary density by increasing VEGF expression in the ischemic muscles of diabetic mice (Biscetti et al., 2010). Consistently, we showed that HMGB1 KO suppressed CXCL5, G-CSF, and VEGF expression in the KSHV-infected cells. Additionally, we also found that PDGF-AA, Emmprin, IL-17A was suppressed by KO of HMGB1 in KSHV-infected endothelial cells. However, the protein expression and mRNA expression levels of these proteins did not display entirely consistent outcomes. The inconsistent results between protein and mRNA expression levels could be due to post-translational modifications or because mRNA expression was only analyzed at a specific time point in our experiments. Nevertheless, because the suppressed cytokines and growth factors in the cytokine array can promote cell proliferation, it is likely that multiple factors work in combination rather than as a single factor to mediate HMGB1-induced cell proliferation in KSHV-infected cells. Although the precise underlying mechanisms by which HMGB1 regulates these cytokines, chemokines, and growth factors are not fully understood, our findings provide insights into the role of HMGB1 in regulating cytokines, chemokines, and growth factors, suggesting that targeting HMGB1 could be a potential strategy for treating KS.

In this study, we found that six cytokines were downregulated in KSHV-infected cells following HMGB1 KO; however, their mRNA expression levels were not consistent with the results of the cytokine array. One possible reason for the lack of a correlation between mRNA expression and cytokine production in KSHV-infected cells is post-transcriptional regulation. Post-transcriptional regulation is a complex process involving various regulatory mechanisms, including microRNA-mediated regulation, which can modulate mRNA stability, translation, and protein synthesis, leading to discrepancies between mRNA expression and protein production. Another possible explanation is the involvement of other regulatory factors. HMGB1 is

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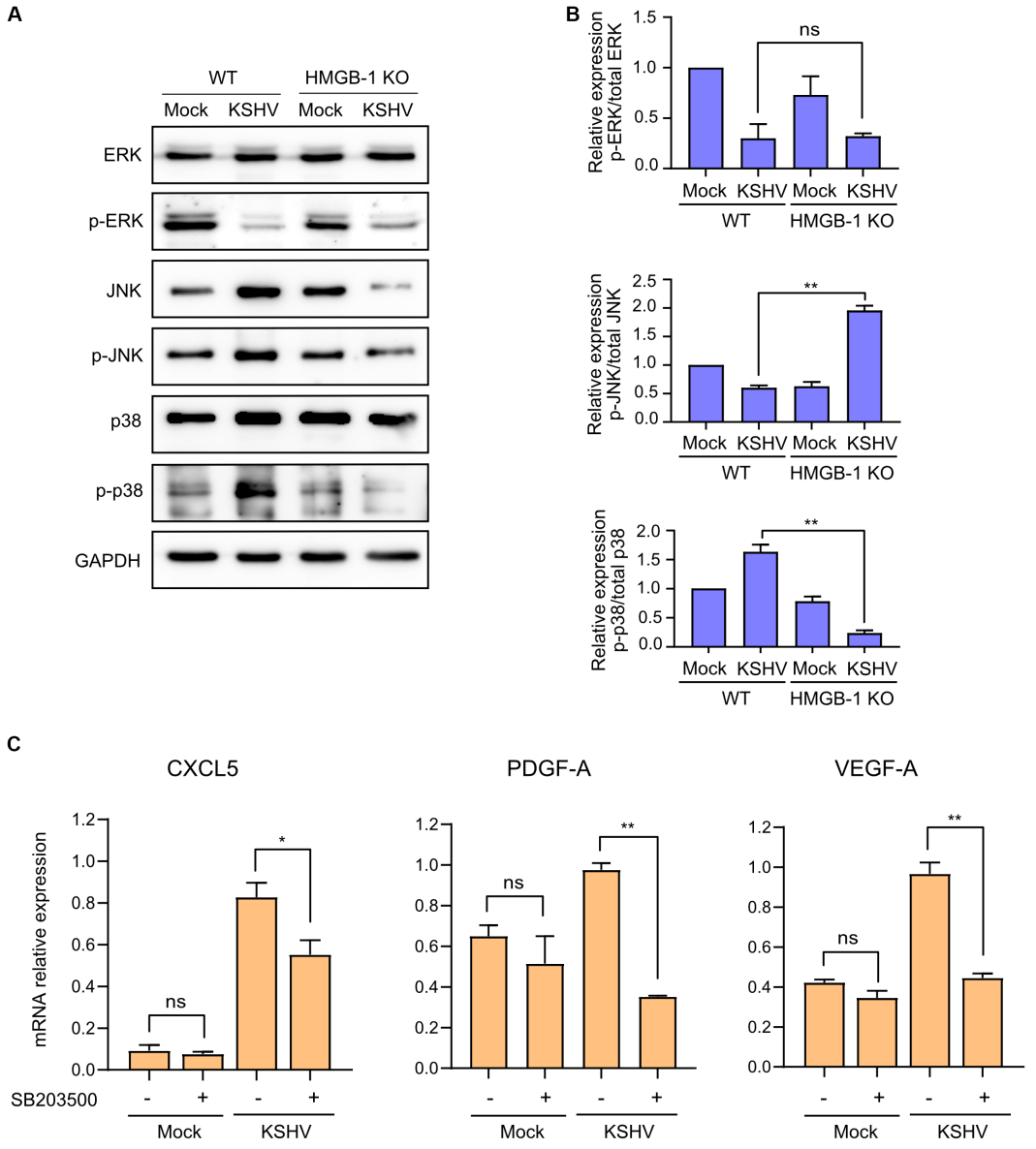

FIGURE 6
Signaling pathways related to KSHV-infected HMGB1 KO HuARLT cells. **(A)** Western blot analysis for HMGB1-associated signaling pathways. GAPDH was used as a housekeeping protein. **(B)** Densitometry analysis for western blotting results. Data are shown as the mean ± SD, $n = 2$, ns, not significant, $^{**}p < 0.01$. **(C)** Relative expression of indicated mRNAs with chemical inhibitors for p38 pathways. SB203500 as a p38 inhibitor (2 μM) were treated in each cell for 24 h. DMSO treatment was used as a negative control. Each mRNA expression was analyzed by quantitative RT-PCR. Data are shown as the mean ± SD, $n = 3$, ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

a multifunctional protein that interacts with various proteins and regulatory factors such as transcription factors, epigenetic modifiers, and signaling molecules. The downregulation of cytokine production in KSHV-infected cells after HMGB1 KO may be due to the disruption of the interaction between HMGB1 and other regulatory factors rather than the direct effect of HMGB1 on cytokine transcription. Although we showed that p38 pathway activated by HMGB1 was associated with VEGF expression, further studies are required to elucidate the exact underlying mechanisms.

This study has a specific limitation regarding its investigation of the role of HMGB1, as it was only conducted *in vitro*. HMGB1 is known to have diverse functions in both inflammation and the development of cancer. Therefore, it is important to recognize that the role of secreted HMGB1 may be more intricate within the microenvironment. To fully understand the precise impact of HMGB1 on KS development, further research using appropriate animal models specifically designed for KS would be necessary. Conducting such studies would provide more detailed insights into the exact contribution of HMGB1 to the development of KS and its implications in a living organism.

In summary, this study investigated the role of HMGB1 in KS, a cancer caused by KSHV infection. The results demonstrated that KSHV infection stimulated the secretion of HMGB1 in human endothelial cells, and that HMGB1 plays a critical role in KS pathogenesis by promoting cytokine and growth factor secretion and facilitating cellular sphere maintenance, suggesting that targeting HMGB1 could be a potential therapeutic strategy for treating KS.

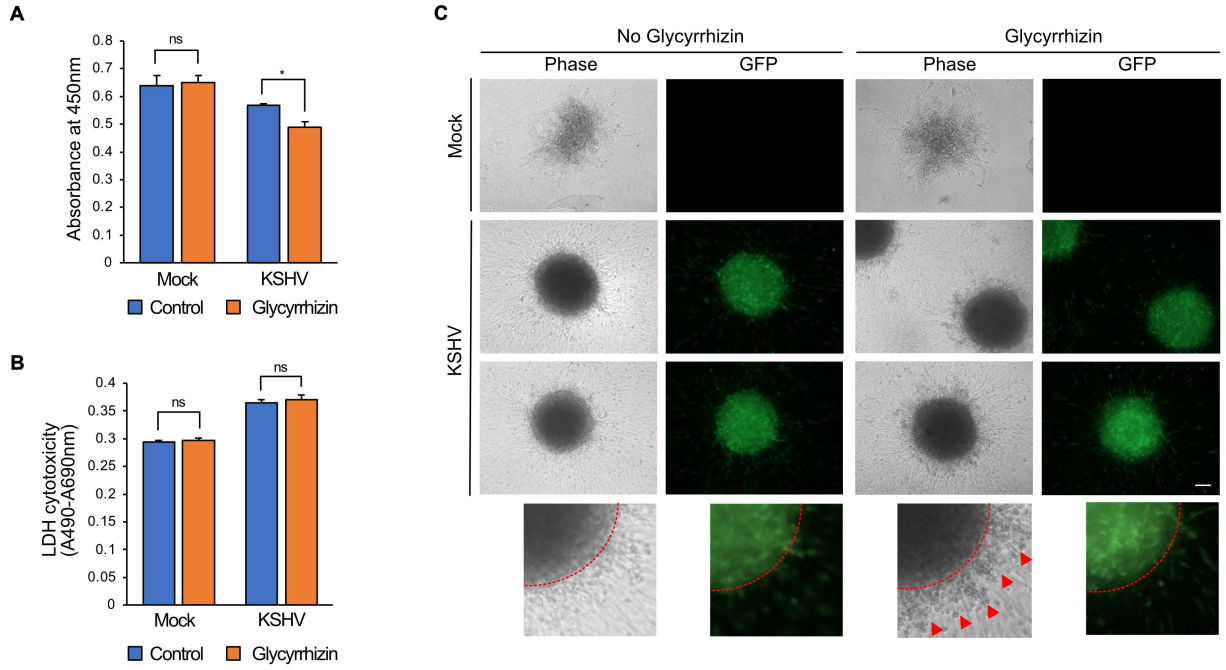

FIGURE 7
The effects of HMGB1 inhibitor, glycyrrhizin, on KSHV-infected HuARLT cells. **(A)** WST-1 cell viability assay for KSHV-infected HuARLT cells with or without glycyrrhizin. Data are shown as the mean ± SD, $n = 3$, ns, not significant, $*p < 0.05$. **(B)** LDH assay for KSHV-infected HuARLT cells with or without glycyrrhizin. Data are shown as the mean ± SD, $n = 3$, ns, not significant. **(C)** 3D culture sphere of mock-or KSHV-infected cells treated with glycyrrhizin. KSHV-infected cells expressed GFP. Red dotted line: the margin of the 3D culture sphere. Red arrows: dead cells or debris. Scale bar, 100 μm.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

M-JL and HK designed the study. M-JL, JP, and SC performed the experiments. M-JL, S-MY, and CP analyzed the data. M-JL, JP, HK, and M-SL wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Biscetti, F., Straface, G., De Cristofaro, R., Lancellotti, S., Rizzo, P., Arena, V., et al. (2010). High-mobility group box-1 protein promotes angiogenesis after peripheral ischemia in diabetic mice through a VEGF-dependent mechanism. *Diabetes* 59, 1496–1505. doi: 10.2337/db09-1507

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., et al. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nat. Med.* 1, 1274–1278. doi: 10.1038/nm1295-1274

Brulois, K. F., Chang, H., Lee, A. S.-Y., Ensser, A., Wong, L.-Y., Toth, Z., et al. (2012). Construction and manipulation of a new Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. *J. Virol.* 86, 9708–9720. doi: 10.1128/JVI.01019-12

Dubich, T., Dittrich, A., Bousset, K., Geffers, R., Büsche, G., Köster, M., et al. (2021). 3D culture conditions support Kaposi's sarcoma herpesvirus (KSHV) maintenance and viral spread in endothelial cells. *J. Mol. Med. (Berl)* 99, 425–438. doi: 10.1007/s00109-020-02020-8

Ganem, D. (2010). KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. *J. Clin. Invest.* 120, 939–949. doi: 10.1172/JCI40567

Harrison, S. M., and Whitehouse, A. (2008). Kaposi's sarcoma-associated herpesvirus (KSHV) Rta and cellular HMGB1 proteins synergistically transactivate the KSHV ORF50 promoter. *FEBS Lett.* 582, 3080–3084. doi: 10.1016/j.febslet.2008.07.055

Hubert, P., Roncarati, P., Demoulin, S., Pilard, C., Ancion, M., Reynders, C., et al. (2021). Extracellular HMGB1 blockade inhibits tumor growth through profoundly remodeling immune microenvironment and enhances checkpoint inhibitor-based immunotherapy. *J. Immunother. Cancer* 9:e001966. doi: 10.1136/jitc-2020-001966

Jeon, H., Kang, S. K., Lee, M. J., Park, C., Yoo, S. M., Kang, Y. H., et al. (2021). Rab27b regulates extracellular vesicle production in cells infected with Kaposi's sarcoma-associated herpesvirus to promote cell survival and persistent infection. *J. Microbiol.* 59, 522–529. doi: 10.1007/s12275-021-1108-6

Jeon, H., Lee, J., Lee, S., Kang, S. K., Park, S. J., Yoo, S. M., et al. (2019). Extracellular vesicles from KSHV-infected cells stimulate antiviral immune response through mitochondrial DNA. *Front. Immunol.* 10:876. doi: 10.3389/fimmu.2019.00876

Kang, S. K., Kang, Y. H., Yoo, S. M., Park, C., Kim, H. S., and Lee, M. S. (2021a). HMGB1 knockout decreases Kaposi's sarcoma-associated herpesvirus virion production in iSLK BAC16 cells by attenuating viral gene expression. *J. Virol.* 95:e0079921. doi: 10.1128/JVI.00799-21

Kang, S. K., Lee, M. J., Ryu, H. H., Lee, J., and Lee, M. S. (2021b). Dimethyl sulfoxide enhances Kaposi's sarcoma-associated herpesvirus production during lytic replication. *Front. Microbiol.* 12:778525. doi: 10.3389/fmicb.2021.778525

Lee, M. S., Jones, T., Song, D. Y., Jang, J. H., Jung, J. U., and Gao, S. J. (2014). Exploitation of the complement system by oncogenic Kaposi's sarcoma-associated herpesvirus for cell survival and persistent infection. *PLoS Pathog.* 10:e1004412. doi: 10.1371/journal.ppat.1004412

Lee, S. A., Kwak, M. S., Kim, S., and Shin, J. S. (2014). The role of high mobility group box 1 in innate immunity. *Yonsei Med. J.* 55, 1165–1176. doi: 10.3349/ymj.2014.55.5.1165

Lee, M. J., Lee, J., Kang, S. K., Wirth, D., Yoo, S. M., Park, C., et al. (2023). CXCL1 confers a survival advantage in Kaposi's sarcoma-associated herpesvirus-infected human endothelial cells through STAT3 phosphorylation. *J. Med. Virol.* 95:28020. doi: 10.1002/jmv.28020

Li, G., Liang, X., and Lotze, M. T. (2013). HMGB1: the central cytokine for all lymphoid cells. *Front. Immunol.* 4:68. doi: 10.3389/fimmu.2013.00068

Lipps, C., Badar, M., Butueva, M., Dubich, T., Singh, V. V., Rau, S., et al. (2017). Proliferation status defines functional properties of endothelial cells. *Cell. Mol. Life Sci.* 74, 1319–1333. doi: 10.1007/s00018-016-2417-5

Martinotti, S., Patrone, M., and Ranzato, E. (2015). Emerging roles for HMGB1 protein in immunity, inflammation, and cancer. *Immunotargets Ther.* 4, 101–109. doi: 10.2147/ITT.S58064

May, T., Butueva, M., Bantner, S., Markusic, D., Seppen, J., MacLeod, R. A., et al. (2010). Synthetic gene regulation circuits for control of cell expansion. *Tissue Eng. Part A* 16, 441–452. doi: 10.1089/ten.TEA.2009.0184

Song, M. J., Hwang, S., Wong, W., Round, J., Martinez-Guzman, D., Turpaz, Y., et al. (2004). The DNA architectural protein HMGB1 facilitates RTA-mediated viral gene expression in gamma-2 herpesviruses. *J. Virol.* 78, 12940–12950. doi: 10.1128/jvi.78.23.12940-12950.2004

Tripathi, A., Shrinet, K., and Kumar, A. (2019). HMGB1 protein as a novel target for cancer. *Toxicol. Rep.* 6, 253–261. doi: 10.1016/j.toxrep.2019.03.002

Wang, P., Hao, P., Chen, X., Li, L., Zhou, Y., Zhang, X., et al. (2022). Targeting HMGB1-NFkappab Axis and miR-21 by glycyrrhizin: role in amelioration of corneal injury in a mouse model of alkali burn. *Front. Pharmacol.* 13:841267. doi: 10.3389/fphar.2022.841267

Wang, S., and Zhang, Y. (2020). HMGB1 in inflammation and cancer. *J. Hematol. Oncol.* 13:116. doi: 10.1186/s13045-020-00950-x

Watanabe, T., Sugimoto, A., Hosokawa, K., and Fujimuro, M. (2018). Signal transduction pathways associated with KSHV-related tumors. *Adv. Exp. Med. Biol.* 1045, 321–355. doi: 10.1007/978-981-10-7230-7_15

Xu, M., Zhou, G. M., Wang, L. H., Zhu, L., Liu, J. M., Wang, X. D., et al. (2016). Inhibiting high-mobility group box 1 (HMGB1) attenuates inflammatory cytokine expression and neurological deficit in ischemic brain injury following cardiac arrest in rats. *Inflammation* 39, 1594–1602. doi: 10.1007/s10753-016-0395-2

Yang, H., Wang, H., and Andersson, U. (2020). Targeting inflammation driven by HMGB1. *Front. Immunol.* 11:484. doi: 10.3389/fimmu.2020.00484

Yoo, S. M., Ahn, A. K., Seo, T., Hong, H. B., Chung, M. A., Jung, S. D., et al. (2008). Centrifugal enhancement of Kaposi's sarcoma-associated virus infection of human endothelial cells in vitro. *J. Virol. Methods* 154, 160–166. doi: 10.1016/j.jviromet.2008.07.026

Yuan, S., Liu, Z., Xu, Z., Liu, J., and Zhang, J. (2020). High mobility group box 1 (HMGB1): a pivotal regulator of hematopoietic malignancies. *J. Hematol. Oncol.* 13:91. doi: 10.1186/s13045-020-00920-3

Zhang, T., Guan, X. W., Gribben, J. G., Liu, F. T., and Jia, L. (2019). Blockade of HMGB1 signaling pathway by ethyl pyruvate inhibits tumor growth in diffuse large B-cell lymphoma. *Cell Death Dis.* 10:330. doi: 10.1038/s41419-019-1563-8

Zhang, Z., Wang, M., Zhou, L., Feng, X., Cheng, J., Yu, Y., et al. (2015). Increased HMGB1 and cleaved caspase-3 stimulate the proliferation of tumor cells and are correlated with the poor prognosis in colorectal cancer. *J. Exp. Clin. Cancer Res.* 34:51. doi: 10.1186/s13046-015-0166-1

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HPV infection and breast cancer risk: insights from a nationwide population study in Taiwan

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Background: The prevalence of cancer, specifically breast cancer, has raised globally. The etiology of breast cancer has been attributed to age, genetic mutations, reproductive history, hormone therapy, lifestyle factors, and viral infections. The human papillomavirus (HPV) has been one of the most widespread sexually transmitted infection in the United States. The role of HPV in breast oncogenesis was hypothesized before, yet the association remained unclear.

Methods: In this study, we employed a nationwide population study using centralized patient data managed by the Ministry of Health and Welfare in Taiwan and the Taiwan Cancer Registry database. The breast cancer incidence rates of the 467,454 HPV patients were compared to twice as many non-HPV patients with matching sex and age. Cumulative breast cancer incidence rates were presented by a Kaplan-Meier curve, and the relative risk of breast cancer for HPV and non-HPV patients were calculated using Cox-regression model.

Results: Our results indicated a crude hazard ratio (HR) and an adjusted hazard ratio (aHR) of 2.336 and 2.271, respectively, when comparing the risk of breast cancer in the HPV and non-HPV group. The risk of breast cancer was comparable or higher than those of head and neck cancer (aHR=1.595) and cervical cancer (aHR=2.225), which both were found to have causal relationships with HPV. The Kaplan-Meier curve further illustrated a higher cumulative risk across 84 months for HPV patients (p<.0001). Besides HPV, age (p<.0001), insurance providers (p<.001), and comorbidities such as abnormal liver function (aHR=1.191, p=.0069) and hyperlipidemia (aHR=1.218, p=.0002) were found to be correlated with higher risks of breast cancer.

Conclusion: A correlation between HPV and breast cancer can be inferred using national health databases. More molecular studies are required to understand the mechanism of the virus-induced oncogenesis of the breast.

KEYWORDS

breast cancer, cancer risks, human papillomavirus - HPV, population-based study, real-world data (RWD)

1 Introduction

Cancer is the leading cause of death and morbidity across the globe. In 2020, approximately 19.3 million new cancer cases were registered worldwide (1), with 122 thousand cases registered in Taiwan, equivalent to 311.34 per 10^6 person (2). In both populations, the most prevalence cancer for female patients was breast cancer, accounting for 11.7% globally and 12.5% in Taiwan of all reported cancer incidents (1, 2). Globally, around 2.26 million female patients were diagnosed of breast cancer, and approximately 685 thousand cases were fatal in 2020 (1). In Taiwan, about 15.3 thousand females were diagnosed with breast cancer, and around 2.66 thousand cases were fatal (2). Notably, elevated risk factors of breast cancer were reported in patients with lower educational attainment and among racial minorities (3). This finding highlights the need to promote accessible screening services and cancer awareness among individuals in lower socioeconomic statuses.

Factors contributing to the etiology of breast cancer include age (4), genetic mutations (5), reproductive history (6), hormone therapy (7), and lifestyle (8). The role of viral infection in oncogenesis has been examined, and 2.2 million new cancer incidents across the world were related to infection in 2018 (9). Some viruses such as hepatitis B virus and hepatitis C virus can cause chronic inflammation which further cell damages and raise the risks of carcinogenesis (10). Others such as Epstein-Barr virus (EBV) were found to upregulate oncogenes and accelerate cell cycles, leading to rapid cell division and cancer development (11). In addition, EBV was also reported to downregulate the tumor suppressor gene through epigenetic, post-transcriptional, and post-translational modifications (12). Importantly, viruses like human papillomavirus (HPV) can integrate their viral DNA to the host genome, resulting in the dysregulation of cellular growth and the subsequent tumorigenesis (13).

HPV is a sexually transmitted virus characterized as developing genital warts on patients. It is the most prevalent sexually transmitted infection in the world, with a global estimation of 1 in 10 women being HPV carriers at any time (14). The oncogenic property of HPV was prominently shown as the leading cause of cervical cancer, reflected by the HPV vaccination campaigns in multiple countries' attempts to eliminate cervical cancer in patients across sexual orientations (15, 16). HPV was also shown to cause a proportion of anal (17) and oropharyngeal cancer (18), and served as a known risk factor for head and neck, vulvar, vaginal, and penile cancer, as well as respiratory and laryngeal tumor (19). Other than HPV, human herpesvirus 8 (HHV-8) has been also reported to be associated with breast cancer in which HHV-8 antibodies were found in the blood sera (20). Furthermore, breast cancer might involve multiple viral infections, such as a combination of HSV-1, HPV, HCMV, EBV, and HHV-8, found in breast tissue samples (21).

Currently, the correlation between HPV and breast cancer remained conflicting. Higher HPV viral loads were detected in breast cancer tissues (22), yet a causal relationship was failed to establish between HPV and breast cancer due to inconsistent association scores across studies and the unspecific infective characteristics of HPV (23). Given the currently available evidence, HPV was proposed as a cofactor or mediator in breast cancer etiology (24). Therefore, more large-scale studies are needed to elucidate the association.

2 Methods

2.1 Data source

Patient data were extracted from a pool of 26 million in the National Health Insurance Research Dataset, 2007-2015, and the Taiwan Cancer Registry, 1979-2015. Both databases were overseen by the Health and Welfare Science Center, Ministry of Health and Welfare, Taiwan. To avoid misclassification of ICD code recorded in the National Health Insurance Research Database and to augment the documentation accuracy of breast cancer, data from the Taiwan Cancer Registry were used to confirm the breast cancer status. Each patient in the datasets possessed an encrypted identification number using their unique Taiwanese National Identification Number. Recorded patient information for each patient included ambulatory care expenditures by visits, inpatient expenditures by admissions, details of ambulatory care orders, details of inpatient orders, registry for beneficiaries, cause of death, and Taiwan Cancer Registry – long/short form.

2.2 ICD-9 international classification of diseases, ninth revision

Cases of human papillomavirus (ICD-9-CM 079.4, 078.1, 795.05, 795.09, 795.15, 795.19, 796.75, 796.79) were included and compared with the non-HPV group. Incidences of female breast cancer (ICD-9 174. X), head and neck cancer (ICD-9 140-149. X), and cervical cancer (ICD-9 180. X) were collected. Ischemic heart disease (IHD, ICD-9 411. 413–4), hypertension (ICD-9 401), ischemic stroke (ICD-9: 433–4,436), diabetes mellitus (DM, ICD-9 250), abnormal liver function (ICD-9 571), renal failure (ICD-9 580–589), gastrointestinal (GI) bleeding (ICD-9 578. X), hyperlipidemia (ICD-9 272. X), chronic kidney diseases (ICD-9 585.9), chronic obstructive pulmonary disease (COPD, ICD-9 492-496), peptic ulcer (ICD-9 533), and gout (ICD-9 274. X) were listed as the comorbidities.

2.3 Study design and ethical considerations

In the population-based cohort study, the index date was designated as the point of origin, and cancer incidence was measured from the index date until December 31, 2015, with the exclusion of the male population from the cervical cancer statistics. The study protocol underwent review and approval by the institutional review board of Chung Shan University Hospital (IRB CS13168) to ensure adherence to ethical considerations. Patient data from the National Health Insurance Research

Dataset were obtained, and de-identification was performed, which waived the need for signed informed consent.

2.4 Statistical analysis

Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, North Carolina) in this study. HPV-infected cases were matched to non-infected cases according to age, sex, and index date in a 1:2 fashion using structured query language (SQL). Patients with a cancer onset before the index date were excluded from the analysis. Comparisons were made between HPV-infected and non-HPV-infected individuals. Demographic data were analyzed using the chi-square test for categorical data and Student's t-test for numerical data. We used the multivariate Cox regression to adjust the potential confounding effect of age, urbanization, insured type, co-morbidities (including ischemic heart disease, hypertension, ischemic stroke, diabetes mellitus, abnormal liver function, renal failure, gastrointestinal bleeding, hyperlipidemia, chronic kidney diseases, chronic obstructive pulmonary disease, peptic ulcer, and gout) to estimate the hazard ratio of breast cancer in patients with HPV infection compared with the non-HPV individuals. We used the Schoenfeld residuals to test the proportional hazard of breast cancer, and the assumption of proportional hazard was not violated. Additionally, Kaplan-Meier curves were utilized to generate cumulative incidence rates of cancer, which were then tested using the log-rank test.

3 Results

About 26 million patients were registered in the National Health Insurance Research Datasets in Taiwan between 2007 to 2015. Among them, 1,103,771 patients were once diagnosed with HPV infection. Patients who had prior history of HPV infection before 2008 were excluded from the study, so were those who developed cancer before the HPV infection. This resulted in a pool of 939,874 HPV patients included in this study [Figure 1 (25)]. As the control, twice as many non-HPV patients with matching age, sex, and index date as the HPV patients (p=1.0000) were selected from the rest of the 25,462,267 non-HPV patients in the National Health Insurance Research Dataset using Structured Query Language (SQL) (Table 1). Nevertheless, several demographic characteristics differed between the HPV positive and negative groups, such as the geological distribution of patients (p<0.0001), the regional levels of urbanization (p<0.0001), the insurance providers (p<0.0001), and several co-morbidities such as ischemic heart disease (p<0.0001), hypertension (p<0.0001), diabetes mellitus (p<0.0001), renal failure (p<0.0001), GI bleeding (p=0.0006), hyperlipidemia (p<0.0001), chronic kidney diseases (p<0.0001), COPD (p<0.0001), peptic ulcer (p<0.0001), and gout (p<0.0001). Subsequently, the breast cancer patients were identified from this pool of HPV positive and negative patients and cross-examined with the Taiwan Cancer Registry to validate the breast cancer status of each selected patient.

The incidence rate of breast cancer on women was 109.67 per 100000 person-years for HPV patients, comparing to 46.97 per 100000 person-years for non-HPV patients, giving a crude hazard-ratio (HR) of 2.336 and an adjusted HR (aHR) of 2.271 (Table 2). Not only did this indicate that the prevalence rate of breast cancer was higher for HPV than non-HPV patients, but the risk of breast cancer was also equivalent or higher than the risk of head and neck cancer and cervical cancer in HPV patients, reflected by an approximately 42% and 2% higher aHR of breast cancer than that of head and neck cancer and cervical cancer, respectively, between HPV and non-HPV patients (Table 2). The cumulative breast cancer incidence rate over the span of 84 months was

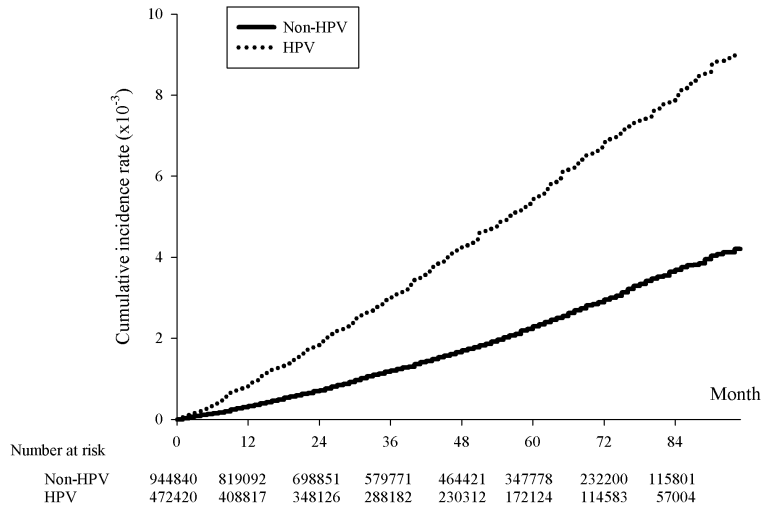

FIGURE 1
Kaplan-Meier curve of the cumulative incidence rates of breast cancer with and without HPV infection. The x-axis represents month since the index date, supplemented with the number of HPV cases and twice the non-HPV cases collected within each span of twelve months, p<.0001. The y-axis indicated the incidence rates in 10^{-3}.

TABLE 1 Baseline characteristics of the HPV and non-HPV populations.

	Non-HPV	HPV	p
Sex			1.0000
Male	944,840 (50.26%)	472,420 (50.26%)	
Female	934,908 (49.74%)	467,454 (49.74%)	
Age			1.0000
<20	513,000 (27.29%)	256,500 (27.29%)	
20-40	669,372 (35.61%)	334,686 (35.61%)	
40-60	469,150 (24.96%)	234,575 (24.96%)	
60-80	191,510 (10.19%)	95,755 (10.19%)	
>=80	36,716 (1.95%)	18,358 (1.95%)	
Urbanization			<.0001
Urban 1	542,433 (28.86%)	319,694 (34.01%)	
2	587,017 (31.23%)	290,511 (30.91%)	
3	340,505 (18.11%)	162,859 (17.33%)	
4	246,844 (13.13%)	109,824 (11.68%)	
5	36,687 (1.95%)	13,197 (1.40%)	
6	70,850 (3.77%)	23,620 (2.51%)	
Rural 7	55,412 (2.95%)	20,169 (2.15%)	
Geographic regions			<.0001
Taipei metropolitan	671,431 (35.72%)	395,713 (42.10%)	
Northern	279,894 (14.89%)	124,964 (13.30%)	
Central	347,664 (18.50%)	180,479 (19.20%)	
Southern	261,434 (13.91%)	107,447 (11.43%)	
Kaohsiung and Pingtung	276,130 (14.69%)	115,919 (12.33%)	
Eastern	43,195 (2.30%)	15,352 (1.63%)	
Insurance Provider			<.0001
Civil Servants' Insurance	111,753 (5.95%)	79,002 (8.41%)	
Labor Union	1,183,084 (62.94%)	618,860 (65.84%)	
Farmers', Fisherman's Association and the water conservancy	237,256 (12.62%)	94,224 (10.03%)	
Low-income household's insurance	20,772 (1.11%)	7,928 (0.84%)	
Township office	294,431 (15.66%)	122,182 (13.00%)	
Others	32,452 (1.73%)	17,678 (1.88%)	
Co-morbidity			
Ischemic heart disease	63,872 (3.40%)	39,334 (4.19%)	<.0001
Hypertension	205,015 (10.91%)	112,709 (11.99%)	<.0001
Stroke	40,087 (2.13%)	20,246 (2.15%)	0.2386
Diabetes mellitus	96,584 (5.14%)	46,782 (4.98%)	<.0001
Abnormal liver function	91,126 (4.85%)	45,661 (4.86%)	0.7009
Renal failure	15,200 (0.81%)	8,191 (0.87%)	<.0001
GI bleeding	10,826 (0.58%)	5,723 (0.61%)	0.0006

(Continued)

TABLE 1 Continued

	Non-HPV	HPV	p
Hyperlipidemia	133,790 (7.12%)	83,114 (8.84%)	<.0001
Chronic kidney diseases	25,740 (1.37%)	14,747 (1.57%)	<.0001
COPD	32,326 (1.72%)	18,607 (1.98%)	<.0001
Peptic ulcer	122,250 (6.50%)	74,302 (7.91%)	<.0001
Gout	49,184 (2.62%)	28,685 (3.05%)	<.0001

significantly higher in HPV positive than negative group as shown in the Kaplan-Meier curve (p<0.001) (Figure 1).

Besides HPV infection with a 2.271 aHR (p<0.0001), other demographic factors might also correlate with the breast cancer incidence rate. The biggest factor was age, where the highest aHR was 4.938 in the group aged 40 to 60 (p<0.0001), followed by 4.058 in the group aged 60 to 80 (p<0.0001), then 1.937 in group aged over and equal to 80 (p<0.0001), and finally 0.011 in group aged less and equal to 20 (p<0.0001), with the age group 20 to 40 served as the control (Table 3). In addition, the breast cancer incidence rates varied among patients covered under different insurance providers. Patients covered by the civil servants' insurance had a higher aHR of 1.234 (p=0.0002), whereas those covered by the insurance of Farmers', Fisherman's Association and the Water Conservancy had a lower aHR of 0.796 (p=0.0009) (Table 3). The incidence rates also differed among breast-cancer patients with co-morbidities. Significantly, the incidence rates of breast cancer were higher in patients experiencing abnormal liver function (aHR=1.191, p=0.0069) and hyperlipidemia (aHR=1.218, p=0.0002).

4 Discussion

This study presented a positive association between HPV infection and the risk of breast cancer using national population data from the Taiwanese single-payer healthcare registry over 84 months. The scale of this study provided an advantage to counter the inconsistency of the past results attempting to associate HPV infection and breast cancer. The 1:2 HPV positive to negative study group design also strengthened the statistical power of the association. Additionally, our study included multiple demographic factors that might serve to offer further insights into the correlation.

The most common transmission route of HPV is through sexual activities. Nevertheless, other transmission mechanisms have been proposed that related to the contact of skin and mucus, including horizontal transfer through non-sexual contact of fomites, fingers, skin, and mouths, self-inoculation presented by female virgins and child with absence of sexual abuse history, and vertical transmission during childbirth (26). How HPV travelled to and resided in breast tissues remained unclear. One theory suggested that HPV can be transmitted from the primary tumor such as cervical neoplasm to the mammary glands through plasma circulation (24). Another proposed that the viral particle can enter the milk ducts and populated in the mammary glands by the means of direct or hand-mediated sexual contact.

On the cellular level, HPV infected the cellular membrane and inserted the L2 capsid proteins into endosome facilitated by a transmembrane protease. The endosome displaying L2 protein protrusion was then trafficked to the Golgi apparatus assisted by cytosolic host factors (27). HPV DNA was then sent to microtubule-organizing center and, finally, it was shipped to chromosome *via* kinesins and spindle fibers during metaphase and anaphase (28).

Molecular studies have also indicated the association between HPV and breast cancer by attempting to provide a plausible transmission mechanism. The comparison between HPV positive and negative breast cancer tissues revealed a downregulation of p53 and an upregulation of BCL2, a hallmark of uninhibited cellular checkpoints (29). The phosphorylation of Erk1/2 and ß-catenin pathway might also be enhanced in breast cancer tissue when HPV L6/L7 cooperated with LMP1 oncoproteins, leading to cell proliferation (30). The proinflammatory cytokine IL-6, which was reported to progress oncogenesis, exhibited increased expression in breast cancer patients with HPV (31). Lastly, the blood-transmission theory of HPV was further supported by the finding

TABLE 2 Risks of cancers.

	Non-HPV			HPV				
	Pm	Event	Incidence rate†	Pm	Event	Incidence rate†	Crude HR	Adjusted HR
Female breast cancer	45,020,697	1,762	46.97 (44.82-49.21)	22,388,511	2,046	109.67 (105.01-114.52)	2.336(2.192-2.490)	2.271(2.129-2.421)
Head and neck cancer	89,136,676	1,064	14.32 (13.49-15.21)	44,357,317	808	21.86 (20.40-23.42)	1.527(1.393-1.673)	1.595(1.453-1.749)
Cervical cancer	45,020,697	712	18.98 (17.63-20.42)	22,388,511	777	41.65 (38.82-44.68)	2.195(1.983-2.430)	2.225(2.008-2.464)

Pm, person-months.
† Crude incidence rate, per 100000 person-years.

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TABLE 3 Adjusted hazard ratios for breast cancers.

	aHR	95% CI	p
HPV	2.271	2.129-2.421	<.0001*
Sex			
Male			
Female			
Age			
<20	0.011	0.005-0.027	<.0001*
20-40	Reference		
40-60	4.938	4.518-5.397	<.0001*
60-80	4.058	3.594-4.581	<.0001*
>=80	1.937	1.429-2.624	<.0001*
Urbanization			
1	Reference		
2	0.983	0.903-1.071	0.6967
3	0.887	0.794-0.990	0.0324
4	0.927	0.816-1.053	0.2442
5	0.714	0.510-1.000	0.0503
6	0.841	0.659-1.073	0.1633
7	0.890	0.687-1.152	0.3755
Geographic regions			
Taipei metropolitan	Reference		
Northern	0.978	0.874-1.095	0.704
Central	0.938	0.847-1.038	0.2156
Southern	0.884	0.785-0.996	0.0428
Kaohsiung and Pingtung	0.934	0.840-1.038	0.2064
Eastern	0.929	0.721-1.197	0.5685
Insurance Provider			
Civil servants' insurance	1.234	1.104-1.379	0.0002*
Labor Union	Reference		
Farmers', Fisherman's Association and Water Conservancy	0.796	0.695-0.911	0.0009*
Low-income household's insurance	0.853	0.565-1.287	0.4492
Township office	1.009	0.919-1.108	0.8532
Others	1.054	0.841-1.321	0.6485
Co-morbidity			
Ischemic heart disease	0.968	0.836-1.121	0.6607
Hypertension	1.095	0.994-1.207	0.065
Stroke	1.114	0.922-1.345	0.2635
Diabetes mellitus	1.084	0.957-1.228	0.206
Abnormal liver function	1.191	1.049-1.351	0.0069*
Renal failure	1.123	0.694-1.818	0.6357

(Continued)

TABLE 3 Continued

	aHR	95% CI	p
GI bleeding	0.829	0.506-1.359	0.4574
Hyperlipidemia	1.218	1.097-1.353	0.0002*
Chronic kidney diseases	1.027	0.711-1.484	0.8876
COPD	1.107	0.882-1.390	0.381
Peptic ulcer	1.071	0.966-1.188	0.1939
Gout	1.074	0.848-1.360	0.5545

Cox regression.
*$p < 0.01$.

of HPV DNA in extracellular vesicles extracted from the serum of breast cancer patients (32).

Other factors obtained from the patient data might confound the association of HPV and breast cancer. Our study reported that age, insurance provider, and co-morbidities such as abnormal liver function and hyperlipidemia influenced the breast cancer incidence rate. Among these confounding factors, our study indicated that the highest risk of breast cancer in the female population was between the ages 40 to 60, compared to all cancer whose risk retained positive correlation across age groups. We postulate that since most menopause occurred at the age of 40 to 60 where hormone homeostasis readjusted and HPV infection rate could also positively associate with menopausal status and negatively with hormone replacement therapy (33), breast cancer incidence rate was likely to be affected by HPV infection due to the alteration of hormone levels. On one hand, the decreased level of estrogen during menopause might result in virginal microbiomes being more favorable for HPV infection (34). On the other hand, molecular studies indicated the potential association of estrogen and apolipoprotein B messenger RNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) family of cytidine deaminases, which served not only to induce antiviral immune response during the HPV infection, but also could mutate host DNA and initiate breast carcinogenesis (35). Estrogen and the lack of p53 were reported to potentially upregulate APOBEC enzymes synergistically in the breast cancer cells containing estrogen receptors (35, 36).

Another confounding factor, the abnormal liver function could be potentially elucidated by the presence of HPV DNA circulating to liver. HPV was found to act cooperatively with hepatitis B virus (HBV) to develop hepatocellular carcinoma, and/or other viral infections might be facilitated by HPV to cause abnormal liver function (37).

The current theory proposed that HPV may be a cofactor or mediator of breast cancer rather than a causative agent, partially due to conflicting results of the presence of HPV in breast cancer tissues (24). Moreover, demographic and other individual factors may influence the possibility of oncogenesis after HPV infection. Our result was consistent with this theory and multiple demographic factors were examined in Table 3. The association of HPV infection status and breast cancer was further supported by increased breast cancer rate found in patients infected by high-risk HPV, including HPV 16, 18, and 33, in a large-scale meta-analysis study (38). Additionally, the association was verified by four different PCR approaches for HPV detection (39). In contrast, studies in different populations across the globe did not yield statistically significant results unanimously to indicate the contribution of HPV on breast cancer development (40, 41). However, the lack of HPV prevalence in breast cancer tissue could potentially be explained by the disappearance of HPV strains in the later stage of cancer (24).

A main obstacle was to understand the route of HPV transmission to breast tissues. Unlike cervical cancer, where HPV infected the epithelial cells of the cervix *via* cervical lesion or the mucous membrane, HPV had less direct routes to infect breast tissues. Although two potential mechanisms where proposed above (24), more anatomic and molecular studies are required to understand the direct and indirect etiologies of HPV on breast cancer.

In summary, our results suggested a significant higher risk of breast cancer in female patients with HPV than those without. The risk of breast cancer in HPV positive patients was reported to be twice as large as HPV negative patients. The accumulated breast cancer incidence rates between HPV positive and negative patients were shown to be significantly different in Kaplan-Meier curve. Despite not being able to conclude a causal relationship, it could be assumed the role of HPV as a contributor of the breast carcinogenesis.

Interestingly, our analysis on patient demographics speculated a potential role of estrogen between HPV infection and breast cancer, which could only be explained by executing further investigations. The circulation of HPV in sera might also implicate other organs such as the liver, but it is beyond the scope of this study.

The hypothesis that breast cancer was associated with HPV was first proposed about 30 years ago (24). However, the link has not been strong enough due to mixed results of HPV prevalence rates in breast cancer patients across studies. Our study utilized large-scale datasets to provide a more robust statistical significance between the incidence rates of breast cancer and HPV status. 26 million people were registered in the Taiwanese Health Registry over the year 2007 to 2015. The breast cancer statuses of the patients were identified in the registry and verified by another database, Taiwanese National Cancer Registry.

It is recognized that a limitation of the study was that the subtype and localization of the HPV was unknown. Investigating the HPV subtype with respect to breast cancer incidence rate may provide clues about the oncogenic properties of high-risk HPV subtype, while data on HPV localization may suggest its possible infectious mechanism in the development of breast cancer. Additionally, it is worth noting that the population-based data used in this study were not collected solely for the purpose of this research. As a result, there is a potential for some ICD outcomes to have been misclassified. Moreover, confounding variables previously reported to be associated with the risk of breast cancer, such as reproductive history, breast feeding, family history of breast cancer, lifestyle, and environmental factors, were not fully accounted for in this study due to the inherent nature of the data collection, which may cause bias in the results. Finally, the results of the study should be interpreted with caution, as the risk factors for breast cancer may differ in different populations.

Nevertheless, a causal relationship between HPV and breast cancer remains unclear. More hypotheses proposed from molecular studies are required to understand the route of HPV transmission to breast tissues as well as the mechanism of viral transmission in breast cells in relation to oncogenesis. Moreover, the HPV subtypes of the breast cancer patients could be examined in the future to provide insights into the mixed HPV prevalence rates in the previous studies of breast cancer tissues. Furthermore, the societal impacts of cancer treatments are becoming more prominent each year. Not only the national treatment cost for cancer was estimated to be 246 billion in the United States by 2030 (42), but also cancer patients pay four times more than patients without cancer on average (43), in addition to the higher risks of psychiatric disorders and mental distress on both cancer patients and their nuclear family members (44). Thus, our study included HPV status, age groups, co-morbidities, and other demographic variables to provide evidence supporting a more stringent approach to breast cancer screening. More research could be done to investigate these factors which could be valuable in drafting novel health initiatives to combat breast cancer.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

Conception or design of the work: J-YH and FC-FL. Data collection: J-YH, CL, SC-ST, FC-FL. Data analysis and interpretation: J-YH, FC-FL. Drafting the article: CL. Critical revision of the article: SC-ST, FC-FL. Approval of the manuscript: J-YH, CL, SC-ST, FC-FL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Publisher's note

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660

2. National Health Insurance Research Database T. *CANCER REGISTRY ANNUAL REPORT, 2020 TAIWAN*. HEALTH PROMOTION ADMINISTRATION, MINISTRY OF HEALTH AND WELFARE TAIWAN (2022). Available at: https://www.hpa.gov.tw/EngPages/List.aspx?nodeid=1070

3. Goding Sauer A, Siegel RL, Jemal A, Fedewa SA. Current prevalence of major cancer risk factors and screening test use in the United States: disparities by education and Race/Ethnicity. *Cancer Epidemiol Biomarkers Prev* (2019) 28:629–642. doi: 10.1158/1055-9965.EPI-18-1169

4. Shoemaker ML, White MC, Wu M, Weir HK, Romieu I. Differences in breast cancer incidence among young women aged 20–49 years by stage and tumor characteristics, age, race, and ethnicity, 2004–2013. *Breast Cancer Res Treat* (2018) 169:595–606. doi: 10.1007/s10549-018-4699-9

5. De Brakeleer S, De Grève J, Loris R, Janin N, Lissens W, Sermijn E, et al. Cancer predisposing missense and protein truncating BARD1 mutations in non-BRCA1 or BRCA2 breast cancer families. *Hum Mutation* (2010) 31(3):E1175-85. doi: 10.1002/humu.21200

6. Hunt SC, Williams RR, Skolnick MH, Lyon JL, Smart CR. Breast cancer and reproductive history from genealogical data. *J Natl Cancer Inst* (1980) 64(5):1047–53.

7. Travis RC, Key TJ. Oestrogen exposure and breast cancer risk. *Breast Cancer Res BCR* (2003) 5:239–247. doi: 10.1186/bcr628

8. Mahmoud R, Marei LS, Shareef MH, Tayyem RF. The association between lifestyle factors and the risk of developing breast cancer in Jordanian women. *Ann Cancer Res Ther* (2020) 28(1). doi: 10.4993/acrt.28.16

9. de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. *Lancet Global Health* (2019) 8(2):e180–90. doi: 10.1016/S2214-109X(19)30488-7

10. Borgia M, Dal Bo M, Toffoli G. Role of virus-related chronic inflammation and mechanisms of cancer immune-suppression in pathogenesis and progression of hepatocellular carcinoma. *Cancers* (2021) 13(17):4387. doi: 10.3390/cancers13174387

11. Chi J-Q, Teng M, Yu Z, Xu H, Su J-W, Zhao P, et al. Marek's disease virus-encoded analog of microRNA-155 activates the oncogene c-Myc by targeting LTBP1 and suppressing the TGF-β signaling pathway. *Virology* (2015) 476:72–84. doi: 10.1016/j.virol.2014.11.027

12. Fierti AO, Yakass MB, Okertchiri EA, Adadey SM, Quaye O. The role of Epstein-Barr virus in modulating key tumor suppressor genes in associated malignancies: epigenetics, transcriptional, and post-translational modifications. *Biomolecules* (2022) 12(1):127. doi: 10.3390/biom12010127

13. McBride AA, Warburton A. The role of integration in oncogenic progression of HPV-associated cancers. *PloS Pathogens* (2017) 13(4):e1006211. doi: 10.1371/journal.ppat.1006211

14. Forman D, de Martel C, Lacey C, Soerjomataram I, Lortet-Tieulent J, Bruni L, et al. Global burden of human papillomavirus and related diseases. *Vaccine* (2012) 30 (Suppl 5):F12–23. doi: 10.1016/j.vaccine.2012.07.055

15. Jassim G, Obeid A, Al Nasheet HA. Knowledge, attitudes, and practices regarding cervical cancer and screening among women visiting primary health care centres in Bahrain. *BMC Public Health* (2018) 18(1):128. doi: 10.1186/s12889-018-5023-7

16. Piróg M, Grabski B, Jach R, Zmaczynski A, Dutsch-Wicherek M, Wróbel A, et al. Human papillomavirus infection: knowledge, risk perceptions and behaviors among SMW and AFAB. *Diagnostics* (2022) 12(4):843. doi: 10.3390/diagnostics12040843

17. Gosens KCM, Richel O, Prins JM. Human papillomavirus as a cause of anal cancer and the role of screening. *Curr Opin Infect Dis.* (2016) 30:87–92. doi: 10.1097/QCO.0000000000000337

18. Berman TA, Schiller JT. Human papillomavirus in cervical cancer and oropharyngeal cancer: one cause, two diseases. *Cancer* (2017) 123(12):2219–29. doi: 10.1002/cncr.30588

19. Zoran S, Arko D, Esih M, Simonović Z, Takač I, Fokter AR. Beyond cervical cancer: human papillomaviruses infection-related neoplasms and their prevention. (2021) 90(3–4):208–18. Available at: https://vestnik.szd.si/index.php/ZdravVest/article/view/3037.

