RECENT DEVELOPMENTS IN THERAPIES AND DIAGNOSTIC TOOLS FOR MELANOMA AND NON-MELANOMA SKIN CANCER

EDITED BY: Taku Fujimura, Yasuhiro Fujisawa, Atsushi Otsuka and Nikolas K. Haass

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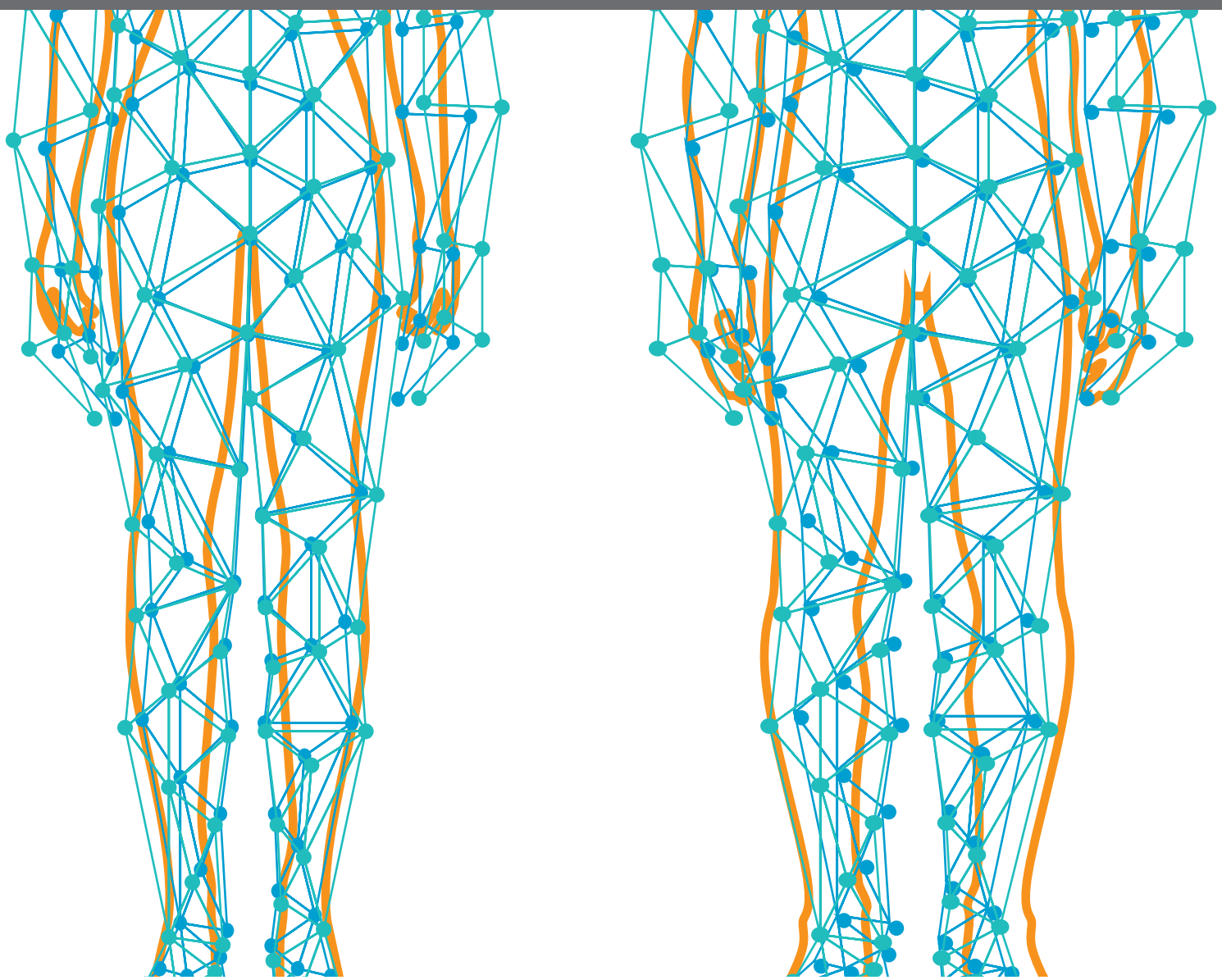

frontiers Research Topics

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RECENT DEVELOPMENTS IN THERAPIES AND DIAGNOSTIC TOOLS FOR MELANOMA AND NON-MELANOMA SKIN CANCER