20. Shakir DM, Abdullah SF, Sharquie IK. Serodiagnosis of human herpesvirus 8 in women with breast cancer. *Biomed Pharmacol J* (2018) 11:391–8. doi: 10.13005/bpj/1385

21. Hsu C-R, Lu T-M, Chin LW, Yang CC. Possible DNA viral factors of human breast cancer. *Cancers* (2010) 2:498–512. doi: 10.3390/cancers2020498

22. Sher G, Salman NA, Kulinski M, Fadel RAA, Gupta VK, Anand A, et al. Prevalence and type distribution of high-risk human papillomavirus (HPV) in breast cancer: a Qatar based study. *Cancers* (2020) 12(6):1528. doi: 10.3390/cancers12061528

23. Usman M, Hameed Y, Ahmad M, Rehman JU, Ahmed H, Hussain MS, et al. Breast cancer risk and human papillomavirus infection: a Bradford hill criteria based evaluation. *Infect Disord Drug Targets* (2022) 22(4):e200122200389. doi: 10.2174/1573401318666220120105931

24. Kudela E, Kúdelová E, Kozubík E, Rokos T, Pribulová T, Holubekova V, et al. HPV-associated breast cancer: myth or fact? *Pathogens* (2022) 11(12):1510. doi: 10.3390/pathogens11121510

25. Huang JY, Lin C, Tsai SC, Lin FC. Human papillomavirus is associated with adenocarcinoma of lung: a population-based cohort study. *Front Med (Lausanne)* (2022) 9:932196. doi: 10.3389/fmed.2022.932196

26. Petca A, Borislavschi A, Zvâncă ME, Petca RC, Ş;andru F, Dumitraşcu MC. Non-sexual HPV transmission and role of vaccination for a better future (Review). *Exp Ther Med* (2020) 20 6:186. doi: 10.3892/etm.2020.9316

27. Harwood MC, Dupzyk AJ, Inoue T, DiMaio D, Tsai B. p120 catenin recruits HPV to γ-secretase to promote virus infection. *PloS Pathogens* (2020) 16(10):e1008946. doi: 10.1371/journal.ppat.1008946

28. Keiffer TR, Soorya SR, Sapp M. Recent advances in our understanding of the infectious entry pathway of human papillomavirus type 16. *Microorganisms* (2021) 9 (10):2076. doi: 10.3390/microorganisms9102076

29. Wang Y-w, Zhang K, Zhao S, Lv Y, Zhu J, Liu H, et al. HPV status and its correlation with BCL2, p21, p53, Rb, and survivin expression in breast cancer in a Chinese population. *BioMed Res Int* (2017) 2017:6315392. doi: 10.1155/2017/6315392

30. Gupta I, Jabeen A, Vranić S, Al Moustafa A-E, Al-Thawadi HA. Oncoproteins of high-risk HPV and EBV cooperate to enhance cell motility and invasion of human breast cancer cells *via* Erk1/Erk2 and β-catenin signaling pathways. *Front Oncol* (2021) 11. doi: 10.3389/fonc.2021.630408

31. Charostad J, Azaran A, Nakhaei M, Astani A, Kaydani GA, Motamedfar A, et al. Upregulation of interleukin-6 in HPV-positive breast cancer patients. *Iranian J Immunol IJI* (2021) 18 4:5. doi: 10.22034/IJI.2021.89107.1930

32. De Carolis S, Storci G, Ceccarelli C, Savini C, Gallucci L, Sansone P, et al. HPV DNA associates with breast cancer malignancy and it is transferred to breast cancer stromal cells by extracellular vesicles. *Front Oncol* (2019) 9. doi: 10.3389/fonc.2019.00860

33. Lai CHE, Chao A, Chang CJ, Huang CC, Wang LCC, Hsueh S, et al. Age factor and implication of human papillomavirus type-specific prevalence in women with normal cervical cytology. *Epidemiol Infect* (2011) 140:466–473. doi: 10.1017/S0950268811000720

34. Reguero-Cadilla L, Dominicci-Maura A, Godoy-Vitorino F, Romaguera J. Effect of menopause on genital human papillomavirus infection and the microbiota in a Hispanic cohort [A320]. *Obstetr Gynecol* (2022) 139:92S–92S. doi: 10.1097/01.AOG.0000825600.95480.64

35. Chong PP. Bacteriology and infectious diseases 2018 - role of human papillomavirus, estrogen and Apobec3B axis in breast cancer initiation. *J Bacteriol Parasitol* (2018) 9. doi: 10.4172/2155-9597-C1-046

36. Ohba K, Ichiyama K, Yajima M, Gemma N, Nikaido M, Wu Q, et al. *In vivo* and *In vitro* studies suggest a possible involvement of HPV infection in the early stage of breast carcinogenesis *via* APOBEC3B induction. *PloS One* (2014) 9(5):e97787. doi: 10.1371/journal.pone.0097787

37. Scinicariello F, Sato TA, Lee CS, Hsu HC, T-s C, Tyring SK. Detection of human papillomavirus in primary hepatocellular carcinoma. *Anticancer Res* (1992) 12 3:763–6.

38. Ren C, Zeng K, Wu C, Mu L, Huang J, Wang M. Human papillomavirus infection increases the risk of breast carcinoma: a large-scale systemic review and meta-analysis of case-control studies. *Gland Surg* (2019) 8 5:486–500. doi: 10.21037/gs.2019.09.04

39. Kroupis C, Markou AN, Vourlidis N, Dionyssiou-Asteriou A, Lianidou ES. Presence of high-risk human papillomavirus sequences in breast cancer tissues and association with histopathological characteristics. *Clin Biochem* (2006) 39 7:727–31. doi: 10.1016/j.clinbiochem.2006.03.005

40. Choi Y-L, Cho EY, Kim JH, Nam SJ, Oh YL, Song SY, et al. Detection of human papillomavirus DNA by DNA chip in breast carcinomas of Korean women. *Tumor Biol* (2008) 28:327–332. doi: 10.1159/000124238

41. Doosti M, Bakhshesh M, Zahir ST, Shayestehpour M, Karimi-Zarchi M. Lack of evidence for a relationship between high risk human papillomaviruses and breast cancer in Iranian patients. *Asian Pacific J Cancer Prev APJCP* (2016) 17 9:4357–61.

42. Mariotto AB, Enewold L, Zhao J, Zeruto CA, Yabroff KR. Medical care costs associated with cancer survivorship in the United States. *Cancer Epidemiol Biomarkers Prev* (2020) 29:1304–1312. doi: 10.1158/1055-9965.EPI-19-1534

43. Park J, Look KA. Health care expenditure burden of cancer care in the United States. *Inquiry: A J Med Care Organization Provision Financing* (2019) 56:46958019880696. doi: 10.1177/0046958019880696

44. Caruso R, Nanni MG, Riba M, Sabato S, Grassi L. The burden of psychosocial morbidity related to cancer: patient and family issues. *Int Rev Psychiatry* (2017) 29:389–402. doi: 10.1080/09540261.2017.1288090

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The correlation between multiple HPV infections and the occurrence, development, and prognosis of cervical cancer

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Cervical carcinoma is the fourth female malignant tumor in the world, and the persistent infection of high-risk human papillomavirus (HPV) is recognized as the most common cause. This article studies the correlation between multiple HPV infections and the occurrence, development, and prognosis of cervical cancer in order to provide more references for clinical diagnosis and treatment. We conducted a retrospective analysis of the clinical data of 400 cervical carcinoma patients admitted to our hospital from 2015 to 2023. The collected patient data include age, HPV infection status, tumor size and morphology, local infiltration depth, diagnostic staging, surgical approach, vascular cancer thrombus status, lymph node status, and postoperative HPV follow-up status. We use SPSS statistical software for data analysis. Our research shows that the high-risk age group for cervical carcinoma is concentrated between 41 and 60 years old, which is basically consistent with the age range of the high incidence of HPV infection. In the statistics for HPV infection types, ~67.7% of patients are single HPV-infected, 25.29% are double infected, and 7.00% are infected with three or more types of HPV. Among the multiple HPV infections, most of the patients are younger than 40 years old and older than 70 years old, with double infection accounting for the majority. The top five HPV subtypes with high detection rates belong to high-risk subtypes, which are the HPV16, 18, 58, 33, and 52 subtypes, respectively. There was no significant relationship between multiple HPV infections and cervical cancer stage, lesion size, pathological tissue type, tissue differentiation degree/vascular cancer thrombus, and lymph node metastasis, and there was no significant difference in the results between the groups. In summary, multiple types of HPV infection in the cervix are common. We found that multiple infections, mainly HPV16, are closely related to cervical cancer. For the HPV16, 18, 58, 33, and 52 subtypes of infection, especially for patients younger than 40 years old and older than 70 years old, priority should be given to prevention and treatment. The relationship between multiple HPV infections and the progression and prognosis of cervical carcinoma requires further research, which could better guide cancer prevention and treatment.

KEYWORDS

multiple HPV infections, cervical cancer, occurrence, development, prognosis

Introduction

Cervical carcinoma is the fourth female malignant tumor in the world, and it is also the most usual malignant tumor of the female reproductive tract. Its morbidity is only next to breast cancer, colorectal cancer, and lung cancer (Hu and Ma, 2018; Arbyn et al., 2020). At present, the morbidity of cervical carcinoma in developing countries is still rising year by year. The occurrence of cervical carcinoma usually goes through a long process, which is the result of multiple factors, multiple genes, and multi-step long-term joint action. The human papillomavirus (HPV) is closely related to the occurrence of cervical carcinoma, and the persistent infection of high-risk HPV is currently recognized as the most common reason for cervical carcinoma (de Freitas et al., 2015). Most women infected with HPV can be cleared by their own immune function, and only a small portion of HPV infection may cause cervical lesions or even cervical cancer (Akeel, 2015; Husain and Ramakrishnan, 2015). Due to the different pathogenic potential of different subtypes of HPV infection (Salazar et al., 2015), the risk of cervical carcinoma and cervical lesions varies. Multiple infections refer to the simultaneous infection of two or more HPV subtypes. Meanwhile, due to the lack of cross antibodies between different subtypes of HPV, multiple HPV infections are common. Therefore, the correlation between multiple HPV infections and cervical carcinoma and cervical lesions is receiving increasing attention. There is currently no consensus on whether multiple HPV infections will increase the incidence of cervical carcinoma, and there is no consensus on their impact on cervical carcinoma. This article studies the correlation between multiple HPV infections and the occurrence, development, and prognosis of cervical cancer in order to provide more reference for clinical diagnosis and treatment.

Methods

A retrospective analysis was conducted on the clinical data of 400 cervical carcinoma patients admitted to our hospital from 2015 to 2023. The inclusion criteria for cases are pathological diagnoses of cervical cancer, while the exclusion criteria are immune system diseases, chronic wasting diseases, and organ transplant surgery. The collected patient data include age, menopausal status, HPV infection status, tumor size and morphology, local infiltration depth, diagnostic staging, surgical approach, postoperative pathological type, interstitial infiltration depth, vascular cancer thrombus status, lymph node status, lymphatic space involvement (LVSI), squamous cell carcinoma antigen (SCC), smoking history, postoperative adjuvant treatment, postoperative HPV follow-up status, tumor-free survival rate, follow-up interval, and mortality rate. All information is obtained by consulting medical records. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University. This study used SPSS software version 25.0 for statistical analysis. Heterogeneity comparison between groups was conducted using the chi-square test, and Fisher's exact test was used when a single sample size was <4. For the remaining analyses, a p-value of < 0.05 was considered statistically significant.

TABLE 1 Clinical and pathological parameters of all subjects.

Parameter		*N*	(%)
Age (years)	≤30	13	3.3
	31–40	61	15.3
	41–50	105	26.3
	51–60	132	33.0
	61–70	76	19.0
	>70	13	3.3
FIGO stage	I	244	61.0
	II	90	22.5
	III	40	10.0
	IV	3	0.8
	Not reported	23	5.8
Tumor size	<4 cm	186	74.70
	≥4 cm	63	25.30
	Not reported	151	-
Histological type	Squamous cell carcinoma (SCC)	343	85.8
	Adenocarcinoma (ADC)	52	13.0
	Other	5	1.3
	Yes	130	40.50
Vascular cancer thrombus	No	191	59.5
	Not reported	79	-
lymph node metastasis	Yes	53	16.25
	No	273	83.74
	Not reported	74	-
Infection type	Single subtype	174	67.7
	Two subtypes	65	25.29
	Three or more subtypes	18	7.00
	Not reported	143	-
Degree of differentiation	Poorly differentiated	38	16.24
	moderately differentiated	190	81.20
	Well differentiated	6	2.56
	Not reported	166	-
HPV reinfection	Yes	17	13.18
	No	112	86.82
	Not reported	271	-

Results

Analysis of clinical data characteristics of cervical cancer patients

We described the clinical data of 400 cervical carcinoma patients admitted to our hospital from 2015 to 2023, as shown in Table 1, including age distribution, tumor size, FIGO stage, pathological type, degree of differentiation, vascular cancer

TABLE 2 Distribution of HPV infection types in different age groups (N %).

Infection type	≤30	31–40	41–50	51–60	61–70	>70	Total
Single subtype	5 (55.6)	30 (61.2)	50 (80.6)	54 (64.3)	33 (70.2)	2 (33.3)	174 (67.7)
Two subtypes	4 (44.4)	16 (32.7)	10 (16.1)	24 (28.6)	10 (21.3)	1 (16.7)	65 (25.3)
Three subtypes	0 (0.0)	2 (4.1)	2 (3.2)	4 (4.8)	1 (2.1)	3 (50)	12 (4.7)
Four subtypes	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.4)	1 (2.1)	0 (0.0)	3 (1.2)
Five subtypes	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	1 (2.1)	0 (0.0)	2 (0.8)
Six subtypes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	0 (0.0)	0 (0.0)
Seven subtypes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.1)	0 (0.0)	1 (0.4)
Total	9 (100.0)	49 (100.0)	62 (100.0)	84 (100.0)	47 (100.0)	6 (100.0)	257 (100.0)

TABLE 3 Distribution of HPV infection types in different age groups (N %).

Infection type	≤30	31–40	41–50	51–60	61–70	>70	total	χ^2	*P*
Single subtype	5 (55.6)	30(61.2)	50(80.6)	54 (64.3)	33(70.2)	2(33.3)	174 (67.7)	26.5	0.003
Two subtypes	4 (44.4)	16(32.7)	10(16.1)	24 (28.6)	10 (21.3)	1(16.7)	65 (25.3)		
Multiple infections	0(0)	3(6.1)	2(3.2)	6(7.1)	4(8.5)	3(50)	18(7)		
Total	9 (100)	49(100)	62(100)	84(100)	47 (100)	6(100)	257 (100)		

thrombus, lymph node metastasis, HPV infection type, and reinfection status. From the table, we can see that the minimum age of the patient is 24 years, the maximum age is 89 years, and the average age is 51.34 years. The high-risk age group for cervical cancer is concentrated between 41 and 60 years. The pathological type of the patient is mainly squamous cell carcinoma. There are 343 cases of SCC, accounting for 85.8%, 52 cases of ADC, accounting for 13%, and 5 cases of other pathological types, accounting for 1.3%. Except for 143 patients who did not undergo standardized HPV examinations before surgery or were unable to track the results, in the HPV infection type statistics, there were 174 patients with a single HPV infection, 65 patients with two types of HPV infection, and 18 patients with three or more types of HPV infection. HPV multiple infections account for approximately one-third of HPV-infected patients, which also proves that HPV multiple infections are relatively common. Among 129 patients who could be traced back to postoperative follow-up results, 17 patients were reinfected with HPV, accounting for ∼13.18%, and 112 patients were persistently negative, accounting for ∼86.82%. The average follow-up time was 28 months.

Correlation analysis between age and HPV infection types and distribution of common infection subtypes

We analyzed the distribution of HPV infection types in different age groups and found that the age group with a high incidence of HPV infection is between 30 and 70 years, with the age group of 41 to 60 years being more significant, which is basically consistent with the distribution of the high incidence age of cervical cancer. Among the multiple infections, most of the patients are younger than 40 years (≤ 30 years old: 44.4%, 31–40 years old: 38.8%) and older than 70 years (66.7%). We speculate that this may be related to factors such as sexual activity, fluctuations in hormone levels, decreased immunity, and an increased probability of causing latent viral infections. Among patients with multiple HPV infections, there are mainly 65 cases of double infection, 12 cases of triple infection, and fewer cases of four or more HPV infections. One patient is simultaneously infected with seven types of HPV. There is a significant difference in the distribution of HPV infection types among different age groups ($P < 0.05$) (Tables 2, 3).

We analyzed the distribution of HPV infection subtypes and found that the top five HPV subtypes with high detection rates in this study belonged to high-risk subtypes, namely, the HPV16, 18, 58, 33, and 52 subtypes (Table 4). Patients with multiple HPV infections are mainly infected with HPV16 mixed with other types of infection (referred to as "high-risk mixed type"), including mixed infections of high-risk and high-risk HPV and mixed infections of high-risk and low-risk HPV. According to the statistics on the number of types of HPV infections, the proportion of double infections is the highest. The top three HPV subtypes in dual infection include 16&18, 16&33, and 16&58 subtypes (Table 5). It is suggested that more attention should be paid to patients infected with the abovementioned HPV subtypes in our clinical studies.

Lymph node metastasis and vascular cancer thrombus

We used SPSS software version 25.0 for statistical analysis of lymph node metastasis and vascular cancer thrombi. A heterogeneity comparison between the groups was conducted using the chi-square test, and Fisher's exact test was used when a single sample size was <4. For the remaining analyses, a *p*-value of<0.05 was considered statistically significant. Table 6 shows the

TABLE 4 Distribution of HPV infection subtypes in different age groups (N).

Infection subtype	≤30	31–40	41–50	51–60	61–70	>70	Total
16	8	35	43	66	33	5	190
18	3	11	8	9	8	0	39
58	1	3	2	7	9	1	23
33	0	5	2	10	2	1	20
52	0	3	3	6	2	1	15
31	0	3	2	3	1	1	10
39	1	2	3	0	0	1	7
53	0	1	0	2	3	1	7
81	0	0	2	0	4	0	6
51	0	0	1	3	1	0	5
59	0	2	1	2	0	0	5
42	0	0	1	2	1	0	4
45	0	1	0	3	0	0	4
56	0	0	1	3	0	0	4
66	0	0	1	0	2	1	4
11	0	2	0	0	1	0	3
35	0	0	1	1	1	0	3
68	0	1	1	0	1	0	3
82	0	1	1	0	0	0	2
CP8034	0	0	0	2	0	0	2
13	0	0	1	0	0	0	1
26	0	0	0	0	0	1	1
43	0	0	0	1	0	0	1
44	0	0	0	0	1	0	1
67	0	0	0	1	0	0	1
73	0	1	0	0	0	0	1

relationship between lymph node metastasis and vascular tumor thrombus with age, HPV infection type, tumor size, and tumor stage. It is found that there is no significant difference between age and HPV infection type groups, while there is a significant difference between tumor size and stage groups. From Table 7, we can see that patients with vascular cancer thrombi are more prone to lymph node metastasis, and there is a significant difference between the two groups.

Correlation analysis between multiple HPV infections and cervical cancer factors

We conducted correlation analysis based on different groups of multiple infections and found that there was no significant relationship between HPV multiple infections and cervical cancer stage, lesion size, pathological tissue type, tissue differentiation degree, vascular cancer thrombus, and lymph node metastasis. There was no significant difference in the results between the groups (Tables 8, 9).

TABLE 5 Distribution of HPV multiple genotypes.

HPV genotypes	Cases, *n* (%)
16‘18	12 (14.5)
16‘33	9 (10.8)
16‘58	9 (10.8)
16‘52	5 (6.0)
16‘39	3 (3.6)
16‘53	3 (3.6)
16‘31	2 (2.4)
18‘31	2 (2.4)
16‘81	2 (2.4)
16‘51	2 (2.4)
16‘CP8034	2 (2.4)
16‘33‘52	2 (2.4)
16‘18‘45	2 (2.4)
Others	29 (34.9)

Discussion

Multiple HPV infections and cervical carcinoma

Cervical carcinoma is a usual malignancy in the female reproductive system. It is one of the malignant tumors with high morbidity and mortality among women in developing countries, threatening the health of many women. According to the statistics, ~48,000 women die from cervical cancer every year (Chen et al., 2016; Bray et al., 2018). At present, it has been determined that the sustained infection of human papillomavirus is a major influencing factor for the occurrence of cervical carcinoma, and we can determine the type and subtype of HPV infection through cervical screening. There are over 200 subtypes of HPV found currently, and 40 subtypes closely related to the reproductive tract (Chunyou and Wang, 2019). According to their risk of causing cervical carcinoma, HPV can be divided into high-risk and low-risk types. The former includes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (Vaccine Immunization Branch of Chinese Preventive Medicine Association, 2019). Persistent infection of high-risk HPV is closely related to cervical carcinoma, while low-risk HPV is more common in benign lesions such as genital warts. According to the reports, persistent infection of high-risk HPV in the cervix can lead to precancerous lesions and can further develop into invasive cancer, accounting for 99.7% of cervical carcinoma cases infected with high-risk HPV (Andersson et al., 2005).

Multiple infections refer to the simultaneous infection of two or more HPV subtypes, which may be secondary, tertiary, or even more. The inconsistent pathogenicity of the different subtypes

TABLE 6 Chi-square test.

Parameter		Lymph node metastasis			Vascular cancer thrombus		
		N (%)	χ^2	*p*	N (%)	χ^2	*p*
Age	30	3(33.3)	8.258	0.143	2(40.0)	1.783	0.878
	31–40	6(14.3)			15(34.1)		
	41–50	17(19.3)			34(40.0)		
	51–60	22(19.3)			48(42.1)		
	61–70	4(6.3)			25(40.3)		
	>70	1(10.0)			6(54.5)		
Infection type	HPV single	17(11.1)	0.056	0.810	59(37.8)	0.037	0.847
	HPV multiple	7(12.3)			22(39.3)		
Tumor size	<4cm	29(16.6)	12.137	0.002	82(48.5)	23.667	0.000
	≥4cm	17(29.8)			30(53.6)		
FIGO stage	I	11(5.5)	144.410	0.000	64(31.4)	25.502	0.000
	II	13(15.3)			46(56.1)		
	III and IV	29(90.6)			19(70.4)		

TABLE 7 Chi-square test.

	Lymph node metastasis			χ^2	*p*
Vascular cancer thrombus		Yes	No	28.862	0.000
	Yes	36	93		
	No	9	159		

of HPV indicates a different risk of cervical lesions. Therefore, many scholars are paying attention to the correlation between multiple HPV infections and cervical carcinoma (Salazar et al., 2015). A recent overseas project showed that 622 cases are multiple infections in 1,216 patients with HPV infection, accounting for almost half of the total (Kim et al., 2021). Our research results also indicate that multiple HPV infections in the cervix are common. However, there is no consensus on whether there is a cooperation between multiple HPV infections and whether it has a promoting effect on the development of cervical carcinoma at various stages. Some scholars believe that multiple HPV infections are related to the progression of cervical lesions (Chowell et al., 2012; Yokomichi et al., 2018; Homaira et al., 2019; Tramuto et al., 2019; Ghimire and Moon-Grady, 2020), but others highlight that there is no correlation between multiple HPV infections and cervical lesions (Xu et al., 2012; Tagarro et al., 2019).

HPV pathogenesis and synergistic effects between multiple infections

HPV infects the cervical epithelial cells through micro damage, which enters and spreads to the basal layer of the cervical epithelium. HPV is released from the capsid and enters the nucleus, becoming a free gene. HPV exists in three forms after infecting cervical epithelial cells, including free type, integrated type, and mixed type. When HPV is in an integrated state, it can exist in the body for a long period of time, making it less likely to be detected by the host immune system and becoming a "persistent infection", which is more common in high-grade intraepithelial neoplasia and cervical carcinoma. Some studies have shown that as the degree of cervical lesions worsens, the proportion of integrated types gradually increases, while the proportion of free types gradually decreases. Especially in cervical cancer, multiple integrated types account for the majority. In cervicitis and CIN, single integration is predominant (Huang et al., 2017; Groves and Coleman, 2018; Xia et al., 2020).

Important genital HPV genotypes belong to α Papillomavirus genus, including α-9, α-7, α-6, and α-5 four categories. α-9 included the high-risk HPV subtypes 16, 31, 33, 35, and 52, and α-7 included the high-risk HPV subtypes 18, 39, 45, 59, and 68. Some studies have shown that multiple infections of genotype α-9 increase the risk of cervical carcinoma by 5.3 times, and multiple infections of genotype α-7 increase the risk of cervical carcinoma by 2.5 times, which also explain that the same type of HPV genotype may have a synergistic effect on co-infection to induce cervical carcinoma (Saslow et al., 2012). Iacobone et al. Iacobone et al. (2019) found that there is a synergistic effect between specific genotypes among different subtypes of HPV, with the association between HPV16 and 53 being more pronounced. However, Vinokurova et al. (Vinokurova et al., 2008) found that HPV16, 18, and 45 have more integration potential compared to HPV31 and 33, which makes them more likely to cause high-grade lesions to progress to cervical carcinoma. HPV16 is present in most multiple infections. HPV16/18 and HPV16/31 are the most common combination of mixed infections (Aleksioska-Papestiev et al., 2018). The research by de Freitas et al. (de Freitas et al., 2015) shows that the most common integrated subtypes in northwestern China are HPV16, 58, and 33. The results of our study show that the incidence of multiple HPV infections is 32.29%, in which HPV16 mixed with other types of infection is the main type, basically consistent with

TABLE 8 Analysis of clinical and pathological parameters of multiple HPV infections and cervical cancer.

Correlations	0–1 grouping	Single double multiple grouping	Expanded subdivision	
Age	Pearson Correlation	0.01	0.067	0.089
	Sig. (2-tailed)	0.878	0.284	0.157
	N	257	257	257
Tumor size	Pearson Correlation	−0.038	−0.019	−0.022
	Sig. (2-tailed)	0.555	0.776	0.731
	N	239	239	239
FIGO stage	Pearson Correlation	0.004	−0.027	−0.033
	Sig. (2-tailed)	0.948	0.681	0.606
	N	240	240	240
Vascular cancer thrombus	Pearson Correlation	0.013	0.031	−0.016
	Sig. (2-tailed)	0.85	0.664	0.825
	N	204	204	204
Lymph node metastasis	Pearson Correlation	0.008	−0.011	−0.026
	Sig. (2-tailed)	0.913	0.876	0.718
	N	202	202	202
Degree of differentiation	Pearson Correlation	−0.010	−0.012	0.020
	Sig. (2-tailed)	0.908	0.886	0.809
	N	144	136	149

the above research results. Due to the limited sample capacity of this study, we need to conduct large-scale multicenter epidemiological surveys in the future to gain a more comprehensive knowledge of the correlation between HPV multiple infections and cervical lesions and cancer.

Multiple HPV infections and age

An abundance of research studies have shown that HPV infection is age-related, and multiple infections show different characteristics at different ages, with a central tendency trend of age (Guo et al., 2007; Saslow et al., 2012). Anna et al. (Iacobone et al., 2019) showed a negative correlation between age and the number of HPV infection types, proving that multiple infections typically happen to sexually active young women. However, some literature studies show that the incidence of HPV multiple infections is higher in elderly patients with cervical carcinoma, which may be related to the decrease in the immune system and significant changes in hormone levels (Saslow et al., 2012). The results of our study show that most of the patients with multiple HPV infections are younger than 40 years ($\leq$ 30 years old: 44.4%, 31–40 years old: 38.8%) and older than 70 years (66.7%). We speculate that this may be related to factors such as sexual activity, fluctuations in hormone levels, decreased immunity, and an increased probability of causing latent viral infections. Our results are consistent with previous reports. In our study, the HPV16, 18, 58, and 33 subtypes were the main subtypes among patients with multiple infections, with HPV16 being the most predominant. Biryukov et al. (Biryukov and Meyers, 2018) found that HPV16 is more likely to adhere to the surface of cervical epithelial cells and enter them, which may be the cause for the higher incidence of infection, compared to other types. Therefore, we believe that multiple infections are mainly high-risk mixed infections, and for mixed infections of the HPV16, 18, 58, and 33 subtypes, we should pay more attention to the clinical treatment.

Multiple HPV infections and prognosis

Joo et al. used *in situ* hybridization and polymerase chain reaction (PCR) to detect HPV integration status and prognosis in 204 patients undergoing chemotherapy for cervical carcinoma. The authors found that HPV integration is a biomarker that predicts disease-free survival after cervical carcinoma (Joo et al., 2017). From this, we can see that the integrated state of HPV may be regarded as one of the markers of disease progression. In addition, there are also reports that HPV integration occurs at the initial stage of cervical lesions, and the mixed type is more likely to predict disease progression than the fully integrated type. Some scholars have found that squamous cell carcinoma has a higher HPV integration rate compared to adenocarcinoma. HPV integration provides a selective growth superiority in infected cells, which is related to the failure of therapy and shortened disease-free survival (Flores, 2006; Guo et al., 2007; Joo et al., 2017). However, the association between the integration status of HPV and the progression of cervical lesions still needs further research. DE BROT et al. (De Brot et al., 2017) suggest that there are multiple HPV infections in cervical lesions; however, there is still a lack of clear correlation between them and cervical histology.

TABLE 9 Analysis of multiple HPV infections and pathological types.

		ADC	SCC	Total	$\chi 2$	p
0–1 grouping	Single subtype	26	145	171	1.493	0.222
	Non-single infection	8	75	83		
Single double multiple grouping	Single subtype	26	145	171	3.335	0.189
	Double subtypes	8	57	65		
	Multiple subtypes	0	18	18		
Expand Subdivision	Single subtype	26	145	171	3.335	0.649
	Double subtypes	8	57	65		
	Three subtypes	0	12	12		
	Four subtypes	0	3	3		
	Five subtypes	0	2	2		
	Six subtypes	0	0	0		
	Seven subtypes	0	1	1		
	Total	34	220	254		

The results of this study suggest that there is no significant difference between single and multiple HPV infections in terms of pathological classification, tissue differentiation, FIGO staging, tumor size, vascular cancer thrombi, and lymph node metastasis. We believe that the prognosis of cervical cancer is influenced by multiple factors such as diagnostic timing, treatment methods, tumor characteristics, and HPV integration and is the result of a combination of multiple factors.

Conclusion

In summary, multiple types of HPV infection in the cervix are common. However, there is currently no consensus at home or abroad on the occurrence, development, and impact of multiple HPV subtypes of infection on cervical lesions and cervical carcinoma. This study found that multiple infections, mainly HPV16, are closely interrelated to cervical carcinoma. For the HPV16, 18, 58, 33, and 52 subtypes of infection, especially for patients younger than 40 years and older than 70 years who have multiple infections with the HPV16, 18, 58, and 33 subtypes, priority should be given to close follow-up, prevention, and active treatment. The relation between multiple HPV infections and the evolvement and prognosis of cervical carcinoma requires a large sample study from multiple centers, which could better guide cancer prevention and treatment.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL and SH processed the case and drafted the manuscript. JN and YL assisted in collecting case data and literature, and conducted all literature and article work. JW made many constructive suggestions during the revision and production process. XW contributes to the statistical analysis of case data. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Akeel, R. A. (2015). Identification of HPV integration and genomic patterns delineating the clinical landscape of cervical cancer. *Asian Pac. J. Cancer. Prev.*16, 80. doi: 10.7314/APJCP.2015.16.18.8041

Aleksioska-Papestiev, I., Chibisheva, V., Micevska, M., and Dimitrov, G. (2018). Prevalence of specific types of human papiloma virus in cervical intraepithelial lesions and cervical cancer in macedonian women. *Med. Arch.* 72, 26–30. doi: 10.5455/medarh.2018.72.26-30

Andersson, S., Safari, H., Mints, M., Lewensohn-Fuchs, I., Gyllensten, U., and Johansson, B. (2005). Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (*CIN). Br. J. Cancer* 92, 2195–2200. doi: 10.1038/sj.bjc.6602648

Arbyn, M., Weiderpass, E., Bruni, L., de Sanjosé, S., Saraiya, M., Ferlay, J., and Bray, F. (2020). Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. *Lancet Glob. Health* 8, e191–e203. doi: 10.1016/S2214-109X(19)30482-6

Biryukov, J., and Meyers, C. (2018). Superinfection exclusion between two high -risk human papilloma virus types during a coinfection. *J. Virol.* 92, e1917–e1993. doi: 10.1128/JVI.01993-17

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin. 68,* 394–424. doi: 10.3322/caac.21492

Chen, W., Zheng, R., Baade, P. D., Zhang, S., Zeng, H., Bray, F. (2016). Cancer statistics in China, 2015. *CA Cancer J. Clin.* 66, 115–132. doi: 10.3322/caac.21338

Chowell, G., Viboud, C., Simonsen, L., Miller, M. A., Echevarría-Zuno, S., González-León, M., and Borja Aburto, V. H. (2012). Impact of antiviral treatment and hospital admission delay on risk of death associated with 2009 A/H1N1 pandemic influenza in Mexico. *BMC Infect. Dis.* 12, 1–11. doi: 10.1186/1471-2334-12-97

Chunyou, H. B., and Wang, H. L. (2019). Clinical analysis of the impact of single and multiple HPV infections on cervical lesions. *Chin. Med. Sci.* 9, 136–138.

De Brot, L., Pellegrini, B., Moretti, S. T., Carraro, D. M., Soares, F. A., Rocha, R. M. (2017). Infections with multiple high-risk HPV types are associated with high-grade and persistent low-grade intraepithelial lesions of the cervix. *Cancer Cytopathol.* 125, 138–143. doi: 10.1002/cncy.21789

de Freitas, A. C., da Conceicao Gomes Leitao, M., and Campos Coimbra, E. (2015). Prospects of molecuarrly-targeted therapies for cervical cancer treatment. *Curr. Drug Targets*16, 77–91. doi: 10.2174/1389450116666141205150942

Flores, R, Papenfuss, M, Klimecki, W. T, Giuliano, A. R. (2006). Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia. *Int. J. Cancer* 118, 1187–1193. doi: 10.1002/ijc.21477

Ghimire, L. V., and Moon-Grady, A. J. (2020). Among pediatric patients hospitalized for influenza infection,pre -existing cardiomyopathy confers significantly higher morbidity and mortality. *Am. J. Cardiol.* 137, 138–139. doi: 10.1016/j.amjcard.2020.10.024

Groves, I. J., and Coleman, N. (2018). Human papillomavirus genome integration in squamous carcinogenesis: what have next-generation sequencing studies taught us? *J. Pathol.* 245, 9–18. doi: 10.1002/path.5058

Guo, M., Sneige, N., Silva, E. G., Jan, Y. J., Cogdell, D. E., Lin, E., et al. (2007). Distribution and viral load of eight oncogenic types of human papillomavirus (HPV) and HPV 16 integration status in cervical intraepithelial neoplasia and carcinoma. *Mod. Pathol.* 20, 256–266. doi: 10.1038/modpathol.3800737

Homaira, N., Briggs, N., Oei, J. L., Hilder, L., Bajuk, B., Snelling, T. (2019). Impact of influenza on hospitalization rates in children with a range of chronic lung diseases. *Influenza Other Respir Viruses* 13, 233–239. doi: 10.1111/irv.12633

Hu, Z., and Ma, D. (2018). The precision prevention and therapy of HPV-related cervical cancer: new concepts and clinical implications. *Cancer Med.* 7, 5217–5236. doi: 10.1002/cam4.1501

Huang, S., Hao, D., Zhang, Y. (2017). The mechanism and clinical detection progress of high-risk HPV DNA integration leading to cervical cancer. *Genetics* 39, 775–83. doi: 10.16288/j.yczz.17-151

Husain, R. S., and Ramakrishnan, V. (2015). Global variation of human papillomavirus genotypes and selected genes involved in cervical malignancies. *Ann Glob Health* 81, 675–683. doi: 10.1016/j.aogh.2015.08.026

Iacobone, A. D., Bottari, F., Radice, D., Preti, E. P, Franchi, D., Urbinati, A. M. V, et al. (2019). Distribution of high-risk human papillomavirus genotypes and multiple infections in preneoplastic and neoplastic cervical lesions of unvaccinated women: a cross-sectional study. *J. Low Genit. Tract. Dis.* 23, 259–264. doi: 10.1097/LGT.0000000000000487

Joo, J., Shin, H. J., Park, B., Park, S. Y., Yoo, C. W., Yoon, K. A., et al. (2017). Integration pattern of human papillomavirus is a strong prognostic factor for disease-free survival after radiation therapy in cervical cancer patients. *Int. J. Radiat. Oncol. Biol. Phys.* 98, 654–661. doi: 10.1016/j.ijrobp.2017.02.226

Kim, S. Y., Hwang, K. A., Ann, J. H., Kim, J. H., and Nam, J. H. (2021). Next-generation sequencing for typing human papillomaviruses and predicting multi-infections and their clinical symptoms. *Microbiol. Immunol.* 65, 273–278. doi: 10.1111/1348-0421.12927

Salazar, K. L., Zhou, H. S., Xu, J., Peterson, L. E., Schwartz, M. R., Mody, D. R., et al. (2015). Multiple human papilloma virus infections and their impact on the development of high-risk cervical lesions. *Acta. Cytol.* 59, 391–398. doi: 10.1159/000442512

Saslow, D., Solomon, D., Lawson, H. W., Killackey, M., Kulasingam, S. L., Cain, J., et al. (2012). American cancer society, American society for colposcopy and cervical pathology, and American society for clinical pathology screening guidelines for the prevention and early detection of cervical cancer. *Am. J. Clin. Pathol.* 137, 516–542. doi: 10.1309/AJCPTGD94EVRSJCG

Tagarro, A., Cruz-Cañete, M., Otheo, E., Launes, C., Couceiro, J. A., Pérez, C., et al. (2019). Oseltamivir for the treatment of influenza in children and adolescents. *Anal Pediatr.* 90, 317. doi: 10.1016/j.anpede.2019.01.008

Tramuto, F., Restivo, V., Costantino, C., Colomba, G. M. E., Maida, C. M., Casuccio, A. (2019). Surveillance data for eight consecutive influenza seasons in Sicily, Italy. *Am. J. Trop. Med. Hyg.* 101, 1232–1238. doi: 10.4269/ajtmh.19-0059

Vaccine and Immunization Branch of Chinese Preventive Medicine Association (2019). Expert consensus on immune prevention of papillomavirus related diseases such as cervical cancer. *Chin. J. Prev. Med.* 53, 761–803. doi: 10.3760/cma.j.issn.0253-9624.2019.08.001

Vinokurova, S., Wentzensen, N., Kraus, I., et al. (2008). Type-de-pendent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer Res.* 68, 307–313. doi: 10.1158/0008-5472.CAN-07-2754

Xia, L., Qiao, Z., Huijie, Z. (2020). The effect of human papillomavirus type 16 DNA integration on cervical carcinogenesis. *Chin. J. Modern Med.* 30, 28–33.

Xu, C. L., Yuan, F., Chen, M., Sun, S. H., Shi, J. H., Wang, L. J. (2012). Risk factors analysis of hospitalized cases of influenza A (H1N1) related pneumonia in people under 18 years old in China from 2009 to 2010. *Chin. J. Epidemiol.* 33, 404–408. doi: 10.3760/cma.j.issn.0254-6450.2012.04.013

Yokomichi, H., Mochizuki, M., Lee, J. J., Kojima, R., Yokoyama, T., and Yamagata, Z. (2018). Incidence of hospitalization for severe complications of influenza virus infection in Japanese patients between 2012 and 2016. *BMJ Open* 179, e024687. doi: 10.1136/bmjopen-2018-024687

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A replicative recombinant HPV16 E7 expression virus upregulates CD36 in C33A cells

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Objective: In past decades, the role of high-risk HPV (HR-HPV) infection in cancer pathogenesis has been extensively studied. The viral E7 protein expressed in pre-malignant cells has been identified as an ideal target for immunological intervention. However, the cultivation of HPV *in vitro* remains a significant challenge, as well as the lack of methods for expressing the HPV E7 protein and generating replication-competent recombinant viral particles, which posed a major obstacle to further exploration of the function and carcinogenic mechanisms of the E7 oncoprotein. Therefore, it is imperative to investigate novel methodologies to construct replication-competent recombinant viral particles that express the HPV E7 protein to facilitate the study of its function.

Methods: We initiated the construction of recombinant viral particles by utilizing the ccdB-Kan forward/reverse screening system in conjunction with the Red/ExoCET recombinant system. We followed the infection of C33A cells with the obtained recombinant virus to enable the continuous expression of HPV16 E7. Afterwards, the total RNA was extracted and performed transcriptome sequencing using RNA-Seq technology to identify differentially expressed genes associated with HPV-induced oncogenicity.

Results: We successfully established replicative recombinant viral particles expressing HPV16 E7 stably and continuously. The C33A cells were infected with recombinant viral particles to achieve overexpression of the E7 protein. Subsequently, RNA-Seq analysis was conducted to assess the changes in host cell gene expression. The results revealed an upregulation of the CD36 gene, which is associated with the HPV-induced oncogenic pathways, including PI3K-Akt and p53 signaling pathway. qRT-PCR analysis further identified that the upregulation of the CD36 gene due to the expression of HPV16 E7.

Conclusion: The successful expression of HPV16 E7 in cells demonstrates that the replicated recombinant virus retains the replication and infection abilities of Ad4, while also upregulating the CD36 gene involved in the PI3K-Akt signaling and p53 pathways, thereby promoting cell proliferation. The outcome of this study provides a novel perspective and serves as a solid foundation for further exploration of HPV-related carcinogenesis and the development of replicative HPV recombinant vaccines capable of inducing protective immunity against HPV.

KEYWORDS

high-risk HPV, HPV16 E7, Ad4, recombinant HPV16 E7 expression virus, CD36

1. Introduction

The human papillomavirus (HPV) is a small (8,000 bp), non-enveloped double stranded circular DNA virus (Pan et al., 2021; Sun et al., 2021; Wang et al., 2022). Persistent infection with HR-HPVs is highly related with various cancers, including cervical cancer (99.7%), head and neck squamous cell carcinomas (60%), anal cancer (93%), vulvar cancer (69%), vaginal cancer (75%), and penile cancers (47%) (Yang et al., 2019; Meznad et al., 2021). As the most prevalent HR-HPV, HPV16 accounts for approximately 50% of cervical cancer cases (Qmichou et al., 2013), its genome is composed of 6 early genes (E6, E7, E1, E2, E4, and E5) and 2 late genes (L1 and L2) (Zhu et al., 2021). Early genes are responsible for regulating viral transcription and genome replication, while late genes encode capsid proteins and glycoproteins. The sustained expression of E6 and E7 in cell lines has been demonstrated to induce immortalization and transformation in various rodent and human cells (Hawley-Nelson et al., 1989; Hoppe-Seyler et al., 2018; Lou et al., 2022). In some rare cases, long-term persistent HR-HPV infection results in the integration of the oncogenes E6 and E7 into the host DNA (Schneider et al., 2020). This integration disrupts host cell apoptosis and promotes continuous cell proliferation, ultimately leading to cancer development. E7 interacts with pRB, resulting in the inactivation of pRB function and the dissociation of E2F from the E2F/pRB complex, which triggers the G1/S transition (Sitarz et al., 2021). This disruption further enhances the proliferation and transformation of epithelial cells. HPV infection-related malignancies remain a significant global public health concern, especially in developing countries. As the most efficient method to prevent HPV infection, HPV prophylactic vaccine has been introduced for more than a decade (Zhou et al., 2019; Yang et al., 2022). However, prophylactic HPV vaccines only target the HPV late genes L1 and/or L2. However, in high-grade lesions or HPV-associated malignancies, the prophylactic vaccine is ineffective since L1 and L2 are lost due to HPV integration into the host genome (Cheng et al., 2018). Previous studies demonstrated that the consistent overexpression of HPV E7 oncoproteins is required throughout the process of cervical epithelial cell carcinogenesis (Domingos-Pereira et al., 2021). Hence, HPV E7 gene is an optimal target for the treatment and prevention of HPV-induced cancers (Domingos-Pereira et al., 2021; Peng et al., 2022). However, since HPV life cycle is tightly linked to the host cell differentiation, the virus is extremely difficult to isolate and culture (Wang et al., 2022). Therefore, the construction of recombinant viruses is of great significance in overcoming these obstacles and paving the way for further exploration of the pathogenesis and treatment of HPV-related cancers.

Adenovirus (Ad) is one of the most commonly used vectors for gene therapy and possesses many advantages over other viral vectors, including high transduction efficiency, broad tissue affinity, and non-integration into the host genome (McKenna et al., 2020; Liu et al., 2021). The utilization of Ad as a vector offers several benefits, such as the effective transduction of target cells at a low multiplicity of infection (MOI), the presence of well-established techniques for manipulation and propagation, and a relatively safe profile due to the viral genome is not integrated into the host genome. Additionally, Ad vectors can deliver large therapeutic genes (approximately 37 kb) (Douglas, 2007; Wu et al., 2015). It is worth mentioning that a previous study indicated that CR1 and CR2 in Ad E1A contain the pRb binding domain, and the Ad E1A (12S) protein shares structural and functional similarities with the HPV E7 protein (Lou et al., 2022; Nouel and White, 2022). The E7 N terminus comprises two regions that exhibit sequence similarity with a segment of conserved region 1 (CR1) and conserved region 2 (CR2) found in the adenovirus E1A protein (Ad E1A) (Nouel and White, 2022). Similar to the Ad E1A antigen, the HPV E7 proteins interact with the retinoblastoma tumor suppressor protein pRB and the related "pocket proteins" p107 and p130 through a conserved LXCXE sequence within CR2 sequences (Dürst et al., 1983; Dyson et al., 1992). These pocket proteins play a crucial role in regulating the activities of the E2F family of transcription factors, which control multiple cell cycle transitions and other cellular activities (Cam and Dynlacht, 2003). The ability of HPV E7 and Ad E1A antigen to associate with pRB plays a critical role in their ability to generate and/or maintain a host cellular milieu conducive to viral genome replication. Several studies have provided evidence that the E6 and E7 proteins of HPV play a role in supporting the DNA replication of Ad that lack the E1A and E1B genes (Steinwaerder et al., 2001), which suggests HPV E7 protein may serve as partial substitutes for Ad E1 proteins in the replication of viral DNA. Hence, we posit that the integration of HPV E7 into the Ad E1A region to generate a replicative recombinant virus capable of expressing HPV E7.

However, due to the significant disparity in size between the Ad genome (approximately 36 kilobases) and the HPV16 E7 gene (a mere 270 base pairs), the precise integration of the HPV16 E7 gene into the E1A region of Ad in a single attempt presents a big challenge. Hence, we designed a novel approach to accurately generate a replicative recombinant HPV16 E7 expression virus (Ad4-HPV16E7), which involved the integration of the HPV E7 gene into the EGFP-Tagged Ad4 E1A region utilizing the ccdB-Kan forward/reverse screening system in conjunction with the Red/ExoCET recombinant system. The establishment of this approach can provide novel ideas and basis for further investigations into the role of HPV16 E7 in cancer pathogenesis, as well as potential therapeutic approaches targeting viral infections and tumor growth.

2. Materials and methods

2.1. Bacterial strains, plasmids, cell culturing

All *Escherichia coli* strains and plasmids used in this study are listed in Supplementary Table S1. *Escherichia coli* strains were grown in LB medium at 30°C or 37°C and selected with appropriate antibiotics [chloramphenicol (Cm), 10 μg/mL; ampicillin (Amp), 10 μg/mL; kanamycin (Kan), 10 μg/mL and tetracycline (Tet), 34 μg/mL]. The concentration of 10% L-arabinose used for induction was 25 mg/mL. Ad4 was purchased from ATCC. C33A cells was purchased from Cell Resource Center of Peking Union Medical College. HEK293T, Siha, Hela and Caski cells were preserved in our laboratory. The cells were maintained in DMEM (EallBio, China) or MEM, RPMI1640 supplemented with 10% FBS and 1% penicillin–streptomycin (P/S) and incubated at 37°C with 5% humidified CO_2.

2.2. Molecular docking

To simulate Ad4 E1A-HPV16 E7 highly accurate 3D homology model structures, Autodock Vina 1.2.2, a silico protein–protein

docking software (Zhang et al., 2023) was employed. The 3D coordinates of Ad4 E1A (PDB ID, 2R7G; resolution, 1.67 Å) and HPV16 E7 (PDB ID, 4YOZ; resolution, 2.25 Å) were downloaded from the PDB.[1] All protein files were converted into PDBQT format with all water molecules excluded and polar hydrogen atoms were added. Molecular docking studies were performed by Autodock Vina 1.2.2.[2]

2.3. Generation of recombinant virus Ad4-HPV16E7

2.3.1. Construction of recombinant HPV16E7 expression virus plasmid

Combination of ccdB screening system and Red-recombination to construct recombinant HPV16E7 expression virus plasmid. Only plasmids carrying ccdB gene can survive in ccdB-resistant GB08Red gyr462 strains. CcdB and Kan genes was inserted into EGFP-Tagged Ad4 E1A region, then electroporated into *E. coli* GB08Red gyr462 electrocompetent cells for Red-recombination (Zhou et al., 2019; Huang et al., 2021; Yang et al., 2022) to construct recombinant ccdB-Kan expression vector, and positive recombinant bacteria were screened in LB solution containing kanamycin. The recombinant ccdB-Kan expression vectors were, respectively, used to construct recombinant HPV16E7 virus plasmid PBR322-Ad4-E1Amut-C16E7P by subsequent ExoCET recombination (Yu et al., 2019; Schneider et al., 2020; Sitarz et al., 2021).

2.3.2. Generation of recombinant virus particles

The recombinant HPV16E7 expression virus plasmid PBR322-Ad4-E1Amut-C16E7P was linearized with *AsisI* digestion to released recombinant viral DNA Ad4-HPV16E7 and purified by Phenol-chloroform extraction followed by ethanol precipitation. Lipo-3000 (ThermoFisher, USA) was used to transfect Ad4-HPV16 E7 in HEK293T cells to produce viral particles. To verify the cervical cancer cells infectivity of the recombinant viruses, SiHa, Caski, HeLa and C33A cells were infected with recombinant virus.

2.3.3. Determination of virus titer

HEK293T cells were infected with different concentrations of Ad4 and Ad4-HPV16E7 virus, and without virus infection as control group. Ten multiple Wells were set up in each group, and the fluorescence number of cells in each well was counted each 2 days until the 10th day of culture. There were 10 multiple wells in each group, and the fluorescence points of each well were counted each 2 days until the 10th day of culture. On the 10th day, the number of fluorescent spots in Ad4 and Ad4-HPV16E7 virus infected cells were counted, and then TCID50 was calculated by the Karber method.

2.4. qRT-PCR analysis

To assess the transcript levels of the E7 gene of the Ad4-HPV16E7 virus, C33A cells were infected with the recombinant virus Ad4-HPV16 and Ad4 (control group) for a duration of 24 h. Subsequently, the RNA of the cells was collected and subjected to qRT-PCR to determine the levels of E7 gene transcripts. Primers for qRT-PCR detection of HPV16 E7 were16E7-F (forward primer, 5-AGGAGGAGGATGAAATAGATGG-3) and 16E7-R (reverse primer, 5-GCACAACCGAAGCGTAGA-3); Primers for qRT-PCR detection of GPDH were GPDH-F (forward primer, 5-GGAAGGTGAAGGTCGGAGTC -3) and GPDH-R (reverse primer, 5- GAAGGGGTCATTGATGGCAAC -3).

2.5. Western blotting

To evaluate Hexon/E7 protein expression levels of Ad4-HPV16E7 virus, HEK293T cells were infected with recombinant virus Ad4-HPV16E7 and Ad4 (control group) respectively for 24 h, without viral infection were used as a blank control group, then the cell total protein was extracted with a cell lysis buffer (120 mM NaCl, 0.5% NP-40, 50 mM Tris–HCl pH 8.0, and 1 mM PMSF) and evaluated by BCA methods. Whole cell extracts (30–45 μg) were eparated by 12% SDS-PAGE and then transferred to NC membranes and incubated with 5% skim milk in TBST at room temperature for 1.5 h. After that, the membranes were probed with primary antibodies at a 1:200–1:500 dilution overnight at 4°C: Rabbit anti-HPV 16 E7 antibody (cat. no. 67017-1-Ig; ProteinTech Group, Inc., Chicago, IL, USA), mouse anti-Hexon antibody (generated in our laboratory), and mouse anti-GPDH antibody (cat. no. 4970; Cell Signaling Technology, Inc., Beverly, MA, USA), then washed with TBST and incubated with goat anti-rabbit (1:5,000; Sigma)/mouse (1:10,000; Sigma) horseradish-peroxidase conjugated secondary antibody for 1 h at room temperature for 2 h. Detection was performed by Gel Detection System (Bio-Rad, USA). Western blotting bands were quantified using ImageJ software.[3]

2.6. Immunofluorescence assays

To confirm the Western Blot results about E7 protein expression levels of Ad4-HPV16E7 virus, HEK293T cells were infected with recombinant virus Ad4-HPV16E7 for 24 h. After that, the cells infected with Ad4-HPV16E7 virus was washed three times with PBS for 10 min per wash, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100, then blocked with 1% BSA. HPV16 E7 in cells was detected with monoclonal rabbit anti-HPV 16 E7 antibody and a secondary goat anti-mouse Alexa Fluor 594 antibody (Invitrogen, 1:1,000) and visualized by fluorescence microscopy.

2.7. RNA-Seq sample collection and preparation

2.7.1. RNA quantification and qualification

C33A cells were subjected to infection with recombinant virus Ad4-HPV16E7 and Ad4 (control group) for a duration of 24 h. A blank control group was also included, which did not undergo viral

1 http://www.rcsb.org/pdb/home/home.do

2 http://autodock.scripps.edu/

3 http://rsbweb.nih.gov/ij/

infection. Total RNA was extracted from all groups using Trizol Reagent (Thermo Fisher Scientific). The RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was employed to assess the quantity and quality of RNA.

2.7.2. Library preparation for transcriptome sequencing

The RNA sample preparations utilized total RNA as the input material. The mRNA was isolated from the total RNA through the utilization of poly-T oligo-attached magnetic beads. Fragmentation was achieved via divalent cations at an elevated temperature in First Strand Synthesis Reaction Buffer (5X). The first strand cDNA was synthesized utilizing random hexamer primer and M-MuLV Reverse Transcriptase, followed by RNA degradation with RNaseH. Subsequently, the second strand cDNA synthesis was performed utilizing DNA Polymerase I and dNTP. The residual overhangs were transformed into blunt ends through the utilization of exonuclease/polymerase activities. Following the adenylation of the DNA fragment's 3′ ends, an adaptor with a hairpin loop structure was ligated to facilitate hybridization. To isolate cDNA fragments ranging from 370 to 420 base pairs in length, the library fragments underwent purification using the AMPure XP system (Beckman Coulter, Beverly, USA). Subsequently, the PCR product was purified using AMPure XP beads, and the library was ultimately obtained via PCR amplification. Following the construction of the library, it was initially assessed using the Qubit2.0 Fluorometer, subsequently diluted to 1.5 ng/μL, and the insert size was determined using the Agilent 2100 bioanalyzer. Upon meeting the expected insert size, qRT-PCR was employed to precisely quantify the effective concentration of the library, ensuring that it surpasses 2 nM and meets the requisite quality standards.

2.7.3. Clustering and sequencing

Once the library has met the necessary qualifications, the various libraries are combined based on their effective concentration and target data volume from the machine, and subsequently sequenced using the Illumina NovaSeq 6000 platform, resulting in a paired-end read of 150 bp. The sequencing process follows the principle of Sequencing by Synthesis, whereby synthesis and sequencing occur simultaneously. The sequenced flowcell was subjected to the addition of four fluorescent labeled dNTP, DNA polymerase, and splice primers, followed by amplification. As the sequence cluster extends the complementary chain, the fluorescence-labeled dNTPs release corresponding fluorescence. The sequencer captures the fluorescence signal and converts it into a sequencing peak through computer software, thereby enabling the acquisition of sequence information pertaining to the fragment under examination.

2.8. RNA-Seq data analysis

2.8.1. Data analysis quality control

The high-throughput sequencer measures image data which is subsequently converted into sequence data (reads) through CASAVA base recognition. The raw data (raw reads) in fastq format undergo initial processing via fastp software, resulting in the acquisition of clean data (clean reads) by eliminating reads containing adapter, N base, and low quality reads from the raw data. Additionally, the Q20, Q30, and GC content of the clean data are computed. The downstream analyses rely on the high-quality clean data.

2.8.2. Permission to reuse and copyright

The reference genome and gene model annotation files were obtained directly from the genome website. The reference genome was indexed using Hisat2 (v2.0.5), and the paired-end clean reads were aligned to it using the same tool. The selection of Hisat2 as the mapping tool was based on its ability to generate a splice junction database from the gene model annotation file, resulting in superior mapping outcomes compared to non-splice mapping tools.

2.8.3. Quantification of gene expression level

The quantification of gene expression levels was performed using featureCounts (v1.5.0-p3) to enumerate the number of reads mapped to each of the two genes. Subsequently, the Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) was computed for each gene, taking into account both the gene length and the mapped reads count. FPKM is a widely adopted method for estimating gene expression levels, as it accounts for the impact of sequencing depth and gene length on the reads count.

2.8.4. Differential expression analysis

The DESeq2 R package (version 1.20.0) was utilized to conduct differential expression analysis between two conditions/groups, each with two biological replicates. DESeq2 employs statistical routines based on the negative binomial distribution to determine differential expression in digital gene expression data. The resulting *p*-values were subjected to the Benjamini and Hochberg's approach for controlling the false discovery rate. Significantly differential expression was defined as padj<=0.05 and|log2(foldchange)| > = 1, which served as the threshold.

2.8.5. Enrichment analysis of differentially expressed genes

The Gene Ontology (GO) enrichment analysis was performed on the differentially expressed genes using the clusterProfiler R package (version 3.8.1), with correction for gene length bias. GO terms that demonstrated a corrected *p*-value of less than 0.05 were considered significantly enriched by the differentially expressed genes. The KEGG database serves as a valuable resource for comprehending the overarching functionalities and utilities of biological systems, encompassing the cell, organism, and ecosystem, through the analysis of molecular-level data, particularly extensive molecular datasets produced by genome sequencing and other advanced experimental methodologies.[4] The statistical enrichment of differential expression genes in KEGG pathways was assessed using the clusterProfiler R package (version 3.8.1). The Reactome database, which consolidates the reactions and biological pathways of human model species, was utilized. Reactome pathways exhibiting a corrected *p*-value of less than 0.05 were deemed significantly enriched by differential expressed genes. Additionally, the DO (Disease Ontology) database, which characterizes the function of human genes and diseases, was employed. DO pathways with a corrected *p*-value of less than 0.05 were also considered significantly enriched by differential expressed genes. The integration of human disease-related genes is facilitated by the DisGeNET database. DisGeNET pathways exhibiting a corrected *p*-value of less than 0.05 were deemed significantly enriched by differential expressed genes. The statistical enrichment of differentially expressed genes in the Reactome

4 http://www.genome.jp/kegg/

pathway, the DO pathway, and the DisGeNET pathway was assessed using the clusterProfiler R package (version 3.8.1).

2.9. Protein–protein interactions network

The STRING database[5] aims to provide a comprehensive assessment and integration of PPIs, encompassing both indirect (functional) and direct (physical) associations (Xia et al., 2020). To evaluate the interactive relationship among differentially expressed genes (DEGs), the DEGs were firstly mapped to STRING. Only experimentally validated interactions with a combined score > 0.4 were considered as significant. Subsequently, the PPIs network was constructed using the Cytoscape software (Ver. 3.7.1). The plug-in Molecular Complex Detection (MCODE) was employed to screen the modules of PPIs network in Cytoscape.

2.10. Statistical analysis

All the experiments were conducted for three independent repeats. Statistical analysis was conducted using GraphPad Prism software (GraphPad Software). Data are presented as the mean ± SD. Student's t test analysis was performed in differences measured variables between experimental and control groups. One-way ANOVA was performed between multiple groups and conditions. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Ad E1A and HPV E7 are capable of forming a stable protein docking model

To validate the feasibility of incorporating HPV E7 into Ad E1A, we conducted molecular docking analysis to assess their binding affinity and interaction modes. The ZDOCK score values and their Pi stacking interaction mode are shown in Table 1. The ZDOCK Score of Ad4 E1 and HPV E7 was 1202.281, and a docking score of ≥1,000 was considered acceptable. HPV E7 forms several Pi stacking interactions with amino acid sites of Ad E1A such as PHE791-TYR792, HIS554-PHE551, which was indicated by the red arrow in Figure 1. Comprehensive analysis revealed that proteins Ad4 E1A and HPV E7 formed a stable protein docking model.

3.2. The recombinant virus Ad4-HPV16E7 express HPV16 E7 gene

We constructed the recombinant virus plasmids PBR322-Ad4-E1Amut-C16E7P using the ccdB-Kan forward/reverse screening system in combination with the Red/ExoCET recombinant system to precisely insert the HPV E7 gene into the EGFP-Tagged Ad4 E1A region (Figure 2).

TABLE 1 Results of molecular docking.

Receptor	Ligand	ZDOCK score	Pi stacking interaction
Ad E1A (2R7G)	HPV E7 (4YOZ)	1202.281	A: PHE791[occupancy 7]- A: TYR792[occupancy 3] A: HIS554[occupancy 4]- A: PHE551[occupancy 7] A: TYR756[occupancy 3]- A: TYR709[occupancy 3]

To obtain the recombinant viruses Ad4-HPV16E7 and Ad4, HEK293T cells were transfected with plasmids pAd4-HPV16E7 and pAd4 for 24–144 h. Fluorescence observations indicated a significantly higher level of GFP expression in cells infected with Ad4-HPV16E7 compared to cells infected with Ad4. The optimal infection time was between 48 and 96 h (Figure 3A). At 96 h, we collected the medium containing Ad4 and recombinant virus Ad4-HPV16E7, labeling them as the P0 generation. Subsequently, we repeated the infection of HEK293T cells using the P0 medium of Ad4 and Ad4-HPV16E7 for 96 h, resulting in the generation of the P1 viral-containing medium. This procedure was repeated until we obtained the P3 generation viral medium. The viral titer of the recombinant virus Ad4-HPV16E7 in the P3 generation exhibited a substantial increase (Ad4: TCID50 = $10^{-7.34}$/0.1 mL, Ad4-HPV16:TCID50 = $10^{-6.75}$/0.1 mL) (Figure 3B). 24-h infection of HEK293T cells yielded an infection efficiency exceeding 80% by utilizing the P3 viral culture supernatant (Figure 3C), which shortened the experimental duration. Consequently, all subsequent experiments involved a 24-h infection of cells using the P3 viral medium. In order to evaluate the infectivity of the recombinant virus in different cell lines, we conducted infection experiments on SiHa (HPV16 positive), Caski (HPV16 positive), HeLa (HPV18 positive), and C33A (HPV negative) cells. The results indicated that after a 24-h infection, all four cell lines were able to express the GFP protein. However, the infection efficiency in Hela cells was significantly lower compared to SiHa, Caski, and C33A cells (Figure 3C).

We utilized the generated recombinant virus Ad4-HPV16E7 to infect the HPV-negative cell line C33A to evaluate the stability of HPV16 E7 gene transcription. RT-qPCR analysis revealed that the presence of the HPV16 E7 gene was exclusively detected in the Ad4-HPV16E7 group, while no amplification signal was observed in the Ad4 group as expected (Figure 3D). These results indicate that the HPV16 E7 gene of the recombinant virus Ad4-HPV16 was effectively transcribed in C33A cells.

Further investigation was conducted to assess the expression of HPV16 E7 protein by the recombinant virus Ad4-HPV16E7. Western blot analysis demonstrated that HEK293T cells infected with Ad4-HPV16E7 exhibited the expression of HPV16 E7 protein, whereas E7 expression was not detected in cells infected with Ad4 virus or in the control group (Figure 3E). To further identify the expression of HPV16 E7 protein by Ad4-HPV16E7, cellular immunofluorescence assay was performed, as shown in Figure 3F. The results demonstrated that the expression of HPV16 E7 protein (bright red) was observed in C33A cells infected with the recombinant virus Ad4-HPV16E7, while no such expression was observed in the control group. The primary capsid protein of adenovirus, known as Hexon (Wodrich et al., 2003), indirectly reflects the virus replication capacity

5 http://string-db.org

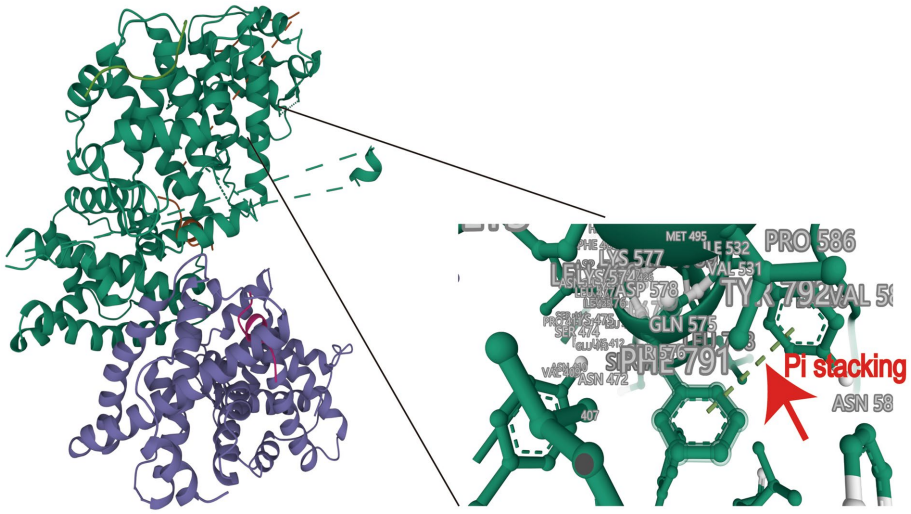

FIGURE 1
Molecular docking of HPV16 E7 onto Ad4 E1A and protein docking model. A docking score of ≥1,000 was considered acceptable.

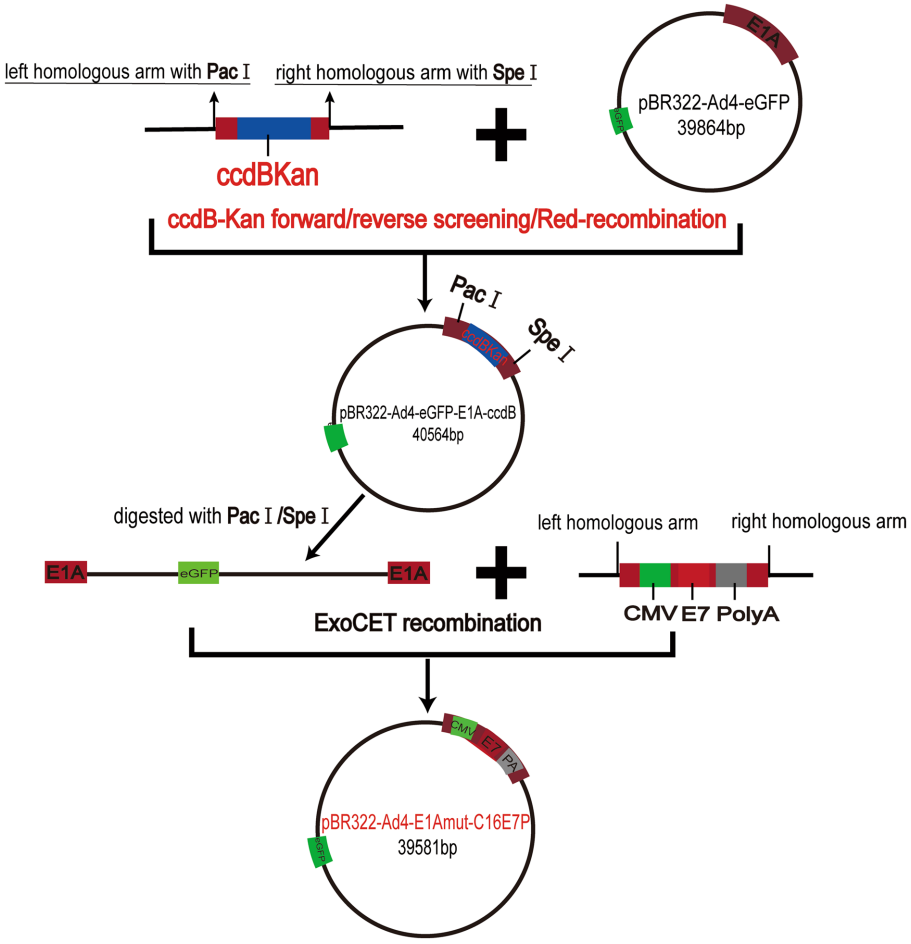

FIGURE 2
Schematic diagram of the recombinant virus plasmids PBR322-Ad4-E1A mut-C16E7P constructs.

through its expression level. Therefore, we also wish to verify whether the recombinant virus maintained the replication ability of Ad4. The expression of Hexon protein in HEK293T cells infected by Ad4 and Ad4-HPV16E7 was evaluated. The results revealed that both Ad4 and Ad4-HPV16E7-infected cells expressed Hexon protein. Interestingly, the expression of Hexon in the recombinant virus Ad4-HPV16E7 was

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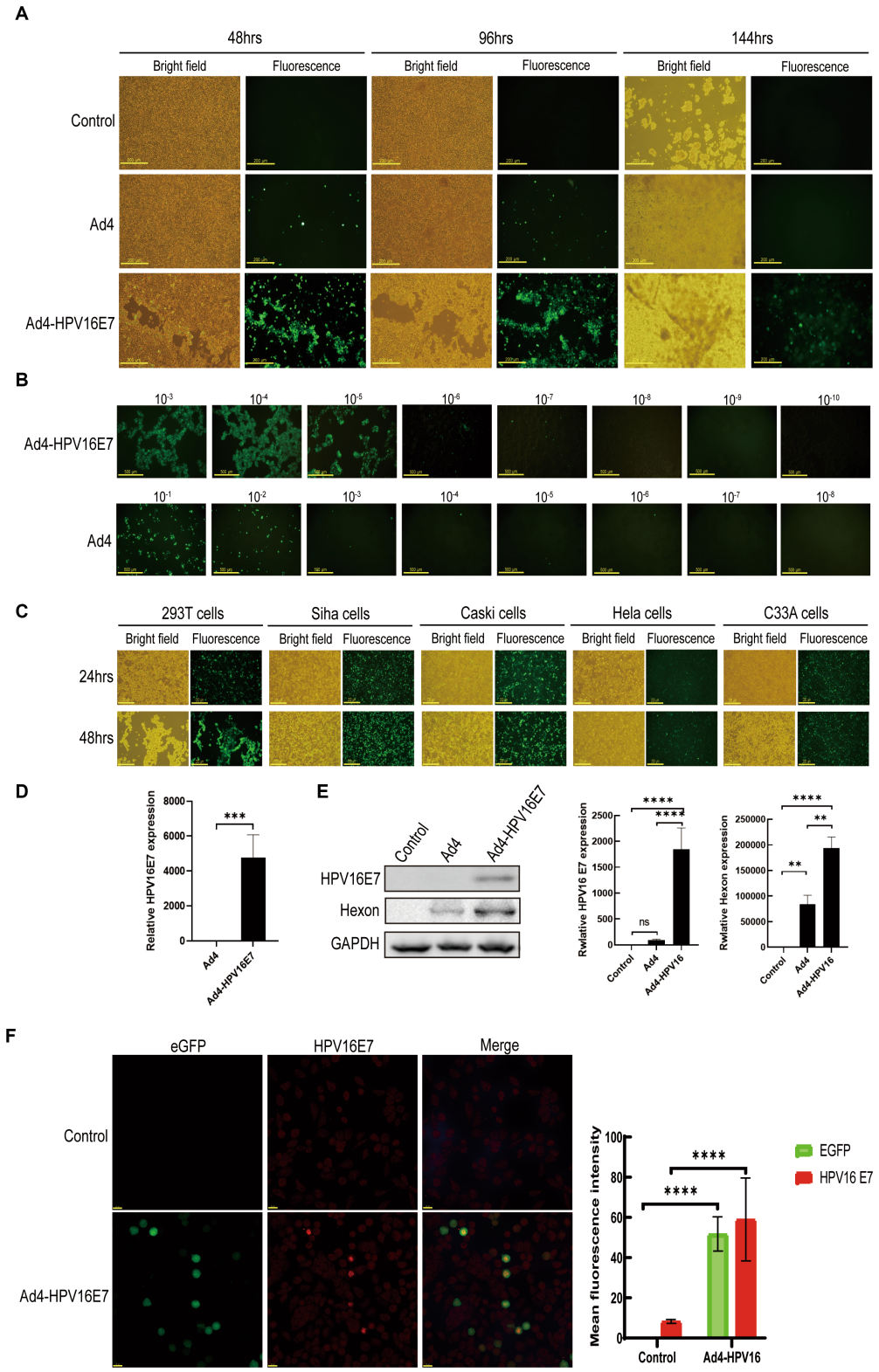

FIGURE 3
Generation of the recombinant virus Ad4-HPV16E7 and evaluation of the HPV16 E7 gene. **(A)** HEK293T cells were transfected with the pAd4-HPV16E7 and pAd4 to obtain the recombinant viruses Ad4-HPV16E7 and Ad4. **(B)** HEK293T cells were infected with eight 10-fold serial dilutions of Ad4-HPV16E7 and Ad4 viruses to calculate the viral titer. **(C)** HEK293T, SiHa, Caski, HeLa, and C33A (HPV negative) cells were able to express the GFP protein. **(D)** RT-qPCR analysis revealed that the HPV16 E7 gene was exclusively detected in C33A cells after the infection of Ad4-HPV16E7 virus. **(E)** The expression of HPV16 E7 and Hexon proteins were detected by Western Blot. **(F)** The expression of HPV16 E7 protein were detected by cellular immunofluorescence assay. Control: cells that are not infected with virus; Ad4, Cells infection with Ad4 virus; Ad4-HPV16E7, Cells infection with Ad4-HPV16E7 virus. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ were considered statistically significant; ns, non-significant.

higher compared to the Ad4 virus (Figure 3E), further suggests that replication capacity of Ad4-HPV16E7 might be better than Ad4 virus.

3.3. A total of 525 genes were up-regulated and 1029 genes were down-regulated in C33A cells infected with the Ad4-HPV16E7 virus compared to that with Ad4

C33A cells were infected by recombinant Ad4-HPV16E7 and Ad4 viruses for 24 h. Total RNA extracted from the cells served as input for RNA sample preparations. Data with an error rate below 0.02% (Figure 4A) and stable GC content were selected for analysis (Figure 4B). Raw sequencing data underwent filtration to ensure data quality and reliability. Following filtration, the clean reads percentages were 96.20, 95.68, and 96.43% for the control, Ad4, and Ad4-HPV16E7 groups, respectively (Figure 4C). The HISAT2 software precisely aligned the clean reads with the reference genome, providing positional information (Dai et al., 2022). Read counts in exonic, intronic, and intergenic regions were determined. The proportion of clean reads in the exon region was 91.15, 88.64, and 93.19% for the control, Ad4, and Ad4-HPV16E7 groups, respectively (Figure 4D).

The expression values of all genes (FPKM) in each sample were computed, and box plots were used to visualize the distribution of gene expression levels in different samples (Figure 5A). Correlation coefficients were calculated based on FPKM values to assess sample correlations within and between groups, which were further utilized to generate the sample correlation heat map (Figure 5B). Subsequently, the comparative analysis of differentially expressed genes (DEGs) was conducted. The statistical histogram revealed 4,309 DEGs in the Ad4 group compared to the control group, while the Ad4-HPV16E7 group exhibited 6,265 DEGs compared to the control group (Figure 5C). To examine the distribution of genes showing significant expression variations between the Ad4-HPV16E7 group and Ad4 group. The volcano plot (Figure 5D) illustrated 525 up-regulated genes and 1,029 down-regulated genes in the Ad4-HPV16E7 group compared to the Ad4 group.

3.4. A biologically interconnection of DEGs as a collective entity

A comprehensive gene search was conducted using OMIM[6] and Genotype[7], resulting in the identification of 39 genes and 377 genes related to HPV infection, respectively. PubMed[8] was extensively reviewed, yielding 1,172 genes associated with HPV infection, and an additional 162 genes were obtained from the human papillomavirus infection pathway (hsa05165) in KEGG. These genes were then cross-screened with significantly different genes obtained from RNA-Seq sequencing results. After filtering, a total of 103 differentially expressed genes (DEGs) were identified as HPV infection-related. The filtered genes were further analyzed using the STRING database to construct a protein–protein interactions (PPIs) network. The resulting network consisted of 100 nodes, 398 edges, and an expected number of 133 edges. The average node degree was 7.96, average local clustering coefficient was 0.566, and the PPI enrichment p-value was $<1.0^{-16}$ (Figure 6). This enrichment suggests a biologically interconnected nature of the proteins within the network as a collective entity.

3.5. HPV-associated up-regulated genes STAT2, CD36, IFIT1, VMP1, and MUC1 of DEGs

This study conducted an analysis of the 103 DEGs genes associated with HPV infection using GO, KEGG, DO, and DisGeNET. The analysis revealed enriched biological processes, metabolic pathways, and disease associations. The results were visualized through bubble diagrams, presenting the top enriched terms and pathways.

The GO enrichment analysis of biological processes (BP) identified that the 103 genes were mainly involved in the reproductive system, positive regulation of cell migration, gland development, cell fate commitment, mesenchyme development, digestive system and cardiac ventricle development. The analysis of cellular components (CC) indicated enrichment in receptor complexes, apical regions of cells, membrane sides, transcription factor complexes, membrane microdomains, cell–cell adherens junctions, and specific granule membranes. The analysis of cellular components (CC) indicated enrichment in receptor complexes, apical regions of cells, membrane sides, transcription factor complexes, membrane microdomains, cell–cell adherens junctions, and specific granule membranes (Figure 7A). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighted 103 genes were involved in MicroRNAs in cancer, PI3K-Akt signaling pathway, breast cancer, proteoglycans in cancer, human T-cell leukemia virus 1 infection, gastric cancer, EGFR tyrosine kinase inhibitor resistance, prostate cancer, thyroid cancer, Rap1 signaling pathway, JAK–STAT signaling pathway and Ras signaling pathway (Figure 7B). The DO pathway analysis indicated these genes were mainly enriched in head and neck cancer, urinary system cancer, breast carcinoma, endocrine gland cancer and musculoskeletal system cancer (Figure 7C). Furthermore, the DisGeNET pathway analysis indicated these genes were mainly enriched in meningioma, invasive carcinoma of breast, papillary thyroid carcinoma, renal carcinoma, precursor T-cell lymphoblastic leukemia-lymphoma, precancerous conditions and tumor initiation (Figure 7D). Cluster analysis was conducted on the expression levels of 103 DEGs genes in the Ad4-HPV16E7, Ad4, and control groups, as depicted in the heat map (Figure 7E). Based on cluster analysis and incorporating previously reported HPV infection-related genes from the literature, we further screened five up-regulated genes (STAT2, CD36, IFIT1, VMP1, and MUC1) in the Ad4-HPV16E7 group that were expressed relative to the Ad4 and control groups (Figure 7F).

3.6. Infection of recombinant virus Ad4-HPV16E7 induces upregulation of CD36 gene in C33A cells

Recent studies indicate that the presence of E7 in HPV-positive cells disrupts the tumor suppressor pRb and affects various signaling

6 https://www.ncbi.nlm.nih.gov/omim
7 https://www.ncbi.nlm.nih.gov/gap/phegeni
8 https://pubmed.ncbi.nlm.nih.gov

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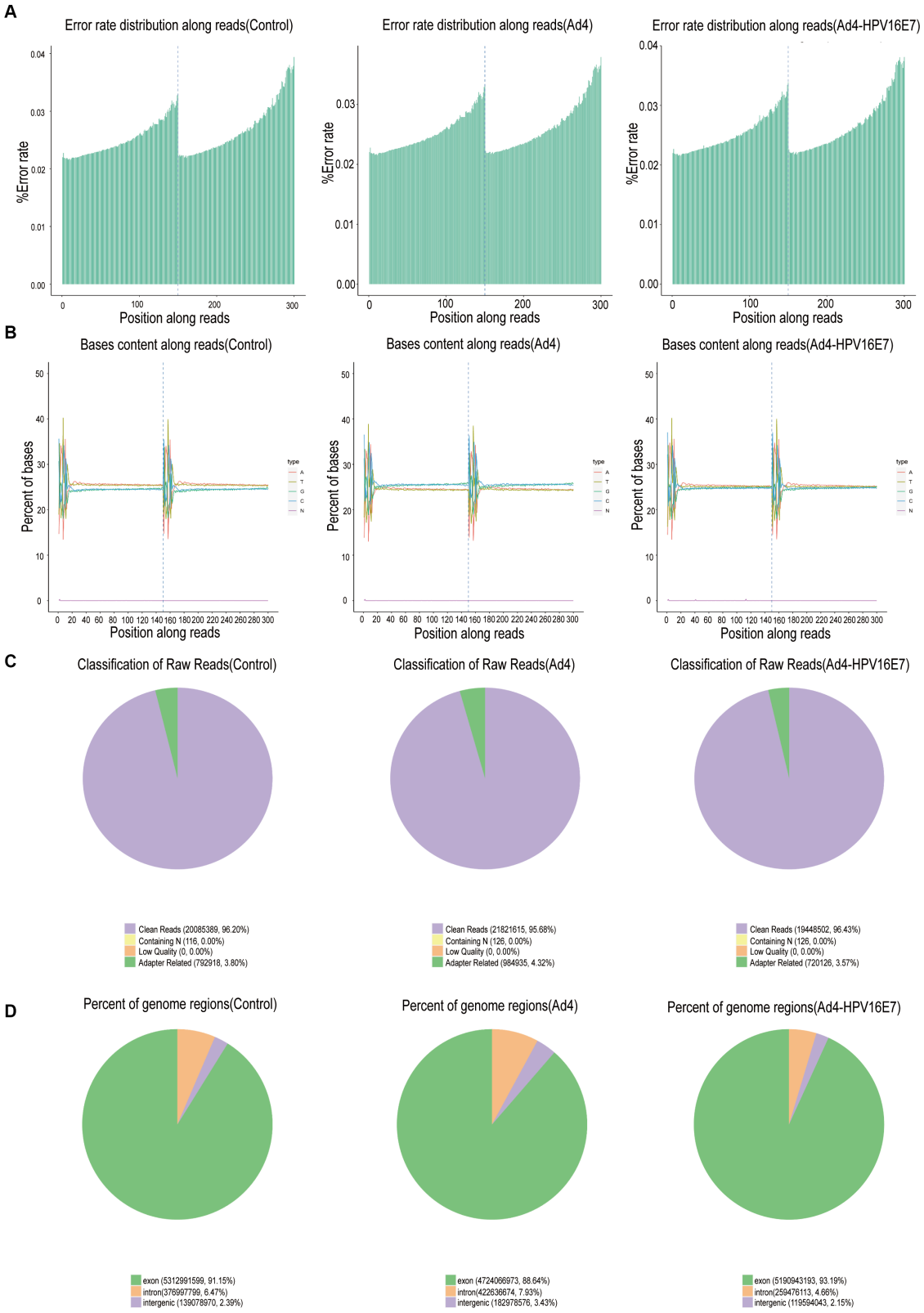

FIGURE 4
Basic information on RNA-Seq. **(A)** Distribution of sequencing data error rate. Data with an error rate below 0.02% was selected for analysis. **(B)** Distribution of GC content. **(C)** The filtered sequencing data of per sample. **(D)** Distribution of sequencing reads in different regions of the genome. The threshold range of the percentage of clean reads greater than 80% indicates that qualified for downstream analyses.

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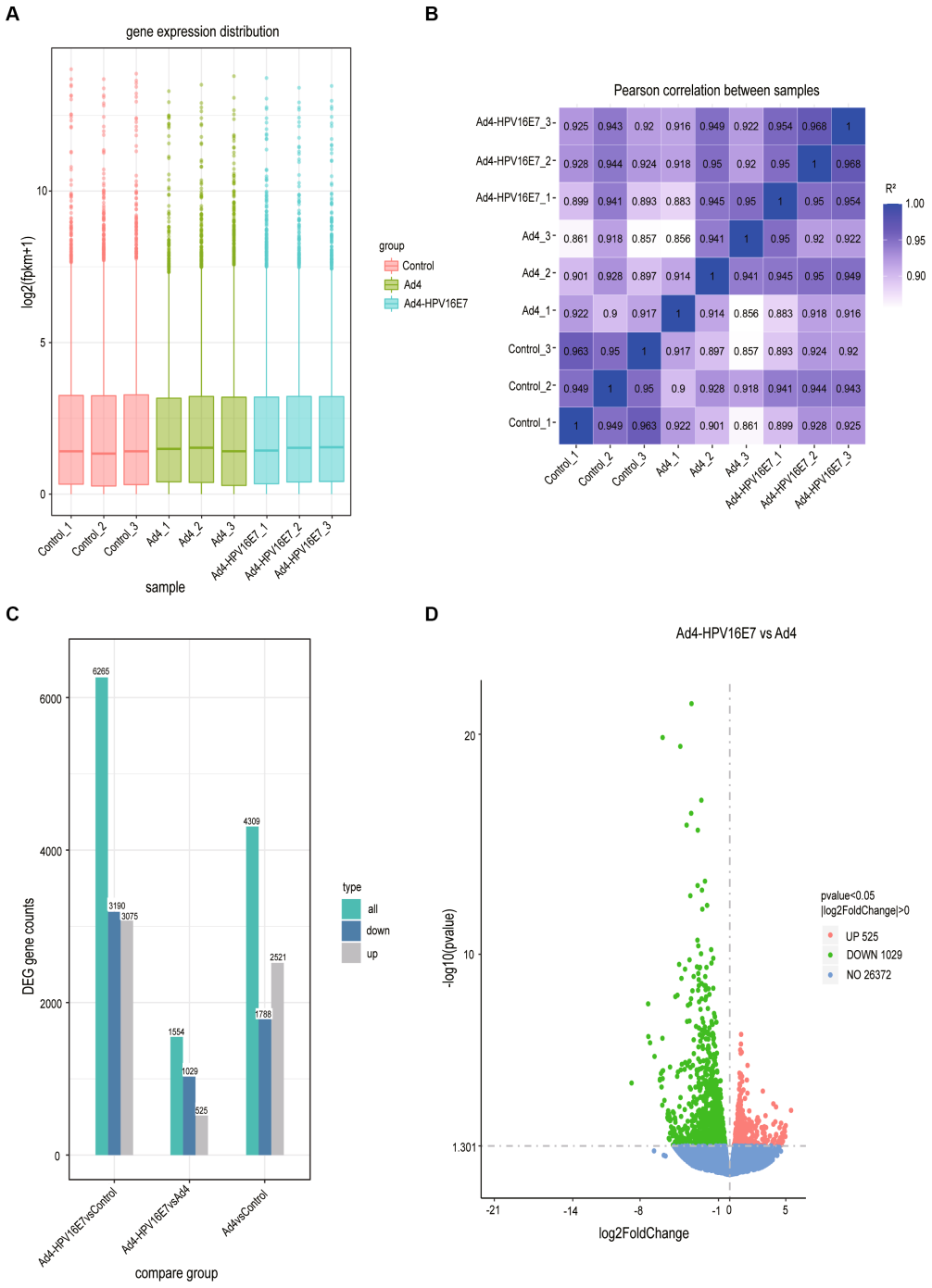

FIGURE 5
The comparative analysis of differentially expressed genes (DEGs). **(A)** Box plot of sample gene expression distribution. **(B)** Heat map of correlation between samples. $R^2 > 0.8$ were considered a strong correlation. **(C)** Statistical histogram of the number of significantly DEGs in the different comparison combination. **(D)** Volcano map of DEGs between the Ad4-HPV16E7 group and Ad4 group.

pathways. The PI3K/Akt signaling cascade is particularly significant in HPV-induced carcinogenesis. Our KEGG pathway analysis confirmed the significant involvement of the 103 genes in the PI3K/Akt signaling pathway. Previous research has highlighted the importance of the PI3K/AKT/mTOR network in mediating communication between HPV-positive cancer cells under normoxic and hypoxic conditions (Bossler et al., 2019).

Moreover, previous studies have provided evidence that the HPV E7 involves in the regulation of the p53 pathway. Activation of the tumor suppressor p53 can induce cell cycle arrest, and the downregulation of cell cycle genes through transcriptional mechanisms is recognized as a primary mechanism in p53-mediated arrest (Nair et al., 2003; Bae et al., 2017; Engeland, 2018). Therefore, we assembled a set of genes associated with

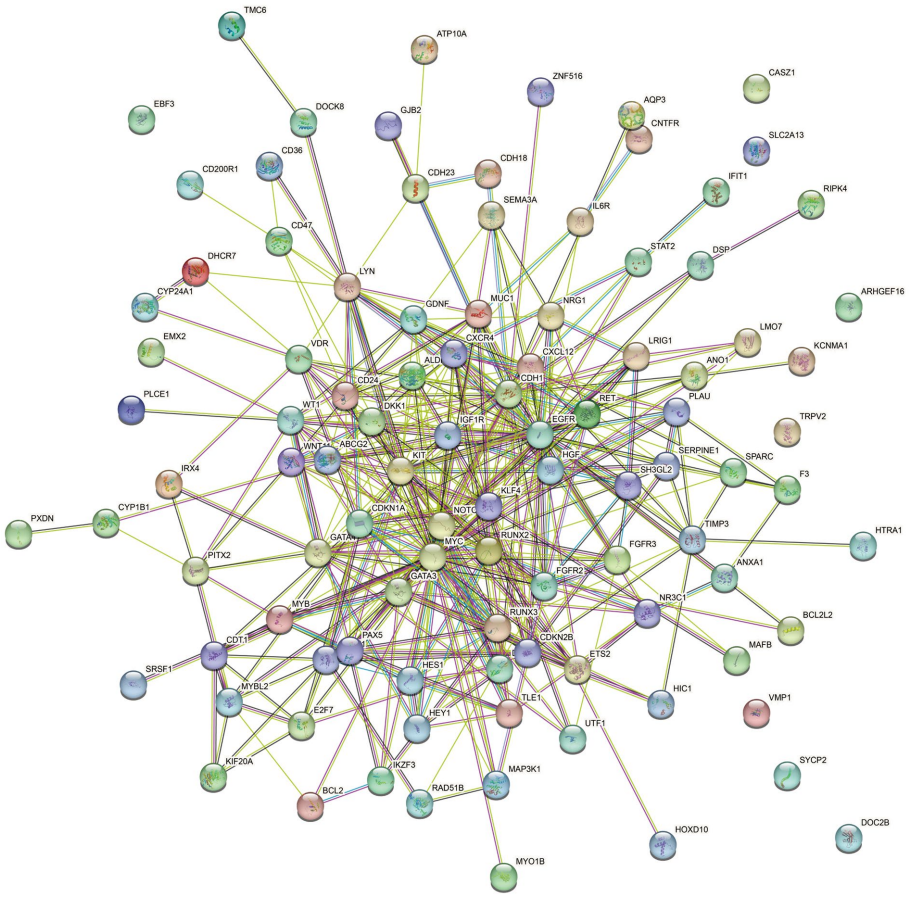

FIGURE 6
STRING protein–protein interaction of the 103 DEGs genes. Each node symbolizes the entirety of proteins generated by an individual gene locus responsible. Colored nodes are query proteins and first shell of interactors. Edges represent protein–protein associations. Associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function.

cervical cancer-related pathways and employed the ssGSEA algorithm to calculate the scores of the five upregulated genes in the PI3K/AKT/mTOR signaling and p53 pathways. Spearman correlation analysis revealed a statistically significant correlation between the CD36 gene and both the PI3K/AKT/mTOR signaling pathway and the p53 pathway (Figures 8A,B). However, STAT2 exhibited a significant correlation with the PI3K/AKT/mTOR signaling pathway but not with the p53 pathway (Figures 8C,D). There was no statistically significant correlation observed between IFIT1, VMP1, MUC1, and these two pathways.

To further investigate the regulatory impact of the Ad4-HPV16E7 virus on STAT2 and CD36 in HPV-negative cervical cancer cells, C33A cells were infected with Ad4 and Ad4-HPV16E7 viruses, and non-infected C33A cells served as the control group. RNA was extracted from the cells, reverse transcribed into cDNA, and specific primers targeting STAT2 and CD36 were used for qRT-PCR. The results showed that the expression of the CD36 gene was upregulated in the Ad4-HPV16E7 group compared to the Ad4 and control groups. However, there was no significant difference observed in the expression of the STAT2 gene (Figure 8E). Gel electrophoresis of the qRT-PCR products of CD36 confirmed the findings (Figure 8F). These results suggest that the Ad4-HPV16E7 virus can upregulate the expression of the CD36 gene in C33A cells.

4. Discussion

The sustained high-level expression of oncogenic proteins E6/E7 is a crucial factor in the development of cancer caused by high-risk HPV infections (Schwartz and Rotter, 1998). The presence of E7 protein is necessary for the malignant transformation of infected tissues, and in many cases of HPV16-associated cancers, the viral genome is integrated into the host genome (Sun et al., 2021). Consequently, these infected cells predominantly express E6/E7 oncogenes, rendering HPV vaccines targeting the L1 and L2 capsid proteins ineffective against established infections (Li et al., 2016; Taghinezhad et al., 2021). Given these unique characteristics of high-risk HPV in carcinogenesis, the E7 proteins emerge as ideal therapeutic targets for HPV-related cancers and represent a focal point for gene therapy research aimed at specifically targeting cells expressing the E7 antigen (He et al., 2019). Targeting E7 not only elicits a specific anti-tumor response in cancer cells but also minimizes

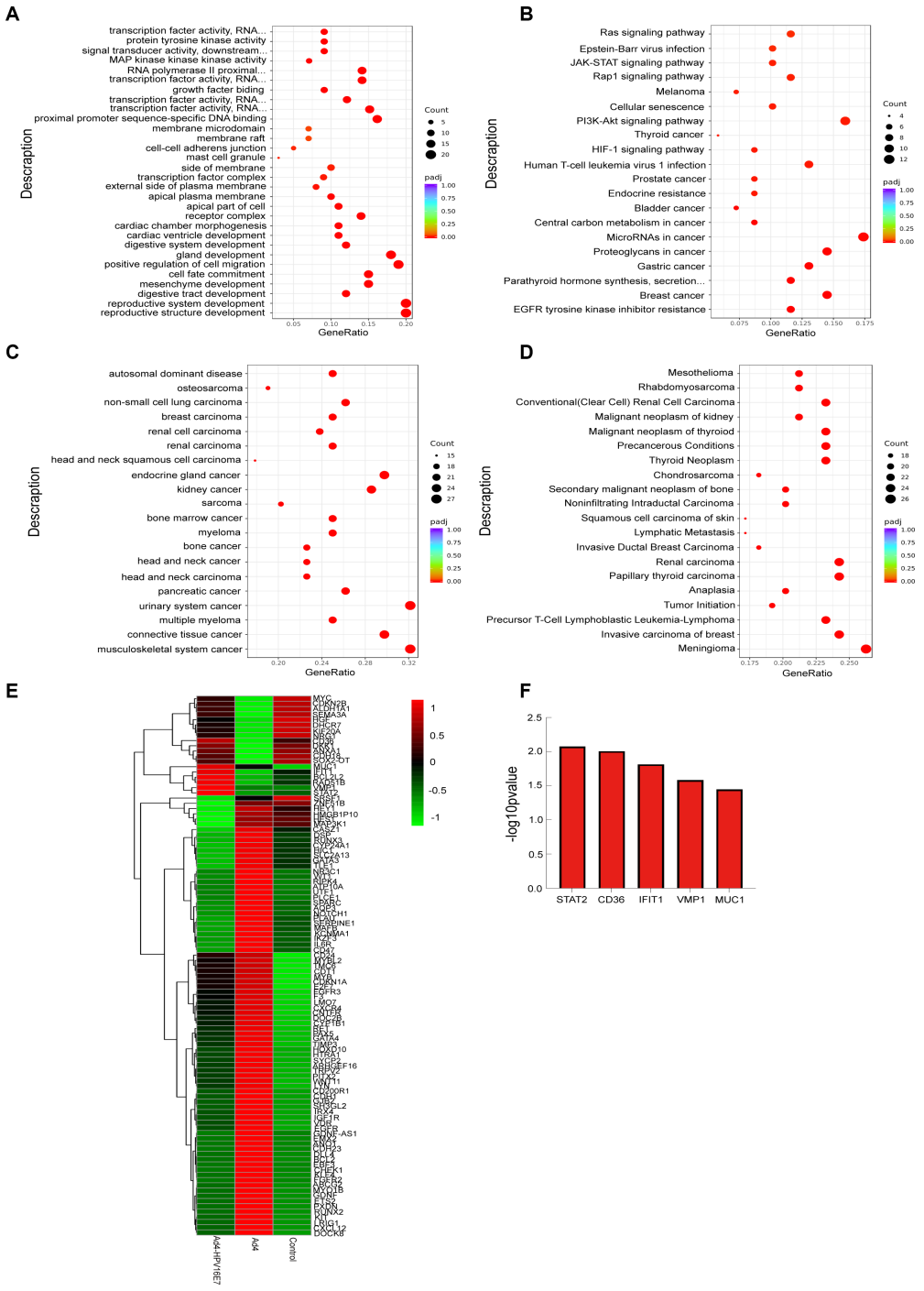

FIGURE 7
Functional and pathway enrichment analysis of identified modules associated with the 103 DEGs. **(A)** Gene Ontology (GO) functional analysis of DEGs. **(B)** KEGG pathway analysis of DEGs. **(C)** DO pathway analysis of DEGs. **(D)** DisGeNET pathway analysis of DEGs. The size of the dots represents the number of genes annotated to the KEGG pathway, and the color from red to purple represents the significance level of KEGG pathway enrichment, the redder the color is, the more significant the result. **(E)** The heat map of the expression levels of 103 genes in the Ad4-HPV16E7, Ad4, and control groups. The greener the color of the heatmap, the lower the gene expression, and the redder the color of the heatmap, the higher the gene expression. **(F)** The five up-regulated genes in the Ad4-HPV16E7 group relative to the Ad4 group and the control group.

the risk of unintended cytotoxicity toward healthy cells. However, the intricate life cycle of HPV and the challenges associated with *in vitro* culturing of the virus have impeded further advancements in related research. To address this limitation, we propose the construction of recombinant viral particles using adenoviral vectors, which provide a stable platform for expressing the HPV16 E7 protein. This approach provides a novel avenue for investigating HPV-associated anti-tumor and antiviral therapies.

The HPV E7 region exhibits homology with Ad E1A, which is known to lack carcinogenic properties in humans. Thus, by inserting

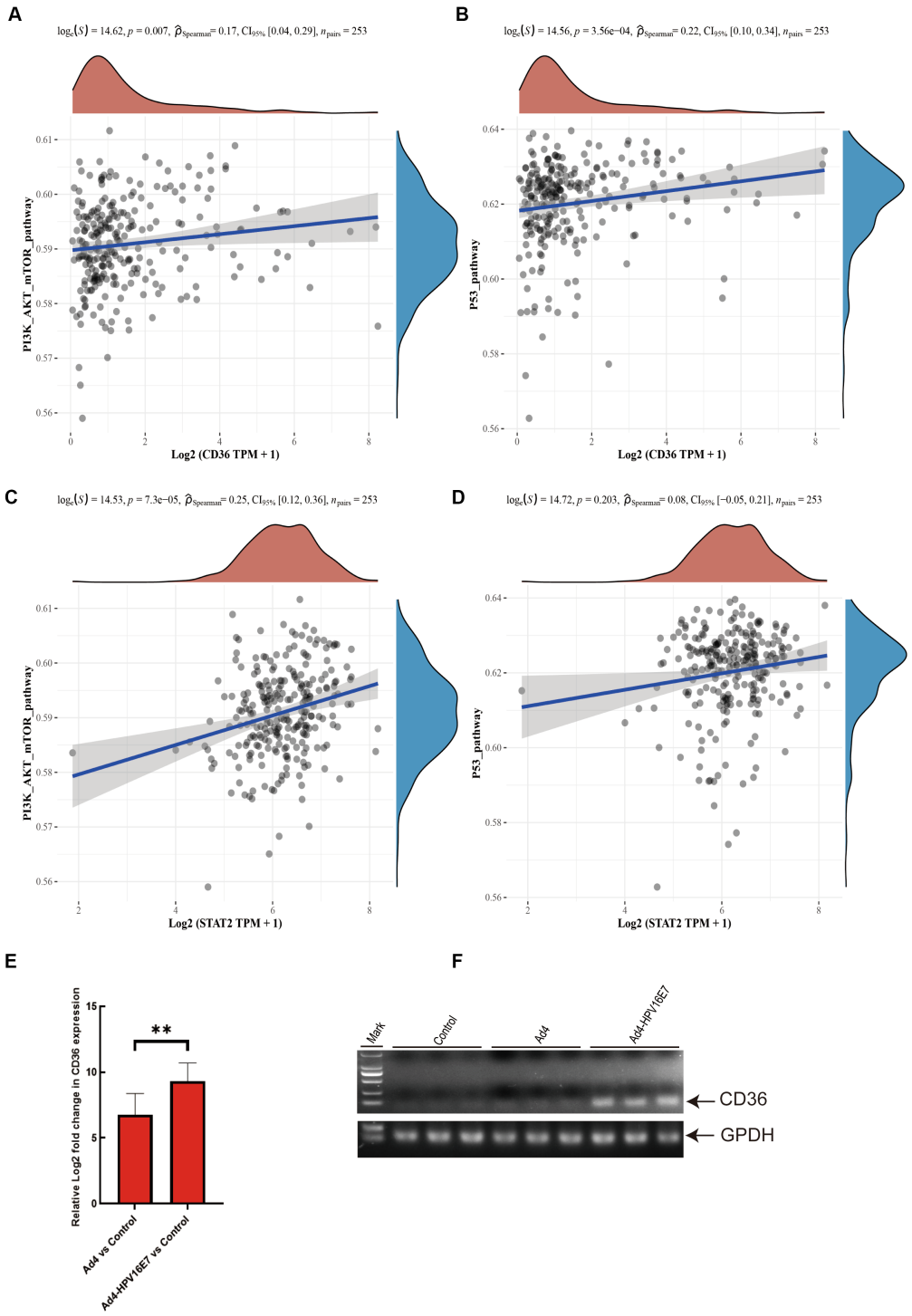

FIGURE 8
Recombinant virus Ad4-HPV16E7 induces upregulation of CD36 gene in C33A cells. **(A,B)** The Spearman correlation analysis was employed to examine the correlations between individual CD36 and PI3K/AKT/mTOR signaling pathway, as well as the P53 pathway score. **(C,D)** The Spearman correlation analysis was employed to examine the correlations between individual STAT2 and PI3K/AKT/mTOR signaling pathway, as well as the P53 pathway score. $P < 0.05$ was considered statistically significant. **(E)** Detection of the relative expression levels of CD36 gene by qRT-PCR. **(F)** Agarose gel electrophoresis of qRT-PCR amplification products. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant.

the HPV16 E7 gene into the EGFP-tagged Ad4 E1A region, we successfully constructed a replication-competent recombinant virus, Ad4-HPV16E7, for the first time. This was achieved through a combination of the ccdB-Kan positive/negative selection system and the Red/ExoCET recombination system. We infected HEK293T, Siha, caski, Hela, and C33A cells with the recombinant virus and observed that while its infectivity was relatively weak in Hela cells, it exhibited strong infectivity in HEK293T, Siha, caski, and C33A cells. In

HEK293T and C33A cells infected with the recombinant virus, HPV16 E7 protein was stably and effectively expressed. Additionally, the expression level of the Hexon protein in the constructed recombinant virus, Ad4-HPV16E7, was higher compared to that of the Ad4 virus, indicating a stronger replication capacity of the recombinant virus relative to Ad4. However, further studies are required to confirm the replication capacity of the recombinant virus.

We performed RNA-Seq analysis to construct libraries and conduct biological analysis of all mRNA transcripts generated by C33A cells after infection with Ad4-HPV16E7 virus. The RNA-Seq results were cross-referenced with reliable sources such as OMIM, Genotype, KEGG, and PubMed to identify genes associated with HPV infection, resulting in the identification of 103 differentially expressed genes (DEGs). Protein–protein interaction (PPI) analysis using STRING network revealed a high level of functional relationship among these 103 DEGs. GO, KEGG, DO, and DisGeNET analyses determined several enriched biological processes and metabolic pathways for these 103 genes. Notably, KEGG pathway analysis indicated significant enrichment of these genes in the PI3K-Akt signaling pathway, which plays a crucial role in HPV-induced carcinogenesis. The clustering analysis of these 103 DEGs indicated that the expression of five upregulated genes (STAT2, CD36, IFIT1, VMP1, and MUC1) in the Ad4-HPV16E7 group was higher than that in the Ad4 and control groups. The selection of these genes was based on the similarity of their expression patterns in the clustering analysis and their association with HPV infection in previous literature. Previous studies have shown that HPV E7 plays a role in regulating the p53 pathway, and activation of the tumor suppressor factor p53 can lead to cell cycle arrest. Additionally, the main mechanism of p53-mediated arrest is the transcriptional downregulation of multiple cell cycle genes. Therefore, we collected genes related to cervical cancer-associated pathways and calculated the correlation scores between these five upregulated genes and the PI3K/AKT/mTOR signaling pathway and the p53 pathway through Spearman correlation analysis. The results indicated a significant correlation between the CD36 gene and both the PI3K/AKT/mTOR signaling pathway and the p53 pathway. The STAT2 gene showed a significant correlation with the PI3K/AKT/mTOR signaling pathway but not with the p53 pathway. IFIT1, VMP1, and MUC1 showed no significant correlation with either of these pathways. qRT-PCR results demonstrated upregulation of the CD36 gene expression in the Ad4-HPV16E7 group compared to the Ad4 and control groups. These findings suggest that the Ad4-HPV16E7 virus has the ability to upregulate CD36 gene expression in C33A cells. Alterations in CD36 expression may be associated with high-risk human papillomavirus infection and may promote the development and progression of cervical cancer. Several studies have shown that CD36 is involved in cell proliferation by regulating the cell cycle. The pathway analysis revealed the correlation between CD36 and the PI3K/AKT/mTOR signaling pathway, which is consistent with previous research and further strengthens the potential role of CD36 in HPV infection and cervical cancer. These results provide important clues for further research into the molecular mechanisms underlying HPV infection and cervical cancer development.

This study investigates a cost-effective and precise approach to constructing a replicative recombinant virus that expresses the HPV16 E7 protein and the successful expression of HPV16 E7 in cells demonstrated that the replicated recombinant virus maintains the replication and infection capabilities of Ad4, while also upregulated the CD36 gene, which is involved in the PI3K-Akt signaling and p53 pathways for promoting cell proliferation. The findings of this study offer a novel perspective for future investigations on HPV-related carcinogenesis/disease and the advancement of replicative recombinant HPV therapeutic vaccines that can elicit protective immunity against HPV. Absolutely, the use of recombinant adenovirus for gene therapy suffers from some challenges. The recombinant adenovirus possesses the tropism of the parental viruses, which infect all cells that possess the appropriate surface receptors, precluding the targeting of specific cell types. Conversely, some cell types that represent important tar gets for gene transfer express only low levels of the cellular receptors, which lead to inefficient infection. Thus, there is a need for addressing attention the rational strategies based on the biology of Ad to further exploit the full potential of the recombinant adenovirus for *in vivo* gene delivery upon systemic administration, thereby provide the basis for researching the replicative recombinant HPV therapeutic vaccines.

Data availability statement

The data presented in the study are deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo), accession number GSE240750.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

YS: Data curation, Investigation, Software, Writing – original draft. YZ: Investigation, Writing – review & editing. HC: Software, Writing – original draft. ZL: Software, Writing – review & editing. PW: Methodology, Resources, Writing – review & editing. CW: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. SL: Participated in the data analysis, Edited the manuscript. FH: Conducted the experiments, Participated in the data analysis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1259510/full#supplementary-material

References

Bae, S. N., Lee, K. H., Kim, J. H., Lee, S. J., and Park, L. O. (2017). Zinc induces apoptosis on cervical carcinoma cells by p 53-dependent and -independent pathway. *Biochem. Biophys. Res. Commun.* 484, 218–223. doi: 10.1016/j.bbrc.2016.12.093

Bossler, F., Hoppe-Seyler, K., and Hoppe-Seyler, F. (2019). PI3K/AKT/mTOR signaling regulates the virus/host cell crosstalk in HPV-positive cervical Cancer cells. *Int. J. Mol. Sci.* 20:2188. doi: 10.3390/ijms20092188

Cam, H., and Dynlacht, B. D. (2003). Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer Cell* 3, 311–316. doi: 10.1016/S1535-6108(03)00080-1

Cheng, M. A., Farmer, E., Huang, C., Lin, J., Hung, C. F., and Wu, T. C. (2018). Therapeutic DNA vaccines for human papillomavirus and associated diseases. *Hum. Gene Ther.* 29, 971–996. doi: 10.1089/hum.2017.197

Dai, C. H., Gao, Z. C., Cheng, J. H., Yang, L., Wu, Z. C., Wu, S. L., et al. (2022). The competitive endogenous RNA (ceRNA) regulation in porcine alveolar macrophages (3D4/21) infected by swine influenza virus (H1N1 and H3N2). *Int. J. Mol. Sci.* 23:1875. doi: 10.3390/ijms23031875

Domingos-Pereira, S., Roh, V., Hiou-Feige, A., Galliverti, G., Simon, C., Tolstonog, G. V., et al. (2021). Vaccination with a nanoparticle E7 vaccine can prevent tumor recurrence following surgery in a human papillomavirus head and neck cancer model. *Onco. Targets. Ther.* 10:1912473. doi: 10.1080/2162402X.2021.1912473

Douglas, J. T. (2007). Adenoviral vectors for gene therapy. *Mol. Biotechnol.* 36, 71–80. doi: 10.1007/s12033-007-0021-5

Dürst, M., Gissmann, L., Ikenberg, H., and Hausen, H. Z. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. U. S. A.* 80, 3812–3815. doi: 10.1073/pnas.80.12.3812

Dyson, N., Guida, P., Münger, K., and Harlow, E. (1992). Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J. Virol.* 66, 6893–6902. doi: 10.1128/jvi.66.12.6893-6902.1992

Engeland, K. (2018). Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death Differ.* 25, 114–132. doi: 10.1038/cdd.2017.172

Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R., and Schiller, J. T. (1989). HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* 8, 3905–3910. doi: 10.1002/j.1460-2075.1989.tb08570.x

He, J., Yang, Y., Chen, Z., Liu, Y., Bao, S., Zhao, Y., et al. (2019). Identification of variants and therapeutic epitopes in HPV-33/HPV-58 E6 and E7 in Southwest China. *Virol. J.* 16:72. doi: 10.1186/s12985-019-1168-y

Hoppe-Seyler, K., Bossler, F., Braun, J. A., Herrmann, A. L., and Hoppe-Seyler, F. (2018). The HPV E6/E7 oncogenes: key factors for viral carcinogenesis and therapeutic targets. *Trends Microbiol.* 26, 158–168. doi: 10.1016/j.tim.2017.07.007

Huang, C. C., Su, Y. C., Chang, C. C., Lee, W. T., Ou, C. Y., Wu, Y. H., et al. (2021). Investigating the association between serum human papillomavirus type 16 E7 antibodies and risk of head and neck cancer. *Cancer Med.* 10, 4075–4086. doi: 10.1002/cam4.3944

Li, L. L., Wang, H. R., Zhou, Z. Y., Luo, J., Wang, X. L., Xiao, X. Q., et al. (2016). C3-Luc cells are an excellent model for evaluation of cellular immunity following HPV16L1 vaccination. *PLoS One* 11:e0149748. doi: 10.1371/journal.pone.0149748

Liu, S., Zhang, B., Rowan, B. G., Jazwinski, S. M., Abdel-Mageed, A. B., Steele, C., et al. (2021). A novel controlled PTEN-knockout mouse model for prostate Cancer study. *Front. Mol. Biosci.* 8:696537. doi: 10.3389/fmolb.2021.696537

Lou, H., Boland, J. F., Li, H., Burk, R., Yeager, M., Anderson, S. K., et al. (2022). HPV16 E7 nucleotide variants found in cancer-free subjects affect E7 protein expression and transformation. *Cancers (Basel)* 14:4895. doi: 10.3390/cancers14194895

McKenna, M. K., Rosewell-Shaw, A., and Suzuki, M. (2020). Modeling the efficacy of oncolytic adenoviruses in vitro and in vivo: current and future perspectives. *Cancers* 12:619. doi: 10.3390/cancers12030619

Meznad, K., Paget-Bailly, P., Jacquin, E., Peigney, A., Aubin, F., Guittaut, M., et al. (2021). The exon junction complex core factor eIF4A3 is a key regulator of HPV16 gene expression. *Biosci. Rep.* 41:BSR20203488. doi: 10.1042/BSR20203488

Nair, P., Somasundaram, K., and Krishna, S. (2003). Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J. Virol.* 77, 7106–7112. doi: 10.1128/JVI.77.12.7106-7112.2003

Nouel, J., and White, E. A. (2022). ZER1 contributes to the carcinogenic activity of high-risk HPV E7 proteins. *mBio* 13:e0203322. doi: 10.1128/mbio.02033-22

Pan, L., Li, B., Chen, J., Zhang, H., Wang, X., Shou, J., et al. (2021). Nanotechnology-based weapons to combat human papillomavirus infection associated diseases. *Front. Chem.* 9:798727. doi: 10.3389/fchem.2021.798727

Peng, S., Xing, D., Ferrall, L., Tsai, Y. C., Roden, R. B. S., Hung, C. F., et al. (2022). Development of a spontaneous HPV16 E6/E7-expressing head and neck squamous cell carcinoma in HLA-A2 transgenic mice. *MBio* 13:e03252-21. doi: 10.1128/mbio.03252-21

Qmichou, Z., Khyatti, M., Berraho, M., Ennaji, M. M., Benbacer, L., Nejjari, C., et al. (2013). Analysis of mutations in the E6 oncogene of human papillomavirus 16 in cervical cancer isolates from Moroccan women. *BMC Infect. Dis.* 13:378. doi: 10.1186/1471-2334-13-378

Schneider, M., Müller, M., Yigitliler, A., Xi, J., Simon, C., Feger, T., et al. (2020). Orf virus-based therapeutic vaccine for treatment of papillomavirus-induced tumors. *J. Virol.* 94:e00398-20. doi: 10.1128/JVI.00398-20

Schwartz, D., and Rotter, V. (1998). p53-dependent cell cycle control: response to genotoxic stress. *Semin. Cancer Biol.* 8, 325–336. doi: 10.1006/scbi.1998.0095

Sitarz, K., Czamara, K., Bialecka, J., Klimek, M., Szostek, S., and Kaczor, A. (2021). Dual switch in lipid metabolism in cervical epithelial cells during dysplasia development observed using Raman microscopy and molecular methods. *Cancers* 13:1997. doi: 10.3390/cancers13091997

Steinwaerder, D. S., Carlson, C. A., and Lieber, A. (2001). Human papilloma virus E6 and E7 proteins support DNA replication of adenoviruses deleted for the E1A and E1B genes. *Mol. Ther.* 4, 211–216. doi: 10.1006/mthe.2001.0447

Sun, X., Fu, P., Xie, L., Chai, S., Xu, Q., Zeng, L., et al. (2021). Resveratrol inhibits the progression of cervical cancer by suppressing the transcription and expression of HPV E6 and E7 genes. *Int. J. Mol. Med.* 47, 335–345. doi: 10.3892/ijmm.2020.4789

Taghinezhad, S. S., Keyvani, H., Bermúdez-Humarán, L. G., Donders, G. G. G., Fu, X., and Mohseni, A. H. (2021). Twenty years of research on HPV vaccines based on genetically modified lactic acid bacteria: an overview on the gut-vagina axis. *Cell. Mol. Life Sci.* 78, 1191–1206. doi: 10.1007/s00018-020-03652-2

Wang, X., Song, Y., Wei, X., Wang, G., Sun, R., Wang, M., et al. (2022). Prevalence and distribution of human papillomavirus genotypes among women attending gynecology clinics in northern Henan Province of China. *Virol. J.* 19:6. doi: 10.1186/s12985-021-01732-8

Wodrich, H., Guan, T., Cingolani, G., Von Seggern, D., Nemerow, G., and Gerace, L. (2003). Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *EMBO J.* 22, 6245–6255. doi: 10.1093/emboj/cdg614

Wu, J., Chen, K. D., Gao, M., Chen, G., Jin, S. F., Zhuang, F. C., et al. (2015). Genetic stability of a recombinant adenovirus vaccine vector seed library expressing human papillomavirus type 16 E6 and E7 proteins. *Exp. Ther. Med.* 9, 1161–1165. doi: 10.3892/etm.2015.2268

Xia, X., Jin, J., Chen, Z. J., Zhou, Z., Chen, H., Zhang, C., et al. (2020). Unraveling the genetic causes in large pedigrees with gout by whole-exome sequencing. *Int. J. Mol. Med.* 45, 1047–1058. doi: 10.3892/ijmm.2020.4501

Yang, R., Klimentová, J., Göckel-Krzikalla, E., Ly, R., Gmelin, N., Hotz-Wagenblatt, A., et al. (2019). Combined transcriptome and proteome analysis of immortalized human keratinocytes expressing human papillomavirus 16 (HPV16) oncogenes reveals novel key factors and networks in HPV-induced carcinogenesis. *mSphere* 4:e00129-19. doi: 10.1128/mSphere.00129-19

Yang, X., Liu, X., Li, J., Zhang, P., Li, H., Chen, G., et al. (2022). Caerin 1.1/1.9 enhances antitumour immunity by activating the IFN-α response signalling pathway of tumour macrophages. *Cancers* 14:5785. doi: 10.3390/cancers14235785

Yu, L., Fei, L., Liu, X., Pi, X., Wang, L., and Chen, S. (2019). Application of p16/Ki-67 dual-staining cytology in cervical cancers. *J. Cancer* 10, 2654–2660. doi: 10.7150/jca.32743

Zhang, X., Wu, X., Hu, H., Liu, X., Kang, Z., and Deng, X. (2023). DT-010 exerts Cardioprotective effects by regulating the crosstalk between the AMPK/PGC-1α pathway and ERp57. *Cardiovasc. Ther.* 2023:8047752. doi: 10.1155/2023/8047752

Zhou, C., Tuong, Z. K., and Frazer, I. H. (2019). Papillomavirus immune evasion strategies target the infected cell and the local immune system. *Front. Oncol.* 9:682. doi: 10.3389/fonc.2019.00682

Zhu, J., Kamara, S., Wang, Q., Guo, Y., Li, Q., Wang, L., et al. (2021). Novel affibody molecules targeting the HPV16 E6 oncoprotein inhibited the proliferation of cervical cancer cells. *Front. Cell Dev. Biol.* 9:677867. doi: 10.3389/fcell.2021.677867

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A novel oncolytic virus-based biomarker participates in prognosis and tumor immune infiltration of glioma

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Background: Glioma is the most common central nervous malignancy. Due to its poor survival outcomes, it is essential to identify novel individualized therapy. Oncolytic virus (OV) treatment is a key therapy regulating tumor microenvironment in malignant glioma. Herein, we aim to identify the key genes after OV infection and its role in glioma.

Methods: Performing an RNA-seq analysis, the differentially expressed genes (DEGs) between EV-A71-infection and mock group were screened with GFold values. DAVID online analysis was performed to identify the functional classification. Overall survival (OS) or disease-free survival (DFS) was evaluated to analyze the relation between PTBP1 expression levels and prognosis of glioma patients. Additionally, the ssGSEA and TIMER algorithms were applied for evaluating immune cell infiltration in glioma.

Results: Following EV-A71 infection in glioma cells, PTBP1, one of the downregulated DEGs, was found to be associated with multiple categories of GO and KEGG enrichment analysis. We observed elevated expression levels of PTBP1 across various tumor grades of glioma in comparison to normal brain samples. High PTBP1 expression had a notable impact on the OS of patients with low-grade glioma (LGG). Furthermore, we observed an obvious association between PTBP1 levels and immune cell infiltration in LGG. Notably, PTBP1 was regarded as an essential prognostic biomarker in immune cells of LGG.

Conclusion: Our research uncovered a critical role of PTBP1 in outcomes and immune cell infiltration of glioma patients, particularly in those with LGG.

KEYWORDS

glioma, oncolytic virus, entrovirus, prognosis, immune infiltration

Introduction

Glioma is a primary malignant tumor that occurs within the brain and has the highest incidence rate among tumors (Chen et al., 2017). Glioblastoma multiforme (GBM), the most aggressive form of brain cancer with a dismal prognosis, represents approximately half of all newly diagnosed gliomas (Le Rhun et al., 2019). In despite of the implementation of optimizing clinical treatment, which involve responsible surgical excision followed by chemoradiotherapy,

low-grade glioma (LGG) patients have an overall survival rate of only about 60 months (Malik et al., 2021). The diffuse invasive growth of glioma cells and the blood–brain barrier are the major contributors to poor prognosis, as it not only impedes surgical tumor excision but also facilitates resistance to chemotherapy and radiotherapy (van Tellingen et al., 2015; Hu et al., 2023). Improving the extended survival rate of glioma patients remains a challenge. Due to the swift advancement of next-generation sequencing, the utilization of pan-cancer investigation has become prevalent in the detection of molecular indicators within tumors, such as aldehyde dehydrogenase (ALDH; Xia et al., 2023), Unc-51 Like Autophagy Activating Kinase 1 (ULK1; Qu et al., 2020) and N6-methyladenine-related genes (Qu et al., 2021a). The identification of novel therapeutic targets based on regulatory mechanism is crucial for enhancing patient outcomes.

Replication-competent virus is a novel oncolytic virotherapy targeting cancer cells (Russell et al., 2012). The oncolytic viruses (OVs) can trigger tumor cell apoptosis via diverse mechanisms, such as apoptosis, pyroptosis, or necroptosis (Rius-Rocabert et al., 2020; Hu et al., 2022). OVs treatment enhance immune cell infiltration and promote inflammation within a highly immunosuppressed tumor microenvironment (TME), which might be critical in breaking the dysimmunity (Ribas et al., 2017; Hemminki et al., 2020). A diverse range of OVs is currently undergoing evaluation in both preclinical and clinical stages for the intervention of glioma, which includes GBM and LGG (Suryawanshi and Schulze, 2021; Li et al., 2022). Notably, a growing pool of highly potential OV candidates, such as adenovirus DNX-2401 (Gállego Pérez-Larraya et al., 2022) and adenovirus ICOVIR17 (Kiyokawa et al., 2021), are currently validating their potential to generate a sustained response in patients with malignant glioma in the postapproval trials (Hulou et al., 2016). The biological mechanisms responsible for OV treatment in glioma are not well understood, and there is a need to identify biomarkers that can offer new insights into treatment options.

In present study, we aimed at identifying the novel biomarkers in OV-infected glioma and providing improved opportunities for glioma diagnosis or prognosis. We carried out a thorough analysis utilizing publicly databases and online analysis tools to examine the impact of EV-A71 infection on gene ontology categories and pathways in glioma. Furthermore, we investigated the role of polypyrimidine tract binding protein 1 (PTBP1) as a prognostic factor in LGG and examined its association with clinicopathological characteristics and immune cell infiltration.

Materials and methods

Data sources and bioinformatical analysis

The mRNA expression data of EV-A71-infected glioma cells were obtained from the Boproject repository (PRJNA562271; https://www.ncbi.nlm.nih.gov/bioproject). Specifically, EV71 infection was infected in glioma cells (CCF-STTG1). To screen differentially expressed genes (DEGs), RNA-seq analysis was conducted using the Illumina HiSeqTM 2000 System. DEGs were identified between the EV-A71 infection group and the mock infection group based on their GFold values. The TCGA glioma datasets, which include LGG and GBM datasets, were analyzed using a bioinformatic tool GEPIA2.[1] The genomic data of Chinese glioma patients was obtained from the CGGA database for this study (http://www.cgga.org.cn/index.jsp; Zhao et al., 2021). The protein expression images were obtained from the Human Protein Atlas (HPA, https://www.proteinatlas.org/). We acquired the distribution of the proteins across normal tissues and HCC tissues in the "Tissue" section and the "Pathology" section. The antibody utilized for performing IHC assay is CAB013507.

Gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis

To evaluate the functional classification, we utilized the DAVID Gene Functional Classification Tool (https://david.ncifcrf.gov/home.jsp; Sherman et al., 2022). The gene list of downregulated or upregulated DEGs was submitted, and we classified the extensive gene list into functional gene groups based on their relatedness.

Survival analysis

To evaluate the association between PTBP1 level and poor prognosis, we conducted an analysis of both overall survival (OS) and disease-free survival (DFS). The glioma cases, encompassing both GBM and LGG patients, were stratified into the groups divided by the levels of PTBP1 TPM: a low PTBP1 TPM level group (bottom 50%) and a high PTBP1 TPM level group (top 50%). Kaplan–Meier curve was utilized to illustrate the survival status, and statistical significance was determined by setting a threshold of p-values less than 0.05.

Immune cell infiltrating estimation

To investigate the association between TPBP1 level and immune cell infiltration in LGG and GBM cells, we utilized the ssGSEA and TIMER algorithms to analyze the TCGA data. The "gene module" available on the TIMER website was employed to visualize the correlation between TPBP1 level and immune infiltration in both GBM and LGG. Furthermore, we utilized the "Survival module" to examine the clinical relevance of immune infiltrates and assess their impact on survival differences. Additionally, we compared the levels of immune infiltrates in glioma with the presence of an IDH mutation using the "Mutation module." Somatic copy number alterations (SCNAs) were defined into four groups by GISTIC 2.0. The SCNA module allows for the comparison of tumor infiltration levels between tumors with different somatic copy number alterations of PTBP1.

Abbreviations: GBM, Glioblastoma multiforme; LGG, Low-grade glioma; OV, Oncolytic virus; TME, Tumor microenvironment; PTBP1, Polypyrimidine tract binding protein 1; DEG, Differentially expressed genes; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; OS, Overall survival; DFS, Disease-free survival; SCNA, Somatic copy number alterations; EV, Entrovirus; BP, Biological processes; codel, Co-deletion.

1 http://gepia2.cancer-pku.cn/#index

Statistical analysis

Student's t-tests or Wilcoxon rank-sum tests were used to detect significant differences in gene expression or immune cell enrichment. One-way ANOVA was used in analyzing gene expression in different cancer stage. Spearman rank correlation was utilized to analyze the correlation between PTBP1 and immune infiltration. A p-value of less than 0.05 was considered statistically significant. All data are reported as the mean ± standard error (SEM).

Results

Analysis of differential expression genes and gene oncology in entrovirus A71-infected glioma cells

Given the interconnection between oncolytic virotherapy and antitumor response in glioma (You et al., 2020; Zhang et al., 2020), we first examined the regulation of entrovirus (EV)-A71 infection on CCF-STTG1 cells. One dataset (PRJNA562271) obtained from Bioproject was included in this study: EV-A71 oncolysis of human malignant gliomas. Genes with GFold > −1 or GFold <1 in RNA-seq analysis were filtered out leaving total of 441 upregulated genes (Supplementary Table S1) and 319 downregulated genes to be analyzed in the research (Supplementary Table S2). Figure 1A shows the total number of DEGs in the comparisons EV-A71 infection vs. mock group. Our results show 22 down-regulated genes with GFold <= −2 and 297 genes with −2 <= GFold < −1, while 331 genes were up-regulated with 1 <= GFold < 2 and 110 genes were up-regulated with GFold >= 2 (Figure 1A). Based on the tumoricidal features of OV in glioma, we included 319 downregulated genes to identify the functional classification using DAVID online tool. According to the results of the functional GO enrichment analysis focusing on biological processes (BP), it was found that the 319 DEGs regulated a total of 9 functional categories. These categories were as follows: negative regulation of transcription (DNA-templated), translation, cytoplasmic translation, positive regulation of calcineurin-NFAT signaling cascade, IRES-dependent viral translational initiation, regulation of alternative mRNA splicing via spliceosome, cellular response to virus, T-helper 1 cell differentiation and immune response (Figure 1B). The KEGG enrichment analysis indicated that the downregulated DEGs showed associations with ribosome, human immunodeficiency virus 1 infection and glutathione metabolism (Figure 1C). Additionally, the result of GO and KEGG enrichment analysis with the 440 upregulated DEGs was illustrated in Supplementary Figure 1.

Upregulation of PTBP1 in human glioma

Considering the involvement of PTBP1 in all categories of GO and KEGG enrichment analysis, we conducted an analysis using the publicly accessible TCGA database to examine the PTBP1 status in normal brain and glioma tissues. The findings from the TCGA database demonstrated a substantial increase in PTBP1 differential transcription levels among GBM (n = 163) or LGG (n = 518) samples when compared to normal (n = 207) samples (p < 0.05, Figure 2A). Additionally, the trends observed in TCGA data were also observed in CGGA dataset. Moreover, an RNA analysis conducted on Chinese human glioma tissues indicated a progressive increase in PTBP1 expression from WHO II to WHO IV stages, providing further confirmation of the distinctive PTBP1 status within glioma (p < 0.001, Figure 2B). Immunohistochemical staining was utilized to measure the protein levels of PTBP1 in human glioma samples. The analysis revealed a pronounced relative overexpression of PTBP1 in glioma tissues when compared to normal cerebral cortex tissues (Figure 2C). Moreover, a stronger intensity and more quantity of PTBP1 protein staining were found in high-grade glioma, compared with low-grade glioma (Figure 2C). These results indicated a positive correlation between PTBP1 level and the pathological stages of glioma, confirming the possibility involvement of PTBP1 in glioma advancement.

Evaluation of PTBP1 as a prognostic biomarker in LGG

The co-deletion status of chromosomal 1p/19q represents a prominent driver in the development of glioma and is diffusely acknowledged as a robust prognostic gene in the study of gene mutations in glioma (Bhattacharya et al., 2021). Here, we explored whether 1p/19q co-deletion (codel) status may correlate with PTBP1 gene expression in glioma. In this regard, we acquired and examined the scaled gene expression profile from the CGGA dataset, arranging genes located on 1p/19q based on the genomic locations (Hu et al., 2017). We observed that in the total cohort of glioma cases, lower levels of PTBP1 significantly correlates with 1p/19q co-deletion (p = 4.5e-11, Figure 3A). Figure 3B shows that PTBP1 gene levels were obviously higher in non-codel group compared to codel group in the WHO III subtype (p = 0.0019). In both the WHO II and WHO IV subtypes, no statistically significant differences were observed in the expression of PTBP1 with respect to the 1p/19q co-deletion status (p > 0.05, Figure 3B). Multiple studies focusing on glioma mutations have consistently reported that the presence of 1p/19q co-deletion in glioma is related to a positive response, leading to improved survival rates (Barthel et al., 2019; Bhattacharya et al., 2021). Patients with high PTBP1 expression (cutoff-high: 50%) had a shorter OS of 60 months compared to 120 months in LGG patients with low PTBP1 level (cutoff-low: 50%; p < 0.001, HR 2.6, Figure 3C). Kaplan–Meier curves were shown in the right panel of Figure 3C, the median DFS was 40 months in patients with high PTBP1 expression, compared to 75 months in patients with low PTBP1 expression (p < 0.001, HR 1.9). However, neither OS or DFS were obviously related to PTBP1 levels in GBM (Figure 3D). Additionally, no statistically significant differences were observed between the groups based on PTBP1 expression status in terms of gender, age and progression status of glioma patients (Supplementary Figure 2; Table 1). Moreover, the areas under the curve (AUCs) of the time-dependent ROC curves were 0.973, indicating a high sensitivity and specificity of PTBP1 signature for predicting OS in glioma (Supplementary Figure 3).

Association of PTBP1 and immune infiltration characteristic in glioma

OV therapy plays a significant role in stimulating the immune response against tumors, making it an essential component in

A

GFold range	Numbers of genes
GFold ⩽ -2	22
-2 < GFold ⩽ -1	297
-1 < GFold < 0	2237
GFold = 0	6089
0 < GFold < 1	2571
1 ⩽ GFold < 2	331
GFold ⩾ 2	110

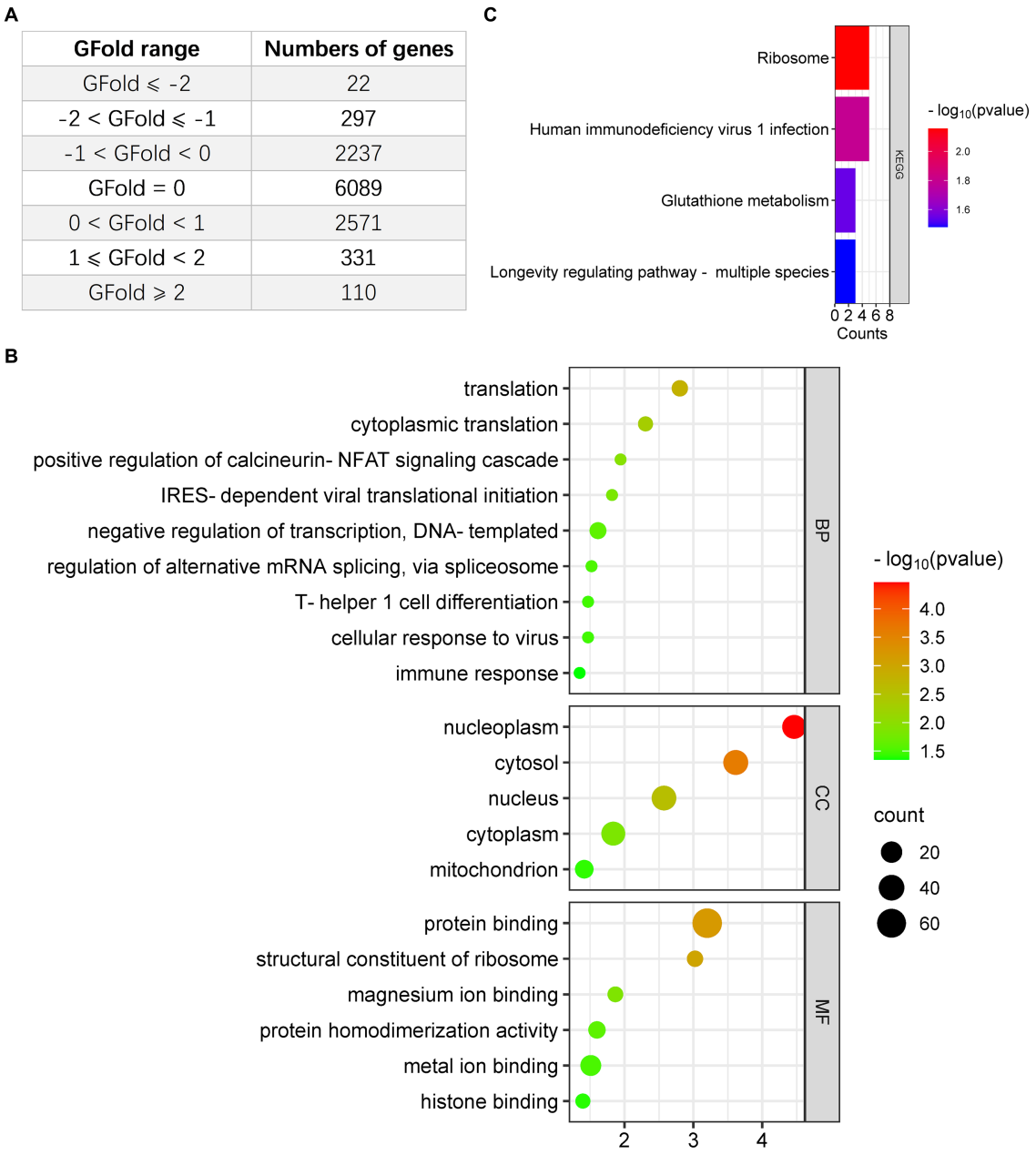

FIGURE 1
Analysis of downregulated DEGs in glioma cells infected with EV-A71, focusing on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). **(A)** The count of DEGs identified in EV-A71-infected glioma cells. **(B)** BioPlanet provided annotations for the prominent Biological Process (BP), Molecular Function (MF), or Cellular Component (CC) categories associated with the downregulated DEGs following EV-A71 infection in glioma cells. **(C)** BioPlanet annotated the top four conserved pathways among the downregulated DEGs following EV-A71 infection.

eradicating cancer cells. To assess its potential anti-tumor efficacy, we subsequently evaluated the impact of PTBP1 expression on immune infiltration in gliomas using the ssGESA algorithm. As illustrated in Figures 4A,C, the correlation results implied that PTBP1 was linked with the presence of T helper cell ($p < 0.001$, R = 0.488), Th2 cells ($p < 0.001$, R = 0.472) and macrophages ($p < 0.001$, R = 0.192), but negatively linked with the presence of NK CD56bright cell ($p < 0.001$, R = −0.378), mast cell ($p < 0.001$, R = −0.349) and Tha1 cells ($p < 0.001$, R = −0.087). The correlation results, as depicted in Figures 4A,C, demonstrated significant associations between PTBP1 and the presence of T helper cells ($p < 0.001$, R = 0.488), Th2 cells ($p < 0.001$, R = 0.472), and macrophages ($p < 0.001$, R = 0.192) in LGG. However, there was a negative association between PTBP1 and the presence of NK CD56bright cells ($p < 0.001$, R = −0.378), mast cells ($p < 0.001$, R = −0.349), and Th1 cells ($p < 0.001$, R = −0.168) in LGG. The analysis of GBM samples presented in Figures 4B,D revealed significant associations between PTBP1 and the presence of Th2 cells ($p < 0.001$, R = 0.427) and NK cells ($p < 0.001$, R = 0.289). Conversely, we observed a negative correlation between PTBP1 expression and the presence of macrophages ($p < 0.001$, R = −0.373), cytotoxia cells ($p < 0.001$, R = −0.352), T cells ($p < 0.001$, R = −0.315), and T cells ($p < 0.001$, R = −0.315).

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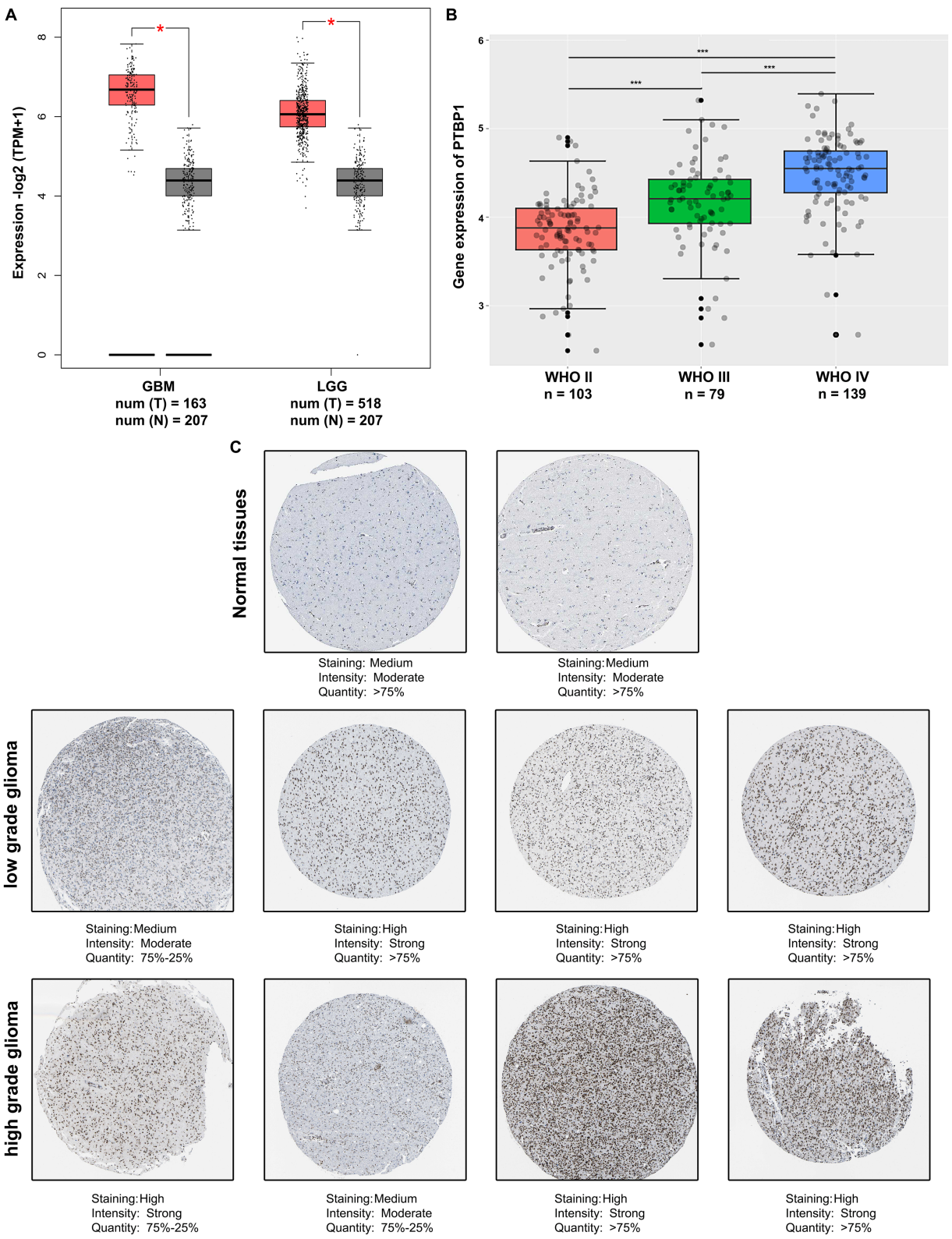

FIGURE 2
PTBP1 was upregulated in human glioma. **(A)** The expression levels of PTBP1 were validated by analyzing the TCGA data in LGG patients (n=518), GBM patients (n=207), and nontumor controls (n=207). Statistical significance was indicated by *p<0.05. **(B)** Analysis of CGGA data revealed the differential expression of PTBP1 mRNA across various glioma subgroups. Statistical significance was indicated by ***p<0.001. **(C)** Representative images of glioma tissue specimens (LGG and GBM) with both low and high PTBP1 levels are presented, as quantified by IHC staining in HPA database.

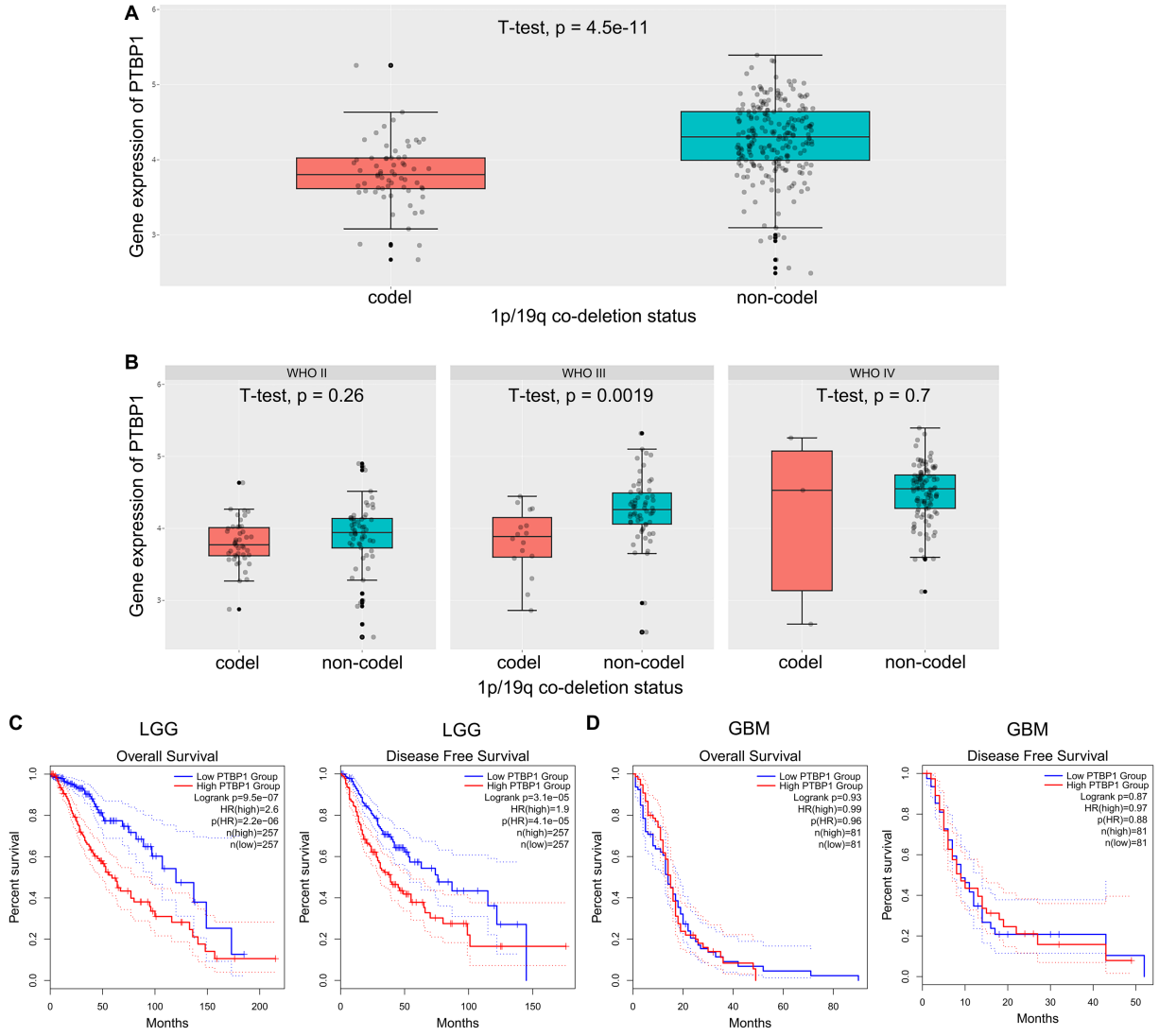

FIGURE 3
Correlation between PTBP1 mRNA expression and clinicopathological features in CGGA data. **(A)** The levels of PTBP1 mRNA expression were compared among glioma cases with different 1p/19q co-deletion statuses. **(B)** The levels of PTBP1 mRNA expression were further analyzed among different 1p/19q co-deletion statuses specifically in WHO II, WHO III, and WHO IV glioma. **(C,D)** The association between PTBP1 expression and survival was assessed among patients with LGG or GBM.

Association of PTBP1 and unfavorable prognosis in LGG immune cells

In addition, we investigated the association between PTBP1 level and immune cell infiltration using the TIMER algorithm. The left-most panel of Figures 5A,B showed that highly expressed PTBP1 levels have positive association with tumor purity in LGG. The data depicted in Figure 5A indicated a positive correlation between PTBP1 levels and the infiltration of B cells ($p = 7.77e\text{-}17$, cor = 0.369), macrophages ($p = 2.05e\text{-}22$, cor = 0.427), and neutrophils ($p = 8.56e\text{-}15$, cor = 0.346) in LGG. This consistent finding was also observed using the ssGSEA computational tool. However, in GBM, no significant association was found between PTBP1 expression levels and immune cell infiltration (Figure 5B). Subsequently, we examined the correlation of immune infiltration with OS in LGG. Interestingly, similar to PTBP1 expression, an obvious correlation between high level of immune cell infiltration and worse overall survival (all p-values <0.001, Figure 5C). These findings suggest that PTBP1 may function as an independent adverse prognostic biomarker in LGG, potentially in relation to immune cell infiltration. Next, we investigated glioma-related chromosomal change or mutation to explore the potential mechanism in immune infiltration. SCNA module of TIMER showed the distributions of immune subset (B cells, CD4+ T cells, macrophage, neutrophil and dendritic cells) at arm-level gain or high amplification of PTBP1 ($p < 0.05$ or $p < 0.001$, Figure 6A). Moreover, mutation module of TIMER demonstrated that the levels of immune infiltrates were related to IDH mutation status in LGG ($p < 0.001$, Figure 6B).

Discussion

The objective of this study was to examine the association between PTBP1 and EV-A71 infection in glioma cells, as well as evaluate the role of PTBP1 in patients diagnosed with LGG and GBM. The findings

TABLE 1 Characteristics of patients with glioma according to PTBP1 expression level.

Characteristics	Low expression of PTBP1	High expression of PTBP1	*p* value
n	349	350	
WHO grade, *n* (%)			< 0.001
G2	170 (26.7%)	54 (8.5%)	
G3	117 (18.4%)	128 (20.1%)	
G4	23 (3.6%)	145 (22.8%)	
IDH status, *n* (%)			< 0.001
WT	49 (7.1%)	197 (28.6%)	
Mut	295 (42.8%)	148 (21.5%)	
1p/19q codeletion, *n* (%)			< 0.001
Non-codel	222 (32.1%)	298 (43.1%)	
Codel	126 (18.2%)	46 (6.6%)	
Gender, *n* (%)			0.785
Female	147 (21%)	151 (21.6%)	
Male	202 (28.9%)	199 (28.5%)	
Age, *n* (%)			< 0.001
<= 60	310 (44.3%)	246 (35.2%)	
> 60	39 (5.6%)	104 (14.9%)	
Histological type, *n* (%)			< 0.001
Astrocytoma	109 (15.6%)	87 (12.4%)	
Oligoastrocytoma	86 (12.3%)	49 (7%)	
Oligodendroglioma	131 (18.7%)	69 (9.9%)	
Glioblastoma	23 (3.3%)	145 (20.7%)	
OS event, *n* (%)			< 0.001
Alive	278 (39.8%)	149 (21.3%)	
Dead	71 (10.2%)	201 (28.8%)	
DSS event, *n* (%)			< 0.001
No	279 (41.2%)	155 (22.9%)	
Yes	66 (9.7%)	178 (26.3%)	
Primary therapy outcome, *n* (%)			< 0.001
PD	49 (10.5%)	63 (13.5%)	
SD	93 (20%)	55 (11.8%)	
PR	42 (9%)	23 (4.9%)	
CR	96 (20.6%)	44 (9.5%)	

from our bioinformatic analysis demonstrated a substantial influence of PTBP1 expression on both survival and immune infiltration among LGG patients. These results indicate the potential clinical relevance of PTBP1 as a biomarker for patients with LGG.

The effect of oncolytic virotherapy in human glioma has been a subject of recent studies, generating a debate (Suryawanshi and Schulze, 2021). Although there is limited evidence supporting oncolytic virotherapy as a potential treatment for the challenges posed by the blood–brain barrier and the immunosuppressive TME, studies have suggested that ongoing clinical trials hold promise in establishing its satisfactory safety and significant immune response (Shoaf and Desjardins, 2022). Activation of the SCARB2/PMAIP1 axis has demonstrated considerable potential in facilitating cell oncolysis and, consequently, eradicating glioma cells (Zhang et al., 2020). In both *in vivo* and *in vitro* experiments, the presence of EV-A71 infection exerts a noteworthy influence in the anti-cancer process. Understanding the molecular networks governing glioma cells has been greatly enhanced by the mechanistic analysis of EV-A71 infection. In the present research, we conducted an evaluation of the DEGs associated with EV-A71 infection to identify potential regulatory processes or genes in glioma. By performing RNA-seq analysis, we observed a significant downregulation of DEGs in EV-A71-infected glioma cells. Importantly, our findings suggested a strong correlation between the downregulated DEGs and the cellular response to the virus, as well as the immune response in the infected glioma cells. These compelling findings prompted further investigations to unravel the mechanisms

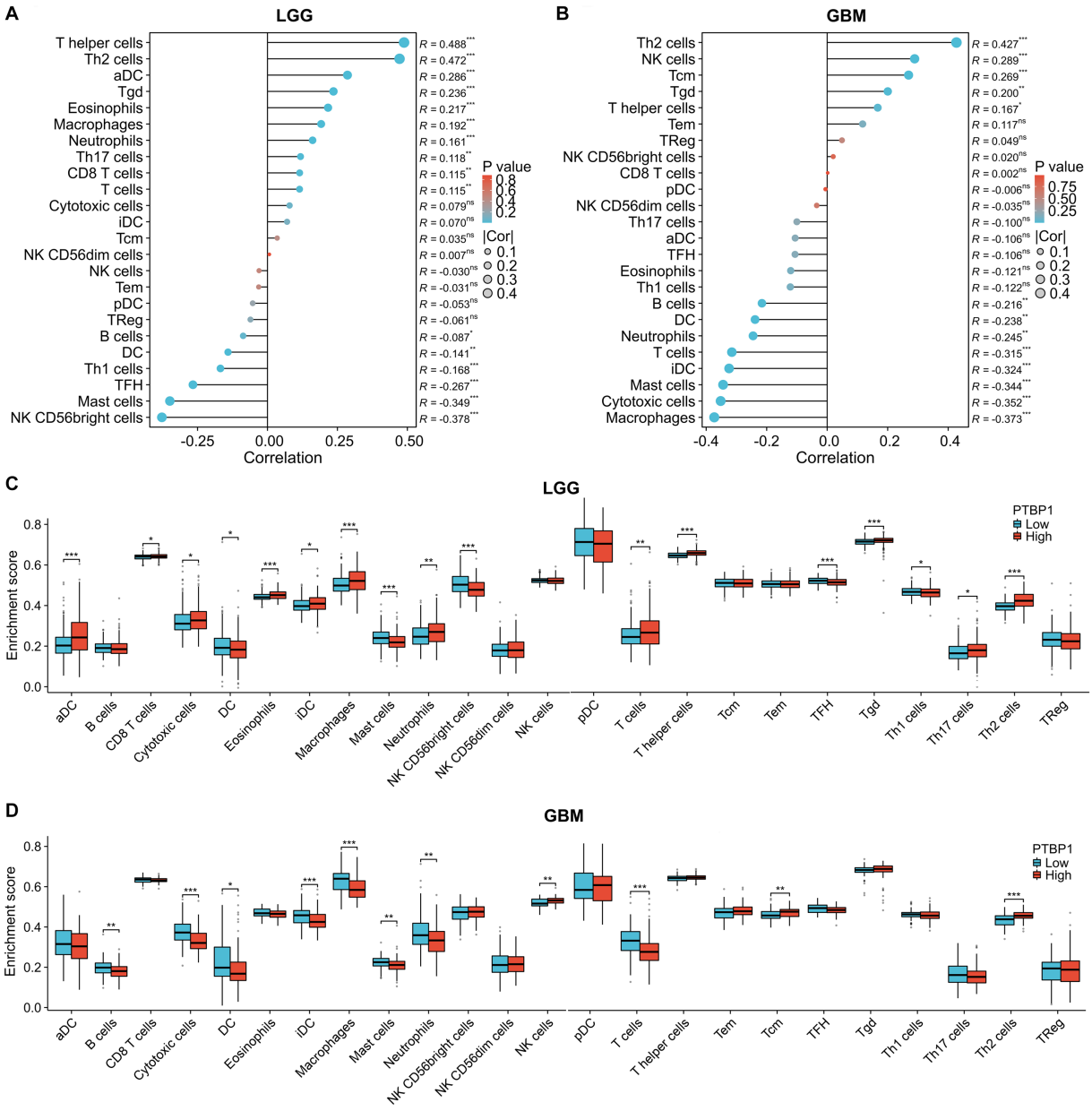

FIGURE 4
Association of PTBP1 expression and immune infiltration characteristic in glioma. To estimate immune cell infiltrations in TCGA data, the ssGESA algorithm was employed. Utilizing Pearson's method, we determined the correlation between PTBP1 levels and immune cells in both **(A)** LGG and **(B)** GBM. Additionally, the enrichment score of immune cells was assessed in PTBP1low and PTBP1high samples, separately for **(C)** LGG and **(D)** GBM. Statistical significance was denoted by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

underlying EV-A71 infection in the regulation and molecular pathogenesis of glioma. Consequently, we shed light on the previously unexplored regulatory role of a top downregulated gene in glioma, which represents one of the hallmarks contributing to tumor progression.

PTBP1 is an RNA-binding protein with multifunctionality, involved in the regulatory process of mRNA splicing and apoptosis in numerous tumors and diseases (Liu et al., 2023). In a study by Gong et al., it was suggested that PTBP1, being upregulated in pan-cancer, plays a role in mediating oncogenesis and immunity by influencing the growth and cell cycle of osteosarcoma cells (Gong et al., 2022). According to previous studies, the downregulation of PTBP1 has been shown to impair immune surveillance, leading to the inhibition of inflammation-induced tumorigenesis (Georgilis et al., 2018). It is noteworthy to mention that inhibiting the expression of PTBP1 does not have an impact on the differentiation of glioblastoma cells, but contributes to the inhibition of cancer growth (Liu et al., 2022; Wang et al., 2022). A previous study has documented a connection between elevated PTBP1 expression levels and the WHO grade in glioma (Liu et al., 2022). Consistent with previous findings, our study revealed a significant correlation between PTBP1 level and advanced tumor stage at both the mRNA and protein expression in glioma. This observation indicates that PTBP1 may play a role in promoting the progression of glioma. In contrast to the unfavorable prognosis typically observed in

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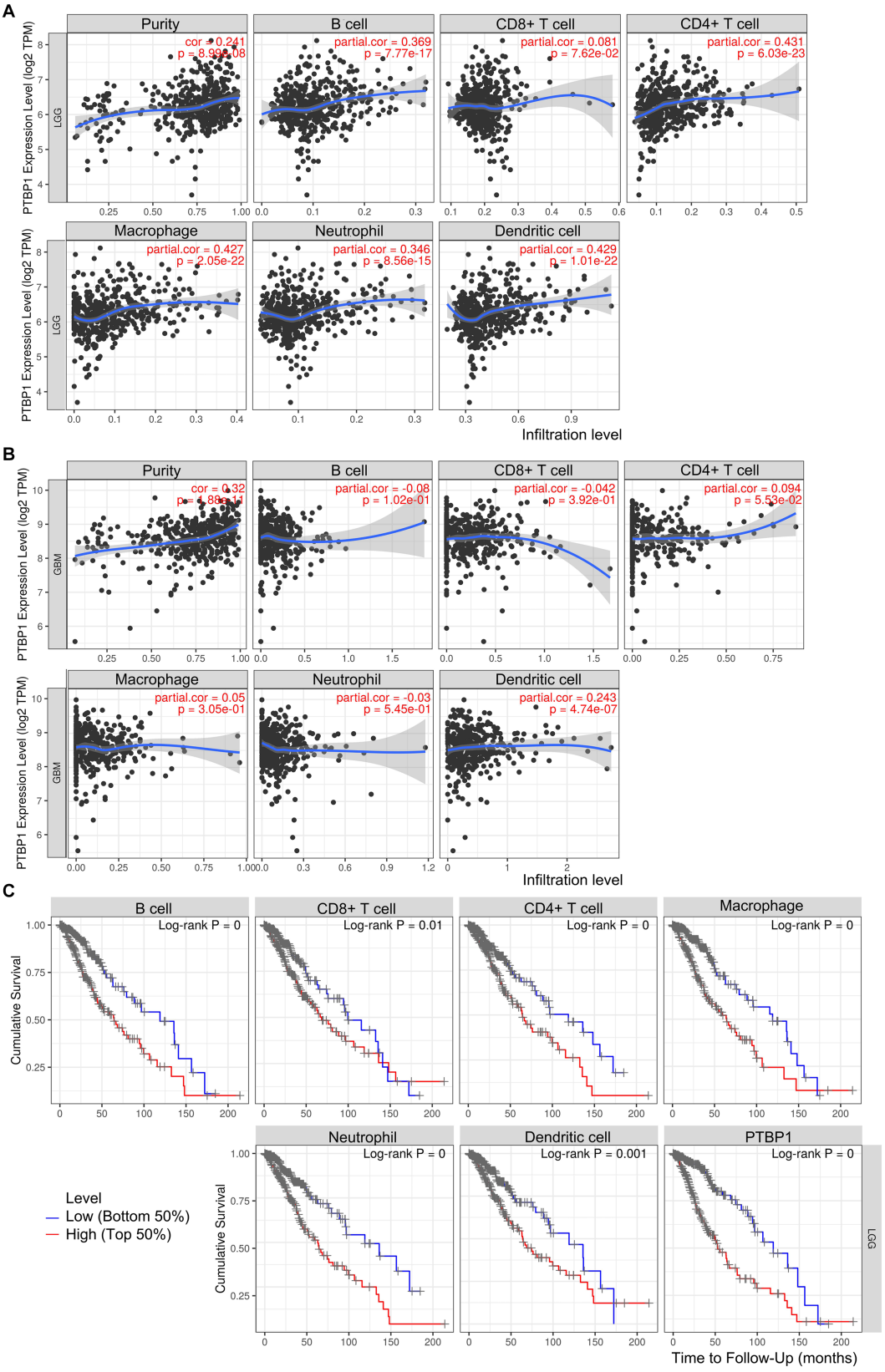

FIGURE 5
Association of PTBP1 expression and unfavorable prognosis in LGG immune cells. The correlation between PTBP1 levels and immune cells was analyzed in both **(A)** LGG and **(B)** GBM using TIMER algorithm. **(C)** Kaplan–Meier survival curves of OS comparing high (top 50%) and low (bottom 50%) immune infiltration in LGG.

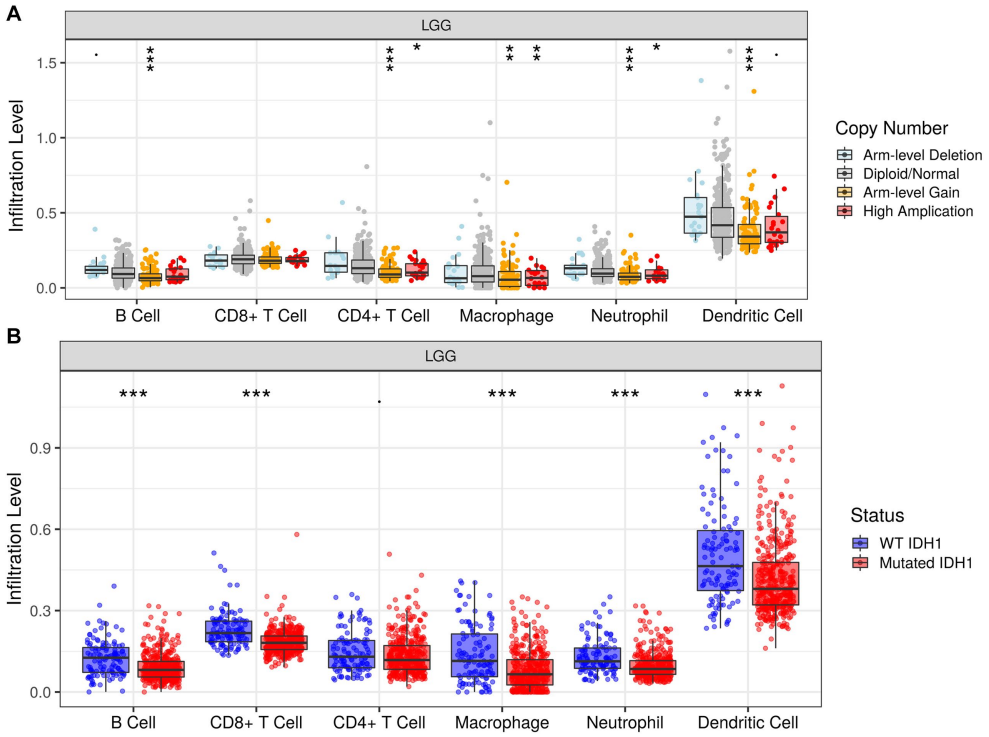

FIGURE 6
Association of infiltration levels and PTBP1 chromosomal change or IDH mutation in LGG cells. Histogram representing the infiltration levels of 6 immune cells (B cell, CD8 + T cell, CD4 + T cell, macrophage, neutrophil and dendritic cell) in different **(A)** copy number groups and **(B)** IDH mutation status.

GBM patients (Liu et al., 2022), these results revealed that high PTBP1 expression was not associated with worse prognosis in GBM. However, we observed that elevated PTBP1 expression was related to a worse prognosis in patients diagnosed with LGG.

Existing studies have documented the ability of OVs and the TME to generate immunostimulatory molecules, which play a crucial role in enabling the efficacy of immune therapies in the treatment of solid tumors (Sivanandam et al., 2019; Hemminki et al., 2020). The application of PD-1 blockade through OV infection has been reported to primarily result in enduring antitumor responses among cancer patients (Ribas et al., 2017). This approach is known to be correlated with the activation of the TME and increased infiltration of immune cells in melanoma (Ribas et al., 2017). An elevated level of immunostimulatory biomolecules is predominantly linked to the accumulation of T regulatory cells in GBM and is acknowledged for its role in compromising the immune system (Sokratous et al., 2017). In our study, we observed a relationship between TPBP1 and the infiltration of several immune cells in LGG. This association is likely to have contributed to the decline in OS. Furthermore, our investigation revealed a strong correlation between TPBP1 level and T helper cells, including CD4+ T cells and Th2 cells. The result suggests that TPBP1 might play a role in the unfavorable prognosis of LGG by interacting with T cells, specifically Th2 cells. The underlying reason for this finding is not yet fully understood; however, one potential explanation revolves around the equilibrium between Th1 and Th2 cells, which disrupts the dynamic balance of the cytokine network in humans and contributes to the initiation of tumor development (Romagnani, 1999; Matia-Garcia et al., 2021).

TME has gained prominence as a pivotal controller influencing the reaction to OV therapy. Owing to the distinct constitution of the extracellular matrix and immunologic surroundings within the cranial region, the brain's TME exhibits distinct characteristics and multiple tumor prognostic markers. For example, EVA1C is thought to be associated with various immune markers, such as B cells and CD4+ T cells. This association indicates that two member of EVA family, EVA1B and EVA1C, might have a role in prognosticating elevated levels of immune infiltration within glioma (Hu and Qu, 2021; Qu et al., 2021b). In addition, a literature analyzed the immune-related signature in glioma and suggested that the expression of OLFML3 might additionally mirror an irregular immune condition (Qu et al., 2023). To gain insight into the mechanisms underlying the association between the risk score and immune cell infiltration, we performed a detailed analysis of the impact of somatic cell copy number alterations (CNAs) in TPBP1. Intriguingly, we found that arm-level deletion had a significant effect on the infiltration levels of B cells, CD4+ T cells, macrophages, and dendritic cells in LGG. The results provided compelling evidence of the pivotal regulatory role of TPBP1 in shaping the TME for patients with LGG.

It is important to note that our study has limitations, as it relied on publicly available data rather than clinical samples, and did not include evidence from *in vitro* and *in vivo* research. The significance of PTBP1 in EV-A71-infected glioma and its potential role in expression levels in glioma have been strongly suggested, highlighting the light spot of this study. As a continuation of this research, we will assess the molecular mechanism of PTBP1 following EV-A71 infection in glioma.

In general, this research contributes to the expanding body of evidence highlighting the significant involvement of EV-A71 infection

in the gene regulation of glioma. Furthermore, our study demonstrated the potential prognostic significance of TPBP1 in LGG, as its overexpression was found to be linked to advanced tumor stage and a poorer prognosis. We also observed a correlation between TPBP1 and immune cell infiltration in LGG, a common occurrence within the immunosuppressive microenvironment of cancer. This study provides insight into a potential regulatory mechanism involved in OV treatment for glioma, laying the groundwork for future investigations aimed at unraveling the prospective molecular function of TPBP1 in glioma.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: PRJNA562271, https://www.ncbi.nlm.nih.gov/bioproject.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

ZH, XY, and HD designed the conception of this study. ZH, XY, RD, and LC were involved in the data analysis. ZH and XY drafted the initial manuscript. The final version of the manuscript was reviewed and revised by RD, LC, CH, and HD. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1249289/full#supplementary-material

References

Barthel, F. P., Johnson, K. C., Varn, F. S., Moskalik, A. D., Tanner, G., Kocakavuk, E., et al. (2019). Longitudinal molecular trajectories of diffuse glioma in adults. *Nature* 576, 112–120. doi: 10.1038/s41586-019-1775-1

Bhattacharya, D., Sinha, N., and Saini, J. (2021). Determining chromosomal arms 1p/19q co-deletion status in low graded glioma by cross correlation-periodogram pattern analysis. *Sci. Rep.* 11:23866. doi: 10.1038/s41598-021-03078-1

Chen, R., Smith-Cohn, M., Cohen, A. L., and Colman, H. (2017). Glioma subclassifications and their clinical significance. *Neurotherapeutics* 14, 284–297. doi: 10.1007/s13311-017-0519-x

Gállego Pérez-Larraya, J., Garcia-Moure, M., Labiano, S., Patiño-García, A., Dobbs, J., Gonzalez-Huarriz, M., et al. (2022). Oncolytic DNX-2401 virus for pediatric diffuse intrinsic pontine glioma. *N. Engl. J. Med.* 386, 2471–2481. doi: 10.1056/NEJMoa2202028

Georgilis, A., Klotz, S., Hanley, C. J., Herranz, N., Weirich, B., Morancho, B., et al. (2018). PTBP1-mediated alternative splicing regulates the inflammatory Secretome and the pro-tumorigenic effects of senescent cells. *Cancer Cell* 34, 85–102.e9. doi: 10.1016/j.ccell.2018.06.007

Gong, H., Jiang, A., Jiang, R., Wang, Y., Zhang, D., Wu, Z., et al. (2022). PTBP1 as a promising predictor of poor prognosis by regulating cell proliferation, immunosuppression, and drug sensitivity in SARC. *Oxidative Med. Cell. Longev.* 2022, 1–26. doi: 10.1155/2022/5687238

Hemminki, O., Dos Santos, J. M., and Hemminki, A. (2020). Oncolytic viruses for cancer immunotherapy. *J. Hematol. Oncol.* 13:84. doi: 10.1186/s13045-020-00922-1

Hu, X., Martinez-Ledesma, E., Zheng, S., Kim, H., Barthel, F., Jiang, T., et al. (2017). Multigene signature for predicting prognosis of patients with 1p19q co-deletion diffuse glioma. *Neuro-Oncology* 19, 786–795. doi: 10.1093/neuonc/now285

Hu, Z., and Qu, S. (2021). EVA1C is a potential prognostic biomarker and correlated with immune infiltration levels in WHO grade II/III glioma. *Front. Immunol.* 12:683572. doi: 10.3389/fimmu.2021.683572

Hu, M., Wang, B., and Wu, C. (2022). Editorial: the role of DNA viruses in human cancers. *Front. Cell. Infect. Microbiol.* 12:1103505. doi: 10.3389/fcimb.2022.1103505

Hu, M., Xing, B., Yang, M., Han, R., Pan, H., Guo, H., et al. (2023). Characterization of a novel genus of jumbo phages and their application in wastewater treatment. *iScience* 26:106947. doi: 10.1016/j.isci.2023.106947

Hulou, M. M., Cho, C. F., Chiocca, E. A., and Bjerkvig, R. (2016). Experimental therapies: gene therapies and oncolytic viruses. *Handb. Clin. Neurol.* 134, 183–197. doi: 10.1016/B978-0-12-802997-8.00011-6

Kiyokawa, J., Kawamura, Y., Ghouse, S. M., Acar, S., Barçın, E., Martínez-Quintanilla, J., et al. (2021). Modification of extracellular matrix enhances oncolytic adenovirus immunotherapy in glioblastoma. *Clin. Cancer Res.* 27, 889–902. doi: 10.1158/1078-0432.CCR-20-2400

Le Rhun, E., Preusser, M., Roth, P., Reardon, D. A., van den Bent, M., Wen, P., et al. (2019). Molecular targeted therapy of glioblastoma. *Cancer Treat. Rev.* 80:101896. doi: 10.1016/j.ctrv.2019.101896

Li, J., Meng, Q., Zhou, X., Zhao, H., Wang, K., Niu, H., et al. (2022). Gospel of malignant glioma: oncolytic virus therapy. *Gene* 818:146217. doi: 10.1016/j.gene.2022.146217

Liu, P., He, G. C., Tan, Y. Z., Liu, G. X., Liu, A. M., Zhu, X. P., et al. (2022). PTBP1 is a novel poor prognostic factor for glioma. *Bio. Med. Res. Int.* 2022, 1–11. doi: 10.1155/2022/7590997

Liu, H. L., Lu, X. M., Wang, H. Y., Hu, K. B., Wu, Q. Y., Liao, P., et al. (2023). The role of RNA splicing factor PTBP1 in neuronal development. *Biochim. Biophys. Acta, Mol. Cell Res.* 1870:119506. doi: 10.1016/j.bbamcr.2023.119506

Malik, N., Geraghty, B., Dasgupta, A., Maralani, P. J., Sandhu, M., Detsky, J., et al. (2021). MRI radiomics to differentiate between low grade glioma and glioblastoma peritumoral region. *J. Neuro-Oncol.* 155, 181–191. doi: 10.1007/s11060-021-03866-9

Matia-Garcia, I., Vadillo, E., Pelayo, R., Muñoz-Valle, J. F., García-Chagollán, M., Loaeza-Loaeza, J., et al. (2021). Th1/Th2 balance in young subjects: relationship with

cytokine levels and metabolic profile. *J. Inflamm. Res.* 14, 6587–6600. doi: 10.2147/JIR.S342545

Qu, S., Chen, Z., Liu, B., Liu, J., and Wang, H. (2021a). N6-methyladenine-related genes affect biological behavior and the prognosis of glioma. *Cancer Med.* 10, 98–108. doi: 10.1002/cam4.3574

Qu, S., Huang, C., Zhu, T., Wang, K., Zhang, H., Wang, L., et al. (2023). OLFML3, as a potential predictor of prognosis and therapeutic target for glioma, is closely related to immune cell infiltration. *VIEW* 4:20220052. doi: 10.1002/VIW.20220052

Qu, S., Liu, S., Qiu, W., Liu, J., and Wang, H. (2020). Screening of autophagy genes as prognostic indicators for glioma patients. *Am. J. Transl. Res.* 12, 5320–5331.

Qu, S., Liu, J., and Wang, H. (2021b). EVA1B to evaluate the tumor immune microenvironment and clinical prognosis in glioma. *Front. Immunol.* 12:648416. doi: 10.3389/fimmu.2021.648416

Ribas, A., Dummer, R., Puzanov, I., Vander Walde, A., Andtbacka, R. H. I., Michielin, O., et al. (2017). Oncolytic virotherapy promotes intratumoral T cell infiltration and improves anti-PD-1 immunotherapy. *Cells* 170, 1109–19.e10. doi: 10.1016/j.cell.2017.08.027

Rius-Rocabert, S., García-Romero, N., García, A., Ayuso-Sacido, A., and Nistal-Villan, E. (2020). Oncolytic Virotherapy in glioma tumors. *Int. J. Mol. Sci.* 21:7604. doi: 10.3390/ijms21207604

Romagnani, S. (1999). Th1/Th2 cells. *Inflamm. Bowel Dis.* 5, 285–294. doi: 10.1097/00054725-199911000-00009

Russell, S. J., Peng, K. W., and Bell, J. C. (2012). Oncolytic virotherapy. *Nat. Biotechnol.* 30, 658–670. doi: 10.1038/nbt.2287

Sherman, B. T., Hao, M., Qiu, J., Jiao, X., Baseler, M. W., Lane, H. C., et al. (2022). DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res.* 50, W216–W221. doi: 10.1093/nar/gkac194

Shoaf, M. L., and Desjardins, A. (2022). Oncolytic viral therapy for malignant glioma and their application in clinical practice. *Neurotherapeutics* 19, 1818–1831. doi: 10.1007/s13311-022-01256-1

Sivanandam, V., LaRocca, C. J., Chen, N. G., Fong, Y., and Warner, S. G. (2019). Oncolytic viruses and immune checkpoint inhibition: the best of both worlds. *Mol. Therapy Oncolytics* 13, 93–106. doi: 10.1016/j.omto.2019.04.003

Sokratous, G., Polyzoidis, S., and Ashkan, K. (2017). Immune infiltration of tumor microenvironment following immunotherapy for glioblastoma multiforme. *Hum. Vaccin. Immunother.* 13, 2575–2582. doi: 10.1080/21645515.2017.1303582

Suryawanshi, Y. R., and Schulze, A. J. (2021). Oncolytic viruses for malignant glioma: on the verge of success? *Viruses* 13:1294. doi: 10.3390/v13071294

van Tellingen, O., Yetkin-Arik, B., de Gooijer, M. C., Wesseling, P., Wurdinger, T., and de Vries, H. E. (2015). Overcoming the blood-brain tumor barrier for effective glioblastoma treatment. *Drug Resist. Updat.* 19, 1–12. doi: 10.1016/j.drup.2015.02.002

Wang, K., Pan, S., Zhao, P., Liu, L., Chen, Z., Bao, H., et al. (2022). PTBP1 knockdown promotes neural differentiation of glioblastoma cells through UNC5B receptor. *Theranostics* 12, 3847–3861. doi: 10.7150/thno.71100

Xia, J., Li, S., Liu, S., and Zhang, L. (2023). Aldehyde dehydrogenase in solid tumors and other diseases: potential biomarkers and therapeutic targets. *Media Commun.* 4:e195. doi: 10.1002/mco2.195

You, L., Chen, J., Liu, W., Xiang, Q., Luo, Z., Wang, W., et al. (2020). Enterovirus 71 induces neural cell apoptosis and autophagy through promoting ACOX1 downregulation and ROS generation. *Virulence* 11, 537–553. doi: 10.1080/21505594.2020.1766790

Zhang, X., Wang, H., Sun, Y., Qi, M., Li, W., Zhang, Z., et al. (2020). Enterovirus A71 Oncolysis of malignant gliomas. *Mol. Ther.* 28, 1533–1546. doi: 10.1016/j.ymthe.2020.04.005

Zhao, Z., Zhang, K.-N., Wang, Q., Li, G., Zeng, F., Zhang, Y., et al. (2021). Chinese glioma genome atlas (CGGA): a comprehensive resource with functional genomic data from Chinese glioma patients. *Genom. Prot. Bioinform.* 19, 1–12. doi: 10.1016/j.gpb.2020.10.005

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A study on the correlation between the prognosis of HPV infection and lesion recurrence after cervical conization

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Introduction: Persistent human papillomavirus infection is an important factor in the development of cervical cancer, which is usually a long process evolving from the development of squamous intraepithelial lesions (SIL), also referred to as cervical intraepithelial neoplasia (CIN). Local treatment of advanced squamous intraepithelial lesions, also regarded as High-Grade Squamous Intraepithelial Lesion, may be effective in preventing cancer.

Objective: To promptly identify high-risk patients with a tendency to recurrence.

Methods: We retrospectively analyzed the clinical data of 300 patients with high-grade squamous intraepithelial lesions of the cervix admitted to the Second Affiliated Hospital of Dalian Medical University from 2019 to 2020 to investigate the relationship between recurrence of cervical lesions and postoperative regression of HPV infection, as well as other related risk factors.

Results: We found that the HPV-negative rates were 81.81, 85.71, and 90.91% at 6, 12, and 24 months, respectively, and the average lesion recurrence rate was 8.16%, with a median time to recurrence of 14 months in patients undergoing CKC for HSIL. The risk of cervical squamous intraepithelial lesions was highest in patients with HPV16. Patients over 61 years of age had the lowest postoperative HPV-negative rate. The conversion rate was significantly lower in patients with multiple HPV genotypes than in those with single HPV infection ($p < 0.05$). The probability of recurrence was higher in patients with the same HPV infection genotype before and after surgery than in patients with different infection genotypes before and after surgery ($p < 0.05$).

Conclusion: Combined with the literature review, we believe that patients aged ≥50 years, with ≥3 pregnancies and births, a history of smoking, and consistent genotypes of preoperative and postoperative HPV infection in cervical conization have more HPV re-infection or persistent infection, and that these factors may be high-risk factors for lesion recurrence. For patients with possible potential high-risk factors, we need to carry out individualized follow-up and focused management, take timely and effective management measures, optimize the treatment plan, reduce the recurrence rate, prevent HSIL and cervical cancer, improve the quality of patient's survival, and improve the prognosis.

KEYWORDS

HPV infections, cervical lesions, cervical conization, recurrence, prognosis

Introduction

At present, cervical cancer has become a public health problem of global concern. It is one of the most common malignant tumors among women in the world, ranking fourth in incidence rate and mortality (Ferlay et al., 2015). High-risk Human Papilloma Virus (HPV) infection is considered the main cause of cervical cancer (Schiffman et al., 2007). Persistent human papilloma virus infection is an important factor in the development of cervical cancer, which is usually a long process evolving from the development of squamous intraepithelial lesions (SIL), also referred to as CIN. Local treatment of advanced squamous intraepithelial lesions, also regarded as High-Grade Squamous Intraepithelial Lesion (HSIL), may be effective in preventing cancer. So cervical cancer is the only malignant tumor that can be prevented by vaccination, and we can achieve early detection, treatment, and prevention through cervical precancerous screening. It is very important to detect cervical lesions in time and block their development. The American Society for Colposcopy and Cervical Pathology (ASCCP) recommends that cervical conization with fertility preservation be the first choice for HSIL with satisfactory Colposcopy examination. Cervical conization includes loop electrosurgical excision procedure (LEEP) and cold knife conization (CKC), but about 15% of patients experience varying degrees of residual or recurrence after surgery (Kocken et al., 2011). How to detect high-risk recurrence patients in time is still inconclusive. Therefore, this article attempts to analyze the prognosis of HSIL cervical conization surgery with HPV infection, explore the relationship between cervical lesion recurrence and HPV prognosis, and other related risk factors, with the aim of timely identify high-risk patients with recurrence tendency, and improve the survival rate and prognosis of patients.

Methods

This article retrospectively analyzed the clinical data of 300 patients with high-grade squamous intraepithelial lesions (HSIL) of the cervix admitted to the Second Affiliated Hospital of Dalian Medical University from 2019 to 2020. The inclusion criteria for cases are those with HPV infection and pathologically diagnosed as HSIL through cervical biopsy. All patients underwent CKC for treatment. We collected age, pathological type, and involvement of glands in the lesion, HPV infection type and genotype, pregnancy and childbirth history, smoking history, postoperative HPV follow-up results at 6, 12, and 24 months, as well as the HPV infection status and lesion characteristics of recurrent patients. The follow-up period was until January 2023. All information is obtained by consulting medical records. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University. We used SPSS statistical software for data analysis, counting data use cases and percentage, and comparing multiple groups using χ^2 tests or Fisher's exact probability method, using Bonferroni correction for pairwise comparison. The difference with $p < 0.05$ is statistically significant.

Results

Analysis of clinical data characteristics of patients with high-grade squamous intraepithelial lesions of the cervix.

TABLE 1 Chi square analysis of gland involvement and HPV negative conversion rate.

Gland involvement	Negative	Positive	Total	χ^2	p
Y(Yes)	118(77.12%)	35 (22.88%)	153	0.043	0.836
N(No)	72 (78.26%)	20 (21.74%)	92		
Total	190 (77.55%)	55 (22.45%)	245		

Among the 300 patients we collected, the average age was 41.13 years (21–70 years). 245 patients completed follow-up, with an overall follow-up rate of 81.67%. 20 patients experienced recurrence within 4 years after surgery, with an average recurrence rate of 8.16% and a median recurrence time of 14 months. Postoperative pathology of 153 patients with cervical cone resection revealed gland involvement. There was no significant difference in the postoperative negative conversion rate between them and patients without gland involvement. We believe that whether the gland is involved or not has no significant effect on the postoperative recurrence rate (Table 1).

Analysis of HPV infection genotypes before and after operation

Table 2 lists the statistics of HPV infection genotypes of HSIL patients before operation, and the top five are genotypes 16, 52, 58, 33, and 31 in turn. 146 cases of cervical high-grade squamous intraepithelial lesions caused by HPV16, accounting for 35.78%. The genotypes of HPV reinfection after cervical conization, and the top five are genotypes 16, 58, 52, 53, and 39. Thus it can be seen that the common HPV infection genotypes in patients with HSIL are 16, 52, and 58 genotypes. Genotypes 16 of HPV has the highest risk of cervical squamous intraepithelial lesions.

Analysis of age and recovery from HPV infection after cervical conization

Figure 1 shows the statistics of HPV retesting after cervical conization in different age groups, and a total of 192 cases were screened with negative results of the last HPV test, with an overall HPV conversion rate of 79.18%. We found that patients over 61 years of age had the lowest postoperative HPV conversion rate of about 73.33%. The conversion rate was also lower in the age group of 51–60 years, at 74.70%, with little difference in the rate between the ages of 30 and 50 years. However, there was no significant difference between the conversion rates of each age group by χ^2 test.

Correlation of multiple HPV infections, HPV genotypes, and recovery from HPV infection after cervical conization

Of the 245 follow-up patients who underwent cervical conization for HSIL, 110 patients were retested for HPV at 6 months postoperatively with a negative rate of 81.81%, 98 patients were retested for HPV at 12 months postoperatively with a negative rate of

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TABLE 2 Statistics of HPV infection genotypes before operation and reinfection genotypes after surgery.

Genotypes before operation	Cases	Percentage (%)	Reinfection genotypes after surgery	Cases	Percentage (%)
16	146	35.78	16	23	19.83
52	41	10.05	58	22	18.97
58	39	9.56	52	13	11.21
33	21	5.15	53	11	9.48
31	18	4.41	39	8	6.90
18	17	4.17	56	7	6.03
35	12	2.94	51	6	5.17
51	12	2.94	CP8304	6	5.17
53	12	2.94	33	5	4.31
68	11	2.70	59	4	3.45
39	10	2.45	68	4	3.45
56	8	1.96	66	3	2.59
66	7	1.72	11	1	0.86
81	7	1.72	31	1	0.86
Not genotyped	14	3.42	45	1	0.86
45	6	1.47	6	1	0.86
59	5	1.23			
6	4	0.98			
42	3	0.74			
82	3	0.74			
11	2	0.49			
Others	10	2.45			

Other genotypes include: 9, 26, 40, 43, 54, 55, 61, 73, 78, 83, etc.

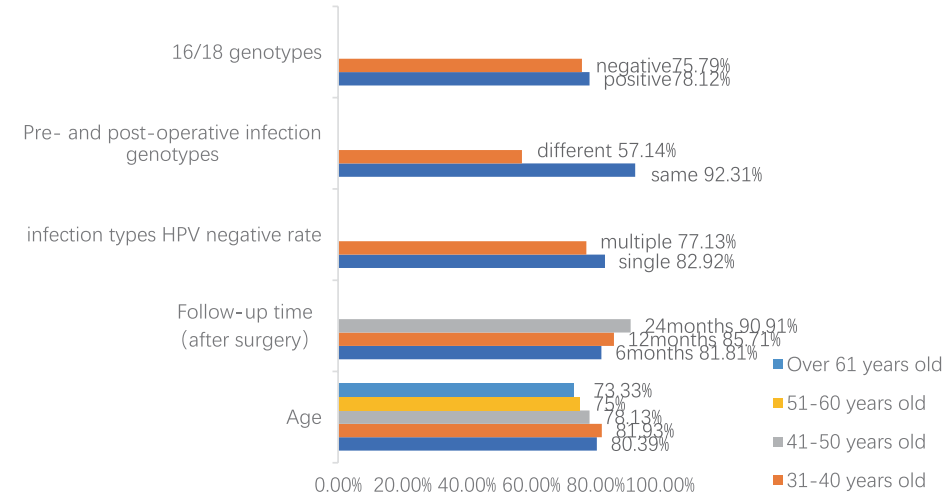

FIGURE 1
Summary of risk factors and negative rate of HPV after cervical conization (Due to the multiple categories of data, the legend only indicates different age groups, and the remaining data are marked as follows: negative = 16/18 genotypes negative, positive = 16/18 genotypes positive, same = the same genotype before and after surgery, different = different genotype before and after surgery, single = single HPV genotype infection, and multiple = multiple HPV genotypes infection.).

85.71%, and 66 patients were retested for HPV at 24 months postoperatively with a negative rate of 90.91%. We can see that the negative rate of HPV test is gradually increasing with time, and the negative rate of HPV test is more than 90% at 2 years after the operation. This also shows that cervical conization is effective in removing HPV. Meanwhile, we found that the negative rate of 49

patients with multiple HPV infections was 77.13%, which was significantly lower than those with HPV mono-infections ($p = 0.049$). We also statistically analyzed the sex-negative rate of patients infected with HPV genotypes 16 or 18 and found that whether they were infected with HPV genotypes 16 or 18 did not significantly affect the negative rate ($p = 0.681$).

For patients who were reinfected with HPV (first postoperative HPV-negative review and second or subsequent HPV-positive review), we sorted the data according to whether the patients were infected with the same HPV genotypes before and after surgery, and we analyzed the effect of the consistency of the preoperative and postoperative infection genotypes on the recurrence of lesions. Due to the small sample size and some subgroup sample counts less than 5, the use of the traditional chi-square test tends to bias the test results, so Fisher's exact test was used for chi-square estimation. The results showed that at the 10% significance level, the probability of recurrence was higher in patients with the same HPV infection genotype before and after surgery than in patients with different infection genotypes before and after surgery.

Correlation between the number of pregnancies and births and HPV infection and regression

We counted the maternal history of 271 patients and found that the number of pregnancies and births had no significant effect on the HPV conversion rate. Due to the small sample size and some subgroup sample counts less than 5, the use of the traditional chi-square test is prone to bias the test results, so Fisher's exact test was used for chi-square analysis (Table 3).

Discussion

HPV and cervical squamous intraepithelial lesions

Persistent human papillomavirus infection is an important factor in the development of cervical cancer, which is usually a long process evolving from the development of squamous intraepithelial lesions (SIL), also known as cervical intraepithelial neoplasia (CIN). Early studies have shown that women with persistent HPV infection have a significantly increased risk of developing cervical squamous intraepithelial lesions, which can progress to cancer if not detected and treated in time (Walboomers et al., 1999; Bosch et al., 2002). In other words, local treatment of advanced squamous intraepithelial lesions, also known as HSIL, may be effective in preventing cancer (International Agency for Research on Cancer, 2005, 2020; Kyrgiou et al., 2020). Local cervical treatments include large loop excision of the transformation zone (LLETZ), cold knife conization (CKC), loop electrosurgical excision procedure (LEEP), or ablation. Because cervical conization allows assessment of the margins of the excised tissue for residual lesions, excisional treatment is currently considered superior to ablation (Perkins et al., 2020). Arbyn et al. showed that the average risk of recurrence after surgery for high-grade lesions was only 6.6%. Among them, the risk of recurrence of HSIL or CIN was 6.7% for LLETZ, while CKC or laser conization had only a 2.1–2.2% risk of recurrence (Arbyn et al., 2017). At the same time, several studies have shown that the recurrence rate after CKC ranges from 1.4 to 2.2% (Santesso et al., 2016; Arbyn et al., 2017; Zhang and Lin, 2022), while the failure rate after LEEP treatment ranges from 7 to 14% (Kreimer et al., 2006; Lubrano et al., 2012; Kuroki et al., 2016). All patients in our study underwent CKC, the recurrence rate of the lesions was 8.16%, the median time to lesion recurrence was 14 months, and the pathological type was LSIL. We believe that CKC and LEEP have their own advantages, and the treatment should be individualized according to the patient's condition. Updates and improvements in surgical approaches need to be further explored and confirmed by multicenter, large-sample prospective studies.

TABLE 3 Chi-square test of the number of pregnancies, births, and HPV negativity rate.

Number of pregnancies	Negative	Positive	Total	χ^2	p
0	33(86.84%)	5 (13.16%)	38		
1–2	131 (82.39%)	28 (17.61%)	159	0.743	0.878
3–4	89 (82.41%)	19 (17.59%)	108		
5 and more	11 (78.57%)	3 (21.43%)	14		
Number of births					
0	53 (84.13%)	10 (15.87%)	63		
1	171 (83.01%)	35 (16.99%)	206	0.090	0.977
2 and more	47 (82.46%)	10 (17.54%)	57		
Total	272 (83.18%)	55 (16.82%)	327		

Analysis of HPV regression after cervical conization

There is an association between the onset, progression, treatment, and prognosis of SIL or CIN and HPV infection (Salvadó et al., 2021). However, HPV infection is asymptomatic in most patients and resolves spontaneously within 1–2 years (Frazer, 2009). The 2-year HPV-negative rate after cervical conization in our study was more than 90%. To reduce the psychological and economic burden on patients, we believe that a 6-month postoperative follow-up period is appropriate. In addition, Alonsol et al. and Perkins et al. (2020) showed that HPV viral load was also strongly related to postoperative residue or recurrence, with higher rates of postoperative residue or recurrence in the group of patients with high preoperative HPV viral load. Thus, persistent HPV infection, as well as HPV viral load, may be a high-risk factor for residue or recurrent postoperative lesions. Whether or not to include HPV viral load as one of the postoperative tests then needs to be supported by more clinical research data. Although surgery removes most of the HPV, it does not remove it completely, and if residual HPV-infected lesions remain after surgery, it may lead to recurrence of the lesions or even eventual progression to cervical cancer.

Age

We counted the HPV testing after cervical conization in different age groups. And we found that there was a relationship between the age of patients and postoperative residual or recurrence. We found

that postoperative HPV-negative rates decreased progressively with increasing age or late menopausal period, although there was no dramatic difference in postoperative negative rates between age groups, with the lowest postoperative HPV-negative rates found in patients over the age of 61 years. It has been shown (Bilibio et al., 2019) that low estrogen levels in postmenopausal women reduce the number of cells secreting interferon-γ and tumor necrosis factor--α, thereby decreasing immune reactivity, and have an accelerating effect on the release of pro-inflammatory cytokines, which in turn promotes lesion progression. Petorius et al. (2006) followed patients after CIN conization and found that the subsequent risk of CIN 3 or cancer increased with patient age. Therefore, we believe that for elderly patients, especially postmenopausal patients, considering that their lesions are mostly hidden in the cervical canal and their immune function is declining, as well as the poor adherence to postoperative follow-up in elderly patients, an individualized plan of treatment should be formulated, and close postoperative follow-up should be performed to detect recurrent cases in time and take appropriate management measures.

Maternity and smoking

Early studies have shown that pregnancy-associated elevated levels of estrogen or growth hormone, such as Human Chorionic Gonadotropin (HCG), may increase the activity of HPV molecules, which in turn may influence the natural history of HPV infection and the progression of lesions (Nair et al., 2005; Delvenne et al., 2007). Jensen et al. (2013) found that childbirth increased the risk of HSIL even more than the risk of persistent HPV infection. The mechanism by which pregnancy influences the progression of HPV infection to clinical lesions may be related to the pregnancy-associated increase in HPV molecular activity due to elevated levels of estrogen or growth hormone, such as human chorionic gonadotropin (HCG). However, there are also studies that show no increased risk of HPV infection during pregnancy (Banura et al., 2008; Schmeink et al., 2012; Trottier et al., 2015). In our study, we counted the maternal history of conization patients and found that the number of pregnancies and births did not have a significant effect on the HPV conversion rate after conization. Therefore, we believe that further prospective studies with large samples are needed to confirm the effects of pregnancy and maternal history on HPV infection and postoperative recurrence.

Smoking has been shown to be an independent risk factor for HSIL in young women after the onset of frequent sexual activity (Collins et al., 2010). Several studies have shown (International Collaboration of Epidemiological Studies of Cervical Cancer et al., 2006; Koshiyama et al., 2019) that smokers have an increased risk of developing cervical cancer compared with nonsmokers, and that the risk of squamous cell carcinoma of smokers increases with the number of cigarettes smoked every day and with decreasing age of smoking initiation. Inamine et al. (2012) found that smoking induces high plasma expression of vascular endothelial growth factor C (VEGF-C) in patients with CIN and that VEGF-C plays a major role in cervical lesions in smoking patients. However, it has also been shown (Hui-Qiong et al., 2016) that there is no statistically dramatic difference in the comparison of postoperative recurrence between smoking and non-smoking patients. The smoking history in our study was only one person, and there are fewer large-sample studies on the effect of smoking on postoperative HPV regression or lesion recurrence, so we believe this requires more data collection and further exploration.

Effect of postoperative re-infection with HPV subtype and type on recurrence

Multiple infections refer to the coexistence of two or more HPV subtypes in patients with cervical cancer, which may be dual, triple or even more. Reinfection with HPV after cervical conization remains a high-risk factor for lesion recurrence, and which subtypes and types of infection are more likely to cause recurrence needs to be further explored. Gök et al. (2007) and Bais et al. (2009) reported that women infected with HPV16 were significantly more likely to develop residual/recurrent CIN than women infected with other HR-HPV genotypes. However, because the study included a small number of residual cases, this does not confirm the correlation between HPV genotype infection and recurrence. Nam et al. (2009) conducted a study on recurrent cases with negative margins and found that pre-taper HPV16 infection was significantly associated with persistence of High Risk-Human Papilloma Virus (HR-HPV) after taper. Furthermore, it has also been reported (Söderlund-Strand et al., 2014; Kang and Kim, 2016) that HPV18 is more likely to be related to recurrence of high-grade CIN ($p < 0.05$). Vintermyr et al. (2014) reported that 95.9% of recurrent CIN2+ patients had persistent HR-HPV infection, of which 74.5% were HPV16 or HPV 18. More than 60% of recurrent CIN2+ occurred within 3 years of initial conization, indicating a high risk of recurrence in patients with persistent HR-HPV infection. In this study, HPV16, HPV52, and HPV58 were the top three infection genotypes in order before conization, and HPV16, HPV58, and HPV52 were the top three infection genotypes in order after conization.HPV16 was the genotype with the highest infection rate before and after conization, but we statistically found that whether or not we were infected with HPV genotypes 16/18 did not have a significant effect on the rate of conversions ($p = 0.836$). Meanwhile, in our study, the negative rate of 49 patients with multiple HPV infections after cervical conization was 77.13%, which was significantly lower than that of those infected with a single HPV genotype. And we found that the probability of recurrence in patients with the same genotype of HPV infection before and after surgery was higher than that in patients with different genotypes of infection before and after surgery. There are still some limitations since some patients in this study were lost to follow-up and the use of LSIL or more defined as residual/recurrent, which may have influenced the statistical results to be biased. Therefore, the relevance between HPV genotype infection and recurrence needs to be confirmed by further studies.

Vaccination and lesion recurrence

Human Papilloma Virus (HPV) vaccination is a primary prevention of cervical cancer and significantly reduces the risk of developing cervical lesions and even cervical cancer. Some scholars believe that HPV vaccination may reduce the risk of recurrence in patients with CIN who undergo surgery (Kang et al., 2013; Ghelardi et al., 2018; Bartels et al., 2020; Jentschke et al., 2020; Lichter et al., 2020; Di Donato et al., 2021), but others believe that vaccination will not play a role in postoperative patients with CIN (Sand et al., 2020).

It is worth noting that in analyzing previous observational studies, we should consider the surgical margins to assess more objectively the effect of HPV vaccination after excisional treatment. The mechanism of prophylaxis after CIN is unclear. Some studies have emphasized a possible cross-immunization effect for initial prophylaxis with vaccines against HPV-related diseases, but the effect on recurrent infections and whether it prevents reactivation of latent HPV infections is unclear (Ault, 2007; Jenkins, 2008; Gravitt and Winer, 2017). We believe that HPV vaccination may decrease the risk of postoperative recurrence in patients with HSIL, but more studies are needed to further confirm the mechanism of action for better clinical application.

Conclusion

In summary, the chances of patients becoming negative for HPV after cervical conization are high, and to reduce the psychological and economic burden on patients, we believe that a postoperative follow-up period of 6 months is appropriate. Whereas the numerous high-risk factors for HPV reinfection or persistent infection are inconclusive. In conjunction with the literature review, we believe that HPV reinfection or persistent infection was more common in patients aged ≥50 years, with ≥3 pregnancies and deliveries, a history of smoking, and consistent genotypes of pre- and postoperative HPV infection in cervical conization, all of which may be high-risk factors for lesion recurrence. For patients with potential high-risk factors, we need to provide individualized follow-up and targeted management, take timely and effective action, optimize treatment plans, reduce recurrence rates, prevent HSIL and cervical cancer, improve the quality of patient survival, and improve prognosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JL: Investigation, Writing – original draft, Writing – review & editing. SH: Data curation, Writing – original draft. JN: Methodology, Writing – review & editing. JW: Project administration, Supervision, Writing – review & editing. YL: Investigation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Arbyn, M., Redman, C. W. E., Verdoodt, F., Kyrgiou, M., Tzafetas, M., Ghaem-Maghami, S., et al. (2017). Incomplete excision of cervical precancer as a predictor of treatment failure: a systematic review and meta-analysis. *Lancet Oncol.* 18, 1665–1679. doi: 10.1016/S1470-2045(17)30700-3

Ault, K. A. (2007). Human papillomavirus vaccines and the potential for cross-protection between related HPV types. *Gynecol. Oncol.* 107, S31–S33. doi: 10.1016/j.ygyno.2007.08.059

Bais, A. G., Eijkemans, M. J. C., Rebolj, M., Snijders, P. J. F., Verheijen, R. H. M., van Ballegooijen, M., et al. (2009). Post-treatment CIN: randomized clinical trial using hrHPV testing for prediction of residual/recurrent disease. *Int. J. Cancer* 124, 889–895. doi: 10.1002/ijc.23824

Banura, C., Franceschi, S., van Doorn, L. J., Arslan, A., Kleter, B., Wabwire-Mangen, F., et al. (2008). Prevalence, incidence and clearance of human papillomavirus infection among young primiparous pregnant women in Kampala, Uganda. *Int. J. Cancer* 123, 2180–2187. doi: 10.1002/ijc.23762

Bartels, H. C., Postle, J., Rogers, A. C., and Brennan, D. (2020). Prophylactic human papillomavirus vaccination to prevent recurrence of cervical intraepithelial neoplasia: a meta-analysis. *Int. J. Gynecol. Cancer* 30, 777–782. doi: 10.1136/ijgc-2020-001197

Bilibio, J. P., Monego, H. I., Binda, M. L. A., and dos Reis, R. (2019). Menopausal status is associated with a high risk for residual disease after cervical conization with positive margins. *PLoS One* 14:e0217562. doi: 10.1371/journal.pone.0217562

Bosch, F. X., Lorincz, A., Munoz, N., Meijer, C. J. L. M., and Shah, K. V. (2002). The causal relation between human papillomavirus and cervical cancer. *J. Clin. Pathol.* 55, 244–265. doi: 10.1136/jcp.55.4.244

Collins, S., Rollason, T. P., Young, L. S., and Woodman, C. B. J. (2010). Cigarette smoking is an inde-pendent risk factor for cervical intraepithelial neoplasia in young women: a longitudinal study. *Eur. J. Cancer* 46, 405–411. doi: 10.1016/j.ejca.2009.09.015

Delvenne, P., Herman, L., Kholod, N., Caberg, J. H., Herfs, M., Boniver, J., et al. (2007). Role of hormone cofactors in the human papillomavirus-induced carcinogenesis of the uterine cervix. *Mol. Cell. Endocrinol.* 264, 1–5. doi: 10.1016/j.mce.2006.10.014

Di Donato, V., Caruso, G., Petrillo, M., Kontopantelis, E., Palaia, I., Perniola, G., et al. (2021). Adjuvant HPV vaccinationto prevent recurrent cervical dysplasia after surgical treatment: a meta-analysis. *Vaccine* 9:410. doi: 10.3390/vaccines9050410

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., et al. (2015). Cancer incidence and mortality worldwide: sources, methods, and major patterns in GLOBOCAN 2012. *Int. J. Cancer* 136, E359–E386. doi: 10.1002/ijc.29210

Frazer, I. H. (2009). Interaction of human papillomaviruses with the host immune system: a well evolved relationship. *Virology* 384, 410–414. doi: 10.1016/j.virol.2008.10.004

Ghelardi, A., Parazzini, F., Martella, F., Pieralli, A., Bay, P., Tonetti, A., et al. (2018). SPERANZA project: HPV vaccination after treatment for CIN2+. *Gynecol. Oncol.* 151, 229–234. doi: 10.1016/j.ygyno.2018.08.033

Gök, M., Coupé, V. M. H., Berkhof, J., Verheijen, R. H. M., Helmerhorst, T. J. M., Hogewoning, C. J. A., et al. (2007). HPV 16 and increased risk of recurrence after treatment for CIN. *Gynecol. Oncol.* 104, 273–275. doi: 10.1016/j.ygyno.2006.10.011

Gravitt, P. E., and Winer, R. L. (2017). Natural history of HPV infection across the lifespan: role of viral latency. *Viruses* 9:267. doi: 10.3390/v9100267

Hui-Qiong, L., Xiu-Hua, G., and Cui, Y. (2016). Analysis of factors associated with regression after LEEP treatment for cervical intraepithelial neoplasia. *Mod. Hosp.* 16, 489–491.doi: 10.3969/j.issn.1671-332X.2016.04.008

Inamine, M., Nagai, Y., Mitsuhashi, A., Nagase, S., Yaegashi, N., Yoshikawa, H., et al. (2012). Cigarette smoke stimulates VEGF-C expression in cervical intraepithelial neoplasia (CIN) 1 and 2 lesions. *Int. J. Clin. Oncol.* 17, 498–504. doi: 10.1007/s10147-011-0322-3

International Agency for Research on Cancer. (2005). *Cervix Cancer Screening: IARC Handbooks of Cancer Prevention*. Lyon: IARC Press, 1–302.

International Agency for Research on Cancer. (2020). *WHO Classification of Tumours: Female Genital Tumours. 5th Edn*. Lyon: IARC Press.

International Collaboration of Epidemiological Studies of Cervical Cancer Appleby, P., Beral, V., de González, A. B., Colin, D., Franceschi, S., et al. Carcinoma of the cervix and tobacco smoking: collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. *Int. J. Cancer* (2006). 118: 1481–1495. doi: 10.1002/ijc.21493

Jenkins, D. (2008). A review of cross-protection against oncogenic HPV by an HPV-16/18 AS04-adjuvanted cervical cancer vaccine: importance of virological and clinical endpoints and implications for mass vaccination in cervical cancer prevention. *Gynecol. Oncol.* 110, S18–S25. doi: 10.1016/j.ygyno.2008.06.027

Jensen, K. E., Schmiedel, S., Norrild, B., Frederiksen, K., Iftner, T., and Kjaer, S. K. (2013). Parity as a cofactor for high-grade cervical disease among women with persistent human papillomavirus infection: a 13-year follow-up. *Br. J. Cancer* 108, 234–239. doi: 10.1038/bjc.2012.513

Jentschke, M., Kampers, J., Becker, J., Sibbertsen, P., and Hillemanns, P. (2020). Prophylactic HPV vaccination after conization: a systematic review and meta-analysis. *Vaccine* 38, 6402–6409. doi: 10.1016/j.vaccine.2020.07.055

Kang, W. D., Choi, H. S., and Kim, S. M. (2013). Is vaccination with quadrivalent HPV vaccine after loop electrosurgical excision procedure effective in preventing recurrence in patients with high-grade cervical intraepithelial neoplasia (CIN2-3)? *Gynecol. Oncol.* 130, 264–268. doi: 10.1016/j.ygyno.2013.04.050

Kang, W. D., and Kim, S. M. (2016). Human papillomavirus genotyping as a reliable prognostic marker of recurrence after loop electrosurgical excision procedure for high-grade cervical intraepithelial neoplasia (CIN2-3) especially in postmenopausal women. *Menopause* 23, 81–86. doi: 10.1097/GME.0000000000000488

Kocken, M., Helmerhorst, T. J., Berkhof, J., Louwers, J. A., Nobbenhuis, M. A. E., Bais, A. G., et al. (2011). Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: a longterm multi-cohort study. *Lancet Oncol.* 12, 441–450. doi: 10.1016/S1470-2045(11)70078-X

Koshiyama, M., Nakagawa, M., and Ono, A. (2019). The preventive effect of dietary antioxidants against cervical Cancer versus the promotive effect of tobacco smoking. *Health* 7:162. doi: 10.3390/healthcare7040162

Kreimer, A. R., Guido, R. S., Solomon, D., Schiffman, M., Wacholder, S., Jeronimo, J., et al. (2006). Human papillomavirus testing following loop electrosurgical excision procedure identifies women at risk for posttreatment cervical intraepithelial neoplasia grade 2 or 3 disease. *Cancer Epidemiol. Biomark. Prev.* 15, 908–914. doi: 10.1158/1055-9965.EPI-05-0845

Kuroki, L. M., James-Nywening, L., Wu, N., Liu, J., Powell, M. A., Thaker, P. H., et al. (2016). High-grade cervical dysplasia after negative loop electrosurgical excision procedure. *J. Low. Genit. Tract Dis.* 20, 300–306. doi: 10.1097/LGT.0000000000000260

Kyrgiou, M., Arbyn, M., Bergeron, C., Bosch, F. X., Dillner, J., Jit, M., et al. (2020). Cervical screening: ESGO-EFC position paper of the European Society of Gynaecologic Oncology (ESGO) and the European Federation of Colposcopy (EFC). *Br. J. Cancer* 123, 510–517. doi: 10.1038/s41416-020-0920-9

Lichter, K., Krause, D., Xu, J., Tsai, S. H. L., Hage, C., Weston, E., et al. (2020). Adjuvant human papillomavirus vaccine to reduce recurrent cervical dysplasia in unvaccinated women: a systematic review and meta-analysis. *Obstet. Gynecol.* 135, 1070–1083. doi: 10.1097/AOG.0000000000003833

Lubrano, A., Medina, N., Benito, V., Arencibia, O., Falcón, J. M., Leon, L., et al. (2012). Follow-up after LLETZ: a study of 682 cases of CIN-CIN3 in a single institution. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 161, 71–74. doi: 10.1016/j.ejogrb.2011.11.023

Nair, H. B., Luthra, R., Kirma, N., Liu, Y. G., Flowers, L., Evans, D., et al. (2005). Induction of aromatase expression in cervical carcinomas: effects of endogenous estrogen on cervical cancer cell proliferation. *Cancer Res.* 65, 11164–11173. doi: 10.1158/0008-5472.CAN-05-1087

Nam, K., Chung, S., Kim, J., Jeon, S., and Bae, D. (2009). Factors associated with HPV persistence after conization in patients with negative margins. *J. Gynecol. Oncol.* 20, 91–95. doi: 10.3802/jgo.2009.20.2.91

Perkins, R. B., Guido, R. S., Castle, P. E., Chelmow, D., Einstein, M. H., Garcia, F., et al. (2020). 2019 ASCCP risk-based management consensus guidelines for abnormal cervical cancer screening tests and cancer precursors. *J. Low. Genit. Tract Dis.* 24, 102–131. doi: 10.1097/LGT.0000000000000525

Petorius, R. G., Peterson, P., Azizi, F., Burchette, R. J. (2006). Subsequent risk and preaentation of cervical intraepithelial neoplasia (CIN3) or cancer after a colposcopic diagnosis of CIN1 or less. *Am. J. Obstet. Gynecol.* 195, 1260–1265. doi: 10.1016/j.ajog.2006.07.036

Salvadó, A., Miralpeix, E., Solé-Sedeno, J. M., Kanjou, N., Lloveras, B., Duran, X., et al. (2021). Predictor factors for conservative management of cervical intraepithelial neoplasia grade 2:cytology and HPV genotyping. *Gynecol. Oncol.* 162, 569–574. doi: 10.1016/j.ygyno.2021.06.019

Sand, F. L., Kjaer, S. K., Frederiksen, K., and Dehlendorff, C. (2020). Risk of cervical intraepithelial neoplasia grade 2 or worse after conization in relation to HPV vaccination status. *Int. J. Cancer* 147, 641–647. doi: 10.1002/ijc.32752

Santesso, N., Mustafa, R. A., Wiercioch, W., Kehar, R., Gandhi, S., Chen, Y., et al. (2016). Systematic reviews and meta-analyses of benefits and harms of cryotherapy, LEEP, and cold knife conization to treat cervical intraepithelial neoplasia. *J. Gynaecol. Obstet.* 132, 266–271. doi: 10.1016/j.ijgo.2015.07.026

Schiffman, M., Castle, P. E., Jeronimo, J., Rodriguez, A. C., and Wacholder, S. (2007). Human papillomavirus and cervical cancer. *Lancet* 370, 890–907. doi: 10.1016/S0140-6736(07)61416-0

Schmeink, C. E., Melchers, W. J., Hendriks, J. C., Quint, W. G., Massuger, L. F., and Bekkers, R. L. (2012). Human papillomavirus detection in pregnant women: a prospective matched cohort study. *J. Women's Health (Larchmt)* 21, 1295–1301. doi: 10.1089/jwh.2012.3502

Söderlund-Strand, A., Kjellberg, L., and Dillner, J. (2014). Human papillomavirus type-specific persistence and recurrence after treatment for cervical dysplasia. *J. Med. Virol.* 86, 634–641. doi: 10.1002/jmv.23806

Trottier, H., Mayrand, M.-H., Baggio, M. L., Galan, L., Ferenczy, A., Villa, L. L., et al. (2015). Risk of human papillomavirus (HPV) infection and cervical neoplasia after pregnancy. *BMC Pregnancy Childbirth* 15:244. doi: 10.1186/s12884-015-0675-0

Vintermyr, O. K., Iversen, O., Thoresen, S., Quint, W., Molijn, A., de Souza, S., et al. (2014). Recurrent high-grade cervical lesion after primary conization is associated with persistent human papillomavirus infection in Norway. *Gynecol. Oncol.* 133, 159–166. doi: 10.1016/j.ygyno.2014.03.004

Walboomers, J. M. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., et al. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189, 12–19. doi: 10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F

Zhang, W., and Lin, Y. (2022). Modified method of cervical conization with hybrid use of a cold knife and an electric knife for high-grade squamous intraepithelial lesions. *J. Int. Med. Res.* 50:030006052211064. doi: 10.1177/03000605221106414

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Human tumor viruses: induction of three-dimensional alterations in the host genome structure

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Certain viruses called tumor viruses or oncoviruses are capable to change the gene expression pattern of distinct human or animal cell types in tissue culture, resulting in uncontrolled proliferation as well as a change in the social behavior of the infected cells: the oncovirus-transformed, immortalized cells are capable to form malignant neoplasms in suitable animal models. At present, seven human viruses are categorized as causative agents of distinct human malignancies. The genomes of human tumor viruses, typically encode viral oncoproteins and non–translated viral RNAs that affect the gene expression pattern of their target cells or induce genetic and epigenetic alterations contributing to oncogenesis. Recently, the application of chromatin conformation capture technologies and three-dimensional (3D) molecular imaging techniques revealed how the gene products or genomes of certain human tumor viruses interact with and induce alterations in the 3D host genome structure. This Mini Review aims to cover selected aspects of these developments. The papers, discussed briefly, describe how insertion of a novel viral binding site for the 3D genome organizer cellular protein CCCTC-binding factor (CTCF) into the DNA of T cells infected by human T-cell lymphotropic virus type 1 (HTLV-1) may contribute to lymphomagenesis, as well as how integration of high risk human papillomavirus genome into the host cell DNA may facilitate cervical carcinogenesis. Recent results regarding the interactions of cellular genomes with the episomal, chromatinized DNA genomes of oncogenic human herpesvirus, Epstein–Barr virus (EBV) will also be summarized, similarly to available data regarding contacts formed by episomal or integrated hepatitis B virus (HBV) DNA with host chromatin. Finally, a putative mechanism of hepatitis C virus (HCV) induced chromatin alterations will be presented, which may solve the riddle, how a cytoplasmic RNA virus without a viral oncogene could induce malingnant transfrormation of hepatocytes.

KEYWORDS

tumor virus, oncogene, transformation, chromatin loop, host cell genome, viral episome, integration

1. Introduction

Application of novel sequencing-based and microscopy-based techniques allowed high-resolution mapping of chromatin contacts and permitted three-dimensional reconstruction of nuclear architecture in various cell types (Lieberman-Aiden et al., 2009; Jerkovic and Cavalli, 2021). Using a refined version (*in situ* Hi.C) of the sequencing-based chromosome conformation capture (3C) method, Rao et al. mapped chromatin interactions, at a 1 Kb resolution, in a human B lymphoblastoid cell line immortalized with Epstein–Barr virus (Rao et al., 2014). Similar interaction

maps were created in 8 other human and mouse cell lines, allowing the identification of six distinct nuclear subcompartments displaying characteristic patterns of histone modifications, and approximately 10, 000 chromatin loops that may allow long-range enhancer-promoter interactions (see reference 3, for a detailed characterization of nuclear subcompartments). Recently, a single chromatin loop situated in the vicinity of the *TCRA* (*T Cell Receptor A*) locus in human chromosome 14 could be visualized by Parteka-Tojek et al., using a combination of FISH staining and interferometric photoactivated localization microscopy (iPALM) (Parteka-Tojek et al., 2022). They used the very same EBV-immortalized human lymphoblastoid cell line (GM12878) that was characterized by Rao et al., who mapped that particular loop by *in situ* Hi-C (Rao et al., 2014). Parteka-Tojek et al. concluded that the physical distance maps generated by single-cell imaging fitted well to the population based genomic data (Parteka-Tojek et al., 2022).

Chromatin loops are formed by the interaction of a ring-shaped protein complex called cohesin and the nuclear protein CCCTC-binding factor (CTCF); reviewed by Beagan and Phillips-Cremins (2020).

Approximately 10% of cancer cases is attributable to virus infections (Plummer et al., 2016). Analysis of whole-genome and whole-transcriptome sequencing data of cancer specimens revealed a high prevalence of certain human tumor-viruses, including Epstein–Barr virus (EBV), hepatitis B virus (HBV) and high risk human papilloma viruses (HPV16 and HPV18) (Zapatka et al., 2020). These viruses carry a **DNA** genome in their virion.

EBV, a human gammaherpesvirus, infects both lymphoid and epithelial cells, and it is regarded as the causative agent of B lymphoproloferative disease; its cell tropism is reflected in its association with Hodgkin lymphoma, Burkitt lymphoma and diffuse large B cell lymphoma (Shannon-Lowe and Rickinson, 2019). In addition, EBV is associated with T/NK lymphoproliferative diseases and T/NK lymphomas/leukemias, as well as with neoplasms of epithelial origin, nasopharyngeal carcinoma and a fraction of gastric carcinomas (Shannon-Lowe and Rickinson, 2019). In rare cases, leiomyosarcomas of smooth muscle origin also carry EBV genomes (Shannon-Lowe and Rickinson, 2019; Zhang et al., 2023).

HBV is one of the causative agent of hepatocellular carcinoma; upon infection of liver cells, the partially double-stranded **DNA** genome carried by the HBV virion is converted to a covalently closed, circular DNA (cccDNA) in the host cell nucleus (Seeger and Mason, 2015). Chronic HBV infections frequently progress to cirrhosis, a risk factor for developing liver cancer (D'souza et al., 2020).

So called high risk human papilloma viruses (HPV16, HPV18) are regarded as causative agents of genital cancers, especially cervical carcinoma; the genomes of these **DNA** tumor viruses encode oncoproteins interacting with so called tumor suppressor proteins that regulate cell proliferation (Zur Hausen, 2009; Pal and Kundu, 2020).

In addition to EBV, HBV, and HPV, two other human viruses that carry a **double-stranded DNA genome** in the virion, were also implicated in tumorigenesis, albeit they cause neoplasms of lower prevalence: Kaposi's sarcoma-associated herpesvirus (KSHV, also called Human gammaherpesvirus 8, HHV-8) is associated with Kaposi's sarcoma and primary effusion lymphoma, whereas the genome of Merkel cell polyomavirus(MCPyV) was frequently detected in cells of a rare, but aggressive malignancy, Merkel cell carcinoma (Mesri et al., 2010; Chakraborty et al., 2012; Czech-Sioli et al., 2020; Pietropaolo et al., 2020).

In contrast to the human tumor viruses listed above, Hepatitis C virus (HCV), a single stranded **RNA virus**, does not carry a viral oncogene in its genome (Yamagishi et al., 2018; Zaki et al., 2022). HCV is considered to be the causative agent of hepatocellular carcinoma (HCC) developing in individuals with chronic HCV infection (Yamagishi et al., 2018; Zaki et al., 2022). Both direct, viral associated, and indirect, immune-associated factors contribute to the development of HCV-associated liver cancer (Zaki et al., 2022). Table 1 summarizes the human tumor viruses and their target cells. The genomes of human tumor viruses typically carry genes coding for viral oncoproteins implicated in tumorigenesis (Vogt, 2012; Chang et al., 2017; Gaglia and Munger, 2018; Krump and You, 2018; Stolz and McCormick, 2020). Oncoproteins frequently target tumor suppressor pathways or host signaling pathways that regulate cell proliferation (Krump and You, 2018). Certain RNA tumor viruses infecting animals do not carry, however, viral oncogenes; they may cause malignant tumors by inserting a DNA copy of their RNA genome into the host cell DNA and activating cellular genes that control cell proliferation *via* the inserted viral promoter or enhancer sequences; see (Robinson and Gagnon, 1986), and references therein. It was suggested that a similar mechanism of insertional tumorigenesis is involved in the initiation and progression of HTLV-1-associated T-cell leukemia-lymphoma as well, as discussed below (see **2**.). Thereafter, I wish to summarize how integrated HPV DNA and episomal HPV-host cell DNA hybrid sequences may affect the 3D structure of host cell chromatin (see **3**.). Latent, episomal EBV genomes also interact with the host cell chromatin in both lymphoid and epithelial cells (see **4**.), whereas extrachromosomal, episomal HBV genomes contact active regions of target cell chromatin at an early stage of infection, followed by integration and loop formation at a later stage (see **5**.), Unexpectedly, it was also observed that HCV, an RNA tumor virus that does not enter the host cell nucleus and does not carry a viral oncogene, may cause misregulation of cohesin, a nuclear protein regulating the 3D genome structure, contributing thereby to a change

TABLE 1 Human tumor viruses and their targets.

Virus family and name	Genome (in the virion)	Target cells
Herpesviridae		
Epstein–Barr virus (EBV)	DNA	lymphoid, epithelial & smooth muscle cells
Kaposi's sarcoma-associated herpesvirus (KSHV)	DNA	endothelial & lymphoid cells
Papillomaviridae		
Human papillomavirus (HPV)	DNA	epithelial cells
Polyomaviridae		
Merkel cell polyomavirus (MCPyV)	DNA	neuroectodermal cells
Hepadnaviridae		
Hepatitis B virus (HBV)	DNA	liver cells
Retroviridae		
Human T-lymphotropic virus, type 1 (HTLV-1)	RNA	T cells
Flaviviridae		
Hepatitis C virus (HCV)	RNA	liver cells

As discussed in this Mini Review, EBV, HPV, HBV, HTLV-I, and HCV are capable to induce alterations in the 3D structure of host cell genomes. Further studies may reveal whether similar mechanisms contribute to oncogenesis by KSHV and MCPyV, too.

of host cell gene expression and development of liver cancer (see **6.**). Finally, I would like to outline briefly how **a new research field**, focusing on tumor virus induced alterations in the 3D host genome, may open new possibilities for diagnostics and therapy viral as well as non-viral malignancies (see **7.**).

2. Insertion of a novel, viral CTCF binding site carried by HTLV-I provirus into the host cell genome may rewire chromatin loops in infected T cells

Using CTCF Chip assays, Satou et al. observed that CTCF, a regulator of chromatin structure, bound to the so called pX region of HTLV-1 provirus in a nonmalignant HTLV-1 infected T cell clone and in a T cell line derived from adult T cell leukemia (ATL) (Satou et al., 2016). Furthermore, similar observations were made using fresh PBMCs (peripheral blood mononuclear cells) from a patient with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic inflammatory disease, and PBMCs of a patient with ATL, indicating that CTCF binds to the HTLV-1 provirus *in vivo* as well (Satou et al., 2016). They compared long-range chromatin interactions in cells carrying either wild type HTLV-I provirus or a mutant HTLV-1 provirus (ΔvCTCF-BS) unable to associate with the zinc finger protein CTCF and demonstrated by quantitative chromosome conformation capture (3C) analysis a decreased interaction frequency in case of the mutant provirus (Satou et al., 2016). In a follow-up study.Melamed et al. applied the method of quantitative 4C (q4C, a modified version of circular chromosome conformation capture) to analyse chromatin contacts formed between HTLV-1 genomes integrated into the DNA of CD4+ T-cell clones, and the relevatnt host cell genome (Melamed et al., 2018). The T-cell clones studied were isolated from HTLV-1-infected individuals, and long-range chromatin contacts could be detected between the HTLV-1provirus and the host genome in 9 of the 10 clones, in parallel with alterations in host gene expression (Melamed et al., 2018). Although these experiments suggested that insertional mutagenesis contributes to ATL pathogenesis, Martinez et al. found that the viral CTCF binding site was dispensable for T-cell immortalization and for persistent infection *in vivo* (Martinez et al., 2019). The situation is complex, because HTLV-1-encoded oncoproteins also contribute to ATL induction, and in infected individuals the provirus is present at unique integration sites in a series of T-cell clones. One may speculate that distinct CTCF-CTCF contacts, formed between integrated HTLV-I genomes and cellular chromatin loops alter the cellular transcriptome in a manner that provides growth advantage for selected T cell clones during ATL initiation or progression.

3. Integration of human papillomavirus (HPV) DNA into the host cell genome: reprogramming of host cell transcription *via de novo* generation of a cellular super-enhancer

So called "high risk" human papillomaviruses, especially HPV type 16 and 18 (HPV16, HPV18) are the causative agents of cervical cancer. Although their circular, double stranded DNA genomes typically persist as extrachromosomal episomes in the nuclei of infected epithelial cells, cervical carcinoma cells usually carry integrated HPV16 and HPV18 DNA sequences in their genome. Genetic and epigenetic consequences of integration result in enhanced expression of the viral oncogenes *E6* and *E7*, as reviewed in (McBride and Warburton, 2017). Warburton et al. observed that multiple, tandemly integrated HPV16 genomes in chromosome 2 of the cervical-derived cell line 20,861 formed a so called super-enhancer (SE) with co-amplified cellular sequences (Warburton et al., 2018). Accordingly, this locus was enriched in Bromodomain-containing protein 4 (BRD4), a regulator of transcription, and in acetylated histone H3 (H3K27ac), another marker of super-enhancers that ensure enhanced expression of distal genes *via* chromatin looping. As a matter of fact, this particular locus directed high level expression of a viral-cellular fusion transcript encoding the HPV oncoproteins E6 and E7 (Warburton et al., 2018). Recently, Tian et al. characterized the sites of HPV integration in HPV-positive cell lines and observed that HPV integration frequently resulted in the generation of cellular super-enhancers called HPV breakpoint-induced cellular SEs (BP-cSEs) (Tian et al., 2023). Furthermore, they showed that some of the BP-cSEs were formed by extrachromosomal, HPV DNA-human DNA hybrid sequences and in the case of HeLa cells they could even trace back the origin of three extrachromosomal DNAs (ecDNAS) to the integration sites of HPV18 genomes on chromosome 8. These sequencing data supported the idea that the hybrid ecDNAs were induced, indeed, by the integration of viral genomes into the host DNA (Tian et al., 2023). Hi-C, Chip-seq and RNA seq data revealed that the episomal, ecDNA based super-enhancers could establish long-range intra- and extra-chromosomal interactions and acted as transcriptional regulators (Tian et al., 2023).

4. Latent Epstein–Barr virus genomes interact with and change the 3D structure of host cell genomes

Latent, growth-transformation associated genes of Epstein–Barr virus (EBV), an oncogenic human herpesvirus, are typically expressed from circular, chromatinized viral episomes in host cell nuclei (Shannon-Lowe and Rickinson, 2019). Although it is well documented that host-cell dependent expression of EBV-encoded latent proteins and non-translated RNAs contrbutes to tumorigenesis, recent studies revealed that direct interactions of the viral episomes with the cellular genome may also play a role in the induction of EBV-associated malignancies. Kim et al. studied Burkitt lymphoma (BL) cell lines using circular chromosome conformation capture (4C), and observed that the EBV episomes preferably associated with intergenic regions of the cellular genome (Kim et al., 2020). They compared the BL data with the results of Rao et al. (2014) who studied an EBV-immortalized B lymphoblastoid cell line (LCL), and found that the EBV genomes formed more contacts with promoter rich regions in the latter (Kim et al., 2020). A re-analysis of public data sets by Wang et al., as well as their own 4C-seq data (4C-seq: circular chromatin conformation capture followed by deep sequencing) supported the view that EBV episomes colocalise with active regions of the host chromatin in LCLs (Wang et al., 2020). I would like to add that besides episome-host genome interactions, EBV-encoded oncoproteins, especially nuclear

antigens also significantly contribute to the reorganization of host cell chromatin during immortalization of resting B cells and establishment of LCLs *in vitro* (Wang et al., 2023).

In addition to lymphoid malignancies, EBV is also associated with certain neoplasms of epithelial origin, including a subset of gastric carcinomas. Okabe et al. found that in EBV-positive gastric carcinoma cell lines the viral episomes form long-range interactions with distinct chromatin domains of the host cell, accompanied by heterochromatin-to euchromatin transitions (Okabe et al., 2020). Okabe et al. suggested that such interactions, called 'enhancer infestation', may induce epigenetic alterations and activate cellular oncogenes (proto-onc genes), contributing to carcinogenesis (Okabe et al., 2020).

5. Chromatin reorganization in hepatitis B virus (HBV) infected hepatoma cells

Using Hi-C sequencing, Guo et al. analysed chromatin interactions in a HBV-infected human hepatoma cell line and its uninfected counterpart (Guo et al., 2023). They observed enhanced chromatin interactions, especially on distinct interphase chromosomes, in HBV-positive cells and recorded characteristic shifts in nuclear subcompartments, accompanied with repression of enhancers regulating inflammatory genes and opening of regions enriched in transposable elements (Guo et al., 2023). These data suggested that reorganization of 3D genome structure may contribute to disease development associated with HBV infection. Yang et al. studied two *in vitro* HBV infection systems corresponding to a *de novo* infection stage with episomal viral genomes and a later stage with integrated HBV genomes (Yang et al., 2020). HBV DNA-host DNA contacts were assessed using 3C-high-throughput genome-wide translocation sequencing (3C-HTGTS), and the results showed that at the initial stage of infection the HBV minichromosome (covalently closed circular DNA, cccDNA) preferably contacts transcriptionally active regions of host cell chromatin, whereas the integrated HBV genome, especially when integrated to a transcriptionally active region on chromosome 2, interacted with the host genome and formed a chromatin loop structure (Yang et al., 2020).

6. Hepatitis C virus (HCV) up-regulates the RAD21 cohesin subunit, a regulator of chromatin structure: implications for hepatocarcinogenesis induced by a cytoplasmic RNA virus

Hepatitis C virus (HCV) is one of the causative agents of hepatocellular carcinoma, its exact role, however, in carcinogenesis is unknown at present (Goossens and Hoshida, 2015). HCV has a single stranded RNA genome, but lacks a viral oncogene, and in contrast to retroviruses that replicate *via* a DNA intermediate integrating into the host cell genome, HCV replication is confined to the cytoplasm. Perhaps for this reason, until recently the interaction of HCV with the host cell chromatin happened to be outside the focus of the research community. As mentioned in the Introduction, chromatin loops are formed by the interaction of a ring-shaped protein complex called **c**ohesin and the nuclear protein CCCTC-binding factor (CTCF) (Beagan and Phillips-Cremins, 2020). Perez et al. studied hepatoma cells and primary hepatocytes infected by hepatitis C virus(HCV) *in vitro*, and observed an increased expression of RAD21, a core component of the cohesin complex, in HCV-infected cells (Perez et al., 2019). In parallel, they documented that cohesin binding sites were enriched at regulator elements of the cellular genome in HCV-infected cells, and there were changes in their transcriptome as well, compared to uninfected controls. Furthermore, HCV also induced centrosome abnormalities, possibly due to cohesin misregulation, Based on preliminary data, Perez et al. argued that NS3/4A, a HCV-encoded protease, may degrade the cohesin regulator WAPL (Wings apart-like protein homolog) protein in HCV-infected cells, resulting in dysregulation of gene expression and induction of chromosomal aberrations (Perez et al., 2019).

7. Perspectives

Tumor viruses or oncoviruses that show a strong association with various neoplastic diseases in humans belong to several virus families (Table 1). Their replication strategies and target cells are heterogenous, and as explored by intense research efforts, a variety of molecular mechanisms contribute to the generation of malignant tumors by oncovirus infected cells. These research efforts had important implications for other areas as well, including cell biology, tumor biology, non-viral carcinogenesis and oncotherapy. As reflected in this Mini Review, the study of tumor virus induced alterations in the 3D host genome is a booming, novel research field that resulted in new concepts and ideas as to the mechanism of malignant transformation, similarly to the studies dealing with changes of the 2D host genome elicited by retroviral insertional mutagenesis (Bushman, 2020). Exploring how tumor viruses affect the 3D structure of host cell gnomes may open new possibilities for molecular-targeted therapy of virus-associated neoplastic diseases, and may have implications for the therapy of non-viral malignancies, too.

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References

Beagan, J. A., and Phillips-Cremins, J. E. (2020). On the existence and functionality of topologically associatingdomains. *Nat. Genet.* 52, 8–16. doi: 10.1038/s41588-019-0561-1

Bushman, F. D. (2020). Retroviral Insertional mutagenesis in humans: evidence for four genetic mechanisms promoting expansion of cell clones. *Mol. Ther.* 28, 352–356. doi: 10.1016/j.ymthe.2019.12.009

Chakraborty, S., Veettil, M. V., and Chandran, B. (2012). Kaposi's sarcoma associated Herpesvirus entry into target cells. *Front. Microbiol.* 3:6. doi: 10.3389/fmicb.2012.00006

Chang, Y., Moore, P. S., and Weiss, R. A. (2017). Human oncogenic viruses: nature and discovery. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 372:20160264. doi: 10.1098/rstb.2016.0264

Czech-Sioli, M., Günther, T., Therre, M., Spohn, M., Indenbirken, D., and Theiss, J. (2020). High-resolution analysis of Merkel cell Polyomavirus in Merkel cell carcinoma reveals distinct integration patterns and suggests NHEJ and MMBIR as underlying mechanisms. *PLoS Pathog.* 16:e1008562. doi: 10.1371/journal.ppat.1008562

D'souza, S., Lau, K. C., Coffin, C. S., and Patel, T. R. (2020). Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma. *World J. Gastroenterol.* 26, 5759–5783. doi: 10.3748/wjg.v26.i38.5759

Gaglia, M. M., and Munger, K. (2018). More than just oncogenes: mechanisms of tumorigenesis by human viruses. *Curr. Opin. Virol.* 32, 48–59. doi: 10.1016/j.coviro.2018.09.003

Goossens, N., and Hoshida, Y. (2015). Hepatitis C virus-induced hepatocellular carcinoma. *Clin. Mol. Hepatol.* 21, 105–114. doi: 10.3350/cmh.2015.21.2.105

Guo, M., Yao, Z., Jiang, C., Songyang, Z., Gan, L., and Xiong, Y. (2023). Three-dimensional and single-cell sequencing of liver cancer reveals comprehensive host-virus interactions in HBV infection. *Front. Immunol.* 14:1161522. doi: 10.3389/fimmu.2023.1161522

Jerkovic, I., and Cavalli, G. (2021). Understanding 3D genome organization by multidisciplinary methods. *Nat. Rev. Mol. Cell Biol.* 22, 511–528. doi: 10.1038/s41580-021-00362-w

Kim, K. D., Tanizawa, H., De Leo, A., Vladimirova, O., Kossenkov, A., and Lu, F. (2020). Epigenetic specifications of host chromosome docking sites for latent Epstein-Barr virus. *Nat. Commun.* 11:877. doi: 10.1038/s41467-019-14152-8

Krump, N. A., and You, J. (2018). Molecular mechanisms of viral oncogenesis in humans. *Nat. Rev. Microbiol.* 16, 684–698. doi: 10.1038/s41579-018-0064-6

Lieberman-Aiden, E., van Berkum, N. L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., et al. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* 326, 289–293. doi: 10.1126/science.1181369

Martinez, M. P., Cheng, X., Joseph, A., Al-Saleem, J., Panfil, A. R., Palettas, M., et al. (2019). HTLV-1 CTCF-binding site is dispensable for in vitro immortalization and persistent infection in vivo. *Retrovirology* 16:44. doi: 10.1186/s12977-019-0507-9

McBride, A. A., and Warburton, A. (2017). The role of integration in oncogenic progression of HPV-associated cancers. *PLoS Pathog.* 13:e1006211. doi: 10.1371/journal.ppat.1006211

Melamed, A., Yaguchi, H., Miura, M., Witkover, A., Fitzgerald, T. W., Birney, E., et al. (2018). The human leukemia virus HTLV-1 alters the structure and transcription of host chromatin in cis. *elife* 7:e36245. doi: 10.7554/eLife.36245

Mesri, E. A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. *Nat. Rev. Cancer* 10, 707–719. doi: 10.1038/nrc2888

Okabe, A., Huang, K. K., Matsusaka, K., Fukuyo, M., Xing, M., Ong, X., et al. (2020). Cross-species chromatin interactions drive transcriptional rewiring in Epstein-Barr virus-positive gastric adenocarcinoma. *Nat. Genet.* 52, 919–930. doi: 10.1038/s41588-020-0665-7

Pal, A., and Kundu, R. (2020). Human papillomavirus E6 and E7: the cervical cancer hallmarks and targets for therapy. *Front. Microbiol.* 10:3116. doi: 10.3389/fmicb.2019.03116

Parteka-Tojek, Z., Zhu, J. J., Lee, B., Jodkowska, K., Wang, P., Aaron, J., et al. (2022). Super-resolution visualization of chroatin loop folding in human lymphoblastoid cells using interferometric photoactivated localization microscopy. *Sci. Rep.* 12:8582. doi: 10.1038/s41598-022-12568-9 Erratum in: Sci Rep. (2022) 12: 22014

Perez, S., Gevor, M., Davidovich, A., Kaspi, A., Yamin, K., Domovich, T., et al. (2019). Dysregulation of the cohesin subunit RAD21 by hepatitis C virus mediates host-virus interactions. *Nucleic Acids Res.* 47, 2455–2471. doi: 10.1093/nar/gkz052

Pietropaolo, V., Prezioso, C., and Moens, U. (2020). Merkel cell Polyomavirus and Merkel cell carcinoma. *Cancers (Basel).* 12:1774. doi: 10.3390/cancers12071774

Plummer, M., de Martel, C., Vignat, J., Ferlay, J., Bray, F., and Franceschi, S. (2016). Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob. Health* 4, e609–e616. doi: 10.1016/S2214-109X(16)30143-7

Rao, S. S., Huntley, M. H., Durand, N. C., Stamenova, E. K., Bochkov, I. D., Robinson, J. T., et al. (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Celll* 159, 1665–1680. doi: 10.1016/j.cell.2014.11.021.Erratum in: Cell. (2015)162: 687-688

Robinson, H. L., and Gagnon, G. C. (1986). Patterns of proviral insertion and deletion in avian leukosis virus-induced lymphomas. *J. Virol.* 57, 28–36. doi: 10.1128/JVI.57.1.28-36.1986

Satou, Y., Miyazato, P., Ishihara, K., Yaguchi, H., Melamed, A., Miura, M., et al. (2016). The retrovirus HTLV-1 inserts an ectopic CTCF-binding site into the human genome. *Proc. Natl. Acad. Sci. U. S. A.* 113, 3054–3059. doi: 10.1073/pnas.1423199113

Seeger, C., and Mason, W. S. (2015). Molecular biology of hepatitis B virus infection. *Virology* 479-480, 672–686. doi: 10.1016/j.virol.2015.02.031

Shannon-Lowe, C., and Rickinson, A. (2019). The global landscape of EBV-associated Tumors. *Front. Oncol.* 9:713. doi: 10.3389/fonc.2019.00713

Stolz, M. L., and McCormick, C. (2020). The bZIP proteins of oncogenic viruses. *Viruses* 12:757. doi: 10.3390/v12070757

Tian, R., Huang, Z., Li, L., Yuan, J., Zhang, Q., and Meng, L. (2023). HPV integration generates a cellular super-enhancer which functions as ec DNA to regulate genome-wide transcription. *Nucleic Acids Res.* 51, 4237–4251. doi: 10.1093/nar/gkad105

Vogt, P. K. (2012). Retroviral oncogenes: a historical primer. *Nat. Rev. Cancer* 12, 639–648. doi: 10.1038/nrc3320

Wang, L., Laing, J., Yan, B., Zhou, H., Ke, L., Wang, C., et al. (2020). Epstein-Barr virus Episome physically interacts with active regions of the host genome in Lymphoblastoid cells. *J. Virol.* 94, e01390–e01320. doi: 10.1128/JVI.01390-20

Wang, C., Liu, X., Liang, J., Narita, Y., Ding, W., and Li, D. (2023). A DNA tumor virus globally reprograms host 3D genome architecture to achieve immortal growth. *Nat. Commun.* 14:1598. doi: 10.1038/s41467-023-37347-6

Warburton, A., Redmond, C. J., Dooley, K. E., Fu, H., Gillison, M. L., and Akagi, K. (2018). HPV integration hijacks and multimerizes a cellular enhancer to generate a viral-cellular super-enhancer that drives high viral oncogene expression. *PLoS Genet.* 14:e1007179. doi: 10.1371/journal.pgen.1007179

Yamagishi, M., Fujikawa, D., Watanabe, T., and Uchimaru, K. (2018). HTLV-1-mediated epigenetic pathway to adult T-cell Leukemia-lymphoma. *Front. Microbiol.* 9:1686. doi: 10.3389/fmicb.2018.01686

Yang, B., Li, B., Jia, L., Jiang, Y., Wang, X., Jiang, S., et al. (2020). 3D landscape of hepatitis B virus interactions with human chromatins. *Cell Discov.* 6:95. doi: 10.1038/s41421-020-00218-1

Zaki, M. Y. W., Fathi, A. M., Samir, S., Eldafashi, N., William, K. Y., and Nazmy, M. H. (2022). Innate and adaptive Immunopathogeneses in viral hepatitis; crucial determinants of hepatocellular carcinoma. *Cancers (Basel).* 14:1255. doi: 10.3390/cancers14051255

Zapatka, M., Borozan, I., Brewer, D. S., Iskar, M., Grundhoff, A., Alawi, M., et al. (2020). The landscape of viral associations in human cancers. *Nat. Genet.* 52, 320–330. doi: 10.1038/s41588-019-0558-9

Zhang, M., Sun, W., You, X., Xu, D., Wang, L., Yang, J., et al. (2023). LINE-1 repression in Epstein-Barr virus-associated gastric cancer through viral-host genome interaction. *Nucleic Acids Res.* 51, 4867–4880. doi: 10.1093/nar/gkad203

Zur Hausen, H. (2009). Papillomaviruses in the causation of human cancers – a brief historical account. *Virology* 384, 260–265. doi: 10.1016/j.virol.2008.11.046

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Analysis of the mRNA export protein ZC3H11A in HCMV infection and pan-cancer

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Background: We have previously reported that human cytomegalovirus (HCMV) infection could promote the progression of glioma. Here we discovered a stress-induced nuclear protein ZC3H11A (ZC3) through high-throughput sequencing after HCMV infection, which has been reported recently by our research group in regulating mRNA export under stress conditions. And also, a thorough analysis of ZC3 in pan-cancer and the omics data of ZC3 are yet to be conducted.

Methods: The transcriptomes of glioma cells after HCMV infection were assessed by RNA sequencing. ZC3 mRNA and protein level following HCMV infection were validated and measured by qRT-PCR and Western-blot. The RNA sequencing and protein expression information of ZC3 across pan-cancer were analyzed and visualized by R packages. The localization of ZC3 protein was assessed by IHC images from HPA. The ZC3 proteomics and transcriptomics data in different cancers were extracted through the CPTAC data portal, and comparisons were conducted with a Python script. The genetic alteration, survival prognosis, immune infiltration analysis of ZC3 in pan-cancer were analyzed by cBioPortal, TCGA, and TIMER2 databases. The protein interaction networks were revealed by STRING, GEPIA2 and TCGA.

Results: Genes in mRNA processing pathways were upregulated after HCMV infection and ZC3 expression in mRNA and protein level was validated. We also discovered that the status of ZC3 were generally at high levels in cancers, although varied among different cancer types. ZC3 protein in tumor cells localized to the nuclear whereas in normal cells it was mainly found in cytoplasmic/membranous. However, from ZC3 proteomics and transcriptomics data in some cancer types, the increase in ZC3 protein was not accompanied by a significant elevation in mRNA level. Additionally, our analysis indicated that elevated ZC3 expression was primarily linked to a negative prognosis in majority cancers but still varied depending on the cancer types. Our annotation analysis suggested that ZC3-related proteins are mainly involved in mRNA processing clusters.

Conclusion: We demonstrated that ZC3 significantly impacted by HCMV infection in gliomas. Furthermore, we identified a set of genes exhibiting analogous expression patterns to ZC3H11A in TCGA pan-cancer cohorts, implying a potential functional role for ZC3H11A in mRNA processing. Our study provided valuable insights into the role of a new mRNA export protein ZC3 in HCMV infection and pan-cancer progression. These results lay the foundation for our next research on the regulatory mechanism of ZC3 in virus-infected tumors.

KEYWORDS

ZC3, HCMV, pan-cancer, mRNA export protein, comprehensive analysis

Introduction

Viral infections have been implicated in the development and progression of various types of tumors through multiple mechanisms. Our previous research have identified that human cytomegalovirus (HCMV) has been implicated as a contributing factor in many cancers (Hu et al., 2017, 2021; Wang et al., 2017; Liang et al., 2019). In the present study, by transcriptomic sequencing and cell line validation, one of the genes that have been shown to be significantly affected by HCMV infection in gliomas is ZC3H11A (ZC3). The discovery of elevated ZC3 expression through HCMV infection of gliomas has provided a unique opportunity to study the expression and function of ZC3 in pan-cancer.

Emerging evidence suggests that ZC3 may have a broader impact on cancer biology beyond gliomas. Our research group recently discovered that the ZC3 gene encodes a stress-induced nuclear protein that is required for efficient reproduction of several nuclear-replicating human viruses (HIV, HSV-1, influenza virus and adenovirus) (Younis et al., 2018). Further, our preliminary results indicate that tumors take advantage of stress-induced ZC3 function(s). A genome-wide association studies (GWAS) in humans also have demonstrated a significant association between the ZC3 gene and susceptibility to breast cancer (Cai et al., 2014). However, there is still a lack of large-scale research to reveal ZC3 expression and function in pan-cancer.

ZC3 is categorized as a CCCH-type zinc finger protein that contains three zinc finger motifs positioned at the N-terminus. Structural prediction programs suggest that a significant portion of the protein, except for the zinc finger and coiled-coil domains, is predominantly intrinsically disordered (Hajikhezri et al., 2020). Zinc finger proteins constitute a vast protein family that possess unique zinc finger (ZnF) domains within their protein sequence. These ZnF domains comprise several ZnF motifs that are short sequences of 30–100 amino acids, coordinating with zinc ions (Zn^{2+}). While zinc ions are the preferred metal, zinc finger motifs in ZC3 can bind to other metal ions, such as copper, cobalt, nickel and cadmium, which compete with zinc ions for binding. The functions of ZC3 have remained elusive for a long time. However, two major proteomics studies have shed light on its potential function, revealing that that ZC3 plays a crucial role in the nuclear export of mRNA as it is one of the essential components of the Transcription-Export (TREX) complex (Dufu et al., 2010; Hein et al., 2015). A comprehensive study of the relationship between ZC3 and various cancers, will contribute to our understanding of the regulation of zinc finger proteins in cancer.

In recent years, increasing evidence indicates that zinc finger proteins play important roles in the development and treatment of cancer. Firstly, research has shown that zinc finger proteins play important roles in regulating cancer cell proliferation, apoptosis, and invasion. For example, zinc finger proteins ZEB1 and ZEB2 can promote cancer cell invasion and metastasis by inhibiting the expression of E-cadherin (Ang et al., 2023; Tan et al., 2023). In addition, some zinc finger proteins can inhibit cancer cell apoptosis, such as Bcl-2 family members, which can promote cancer cell survival by inhibiting the mitochondrial apoptotic pathway (Borden, 2020). Secondly, zinc finger proteins are also involved in the regulation of signaling pathways in cancer development. For example, zinc finger protein Nrf2 can promote cancer cell metastasis and proliferation by regulating the oxidative stress response, while GATA3 can inhibit cancer cell angiogenesis and invasion (Wu et al., 2023).

Several cancers have been linked to abnormal ZC3 expression or protein interactions. For instance, ZC3 expression levels are considerably higher in breast cancer tissues than in normal tissues (Cai et al., 2014; Tabl et al., 2018). Moreover, a significant upregulation of ZC3 has been observed in KRAS-mutant lung adenocarcinomas (Grzeskowiak et al., 2018), where KRAS mutations are frequently observed molecular alterations in non-small cell lung cancers and serve as a predictor of poor prognosis (Buscail et al., 2020). In addition, ZC3 has been reported to interact with a mutant form of the nuclear matrix protein Matrin-3, which is present in amyotrophic lateral sclerosis (ALS) patients. This study demonstrated that ALS-associated mutations increase the co-localization of Matrin-3 with components of the TREX complex, possibly explaining the nuclear mRNA export defects observed in ALS patients (Boehringer et al., 2017). The export of mRNAs is a process that responds to a range of cellular stimuli and stressors. Primary human cancer specimens have exhibited abnormal RNA export. These exported RNAs encode factors that play crucial roles in almost all aspects of malignancy (Borden, 2020). Therefore, the function of ZC3 in regulating mRNA nuclear export may potentially have an impact on tumors.

Herein, in HCMV infected glioma, we identified an abnormal expression of ZC3, an mRNA nuclear export gene, by transcriptome sequencing and subsequent qRT-PCR and western-blot verification in cell lines. We also established a systematic analysis to investigate the association of ZC3 in primary tumors using the TCGA pan-cancer data, and compared the ZC3 proteomics and transcriptomics data for each type of pan-cancer patients from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset. The results of this study offered insights into the panoramic view of ZC3 in cancer, including its underlying causes and potential consequences. It also deepened the understanding of the relationship between RNA-binding proteins and tumors. These research findings are significant for targeting ZC3 in cancer treatment as they identify vulnerable cancer types and potential biomarkers that can be further investigated for therapeutic purposes.

Abbreviations: TCGA, The Cancer Genome Atlas; BLCA, bladder cancer; BRCA, breast cancer; COAD, colon adenocarcinoma; ECM, extracellular matrix; EMT, epithelial mesenchymal transition; ESCA, esophageal cancer; FC, fold change; HIF, hypoxia induced factor; HNSC, head and neck cancer; HR, hazard ratio; KICH, kidney chromophobe; KIRC, renal clear cell cancer; KIRP, renal papillary cancer; LIHC, hepatocellular cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cancer; MMP, matrix metalloproteases; PRAD, prostate adenocarcinoma; pval, *p*-value; ROC, receiver operator characteristic; STAD, stomach adenocarcinoma; THCA, thyroid cancer; UCEC, uterine corpus endometrial carcinoma; and VHL, Von Hippel-Lindau.

Materials and methods

Cell culture and virus infection

The U87 glioma cell line was acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was maintained in MEM at 37°C under 5% CO_2. The HCMV strain AD169 and virus infection procedures were conducted as described previously (Hu et al., 2021). Cells were grown in a serum-free medium and exposed to HCMV at the multiplicity of infection (MOI) of 1. After a 2-h incubation at 37°C, the medium was replaced with a complete one. Simulated infections were carried out separately and concurrently.

RNA-seq and bioinformatics

U87 glioblastoma cells were infected with human cytomegalovirus (HCMV) at a MOI of 1. The cells were then incubated for a period of 2 days. Total RNA was extracted from the cells using TRIzol, following the protocol provided by the manufacturer. To prepare the mRNA libraries, the Illumina TruSeq strand-specific mRNA-seq library preparation kit was used. The libraries were subsequently sequenced on the NextSeq 500 platform, generating paired-end reads. The mRNA sequencing data have been deposited in the European Nucleotide Archive (ENA) under the Accession Number PRJEB30943. All subsequent analyses were performed on clean data, as described in detail in the Results section. We computed the significance of genes remained after filtering with FDR < 0.05 as a threshold by DESeq2 R package. All data analysis was conducted on a server running the CentOS7 operating system, provided by Qingdao University.

Reverse transcription and quantitative real-time PCR

The extraction of RNA was conducted utilizing Trizol reagent (Invitrogen) in accordance with the guidelines provided by the manufacturer. For the synthesis of first-strand cDNA, the first strand cDNA synthesis kit (MBI Fermentas, St Leon Roth, Germany) was employed. The quantification of reverse transcription-polymerase chain reaction (qRT-PCR) was carried out using the Bio-Rad iCycler IQ5 system in conjunction with SYBR Green Master mix. The expression levels of mRNA in each sample were adjusted to the internal control of endogenous origin. The forward and reverse primers for ZC3 detection were 5′-AAGGAAGGACTTACCCATTTTGATATT-3′ and 5′-TGGGTCAGATTTCCCTATGAGAA-3′, respectively. The forward and reverse primers for HCMV UL122 detection were 5′-TGACCGAGGATTGCAACGA-3′ and 5′-CGGCATGATTGACAGCCTG-3′, respectively. The forward and reverse primers 5′-TGGAACGGTGAAGGTGACAG-3′ and 5′-GGCTTTTAGGATGGCAAGGG-3′ were used to detect β-actin as the internal control.

Western blot analysis

Cell harvesting and protein extraction were carried out using RIPA with PMSF. SDS-polyacrylamide gel electrophoresis was employed to separate the extracted proteins. Following separation, the proteins were transferred onto a PVDF membrane (Merck Millipore, USA) and blocked using 5% skim milk. The blots were then probed with the ZC3 primary antibody (ab99930, Abcam, USA) at a dilution of 1:1,000 and the HCMV IE antibody (ab53495, Abcam, USA) at a dilution of 1:1,000. The β-actin antibody (bs-10966R, Bioss, China) was used as an internal reference at a dilution of 1:2,000. Subsequently, the secondary antibodies and ECL western blotting detection were performed following established protocols (Hu et al., 2021).

An analysis of ZC3 expression across pan-cancer

We obtained the ZC3 (ENSG00000058673) RNAseq data for TCGA gene expression (level3, HTSeq-FPKM). The fregments per kilobase per million (FPKM) were subsequently transformed and normalized to transcripts per million (TPM). All cancer types were identified using their TCGA four-letter abbreviation (refer to abbreviations). The R language for statistical computing (version 3.6.3) was utilized to conduct all statistical analyses. The ggplot2 package was used for data visualization. To achieve a normal distribution, the normalized counts were $\log_2$ transformed prior to conducting any statistical analyses. Box or violin plots were created using expression data that had been transformed with $\log_2$ (TPM + 1). The normalized cancer and adjacent normal data for visualization has been uploaded as Supplementary material as median with the first and third quartile (Q1, Q3).

To analyze protein expression in primary tumor and normal tissues from the CPTAC dataset, we utilized the UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html), an interactive web resource. Our analysis focused on examining total protein and phosphorylation levels of ZC3. *Z*-values were used to represent standard deviations from the median across samples for the specific cancer type. Log_2 Spectral count ratio values from CPTAC were normalized within each sample profile and across samples. The normalized statistical significance data between cancer and normal sample for visualization has been uploaded as Supplementary material include maximum, minimum, median and the first and third quartile (Q1, Q3).

To assess localization in ZC3 expression on a protein level, we obtained immunohistochemistry (IHC) images of ZC3 protein expression from both normal and tumor tissues. We accessed these images from the Human Protein Atlas website (http://www.proteinatlas.org/) for analysis.

For the proteomics vs. transcriptomics comparison, raw datasets in the field of proteomics and transcriptomics are accessible through the CPTAC data portal in a Python programming environment. We imported the Omics Data package and loaded the data with a standard Python import statement. Then we extracted the data and compared the proteomics and transcriptomics data from different type of cancer patients

for the ZC3 gene. Finally, we plotted the transcriptomics data against the proteomics data with Seaborn library. A Python script for processing colon cancer has been uploaded in the Supplementary material.

Genetic alteration analysis

For genetic alteration analysis, the cBioPortal tool (https://www.cbioportal.org) was used to gather information on the genetic alterations of ZC3 in all TCGA tumors. The "TCGA Pan Cancer Atlas Studies" option in the "Quick select" section was selected, and the genetic alteration characteristics of the ZC3 gene were queried. The "Cancer Types Summary" module provided results on mutation type, alteration frequency, and copy number alteration (CNA) across all TCGA tumors. The "Mutations" module was consulted for mutated site information, protein structure schematic diagrams, or 3D structures. The "comparison" module was used to gather data on overall, disease-free, progression-free, and disease-free survival differences among TCGA cancer cases with or without ZC3 genetic alterations. Kaplan–Meier plots were generated, and log-rank *p*-values were computed.

Survival prognosis analysis

The "Survival Map" module of GEPIA2 was employed to obtain the OS (Overall survival) and DFS (Disease-free survival) significance map data of ZC3 in all TCGA tumors. We utilized expression thresholds of cutoff-high (50%) and cutoff-low (50%) to divide cohorts into high-expression and low-expression groups. The log-rank test was conducted to evaluate the hypothesis, and survival plots were generated using the "Survival Analysis" module of GEPIA2.

Immune infiltration analysis

For immune infiltration analysis, the "Immune-Gene" module of the TIMER2 web server was utilized to evaluate the correlation between ZC3 expression and immune infiltrates across all TCGA tumors, with a focus on $CD8^+$ T-cells and cancer-associated fibroblasts. Immune infiltration was estimated using algorithms such as MCPCOUNTER, CIBERSORT, TIMER, CIBERSORT-ABS, QUANTISEQ, XCELL, and EPIC. The purity-adjusted Spearman's rank correlation test provided *p*-values and partial correlation (cor) values. Data was presented through scatter plots and heatmaps.

ZC3-related proteins enrichment analysis

To analyze the protein-protein interaction network, we used the STRING website (https://string-db.org/) and set the following main parameters for analysis: "Low confidence (0.150)" for minimum required interaction score, "evidence" for meaning of network edges, "no more than 50 interactors" in 1st shell for max number of interactors to show, and "experiments" for active interaction sources.

We utilized GEPIA2 to collect the leading 100 ZC3-linked genes from the comprehensive TCGA tumor and normal tissue datasets. Following this, we performed a gene-gene Pearson correlation analysis between ZC3 and the chosen genes. Furthermore, we generated a heatmap to visually represent the expression profile of the selected genes alongside the purity-adjusted Spearman's rank correlation test results for partial correlation (cor) and *p*-value.

To conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, two datasets were merged and filtered. To visualize the enriched pathways, we utilized the "tidyr" and "ggplot2" R packages. We performed this analysis using R-3.6.3, 64-bit, which was deemed appropriate for our study. A two-tailed *p*-value of <0.05 was deemed statistically significant for this analysis.

Statistical analysis

Statistical comparisons were performed using unpaired student *t*-test or one-way analysis of variance (ANOVA). Data normality was assessed using the Shapiro-Wilcoxon normality test and D'Agostino & Pearson test. The data were presented as means ± standard deviation (SD), and a *p*-value of <0.05 was considered statistically significant. GraphPad Prism software version 8 (La Jolla, CA) was used for data analysis.

Results

HCMV infection upregulates ZC3H11A expression in glioma cells

Our recent study has provided evidence that infection with human cytomegalovirus (HCMV) can induce specific reactions within cells and contribute to the advancement of glioma. These reactions are characterized by an elevation in the growth and proliferation of glioma cells, along with a heightened resistance to apoptosis. In order to gain a comprehensive understanding of the cellular responses involved, we utilized a genome-wide transcriptome analysis. In this study, we infected glioma cells with HCMV and conducted a transcriptome analysis comparing infected cells to mock-infected cells at 2 days post-infection (dpi). This time point was chosen based on our previous demonstration that the host response is already induced and reaches its peak at this time (Dufu et al., 2010). By focusing on the genes that were most significantly upregulated in HCMV-infected glioma cells, we observed a substantial increase in the expression of stress-induced genes, as well as mRNA processing pathways. In our study, we made an interesting discovery regarding the gene ZC3, which showed minimal expression in cells not infected with HCMV. The Gene ontology (GO) pathway analysis was performed using ClusterProfiler and org.Hs.eg.db R package and enriched functional groups following HCMV infection are depicted in bubble plot (Figure 1A). This finding is particularly noteworthy because HCMV infection is known to cause a global

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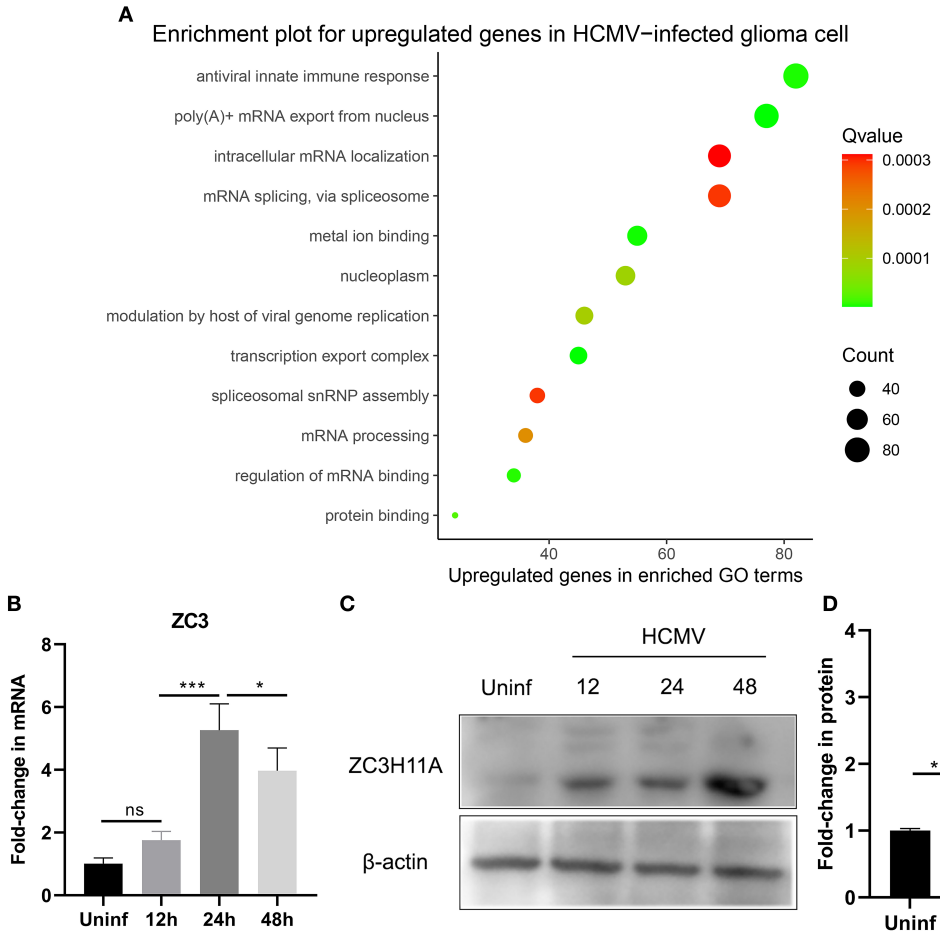

FIGURE 1
Effect of HCMV infection on the transcriptome and expression of ZC3 in glioma cells. **(A)** The enriched functional pathways for upregulated genes after HCMV infection. **(B)** Quantitative PCR analysis of ZC3H11A mRNA expression levels after HCMV infection. **(C, D)** Immunoblot detection of ZC3H11A protein levels during HCMV infection. *$p < 0.05$, ***$p < 0.0001$.

decrease in the levels of host cell mRNA (Isler et al., 2005). Previous research on ZC3 has primarily focused on its function in adenovirus, and its role in HCMV infection has not been explored before.

In order to validate these findings, we utilized quantitative real-time reverse transcription-PCR (RT-PCR) to analyze the RNA extracted from both infected and mock-infected glioma cells. The results of this analysis provided further evidence supporting the transcriptome analysis that revealed the upregulation of ZC3 expression in glioma cells following HCMV infection (Figure 1B). Interestingly, HCMV infection also resulted in a significant increase in the ZC3H11A protein at late time points of infection (Figures 1C, D). Since HCMV induces a widespread inhibition of the host's protein production, while simultaneously facilitating the translation of its own viral mRNAs. The results suggest that ZC3 is significantly impacted by HCMV infection in gliomas.

Analysis of ZC3 mRNA and protein expression in pan-cancer

In order to verify our hypothesis of ZC3, in this section we aimed to provide an in-depth evaluation of ZC3 expression across TCGA normal tissues and TCGA cancer types. UCSC TOIL was used to correct for batch effects and merge samples. RNAseq data in transcripts per million reads (TPM) format was subjected to analysis and comparison after $\log_2$ transformation. Twenty-four sets of comparable tumors and normal tissues were eventually generated (Supplementary Table S1). In general, ZC3 mRNA exhibited a distinct elevated expression pattern in pan-cancer compared to the corresponding normal tissue. As determined by *T*-test, ZC3 mRNA expression was significantly higher in 11 types (11/24, $p < 0.01$) of tumors compared to normal tissues. In contrast, ZC3 mRNA expression was lower in tumor tissues than in normal in 3 (3/24, $p < 0.01$) types

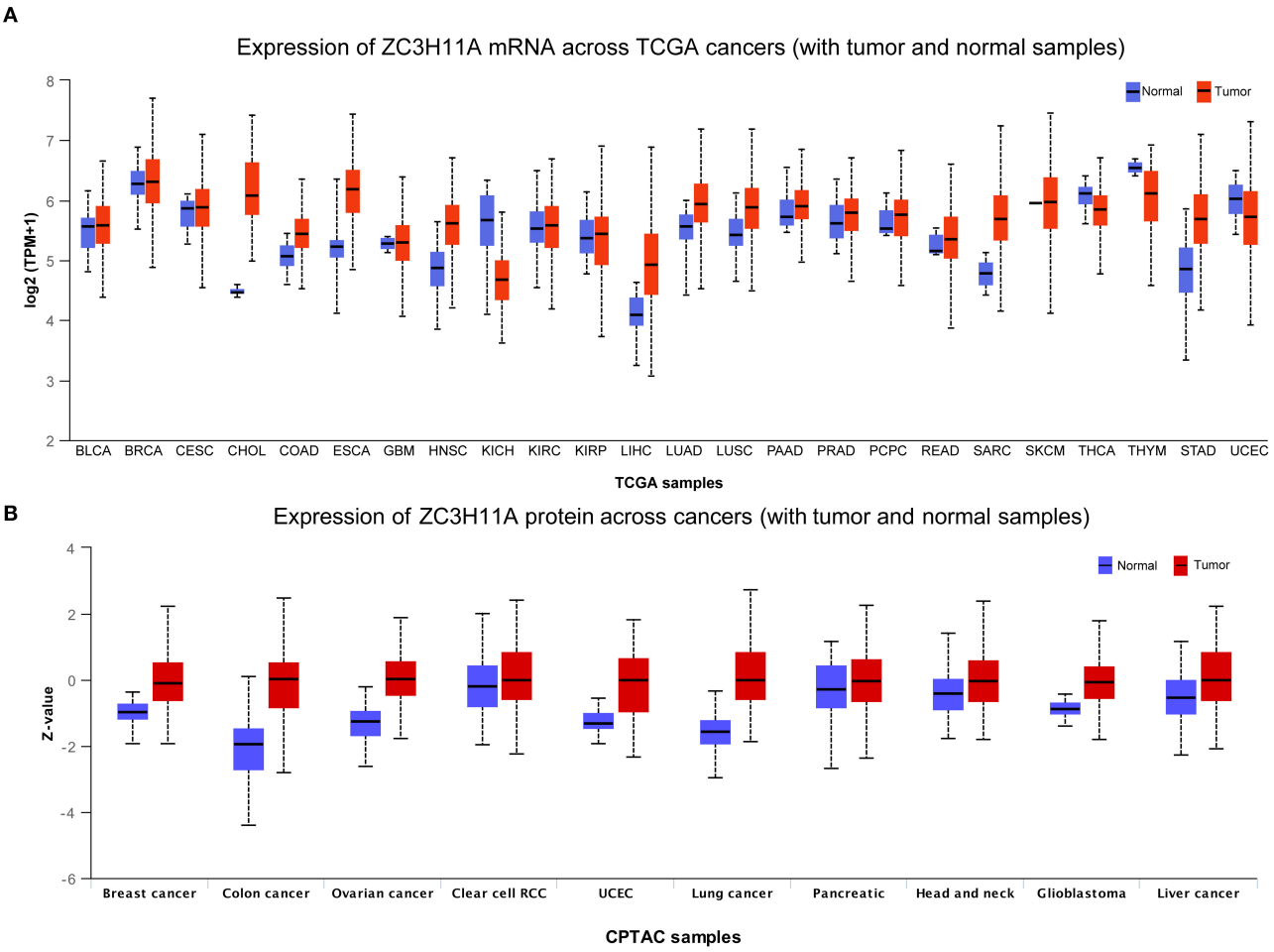

FIGURE 2
The mRNA and protein expression of ZC3 in human tumors. **(A)** The mRNA expression [$\log_2$ (TPM + 1)] of ZC3 in TCGA tumors vs. adjacent tissues as visualized by ggplot2. The sample lines indicate the medians and quartiles. **(B)** The protein level of ZC3 in normal tissue and primary tumors. The analysis of protein data was performed using CPTAC.

tumors (Figure 2A, Supplementary Table S2). Comparing across cancer types, ZC3 expression varied broadly suggesting that high ZC3-expressing cancers may be driven by unique genetic features (Figure 2A). Interestingly, among the high ZC3 expression cancer types, we noticed a more significant differences in gastrointestinal neoplasms and digestive system cancers (CHOL, ESCA, LIHC and STAD). Moreover, according to the interquartile range (IQR), ZC3 mRNA expression was more widespread in some types of cancer than others; e.g., bile duct cancer (CHOL) had a wide spread while pancreatic cancer (PAAD) had a narrow spread.

Next, a comparison of ZC3 protein expression across the TCGA was also conducted. Although the expression of ZC3 protein varied across tumor types, unlike ZC3 mRNA expression, ZC3 protein was much higher in tumor tissue than normal tissue in almost all cancers (Figure 2B, Supplementary Table S3). ZC3 protein followed the same expression patterns throughout most tumor types.

Additionally, we localized the ZC3 protein expression using tumor histological staining obtained from HPA. As expected, ZC3 protein staining was positive in all selected tumor tissue showing moderate and/or strong staining intensity. In contrast, ZC3 was mainly detected in a salt-and-pepper like pattern with low staining or not detected in corresponding normal tissue (Figure 3). The neuronal cells in cerebral cortex and tubular cells in kidney were two exceptions, because these cells showed moderate intensity with around 75% quantity for ZC3 staining. The cervical adenocarcinoma cells displayed continuous positive for ZC3 while ovarian stroma cells displayed discontinuous staining. Interestingly, ZC3 protein in tumor cells localized to the nuclear whereas in normal cells it was mainly found in cytoplasmic/membranous (Figure 3).

As our group previously demonstrated that ZC3 protein levels were significantly elevated under stress condition, while mRNA levels were not elevated apparently. In order to determine this correlation in tumor, we compared the ZC3 proteomics and transcriptomics data for each type of pan-cancer patients with Python code from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset. The results showed that ZC3 Protein and mRNA changes were highly correlated for most cancer types (5/8, Figures 4A–E). However, in glioblastoma (GBM), kidney clear cell carcinoma (ccRCC) and

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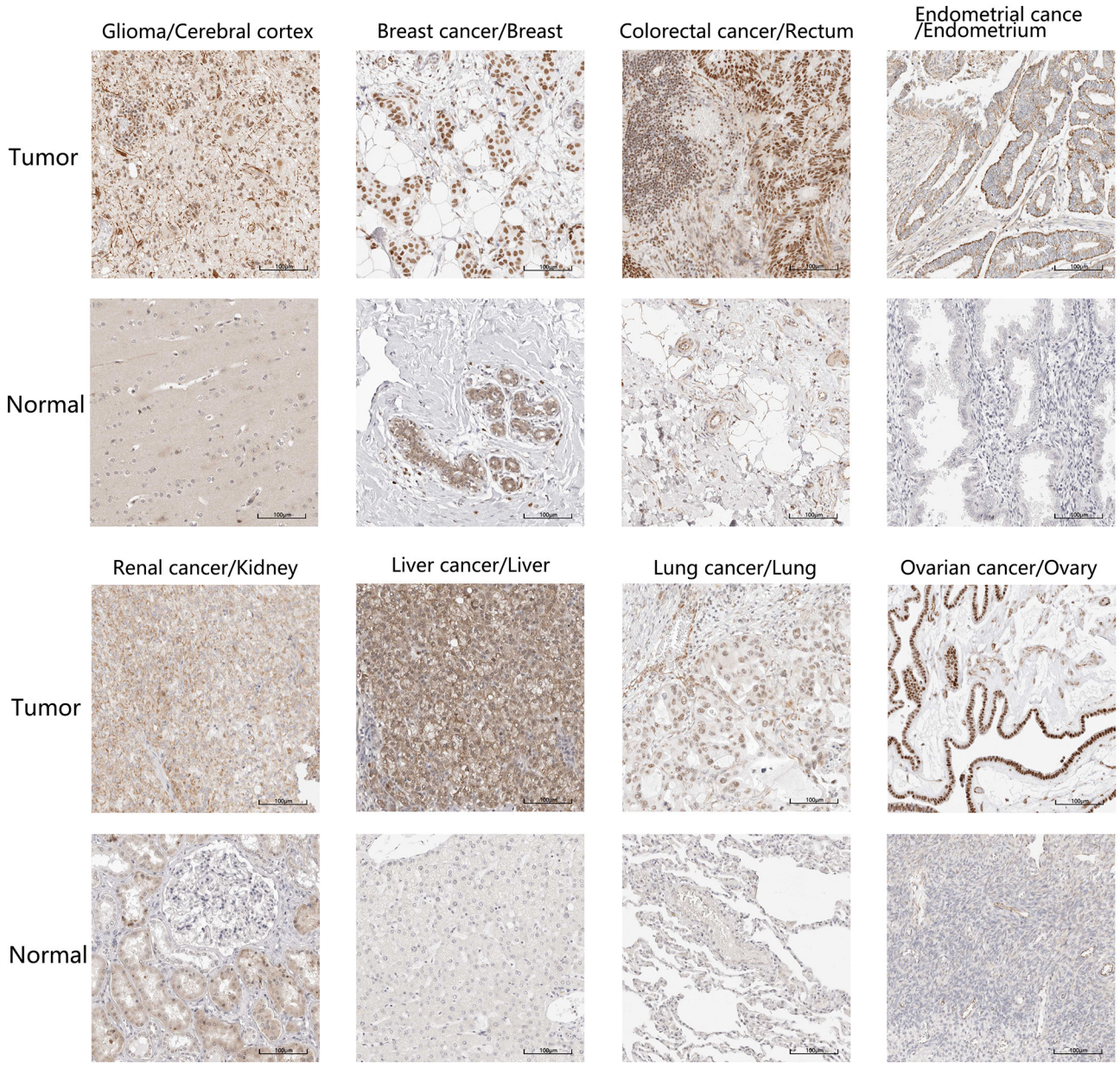

FIGURE 3
The expression and intracellular localization of ZC3 protein. The expression and intracellular localization of ZC3 protein that assessed by immunostaining in tumors and the corresponding normal tissues from HPA.

pancreatic ductal adenocarcinoma (PDAC), the increase in ZC3 protein was not accompanied by a significant elevation in mRNA level (Figures 4F–H). This observation indicates that ZC3 protein may be more dynamically involved in post-translational levels in certain cancer types.

Analysis of ZC3 phosphorylation in certain cancer types

Since ZC3 may undergo various posttranslational modifications, including extensive regulation via phosphorylation, in three cancer types (ccRCC, GBM, and PDAC) with low correlation between ZC3 mRNA expression and protein abundance. We next compared the phosphorylation of ZC3 between primary tumors and normal tissues. Based on mass spectrometry analysis and phospho-proteomics data, ZC3 unique phosphorylation sites have been identified in cancers (Beausoleil et al., 2006; Dephoure et al., 2008; Zhou et al., 2013). As summarized in Figure 5A, the phosphorylation level at S108 (p = 6.796E-10) and S290 (p = 6.507E-04) was both reduced in PDAC, while the phosphorylation level at these same sites was significantly increased in GBM compared to normal tissues (p = 6.888E-07 and p = 1.85E-03; Figures 5E–I). In ccRCC the phosphorylation level was reduced at S108 (p = 4.347E-11) and

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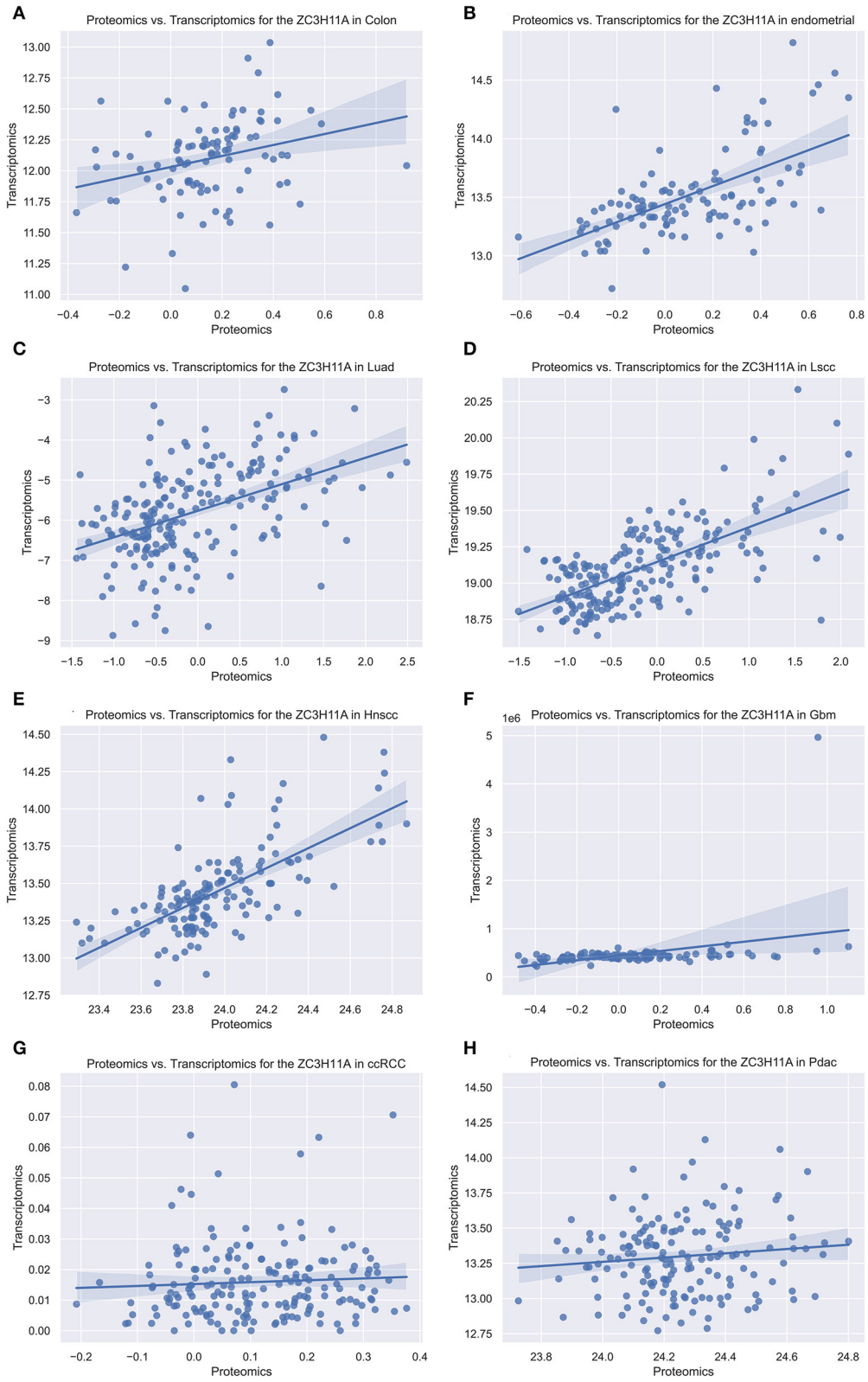

FIGURE 4
The scatterplot comparing transcriptomics and proteomics of ZC3 in pan-cancer with CPTAC dataset. The proteomics and transcriptomics data from the pan-cancer patients were compared using the National Cancer Institute's CPTAC program as a Python package. **(A–H)** Represent different cancer types and the abbreviated names are listed on each title.

S129 ($p = 3.991E-10$) and was no significant differences at S171 ($p = 0.703$; Figures 5B–D). The phosphorylation level of ZC3 protein can generally be categorized as being tumor specific compared to normal tissues.

Genetic alteration analysis of ZC3 in pan-cancer

Genetic alterations are responsible for the development of human cancers. We proceeded to investigate the genetic alterations in ZC3 in human tumor samples. Based on findings from TCGA Pan-cancer Studies, the alteration frequency of ZC3 (>9%) was observed to be highest in breast cancer, with "Amplification" identified as the primary type. Endometrial Cancer had the second highest alteration frequency (>9%) of ZC3 with the incidence of "Mutation" as the primary type (Figure 6A). Figure 6A also showed the alteration frequency across other types of cancer. The main features of the mutations type were "Mutation" and "Amplification." We further presented additional mutations and their location within ZC3 (Figure 6B). It appeared that there was no main type of genetic alteration and the genetic alteration were rather sporadic with some locating in ZnF motifs (C3H1) and colied coil motifs. Figure 6C shows the visualized mutated amino acids G293C within a crystal structure of ZC3.

Next, a systematical investigation was conducted to determine the correlation between clinical survival prognosis and certain genetic alterations of ZC3 in various types of tumors. Based on all combined TCGA PanCancer Atlas, altered ZC3 (red line) was not associated with overall survival (OS, $p = 0.460$) or disease-free survival (DFS, $p = 0.447$; Figures 6D, E). Conversely, the OS and DFS in patients with ZC3 alterations was much significant in GBM cases (Figures 6F, G). Together, these findings suggest ZC3 genetic alterations may play a role in the development of some special types of tumors.

Association of ZC3 expression with patient survival

We then sought to investigate the correlation between ZC3 expression and prognosis in pan-cancer. Based on ZC3 expression level, cancer cases were divided into two groups: low ZC3 expression and high ZC3 expression. Our analysis of the GEPIA2 TCGA data set revealed the correlation between ZC3 expression and patient OS or DFS varied depending on the cancer type tested as shown in Figure 7. More specifically, increased expression of ZC3 was primary associated with overall survival (OS) disadvantage and predicted poor prognosis of patients with, Cervical Squamous and Endocervical Adenocarcinoma (CESC), Kidney Papillary Cell Carcinoma (KIRP), Adrenocortical Carcinoma (ACC) and Lower Grade Glioma (LGG; Figure 7A). In contrast, increased ZC3 expression was associated with increased survival advantage in Head and Neck Squamous Cell Carcinoma (HNSC). While increased ZC3 expression only predicted poor prognosis with disease free survival (DFS) of patients with ACC, Bladder Urothelial Carcinoma (BLCA), CESC and KIRP (Figure 7B).

In order to confirm these results, we also used Starbase and Kaplan-Meier plotter tool to analyze the survival data. The results were similar to those with GEPIA2, that a primary correlation between high ZC3 expression and OS disadvantage was noted in Starbase, where ZC3 predicted poor prognosis of ACC, KIRP, LGG and moreover Kidney Chromophobe (KICH; Supplementary Figure S1A). The Kaplan-Meier plotter tool showed an increased survival disadvantage with ZC3 high expression in CESC, KIRP, and Liver Hepatocellular Carcinoma (LIHC) but predicted better survival for patients with HNSC and moreover Esophageal Carcinoma (ESCA; Supplementary Figure S1B and Supplementary Table S4). The results showed that ZC3 high expression was primary associated with poor prognosis in majority cancers but still varied depending on the cancer types.

Association between ZC3 expression and immune infiltration

Stromal cells which surrounding cancer cells caner support cancer cell malignancy and are essential for tumor progression. Among the stromal cells, cancer-associated fibroblasts (CAFs) are the most abundant. Therefore, we chose CAFs, NK cells and $CD8^+$ T cells to investigate the relationship between ZC3 expression and the infiltration level of pan-cancer types. The correlation was calculated by a variety of algorithm for the quantification of immune cells such as EPIC, XCELL, quanTIseq, TIMER, and so forth (Figure 8). As shown, a significant positive correlation between ZC3 gene expression and the presence of cancer-associated fibroblast (CAF) cells (Figure 8A) was observed in almost all types of cancer, suggesting that high ZC3 gene expression is linked to an active stromal component. For NK cells (Figure 8B), compared to EPIC and CIBERSORT methods, quanTIseq robustly obtained positive correlations across all cancer types. In contrast, MCPcounter and quanTIseq had a significant positive correlation. Because tumor purity and immune infiltration are inevitably negatively correlated for techniques like EPIC, which provide cell fractions referred to as total cells. In the minor cluster consisting of HNSC, THYM and UCEC, a significant negative correlation was observed between ZC3 expression and $CD8^+$ T cells, but weak or no correlation in the other clusters as LUAD, LUSC, MESO, and OV (Figure 8C).

It is worth noting, in nearly all cancer types, innate immune system and stromal component exhibited a positive correlation with ZC3 expression, providing further evidence that samples displaying a hot Targeting Tumor Microenvironment (TME) may be linked to increased ZC3 gene expression.

The main function of ZC3-related proteins was annotated to mRNA processing cluster

To date, only few studies have addressed the ZC3 interactions. We explored large-scale proteomics and STRING database to discover protein–protein interactions and potential functional

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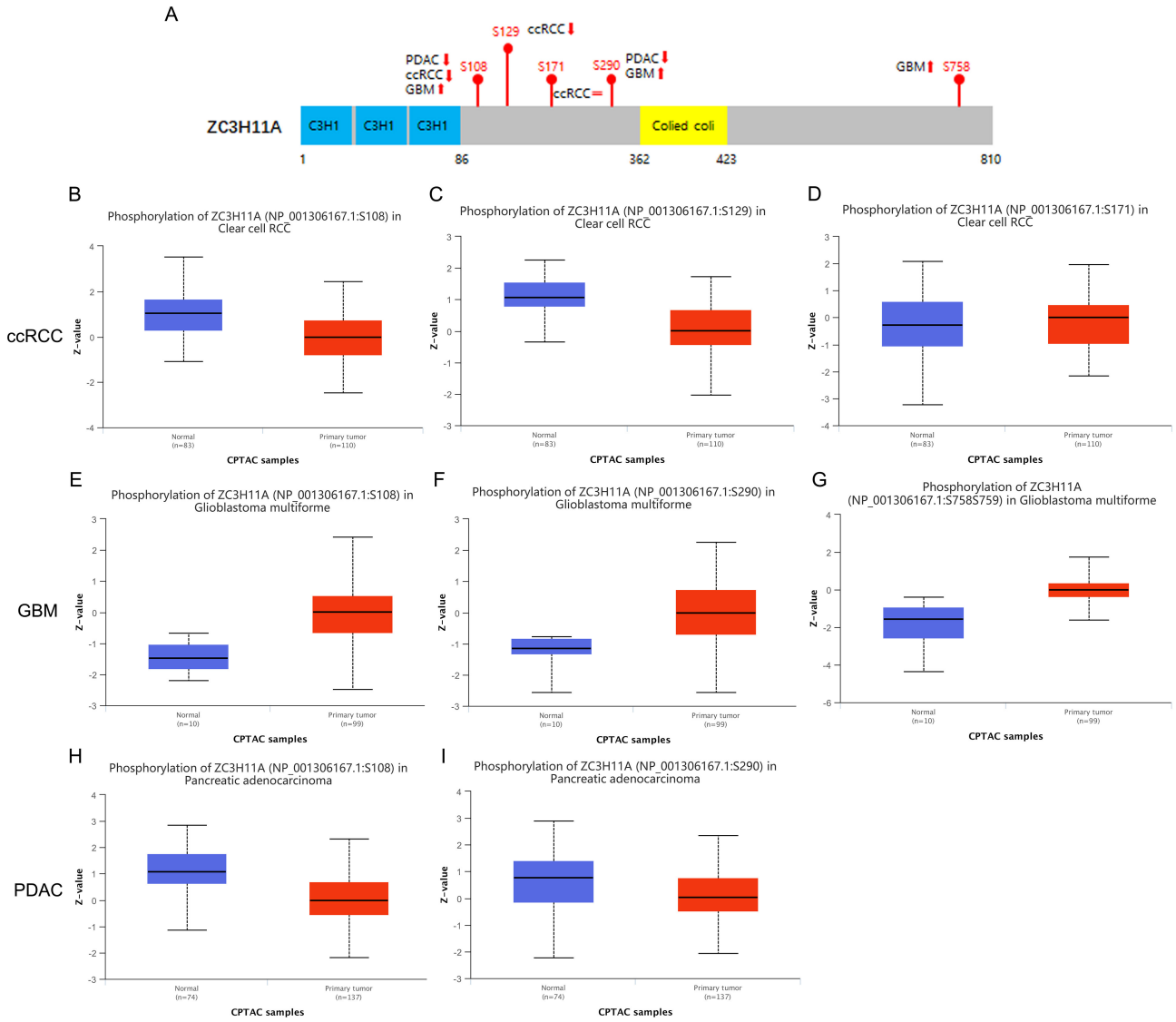

FIGURE 5
Tumor-associated protein phosphorylation of ZC3. **(A)** A schematic diagram of ZC3 phosphoprotein sites and summarized phosphorylation levels in primary tumors. **(B–D)** Box plot representation of ZC3 phosphorylation levels at different sites in ccRCC. **(E–G)** Box plot representation of ZC3 phosphorylation levels at different sites in GBM. **(H, I)** Box plot representation of ZC3 phosphorylation levels at different sites in PDAC. *Z*-values represent standard deviations from the median across samples for the given cancer type.

clustering. By mining the reported proteomics studies, we identified 14 confirmed interactions, and most of these are the Transcription and EXport (TREX) complex (Prigge et al., 2009; Dufu et al., 2010; Hein et al., 2015). The STRING analysis result revealed more interactions which are also associated with TREX complex or mRNA processing (Figure 9A). We selected the shared proteins between the two sources and computed the expression correlation analysis with ZC3 in pan-cancer types. We observed a pan-cancer positive correlation between ZC3 and many TREX complex proteins (Figures 9B–E). We also picked up the 14 interacted proteins from reported proteomics and the experimentally determined interactions from STRING to perform gene ontology (GO) functional annotation clustering analysis. The result revealed enriched terms for mRNA processing, spliceosome and mRNA export (Figure 9F).

In addition to direct interactions, we further identified a list of partners which have a similar expression pattern with ZC3 under TCGA pan-cancer types. Consistent with our prediction, the functional clustering of these genes robustly enriched in mRNA processing or splicing terms (Figure 9G). Interesting, GO functional clustering results also showed clusters of herpes virus infection and stress response (Figure 9G). The results of this section strongly imply a functional role for ZC3 in mRNA processing.

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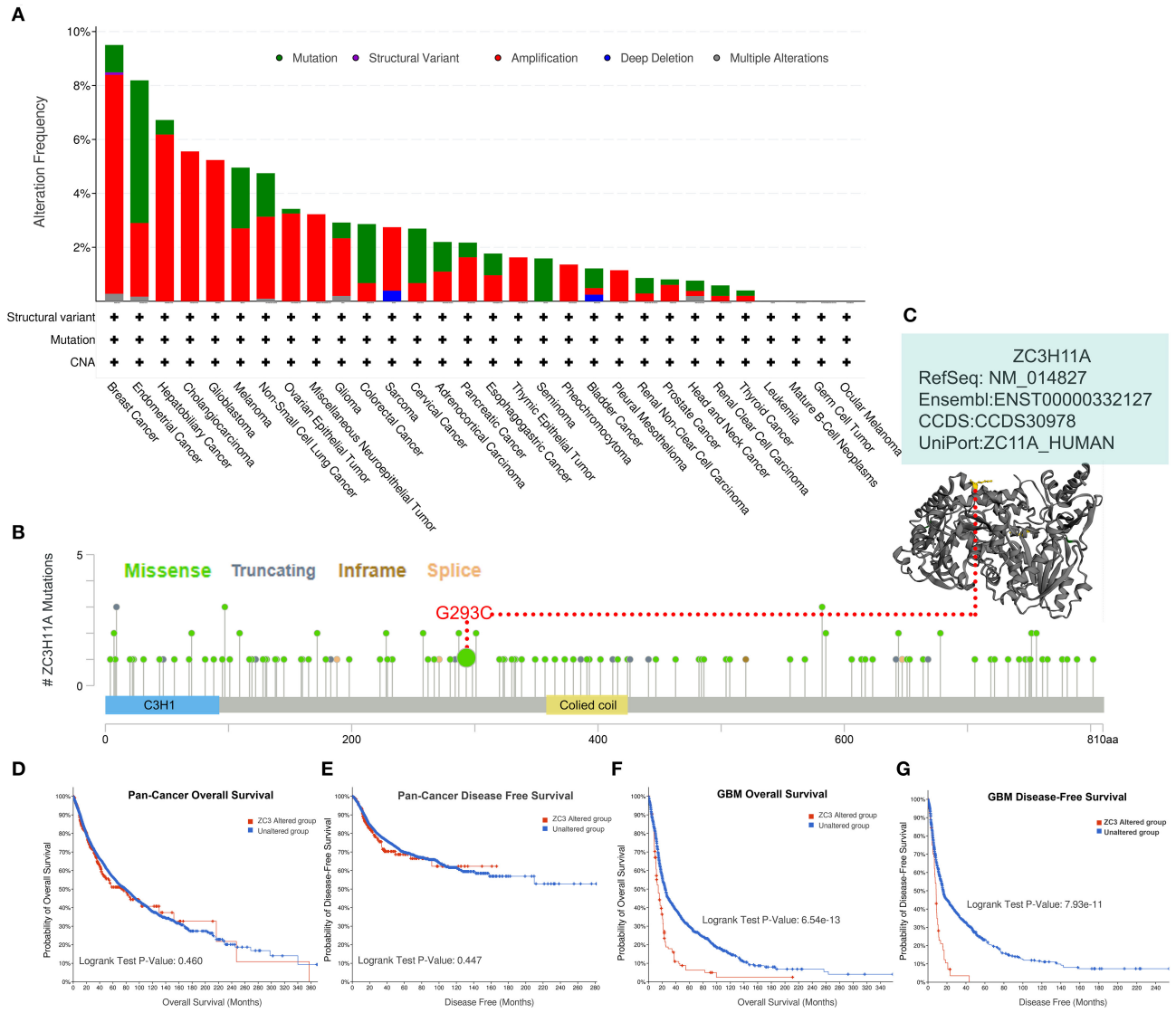

FIGURE 6
Genetic alteration status of ZC3 in pan-cancer. **(A)** The alteration frequency and mutation type of ZC3 in different cancer types. **(B)** The amino acid mutation sites of ZC3 protein and **(C)** a mutated crystal structure of aminotransferase in 293aa. **(D, E)** The correlation between ZC3 alteration status and pan-cancer overall survival or disease-free survival. **(F, G)** The correlation between ZC3 alteration status and GBM overall survival or disease-free survival.

Discussion

The complex interplay between viral infections and oncogenesis has been a subject of considerable interest in recent years. Employing a global transcriptome analysis of infected human glioma cells, we found that the ZC3 gene was one of the most profoundly upregulated mRNA export genes following HCMV infection compared to uninfected controls. Importantly, at 48 h post infection, the elevation of ZC3 protein was more pronounced than the mRNA level. This observation suggests that HCMV infection plays a crucial role in the dysregulation of ZC3 in gliomas, potentially contributing to tumor development and progression.

There are several reasons why we believe that the ZC3 may be important for cancer biology. Firstly, ZC3 is a stress-induced protein and may therefore be essential during tumor growth, for instance due to hypoxia. Secondly, ZC3 is a member of the zinc finger CCCH-type containing protein family, which recently has been demonstrated to play a role in RNA processing and splicing (Younis et al., 2018; Hajikhezri et al., 2020). The research on RNA regulatory proteins in tumors is by far not fully explicated (Hu et al., 2022). This study provides a comprehensive analysis of the relationship between ZC3 and pan-cancer, with a focus on mRNA and protein expression, proteomics and transcriptomics correlation, genetic alterations, patient survival, post-translational modification, and immune infiltration.

Firstly, our study showed a significant upregulation of ZC3 mRNA and protein expression in most cancer types compared to normal tissues, indicating that the over-expression of ZC3 is a common phenotype in cancer, although the pattern and

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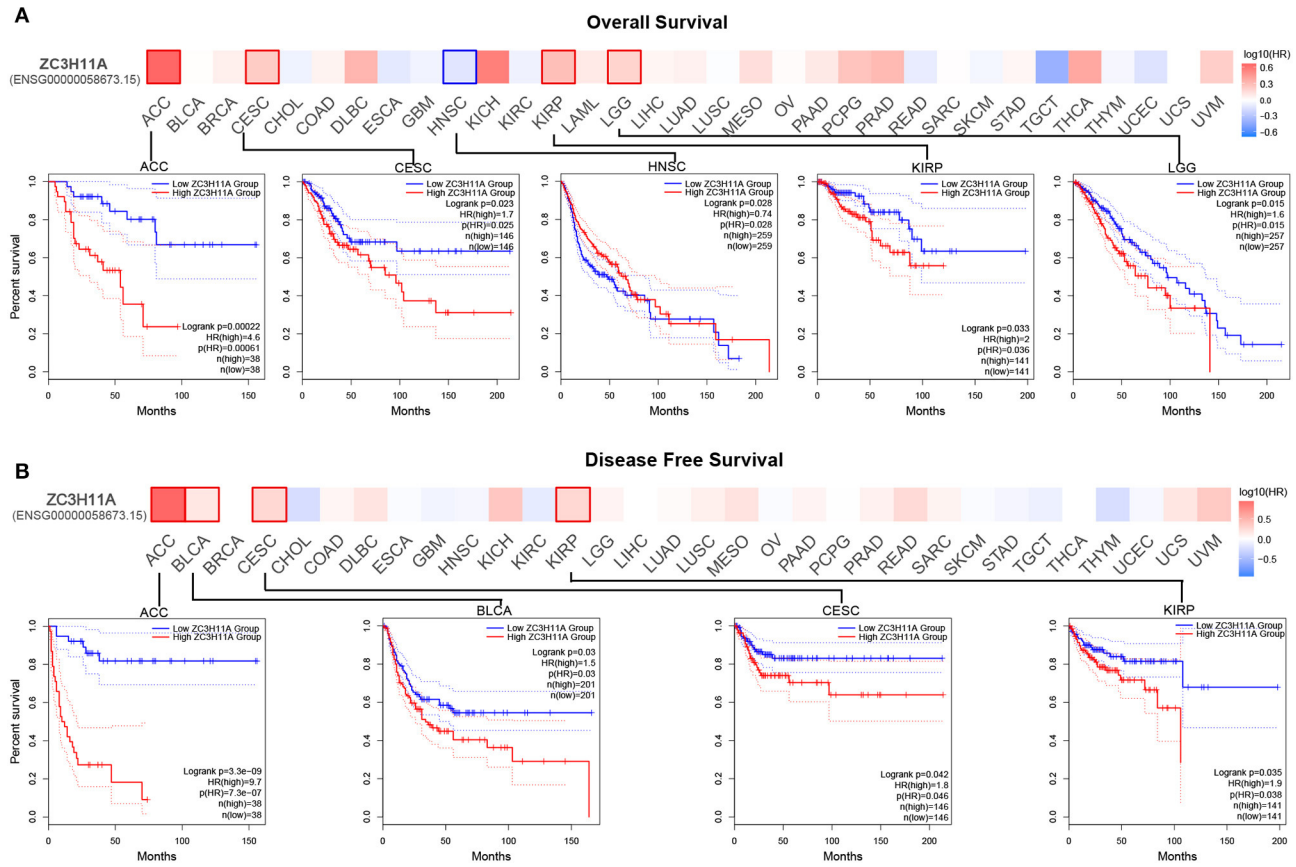

FIGURE 7
The correlation between ZC3 expression and the patient survival rate across different types of cancer. **(A)** The correlation between ZC3 expression and patient overall survival by Gepia2 tool analysis. **(B)** The correlation between ZC3 expression and patient disease-free survival by Gepia2 tool analysis.

extent of this loss may differ depending on the cancer type. We identified that ZC3 mRNA expression was more widespread in some types of cancer than others. This could be because some cancers have multiple clearly defined subtypes, increasing their genetic diversity. This indicates that ZC3 may be playing diverse roles in different cancer types, possibly due to variations in their molecular characteristics. Interestingly, ZC3 protein in tumor cells localized to the nuclear whereas in normal cells it was mainly found in cytoplasmic/membranous. This result is consistent with the RNA processing function of ZC3 and suggests that tumor cells may require more ZC3 to play a specific role in RNA processing within the nucleus, including transcription and RNA export. The data on subcellular localization serve as a valuable asset in defining the protein composition of different compartments and can thus constitute a starting point for further in-depth functional studies.

Next, we compared the ZC3 proteomics and transcriptomics data for each type of pan-cancer patients from the CPTAC to provide complementary information on the protein expression and gene expression profiles. Our results showed that the correlation between ZC3 protein expression and mRNA expression was moderate. This correlation varies among different cancer types, with the highest correlation observed in head and neck squamous cell carcinoma and the lowest correlation observed in glioblastoma. Particularly, the increase in ZC3 protein accumulation did not coincide with a corresponding increase in ZC3 mRNA expression in GBM, ccRCC and PDAC. The discrepancy between ZC3 proteomics and transcriptomics data may reflect the complex regulatory mechanisms that control post-transcriptional gene expression, including RNA stability, splicing, and translation efficiency. In addition, the protein expression levels of ZC3 may be influenced by post-translational modifications, such as ubiquitination and phosphorylation, which are not directly reflected in mRNA expression levels.

We further revealed the phosphorylation of ZC3 in three cancer types (ccRCC, GBM, and PDAC) which has low correlation between ZC3 mRNA expression and protein abundance. Our results showed that ZC3 is phosphorylated at several sites, including Ser-108, Ser-290, and Ser-313, in GBM, but not in others. Phosphorylation of ZC3 may affect its interaction with other proteins and RNAs that play a role in cancer progression. For example, ZC3 has been shown to interact with the mRNA decay machinery, including the exosome and decapping enzymes (Singh et al., 2014). Phosphorylation of ZC3 may affect its recruitment to these complexes and therefore alter mRNA stability and decay rates.

Genetic alterations, such as mutations and copy number variations, are common events in cancer that can affect the

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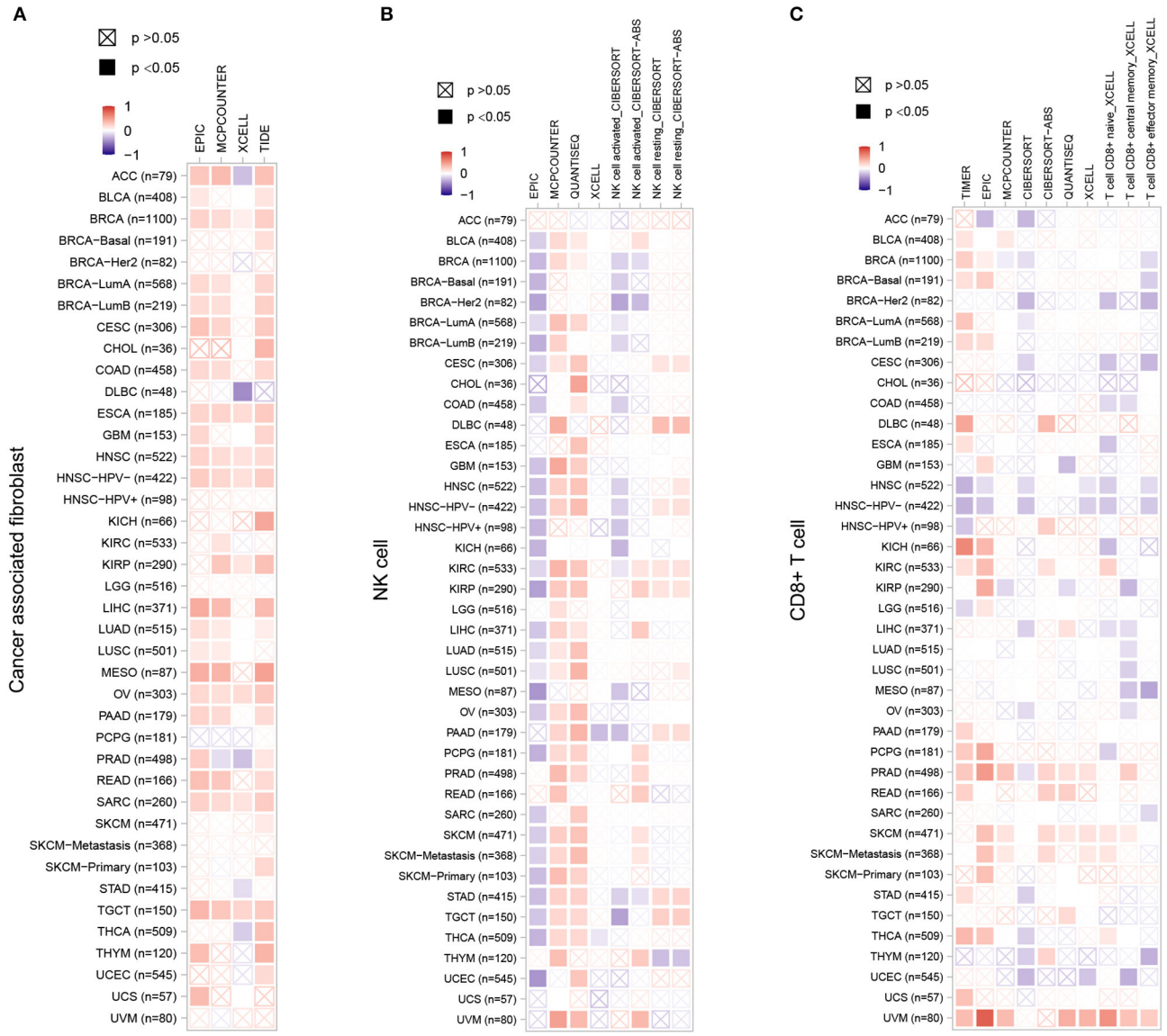

FIGURE 8
The correlation between ZC3 expression levels and infiltration of immune cells. For the correlative analysis of the ZC3 gene and CAFs **(A)**, NK cells **(B)**, and CD8$^+$ T cells **(C)** across all tumors in TCGA. The following algorithms were utilized: EPIC, TIDE, quanTIseq, CIBERSORT, TIMER, XCELL, and MCPCOUNTER. A positive correlation (0–1) is indicated in red, while a negative correlation (−1 to 0) is depicted in blue. A correlation with a *p*-value <0.05 is considered statistically significant. Correlation values that are statistically non-significant are marked with a cross.

tumor biology behavior. ZC3 gene was altered in some cancer types, such as breast cancer and endometrial cancer, with a frequency ranging from 1 to 9%. The most frequent genetic alteration was amplification, followed by missense mutation, and deep deletion. The association between ZC3 genetic alteration and cancer progression remains unclear. However, our finding suggests that mutations in the ZC3 gene may play a role in tumor development and warrants further investigation.

The discrepancy in the correlation between ZC3 expression and patient survival may be due to the different roles of ZC3 in different cancer types. However, in most case, the higher expression of ZC3 was associated with worse overall survival. ZC3 may promote tumor growth and metastasis by facilitating RNA processing and/or interacting with splicing factors. The correlation between ZC3 expression and immune infiltration may reflect the role of ZC3 in regulating immune-related genes and pathways. For example, ZC3 has been shown to interact with several immune-related genes, including interleukin-6 and a large group of interferon-stimulated genes (Darweesh et al., 2022), which play a role in anti-tumor immune response.

ZC3 is a member of the zinc finger CCCH-type containing protein family, which includes several proteins that are involved in RNA processing, splicing, and decay. Our results also showed that the main function of ZC3-related proteins was annotated as the mRNA processing cluster. This cluster includes several functional annotation groups, such as RNA binding, mRNA splicing, mRNA transport, and mRNA decay pathways. This functional annotation

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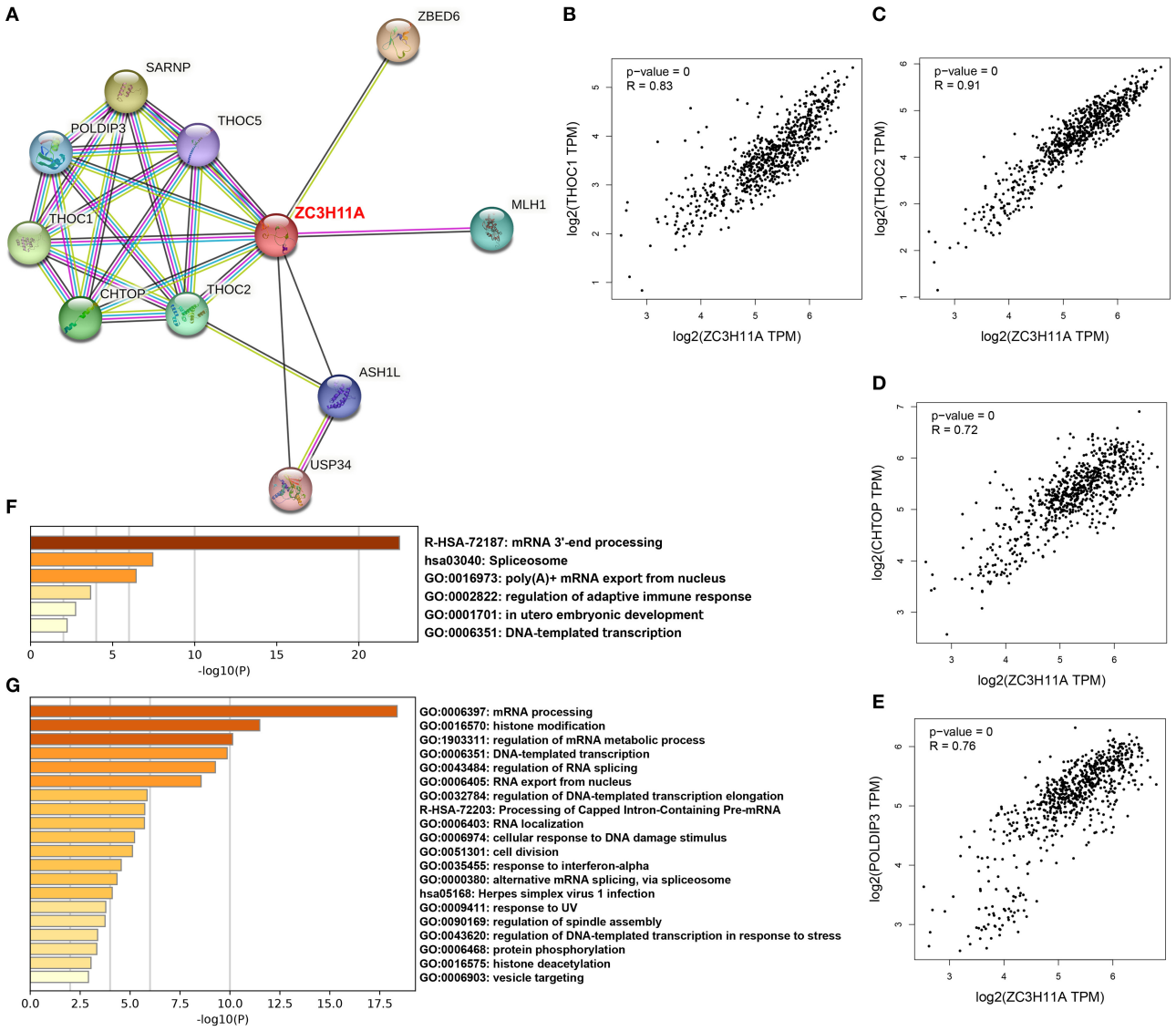

FIGURE 9
ZC3-related proteins and functional clustering analysis. **(A)** An experimentally determined protein-protein interaction network of ZC3 based on STRING database. The identified proteins are indicated by the colored nodes. **(B–E)** The expression correlation in pan-cancers between ZC3 and ZC3-interacted proteins. **(F)** The GO functional annotation clustering of ZC3-interacted proteins. **(G)** The GO functional annotation clustering of proteins which have a similar expression pattern with ZC3 in pan-cancers.

suggests that ZC3-related proteins are vital in regulating RNA post-transcriptional gene expression and metabolism, which are critical processes in cancer development and progression.

In summary, the findings of this study offer novel insights into the relationship among virus infection, ZC3 and pan-cancer. We also highlight the importance of multi-omics analysis in cancer research, which involves the integration of transcriptomic and proteomic data to achieve a comprehensive understanding of ZC3 underlying cancer development and progression. Further investigations are required to clarify the molecular mechanisms that underlie the correlation between ZC3 and cancer in-depth and to explore its potential as a diagnostic and therapeutic target in cancer.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JL: Writing—original draft. MS: Writing—original draft, Data curation, Methodology. ZL: Writing—original draft, Software. FN: Formal analysis, Project administration, Writing—original draft. BW: Validation, Writing—review & editing. DQ: Investigation,

Resources, Writing—original draft. MH: Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1296725/full#supplementary-material

SUPPLEMENTARY FIGURE S1
The relationship between ZC3 expression and prognosis verified by Starbase and Kaplan-Meier plotter tool. **(A)** The correlation between ZC3 expression and patient overall survival by starbase. **(B)** The correlation between ZC3 expression and patient overall survival by Kaplan–Meier plotter tool.

SUPPLEMENTARY TABLE S1
Descriptive statistics of ZC3 mRNA expression in TCGA cancer and normal tissues.

SUPPLEMENTARY TABLE S2
Independent samples *t*-tests of ZC3 mRNA between tumor and normal tissues.

SUPPLEMENTARY TABLE S3
Descriptive statistics of ZC3 protein expression in TCGA cancer and normal tissues.

SUPPLEMENTARY TABLE S4
Survival analysis table of pan-cancer analysis for ZC3H11A from starbase.

References

Ang, H. L., Mohan, C. D., Shanmugam, M. K., Leong, H. C., Makvandi, P., Rangappa, K. S., et al. (2023). Mechanism of epithelial-mesenchymal transition in cancer and its regulation by natural compounds. *Med. Res. Rev.* 43, 1141–1200. doi: 10.1002/med.21948

Beausoleil, S. A., Villen, J., Gerber, S. A., Rush, J., and Gygi, S. P. A. (2006). probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* 24, 1285–1292. doi: 10.1038/nbt1240

Boehringer, A., Garcia-Mansfield, K., Singh, G., Bakkar, N., Pirrotte, P., Bowser, R., et al. (2017). Associated mutations in matrin 3 alter protein-protein interactions and impede mRNA nuclear export. *Sci. Rep.* 7, 14529. doi: 10.1038/s41598-017-14924-6

Borden, K. L. B. (2020). The nuclear pore complex and mRNA export in cancer. *Cancers* 13, 42. doi: 10.3390/cancers13010042

Buscail, L., Bournet, B., and Cordelier, P. (2020). Role of oncogenic KRAS in the diagnosis, prognosis and treatment of pancreatic cancer. *Nat. Rev. Gastroenterol. Hepatol.* 17, 153–168. doi: 10.1038/s41575-019-0245-4

Cai, Q., Zhang, B., Sung, H., Low, S.-K., Kweon, S.-S., Lu, W., et al. (2014). Genome-wide association analysis in East Asians identifies breast cancer susceptibility loci at 1q32. 1, 5q14. 3 and 15q26. 1. *Nat. Genet.* 46, 886–890. doi: 10.1038/ng.3041

Darweesh, M., Younis, S., Hajikhezri, Z., Ali, A., Jin, C., Punga, T., et al. (2022). ZC3H11A loss of function enhances NF-kappaB signaling through defective IkappaBalpha protein expression. *Front. Immunol.* 13, 1002823. doi: 10.3389/fimmu.2022.1002823

Dephoure, N., Zhou, C., Villen, J., Beausoleil, S. A., Bakalarski, C. E., Elledge, S. J., et al. (2008). A quantitative atlas of mitotic phosphorylation. *Proc. Natl. Acad. Sci. USA.* 105, 10762–10767. doi: 10.1073/pnas.0805139105

Dufu, K., Livingstone, M. J., Seebacher, J., Gygi, S. P., Wilson, S. A., Reed, R., et al. (2010). ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex. *Genes Dev.* 24, 2043–2053. doi: 10.1101/gad.1898610

Grzeskowiak, C. L., Kundu, S. T., Mo, X., Ivanov, A. A., Zagorodna, O., Lu, H., et al. (2018). *In vivo* screening identifies GATAD2B as a metastasis driver in KRAS-driven lung cancer. *Nat. Commun.* 9, 2732. doi: 10.1038/s41467-018-04572-3

Hajikhezri, Z., Darweesh, M., Akusjarvi, G., and Punga, T. (2020). Role of CCCH-type zinc finger proteins in human adenovirus infections. *Viruses* 12, 1322. doi: 10.3390/v12111322

Hein, M. Y., Hubner, N. C., Poser, I., Cox, J., Nagaraj, N., Toyoda, Y., et al. (2015). A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell.* 163, 712–723. doi: 10.1016/j.cell.2015.09.053

Hu, M., Wang, B., Qian, D., Wang, M., Huang, R., Wei, L., et al. (2017). Human cytomegalovirus immediate-early protein promotes survival of glioma cells through interacting and acetylating ATF5. *Oncotarget* 8, 32157–32170. doi: 10.18632/oncotarget.17150

Hu, M., Wang, B., and Wu, C. (2022). The role of DNA viruses in human cancers. *Front. Cell. Infect. Microbiol.* 12, 1103505. doi: 10.3389/fcimb.2022.1103505

Hu, M., Yu, B., Zhang, B., Wang, B., Qian, D., Li, H., et al. (2021). Human cytomegalovirus infection activates glioma activating transcription factor 5 via microRNA in a stress-induced manner. *ACS Chem. Neurosci.* 12, 3947–3956. doi: 10.1021/acschemneuro.1c00576

Isler, J. A., Skalet, A. H., and Alwine, J. C. (2005). Human cytomegalovirus infection activates and regulates the unfolded protein response. *J. Virol.* 79, 6890–6899. doi: 10.1128/JVI.79.11.6890-6899.2005

Liang, S., Yu, B., Qian, D., Zhao, R., Wang, B., Hu, M., et al. (2019). Human cytomegalovirus ie2 affects the migration of glioblastoma by mediating the different splicing patterns of RON through hnRNP A2B1. *Neuroreport* 30, 805–811. doi: 10.1097/WNR.0000000000001277

Prigge, J. R., Iverson, S. V., Siders, A. M., and Schmidt, E. E. (2009). Interactome for auxiliary splicing factor U2AF(65) suggests diverse roles. *Biochim. Biophys. Acta.* 1789, 487–492. doi: 10.1016/j.bbagrm.2009.06.002

Singh, A., Minia, I., Droll, D., Fadda, A., Clayton, C., Erben, E., et al. (2014). Trypanosome MKT1 and the RNA-binding protein ZC3H11: interactions and potential roles in post-transcriptional regulatory networks. *Nucleic Acids Res.* 42, 4652–4668. doi: 10.1093/nar/gkt1416

Tabl, A. A., Alkhateeb, A., Pham, H. Q., Rueda, L., ElMaraghy, W., and Ngom, A. (2018). A novel approach for identifying relevant genes for breast cancer survivability on specific therapies. *Evol. Bioinform. Online.* 14, 1176934318790266. doi: 10.1177/1176934318790266

Tan, X., Xiao, G. Y., Wang, S., Shi, L., Zhao, Y., Liu, X., et al. (2023). EMT-activated secretory and endocytic vesicular trafficking programs underlie a vulnerability to PI4K2A antagonism in lung cancer. *J. Clin. Investig.* 133, e165863. doi: 10.1172/JCI165863

Wang, X., Hu, M., Xing, F., Wang, M., Wang, B., Qian, D., et al. (2017). Human cytomegalovirus infection promotes the stemness of U251 glioma cells. *J. Med. Virol.* 89, 878–886. doi: 10.1002/jmv.24708

Wu, C. H., Feng, C. W., Wang, C. L., Wen, Z. H., Long, C. Y., Tang, F. H., et al. (2023). Zinc finger protein 90 knockdown promotes cisplatin sensitivity via Nrf2/HO-1 pathway in ovarian cancer cell. *Cancers* 15, 1586. doi: 10.3390/cancers 15051586

Younis, S., Kamel, W., Falkeborn, T., Wang, H., Yu, D., Daniels, R., et al. (2018). Multiple nuclear-replicating viruses require the stress-induced protein ZC3H11A for efficient growth. *Proc. Natl. Acad. Sci. USA*. 115, E3808–E3816. doi: 10.1073/pnas.1722333115

Zhou, H., Di Palma, S., Preisinger, C., Peng, M., Polat, A. N., Heck, A. J., et al. (2013). Toward a comprehensive characterization of a human cancer cell phosphoproteome. *J. Proteome Res.* 12, 260–271. doi: 10.1021/pr300630k

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Clinical significance of anti-Epstein–Barr virus antibodies in systemic chronic active Epstein–Barr virus disease

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Systemic chronic active Epstein–Barr virus disease (sCAEBV) is a rare and fatal neoplasm, involving clonally proliferating Epstein–Barr virus (EBV)-infected T cells or natural killer cells. Patients with sCAEBV have abnormal titers of anti-EBV antibodies in their peripheral blood, but their significance is unknown. We retrospectively investigated titers and their relationship with the clinical features of sCAEBV using the data collected by the Japanese nationwide survey. Eighty-four patients with sCAEBV were analyzed. The anti-EBV nuclear antigen (EBNA) antibody, targeting EBNA-expressing EBV-positive cells, was found in 87.5% of children (<15 years old), 73.7% of adolescents and young adults (15–39 years old), and 100% of adults (≥40 years old). Anti-EBNA antibody titers were significantly lower and anti-VCA-IgG antibody titers significantly higher in patients with sCAEBV than those in healthy controls ($p < 0.0001$). Patients with high anti-VCA-IgG and anti-early antigen-IgG antibody (antibodies against the viral particles) levels had significantly better 3-year overall survival rates than those with low titers, suggesting that patients with sCAEBV have a reduced immune response to EBV-infected cells.

KEYWORDS

systemic chronic active Epstein–Barr virus disease, sCAEBV, anti-Epstein–Barr virus antibody, VCA-IgG, EBNA

Introduction

Systemic chronic active Epstein–Barr virus disease (sCAEBV) is an Epstein–Barr virus (EBV)-infected T-or natural killer (NK) cell neoplasm, according to the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues in 2022 (Alaggio et al., 2022). Why EBV persistently infects T or NK cells, leading to their immortalization and clonal expansion in sCAEBV, is unknown. Moreover, no effective drug has been developed to eradicate EBV-infected T or NK cells in patients with sCAEBV (Yonese et al., 2020). Thus, there is an urgent need to elucidate the pathological mechanism and develop therapeutic agents.

In sCAEBV, dysfunction of EBV-specific cytotoxic T cells occurs (Fujieda et al., 1993; Shibayama et al., 2017). In addition, congenital immunodeficiency with *FAS, IL2RG*, or *PRF1* gene mutations can be complicated by sCAEBV-like EBV-positive T- or NK-cell lymphoproliferative disorders (Katano et al., 2004; Sekinaka et al., 2017; Ishimura et al., 2019; Tanita et al., 2019). These results indicate that immunodeficiency against EBV or EBV-infected cells underlies the pathogenesis of sCAEBV. However, patients with sCAEBV present with hypergammaglobulinemia and elevated anti-EBV antibody levels, particularly those against viral capsid antigen (VCA), a viral protein, thereby suggesting a hyperimmune state of the disease (Okano et al., 2005).

EBV is a ubiquitous virus and belongs to the human herpes virus family. EBV mainly targets B lymphocytes, but also infects T cells and NK cells. The genome of EBV encodes a variety of genes, and the humoral response produces antibodies to the products of these genes. Anti-VCA antibodies involve three immunoglobulin classes, IgG, IgA and IgM. All anti-VCA antibodies appear in the acute phase of EBV infection, and then only the anti-VCA-IgG antibody remains positive after the acute phase. EBNA proteins are synthesized in the latent phase. Anti-EBNA antibodies appear 3–6 weeks after EBV infection and remain present throughout life. Thus, the detection of anti-EBNA antibody indicates past EBV infection or recovery. Early antigen-diffuse and restrict complex (EA-DR) is synthesized in the lytic phase of the EBV replication. EA-DR is composed of two components, EA-D and EA-R. Usually, Anti-EA antibodies appear in the acute phase and then decline to undetectable levels. The high titers of anti-EA antibody can be detected in the different diseases and in healthy individuals (Smatti et al., 2018). Not all patients with sCAEBV present with unusual patterns of anti-EBV antibodies (Xiao et al., 2016). The association between EBV-associated antibodies and sCAEBV is unclear, and there is no common pattern in all cases (Kimura et al., 2001; Okano et al., 2005; Kimura, 2006).

To evaluate the significance of EBV antibody titers in sCAEBV, we investigated the association between EBV antibody titers in serum samples and clinical features, including prognosis, in patients with sCAEBV according to the WHO 2022 classification.

Materials and methods

Study design

This is an observational study of clinical information. We conducted a retrospective study based on a nationwide survey conducted from 2016 to 2018 by the Japanese Study Group of sCAEBV, supported by the Japanese Agency for Medical Research and Development, to identify the clinical features and treatments for sCAEBV. The details of patient collection have been previously described (Yonese et al., 2020). For the analysis, we selected patients whose anti-EBV antibody titers measured using the fluorescent-labeled antibody (FA) methods in standardized laboratories were available. Since the quantification of the titers of anti-EBV antibodies is covered by health insurance and widely performed as a general examination, the diagnostic agents are verified annually by The Japanese Society of Clinical Virology. Specifically, each antibody was reacted in serum with cultured cells and fixed on a glass slide, expressing each antigen of human origin. After washing off excess serum, anti-human/rabbit polyclonal antibodies labeled with fluorescent dyes were added. Each antibody was detected by observation under a fluorescence microscope and the antibody titer was quantified at the highest dilution of the detected sample. Antibody titers of patients were measured by SRL, Inc., BML, Inc., and other Japanese clinical laboratories. Plasma of healthy individuals was measured by BML.

Patients with anti-VCA-IgG antibodies were considered to be EBV seropositive. This is because anti-VCA-IgG antibodies are known to gradually rise during the acute phase of EBV infection and persist throughout life, whereas anti-EBNA antibodies are persistently detected during recovery from the initial EBV infection. If either one is positive, the patient is considered to be already infected with EBV (Schillinger et al., 1993). However, anti-EBNA antibody was reported to be negative in 5–10% of healthy individuals who were positive for the anti-VCA-IgG antibody (Smatti et al., 2018). Thus, the anti-VCA-IgG antibody more accurately indicates past infection with EBV.

Diagnostic criteria

The patients were diagnosed with sCAEBV based on the following diagnostic criteria, in line with the WHO 2022 classification (Kimura et al., 2012; Yonese et al., 2020; Alaggio et al., 2022):

1. Elevated EBV DNA load in peripheral blood (PB) > $10^{2.5}$ copies/μg DNA,
2. EBV infection of T or NK cells in the PB or affected tissues,
3. Systemic inflammatory symptoms, such as infectious mononucleosis-like conditions persisting for >3 months, and.
4. Exclusion of other possible diagnoses known immunodeficiency, malignancy, or autoimmune disorders.

Healthy controls

In this study, we included 220 healthy student volunteers from the adolescent and young adult (AYA) generation. Students with any disease were excluded. We collected their blood samples from 2018 to 2020. Among them, 178 volunteers (103 males and 75 females) who were positive for the anti-VCA-IgG antibody, were used as EBV-positive healthy controls. We measured anti-EBV antibodies, the anti-VCA-IgG antibody and anti-EBV nuclear antigen (EBNA) antibody, using the FA methods in standardized laboratories. All fresh samples were measured on the collection day.

Statistical analysis

The Mann–Whitney U test was used to compare the distributions of anti-VCA-IgG and anti-EBNA antibodies between healthy individuals and patients. In the group of patients, data of three age groups [children (< 15 years), AYA (15–39 years), and adults (> 39 years)] were compared. The log-rank test was used for survival analysis. Statistical significance was set at $p < 0.05$. All statistical analyzes were performed using GraphPad Prism version 8 (GraphPad Software Inc., Boston, MA, United States).

Ethics statement

The study protocol of the nationwide survey of the sCAEBV was approved by the ethics committees of Tokyo Medical and Dental University and St. Marianna University School of Medicine.

The analysis of the samples from healthy donors was approved by the ethics committees of Tokyo Medical and Dental University, St. Marianna University School of Medicine, and Tokyo Kasei University and registered at University hospital Medical Information Network (UMIN) Center (#UMIN000032099). The donors provided written informed consent to participate in this study.

Data sharing statement

The data that support the findings of this study are available upon request from the corresponding author, AA. The details of the nationwide survey have been published previously (Yonese et al., 2020).

Results

Clinical features of sCAEBV patients

Among the 100 patients analyzed in the nationwide survey of patients with sCAEBV, 84 patients whose anti-EBV antibodies were examined using the FA method were included in this study. A flowchart of patient selection is shown in Supplementary Figure S1. The clinical findings of the patients are summarized in Table 1. The study included 43 males and 41 females, aged 3–78 years (median: 23.0 years). The patient samples were collected at multiple facilities, and the methods of detecting EBV-infected lymphocyte phenotypes were as follows: antibody-conjugated magnetic bead sorting ($n = 55$), flow cytometry ($n = 14$), and histopathology using *in situ* hybridization for EBV-encoded small RNA ($n = 8$). Detailed methods for four patients were not available. The EBV-infected cell phenotypes were as follows: CD4-positive cells (23, 27.4%), CD8-positive cells (11, 13.1%), CD56-positive cells (25, 29.8%), and γδT-cells (2, 2.4%). EBV was detected in multiple T and NK cells from 18 patients. Hypersensitivity to mosquito bites and hydroa vacciniforme-like skin eruptions were observed in 20 (23.8%) and 5 (6.0%) patients, respectively. Moreover, 21 patients (25.0%) had accompanied hemophagocytic lymphohistiocytosis, a fatal complication of sCAEBV. No significant differences in clinical features were observed between the patients selected for the present study and 100 patients of the nationwide survey, the population group. A total of 220 donors, were considered as healthy controls, of which 178 had anti-VCA-IgG antibodies and were considered as EBV seropositive.

EBV-associated antibodies in sCAEBV patients

We investigated the positive rates of five EBV-associated antibodies in patients with sCAEBV to clarify the characteristics of each antibody in this disease. As our previous study revealed that the prognosis and sex differed based on the age of patients with sCAEBV, we examined the antibody titers in different age groups (Yonese et al., 2020). The number of patients in each age group is presented in Table 1. The positive rate of anti-EBV antibodies based on age is shown in Figures 1A–E. All patients tested positive for anti-VCA-IgG antibodies (Figure 1A); however, the positive rates of the anti-VCA-IgA antibody differed based on age as follows: 78.6% in children, 63.6% in AYA, and 50.0% in adults (Figure 1B). Although the difference was not statistically significant, the positive rates of the anti-VCA-IgA antibody tended to decrease with age. In contrast, 87.5, 73.7, and 100% of the children, AYA, and adults (Figure 1C), respectively, tested positive for the anti-EBNA antibody; thus, the positive rate in adults tended to increase by age. As shown in Figures 1D–E, no significant differences were observed in the positive rate of the anti-early antigen (EA) antibodies based on age. The sex distribution of the patients by age are shown in Figures 1F–H. Briefly, 65, 55, and 19% of children, AYA, and adults respectively, were males, indicating that childhood and adult sCAEBV cases have different characteristics.

As shown in Figure 1C, no anti-EBNA antibody-negative patients were over 40 years of age. The positive rates of the anti-EBNA antibodies by generation and sex are shown in Supplementary Figure S2A. In males, 82.4, 80.0, and 100% of children, AYA, and adults, respectively, tested positive for the anti-EBNA antibody. In females, 100, 66.7, and 100% of children, AYA, and adults, respectively, tested positive for the anti-EBNA antibody. Anti-EBNA antibody titers classified by generation and sex are shown in Supplementary Figure S2B. No significant differences were observed between the groups ($p = 0.3885$). Anti-VCA-IgG and anti EA-IgG antibody titers classified by sex are shown in Supplementary Figures S2C,D. No significant differences were observed between the groups ($p = 0.9183$, 0.3617). In addition, there were no significant differences in antibody titers with age (Supplementary Figure S3). As shown in previous reports (Kimura et al., 2001), this study also confirmed that anti-VCA-IgG and anti-EA-IgG antibody titers were significantly higher in the T-cell type of sCAEBV than in the NK-cell type of sCAEBV (Supplementary Figure S4).

Comparison of EBV antibody titers between age-matched patients with sCAEBV and healthy controls

Next, we compared the titers of the anti-VCA-IgG and anti-EBNA antibody between the healthy controls and age-matched patients with AYA sCAEBV (15–39 years old). Healthy controls consisted of 129 males and 91 females (Figure 2A). As shown in Figure 2B, the anti-VCA-IgG antibody titers were significantly higher in patients with sCAEBV than that in healthy controls ($p < 0.0001$; Figure 2B). In contrast, the anti-EBNA antibody titer of the healthy controls was higher than that of the patients (Figure 2B, $P < 0.0001$). A significant difference was observed in the negative rate of anti-EBNA antibodies between healthy controls and patients ($p < 0.0001$): 2.8% of the healthy controls tested negative for the anti-EBNA antibody (Figure 2D), while 26.3% of the patients with sCAEBV tested negative for this antibody (Figure 2E). To examine sex preference with respect to the positive rate of anti-EBNA antibodies, we analyzed the rate by sex in the healthy control group. No significant difference was observed in the rates and titers of anti-EBNA antibodies between males and females in the control group (Figures 2F,G).

TABLE 1 Comparison of patient characteristics and clinical findings between this study and a previous report (Yonese et al., 2020).

	Patients (*N* = 84)	The population (*N* = 100) *	*p***
Male/female sex, *n*	43/41	53/47	0.8824
Age at diagnosis, range (median), y	3–78 (23)	1–78 (21)	
Child (aged <15 y)	26 (17/9)	37 (25/12)	0.4370
AYA (15 ~ 39 y)	42 (23/19)	46 (24/22)	0.6573
Adult (aged >39 y)	16 (3/13)	17 (4/13)	0.8473
EBV-infected cell type			
CD4	23 (27.4%)	25 (25.0%)	0.7385
CD8	11 (13.1%)	13 (13.0%)	>0.9999
CD56	25 (29.8%)	28 (28.0%)	0.8705
γδT	2 (2.4%)	3 (3.0%)	>0.9999
CD56- NK	0 (0.0%)	2 (2.0%)	0.5011
CD4 + CD8	6 (7.1%)	6 (6.0%)	0.7733
CD4 + CD56	3 (3.6%)	4 (4.0%)	>0.9999
CD8 + CD56	1 (1.2%)	1 (1.0%)	>0.9999
CD4 + CD8 + CD56	4 (4.8%)	6 (6.0%)	0.7568
Others	4 (4.8%)	7 (7.0%)	0.8203
NA	5 (6.0%) ***	5 (5.0%) ***	
Symptoms and signs at diagnosis			
Fever	70 (83.3%)	85 (85.0%)	0.8400
Hepatosplenomegaly	59 (70.2%)	64 (70.3%)	>0.9999
Lymphadenopathy	44 (52.4%)	48 (52.7%)	>0.9999
Cardiac dysfunction	7 (8.3%)	8 (8.8%)	>0.9999
Aneurysm	7 (8.3%)	8 (8.8%)	>0.9999
Gastrointestinal symptom	7 (8.3%)	7 (7.7%)	>0.9999
Neurological symptoms	7 (8.3%)	7 (7.7%)	>0.9999
Vasculitis	6 (7.1%)	6 (6.6%)	>0.9999
Uveitis	4 (4.8%)	4 (4.4%)	>0.9999
HLH	21 (25.0%)	24 (26.4%)	0.8641
HMB	20 (23.8%)	25 (25.0%)	0.8653
HV-like eruption	5 (6.0%)	9 (9.0%)	0.5794
Neutropenia (< 1,000 /μL)	11 (13.9%)	14 (15.6%)	0.6708
Anemia (< 9 g/dL)	12 (14.6%)	12 (13.6%)	>0.9999
Thrombocytopenia (< 10×10^4/μL)	23 (27.7%)	27 (30.3%)	0.7391
High ALT (> 128 U/L)	26 (31.7%)	27 (30.7%)	>0.9999
High sIL-2R (> 2,400 U/mL)	16 (25.8%)	17 (26.6%)	>0.9999
Chemotherapy	20	20	0.8619
Chemotherapy + HSCT	43	47	
HSCT	12	12	

NA, the detailed phenotype of the infected cells was not available; HLH, hemophagocytic lymphohistiocytosis; HMB, Hypersensitivity to mosquito bites; HV, hydroa vacciniforme; HSCT, hematopoietic stem cell transplantation; ALT, alanine transaminase; AYA, adolescent and young adult. *Patients analyzed in the nationwide survey (Yonese et al., 2020). **Fisher's exact test. ***1 and 4 patients were described to be CD3- and NK-infected, respectively; derailed phenotypes were not available.

Relation between prognosis and anti-EBV antibody titers of patients with sCAEBV

Finally, we examined the relation between EBV antibody titers and prognosis in patients with sCAEBV. The distribution of anti-EBV antibody titers is shown in Supplementary Figure S5. Patients were categorized into two groups based on the titer of each antibody: the cutoff values were ≥ 1,280, ≥ 640, and ≥ 40 for the anti-VCA-IgG antibody, anti-EA-IgG antibody, and anti-EBNA antibody, respectively. The survival curves for the anti-EBV antibody titers are shown in

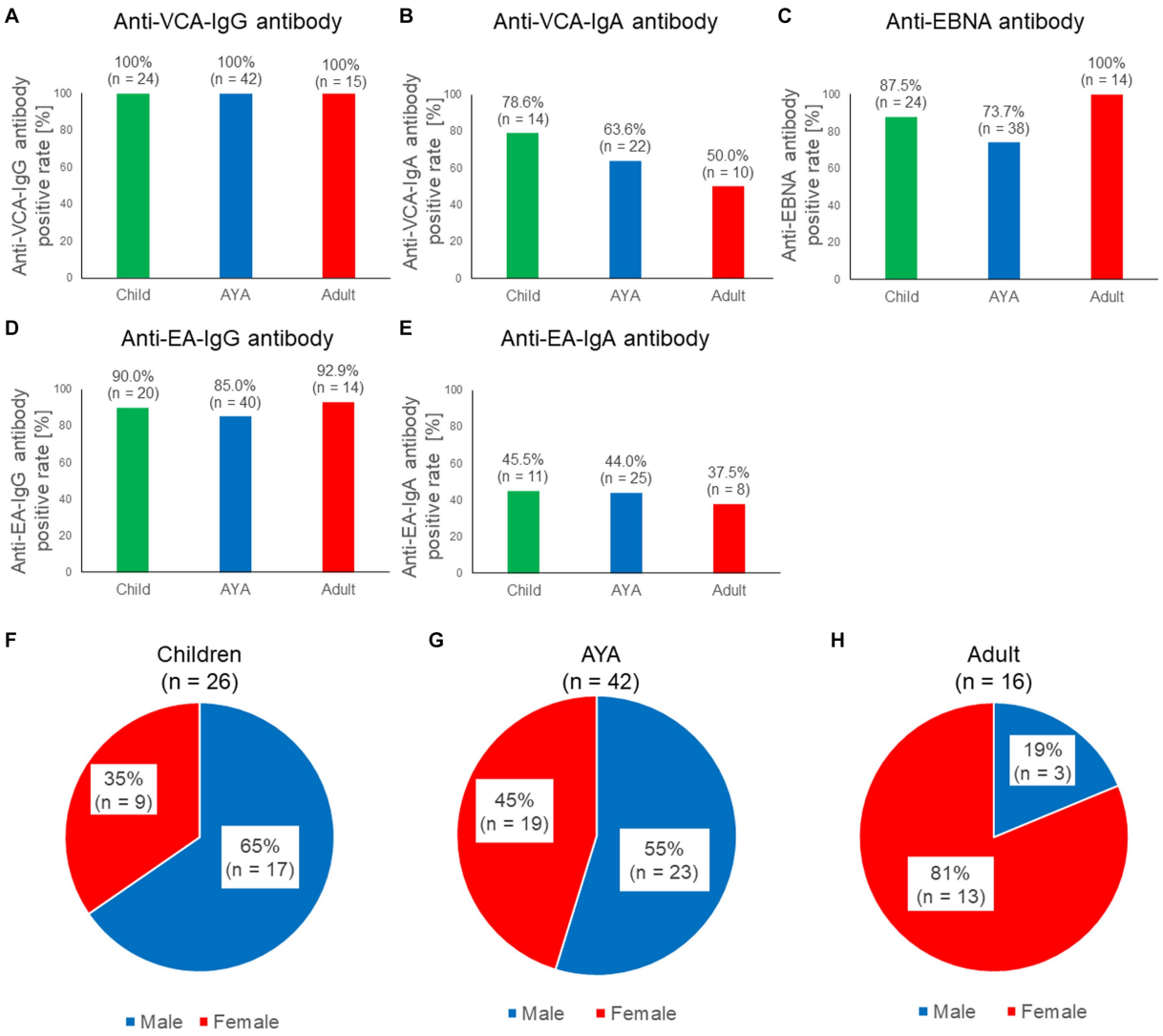

FIGURE 1
Characteristics of the five anti-EBV antibodies in patients with sCAEBV. **(A–E)** Comparisons of the seropositive rate among three generations for five anti-EBV antibodies: anti-VCA-IgG antibody, anti-VCA-IgA antibody, anti-EBNA antibody, anti-EA-IgG, and anti-EA-IgA. Green indicates children, blue indicates AYA, red indicates adults. The number of available samples is shown at the top of the graphs. **(F–H)**. Ratios of males and females with sCAEBV among children, AYA, and adults. Blue indicates males, red indicates females. AYA, adolescent and young adult; EA, anti-early antigen; EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus; sCAEBV, systemic chronic active Epstein–Barr virus disease; VCA, viral capsid antigen.

Figure 3. The survival rates for the low-titer groups of the anti-VCA-IgG (Figure 3A) and anti-EA-IgG (Figure 3B) antibodies were significantly lower than those for the high-titer groups ($p = 0.0236$ and 0.0123, respectively). However, no significant difference was detected ($p = 0.1689$) between the low-titer and high-titer of the anti-EBNA antibody group (Figure 3C). Similarly, no significant differences were detected between the low-titer and high-titer of the anti-EBNA, anti-EA-IgG, and anti-EA-IgA antibodies groups when we set different cutoff values (Supplementary Figure S6). Significant differences might be obtained by increasing the number of samples.

Discussion

To the best of our knowledge, this is the first report to analyze the significance of anti-EBV antibodies in patients with sCAEBV. We found that anti-EBV antibody titers were significantly different between patients and seropositive healthy controls. In addition, anti-VCA-IgG and anti-EA-IgG antibody titers were correlated with prognosis. These results suggest the hypothesis: anti-EBV antibodies are potent prognostic markers and may be the key to understanding the pathogenesis of sCAEBV.

Adult patients tended to have lower rates of positivity for anti-EA-IgA and anti-VCA-IgA antibodies (Figures 1B,E). IgA is an isotype antibody which is associated with local immunity within the mucosa. Anti-EA-IgA and anti-VCA-IgA antibodies are characteristically present in EBV-positive nasopharyngeal carcinoma (Liu et al., 2012, 2013; Tan et al., 2020). Thus, the presence of anti-EBV-IgA antibodies may reflect the presence of EBV-infected cells in the pharyngeal mucosa. Sometime after the initial EBV infection, adult patients may have fewer EBV-infected cells in the mucosa, resulting in a lower rate of anti-EBV-IgA antibody positivity.

A

	Male	Female	Total
Healthy controls	129	91	220
Patients with sCAEBV	23	19	42

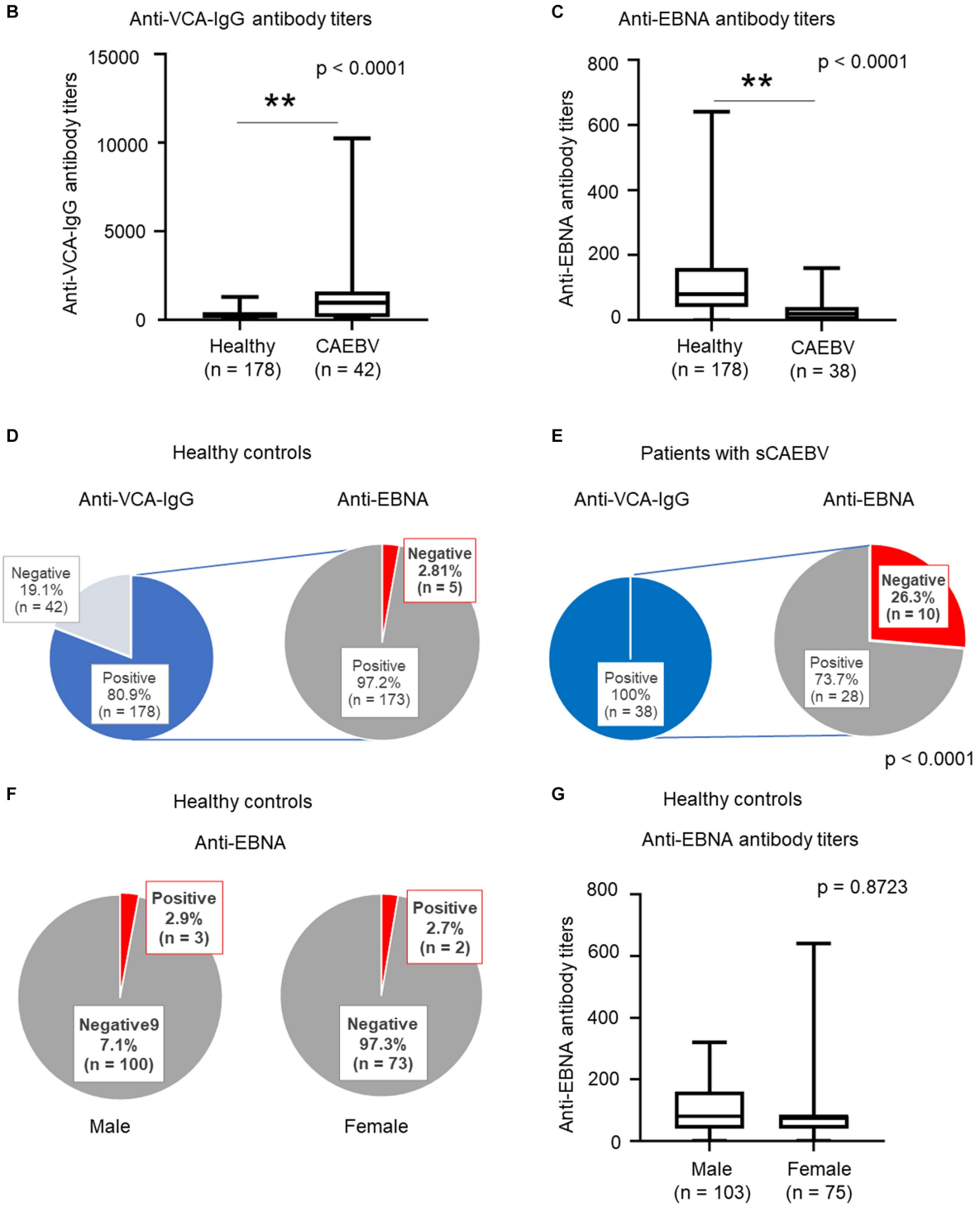

FIGURE 2
Comparison of anti-EBV antibodies among healthy young adults and age-matched AYA patients with sCAEBV **(A)** Sex of healthy controlsand patients with sCAEBV. **(B,C)** Comparison of the distribution of anti-VCA-IgG and anti-EBNA antibody titers carried out using the Mann–Whitney U test** $p < 0.001$ compared with the control. **(D,E)** Comparison of seroprevalence of anti-VCA-IgG and anti-EBNA antibody. The left graphs show the seroprevalence of anti-VCA-IgG. Bule indicates positive rate, while light gray indicates negative rate. The graphs on the right show the negative rates of the anti-EBNA antibody among individuals who were positive for the anti-VCA-IgG antibody. Red indicates the negative rate for the anti-EBNA

(Continued)

FIGURE 2 (Continued)
antibody among individuals with the anti-VCA-IgG antibody. Dark gray indicates the positive rate for the anti-EBNA antibody or for both the anti-VCA-IgG and anti-EBNA antibody. **(F,G)** Comparison of anti-EBNA antibody positive rates and titers based on sex in healthy controls. EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus; sCAEBV, systemic chronic active Epstein–Barr virus disease; VCA, viral capsid antigen.

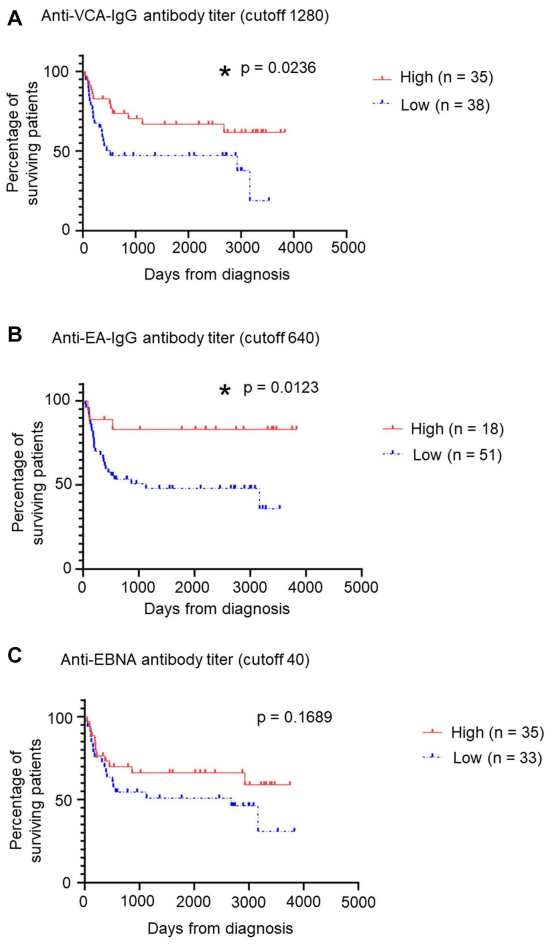

FIGURE 3
Relation between patients' survival and some anti-EBV antibody titers. **(A)** Relation between patients' survival and anti-VCA-IgG antibody titers using a survival curve. We established a cutoff value of 1,280. Red indicates the "High" group, including patients with >1,280 titers, while blue indicates the "Low" group. *$p < 0.05$ compared with the control. **(B)** Relation between patients' survival and anti-EA-IgG antibody titers using a survival curve from GraphPad Prism 8. We established a cutoff value of 640. Red indicates the "High" group, including patients with >640 titers, while blue indicates the "Low" group. *$p < 0.05$ compared with the control. **(C)** Relation between patients' survival and anti-EBNA antibody titers using a survival curve. We established a cutoff value of 40. Red indicates the "High" group, including patients with >40 titers, while blue indicates the "Low" group. EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus; sCAEBV, systemic chronic active Epstein–Barr virus disease; VCA, viral capsid antigen.

In this study, we compared antibody titers between healthy controls, who were previously infected with EBV, and patients with sCAEBV. Serum EBV antibody titers and antibody positivity were compared between the control and age-matched AYA patients with sCAEBV, and significant differences in anti-EBNA and anti-VCA-IgG antibody titers were observed (Figures 2B–E). Patients with sCAEBV had lower anti-EBNA antibody positivity and lower anti-EBNA antibody titers than those in healthy controls. Anti-EBNA antibodies include all classes of antibodies: IgG, IgA and IgM. To investigate the clinical significance of anti-EBNA antibody titers in sCAEBV, future analyzes of each class of antibodies will be interesting. Interestingly, all anti-EBNA-negative sCAEBV patients were under 40 years of age. A higher occurrence in males and relatively low rate of anti-EBNA antibodies in childhood suggests the possibility of X-linked immune diseases. However, future analyzes are required to confirm these findings.

Another reason for the low anti-EBNA antibody positivity and low titers in patients with sCAEBV may be the low expression of EBNA in EBV-infected cells. EBV infection in T or NK cells is reported to be a type-2 latent infection without virus replication (Münz, 2019). EBNA1 is expressed in EBV-infected cells of type 2 latent infection. However, Iwata et al. (2010) analyzed *EBNA1* expression in 24 young patients with sCAEBV using real-time RT-PCR, and found that *EBNA1* expression was lower in the PB mononuclear cells (PBMCs) of sCAEBV patients compared to that in EBV-positive T or NK tumor cell lines. In addition, 14 of the 24 patients had undetectable *EBNA1* gene expression in the PBMCs (Iwata et al., 2010). In the present study, anti-EBNA antibody-negative cases were particularly common among younger patients who may have had low EBNA1 protein expression. Low EBNA1 protein expression in EBV-positive T and NK cells of patients with sCAEBV may contribute to host immune evasion mechanisms, resulting in the failure to eliminate EBV-infected cells and sCAEBV development. EBNA1 protein expression in EBV-infected cells and its regulatory mechanisms in sCAEBV should be investigated in a large number of patients.

Furthermore, EBNA-1 is an intracellular antigen. sCAEBV is associated with defects in cytolytic mediators (Fujieda et al., 1993; Shibayama et al., 2017), which lead to reduced apoptosis of EBV-infected cells. Intracellular antigens can be exposed on membrane blebs of apoptotic cells, where they can be recognized by the B-cell receptor of B cells and induce a B-cell and antibody response. Defective cytolysis could lead to lower EBNA1 exposure to extracellular milieu and lower antibodies formation and production.

While anti-EBNA antibody levels tended to be lower in patients with sCAEBV than in healthy controls, anti-VCA-IgG antibody titers were significantly higher in sCAEBV patients. VCA is a protein found in EBV viral particles (Johannsen et al., 2004; Wang et al., 2011); however, because the virus does not replicate in patients with sCAEBV, VCA is not detected in their PB (Münz, 2019). Therefore, we assumed that the high anti-VCA-IgG antibody titer did not reflect EBV levels. Polyclonal hypergammaglobulinemia, which is observed in patients with sCAEBV, is associated with persistent stimulation of B cells by activated EBV-infected T cells or NK cells (Wakiguchi et al., 2000; Okano et al., 2005; Nomura et al., 2011). In other words, anti-VCA-IgG antibody titers may reflect an enhanced nonspecific

immune response activated by EBV-infected cells. Furthermore, higher anti-VCA-IgG antibody titers and anti-EA-IgG antibodies, which are also antibodies against EBV virus particles, were associated with significantly better prognosis in patients with sCAEBV. These results suggest that the patients' comprehensive immunity influences the prognosis of sCAEBV. However, we could not determine the relationship between anti-EBNA antibody titers and prognosis. The positive rate of anti-EBNA antibodies varied widely by age, and the differences in sex preference and clinical findings by age indicate that the pathogenesis of sCAEBV differs by age. Thus, in future, the significance of the relationship between anti-EBNA1 antibody titer and prognosis should be analyzed in a large number of patients, according to age.

Anti-VCA-IgG and anti-EA-IgG antibodies are significantly higher in EBV-positive B-cell lymphomas, such as Burkitt lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma, than in healthy controls (Xie et al., 2023). This study showed that sCAEBV patient specimens also had similarly high anti-VCA-IgG antibody titers. Interestingly, these EBV-positive B-cell lymphomas have higher anti-EBNA-IgG antibody titers than do healthy controls, whereas the anti-EBNA antibody titers of sCAEBV patients in this study were lower than those of the healthy controls. The mechanism for the lower anti-EBNA antibody titer and positivity in sCAEBV is not clear but is interesting compared to other EBV-positive B-cell lymphomas. The abovementioned hypothesis in sCAEBV needs further evidence to be proven.

This study had limitations. This was a retrospective analysis based on a questionnaire. The timing and clinical laboratories that measured the antibody titers were variable as described in the Materials and methods: they are validated and standardized but may have introduced a bias. In the future, the results should be verified by unifying methods and measuring simultaneously. In addition, the sample of healthy controls was limited to individuals in their 20s. Thus, whether differences in antibody positivity and titers between patients and healthy controls are also observed in other age groups, especially in adults, should be verified by inclusion of all age groups in the sample.

Recently, research on immunotherapies such as PD-1 antibody and T cell activation therapy is advancing, allowing EBV-positive T or NK-cell neoplasms to be targeted by treatment (Bollard et al., 2014; Kwong et al., 2017). Further studies, focusing on the immune system in a large number of patients, will be useful in understanding the pathogenesis and developing treatment methods for sCAEBV.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the ethics committees of Tokyo Medical and Dental University, St. Marianna University School of Medicine, and Tokyo Kasei University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MN: Conceptualization, Investigation, Project administration, Writing – original draft. MiS: Formal analysis, Investigation, Writing – original draft. MY: Investigation, Writing – review & editing. YK: Formal analysis, Writing – review & editing. AO: Investigation, Writing – review & editing. ES: Formal analysis, Writing – review & editing. IY: Resources, Writing – review & editing. MeS: Resources, Writing – review & editing. AA: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1320292/full#supplementary-material

References

Alaggio, R., Amador, C., Anagnostopoulos, I., Attygalle, A. D., Araujo, I. B. O., Berti, E., et al. (2022). The 5th edition of the World Health Organization classification of Haematolymphoid Tumours: lymphoid neoplasms. *Leukemia* 36, 1720–1748. doi: 10.1038/s41375-022-01620-2

Bollard, C. M., Gottschalk, S., Torrano, V., Diouf, O., Ku, S., Hazrat, Y., et al. (2014). Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J. Clin. Oncol.* 32, 798–808. doi: 10.1200/JCO.2013.51.5304

Fujieda, M., Wakiguchi, H., Hisakawa, H., Kubota, H., and Kurashige, T. (1993). Defective activity of Epstein-Barr virus (EBV) specific cytotoxic T lymphocytes in children with chronic active EBV infection and in their parents. *Acta Paediatr. Jpn.* 35, 394–399. doi: 10.1111/j.1442-200X.1993.tb03079.x

Ishimura, M., Eguchi, K., Shiraishi, A., Sonoda, M., Azuma, Y., Yamamoto, H., et al. (2019). Systemic Epstein-Barr virus-positive T/NK lymphoproliferative diseases with SH2D1A/XIAP Hypomorphic gene variants. *Front. Pediatr.* 7:183. doi: 10.3389/fped.2019.00183

Iwata, S., Wada, K., Tobita, S., Gotoh, K., Ito, Y., Demachi-Okamura, A., et al. (2010). Quantitative analysis of Epstein-Barr virus (EBV)-related gene expression in patients with chronic active EBV infection. *J. Gen. Virol.* 91, 42–50. doi: 10.1099/vir.0.013482-0

Johannsen, E., Luftig, M., Chase, M. R., Weicksel, S., Cahir-McFarland, E., Illanes, D., et al. (2004). Proteins of purified Epstein-Barr virus. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16286–16291. doi: 10.1073/pnas.0407320101

Katano, H., Ali, M. A., Patera, A. C., Catalfamo, M., Jaffe, E. S., Kimura, H., et al. (2004). Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. *Blood* 103, 1244–1252. doi: 10.1182/blood-2003-06-2171

Kimura, H. (2006). Pathogenesis of chronic active Epstein-Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev. Med. Virol.* 16, 251–261. doi: 10.1002/rmv.505

Kimura, H., Hoshino, Y., Kanegane, H., Tsuge, I., Okamura, T., Kawa, K., et al. (2001). Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 98, 280–286. doi: 10.1182/blood.V98.2.280

Kimura, H., Ito, Y., Kawabe, S., Gotoh, K., Takahashi, Y., Kojima, S., et al. (2012). EBV-associated T/NK-cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases. *Blood* 119, 673–686. doi: 10.1182/blood-2011-10-381921

Kwong, Y. L., Chan, T. S. Y., Tan, D., Kim, S. J., Poon, L. M., Mow, B., et al. (2017). PD1 blockade with pembrolizumab is highly effective in relapsed or refractory NK/T-cell lymphoma failing l-asparaginase. *Blood* 129, 2437–2442. doi: 10.1182/blood-2016-12-756841

Liu, Y., Huang, Q., Liu, W., Liu, Q., Jia, W., Chang, E., et al. (2012). Establishment of VCA and EBNA1 IgA-based combination by enzyme-linked immunosorbent assay as preferred screening method for nasopharyngeal carcinoma: a two-stage design with a preliminary performance study and a mass screening in southern China. *Int. J. Cancer* 131, 406–416. doi: 10.1002/ijc.26380

Liu, Z., Ji, M. F., Huang, Q. H., Fang, F., Liu, Q., Jia, W. H., et al. (2013). Two Epstein-Barr virus-related serologic antibody tests in nasopharyngeal carcinoma screening: results from the initial phase of a cluster randomized controlled trial in southern China. *Am. J. Epidemiol.* 177, 242–250. doi: 10.1093/aje/kws404

Münz, C. (2019). Latency and lytic replication in Epstein-Barr virus-associated oncogenesis. *Nat. Rev. Microbiol.* 17, 691–700. doi: 10.1038/s41579-019-0249-7

Nomura, K., Kanegane, H., Otsubo, K., Wakiguchi, H., Noda, Y., Kasahara, Y., et al. (2011). Autoimmune lymphoproliferative syndrome mimicking chronic active Epstein-Barr virus infection. *Int. J. Hematol.* 93, 760–764. doi: 10.1007/s12185-011-0877-9

Okano, M., Kawa, K., Kimura, H., Yachie, A., Wakiguchi, H., Maeda, A., et al. (2005). Proposed guidelines for diagnosing chronic active Epstein-Barr virus infection. *Am. J. Hematol.* 80, 64–69. doi: 10.1002/ajh.20398

Schillinger, M., Kampmann, M., Henninger, K., Murray, G., Hanselmann, I., and Bauer, G. (1993). Variability of humoral immune response to acute Epstein-Barr virus (EBV) infection: evaluation of the significance of serological markers. *Med. Microbiol. Lett.* 2, 296–303.

Sekinaka, Y., Mitsuiki, N., Imai, K., Yabe, M., Yabe, H., Mitsui-Sekinaka, K., et al. (2017). Common variable immunodeficiency caused by FANC mutations. *J. Clin. Immunol.* 37, 434–444. doi: 10.1007/s10875-017-0396-4

Shibayama, H., Imadome, K. I., Onozawa, E., Tsuzura, A., Miura, O., Koyama, T., et al. (2017). Virus-specific cytotoxic T cells in chronic active Epstein-Barr virus infection. *Rinsho Ketsueki* 58, 583–588. doi: 10.11406/rinketsu.58.583

Smatti, M. K., Al-Sadeq, D. W., Ali, N. H., Pintus, G., Abou-Saleh, H., and Nasrallah, G. K. (2018). Epstein-Barr virus epidemiology, serology, and genetic variability of LMP-1 oncogene among healthy population: an update. *Front. Oncol.* 8:211. doi: 10.3389/fonc.2018.00211

Tan, L. P., Tan, G. W., Sivanesan, V. M., Goh, S. L., Ng, X. J., Lim, C. S., et al. (2020). Systematic comparison of plasma EBV DNA, anti-EBV antibodies and miRNA levels for early detection and prognosis of nasopharyngeal carcinoma. *Int. J. Cancer* 146, 2336–2347. doi: 10.1002/ijc.32656

Tanita, K., Hoshino, A., Imadome, K. I., Kamiya, T., Inoue, K., Okano, T., et al. (2019). Epstein-Barr virus-associated γδ T-cell lymphoproliferative disorder associated with Hypomorphic IL2RG mutation. *Front. Pediatr.* 7:15. doi: 10.3389/fped.2019.00015

Wakiguchi, H., Hisakawa, H., Hosokawa, T., Kubota, H., and Kurashige, T. (2000). Analysis of IgG subclasses in chronic active Epstein-Barr virus infection. *Pediatr. Int.* 42, 21–25. doi: 10.1046/j.1442-200x.2000.01175.x

Wang, W. H., Chang, L. K., and Liu, S. T. (2011). Molecular interactions of Epstein-Barr virus capsid proteins. *J. Virol.* 85, 1615–1624. doi: 10.1128/JVI.01565-10

Xiao, H. J., Li, J., Song, H. M., Li, Z. H., Dong, M., and Zhou, X. G. (2016). Epstein-Barr virus-positive T/NK-cell lymphoproliferative disorders manifested as gastrointestinal perforations and skin lesions: a case report. *Medicine (Baltimore)* 95:e2676. doi: 10.1097/MD.0000000000002676

Xie, C., Zhong, L. Y., Bu, G. L., Zhao, G. X., Yuan, B. Y., Liu, Y. T., et al. (2023). Anti-EBV antibodies: roles in diagnosis, pathogenesis, and antiviral therapy. *J. Med. Virol.* 95:e28793. doi: 10.1002/jmv.28793

Yonese, I., Sakashita, C., Imadome, K. I., Kobayashi, T., Yamamoto, M., Sawada, A., et al. (2020). Nationwide survey of systemic chronic active EBV infection in Japan in accordance with the new WHO classification. *Blood Adv.* 4, 2918–2926. doi: 10.1182/bloodadvances.2020001451

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