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EDITORIAL
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Editorial: Recent Developments in Therapies and Diagnostic Tools for Melanoma and Non-melanoma Skin Cancer

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Keywords: biomarker (BM), immune checkpoint antibodies, dermoscopy (DS), skin imaging, T-cell lymphoma cutaneous, sentinel lymph node biopsy (SLNB), deep-learning (DL), convolutional neural network (CNN)

Editorial on the Research Topic

Recent Developments in Therapies and Diagnostic Tools for Melanoma and Non-melanoma Skin Cancer

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UPDATES ON BIOMARKERS FOR IMMUNE CHECKPOINT THERAPY OF ADVANCED MELANOMA

In the past decade, there has been a paradigm shift for the treatment of skin cancer, especially of advanced melanoma, due to the unprecedented success of MAPK pathway and immune checkpoint inhibitors (MAPKi, ICi). In this Research Topic, we focus on the latter. The most commonly used ICi are monoclonal antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; ipilimumab), programmed cell death 1 (PD-1; nivolumab and pembrolizumab) and, more recently, programmed death-ligand 1 (PD-L1; atezolizumab, durvalumab, and avelumab). In addition to multiple described resistance mechanisms to ICi (1), the lack of reliable biomarkers for both drug efficacy and immune-related adverse events (irAEs) has been a major clinical concern and is reviewed here extensively [Kambayashi et al.; Nakamura (a)]. Importantly, although many predictive biomarkers for both ICi tumor response and irAEs have been identified, no single biomarker has shown to be predictive on its own; therefore, a combination of multiple biomarkers should be utilized [Kambayashi et al.; Nakamura (a)]. Furthermore, irAEs and tumor response can be interconnected, but often are not; thus, irAEs or biomarkers for irAEs cannot be reliably used as biomarker for tumor response [Kambayashi et al.; Nakamura (a)]. Further studies are needed to improve biomarkers for predicting the efficacy of ICi treatment [Kambayashi et al.; Nakamura (a)]. To this end, Kümpers et al., show that PD-L1 expression on primary tumors and melanoma metastases is not associated with the clinical response of anti-PD1 antibodies, while several previous studies suggest an association of PD-L1 status and response to anti-PD1 antibodies (2). Instead, immune cell infiltration in the primary melanoma, measured by the Immunoscore, was associated with a significantly improved response to ICi in terms of increased overall survival

(Kümpers et al.). Another research paper in this Research Topic suggests that baseline serum levels of CXCL5, which have been reported previously as a biomarker for autoimmune disease, could be a predictive marker for the efficacy of anti-PD1 antibodies (Fujimura, Sato et al.). While ICi were initially only used for definitive therapy, the field has quickly moved to adjuvant and, more recently, to neoadjuvant therapy (3). Combination of nivolumab plus ipilimumab (N+I) is amongst the most effective therapies against both BRAF-mutated and BRAF-wildtype advanced melanoma, but leads to a high frequency of irAEs [Nakamura (a)]. Fujimura, Kambayashi et al. show in a case report that N+I combination therapy for BRAF-mutated advanced melanoma before primary tumor resection strikingly increased CD8+ cytotoxic T cells in the primary tumor, leading to induced anti-melanoma immune response in metastases in six different organs, but also induced serious AEs after administration of N+I combination therapy (4). In addition, previous reports suggest that the efficacy of ipilimumab among patients with anti-PD1 antibody-resistant melanoma is extremely low after objective tumor progression (5). In summary, the optimization of immunotherapy using ICi is still challenging, but rapidly developing, and it is exciting to see how the landscape of melanoma biomarkers has changed within a decade (6).

UPDATES ON SYSTEMIC THERAPIES FOR NON-MELANOMA SKIN CANCERS

On the heels of the success of systemic therapies for melanoma, similar approaches are being tested in non-melanoma skin cancers (NMSC). Tanese et al. review systemic therapy options, including hedgehog inhibitors for basal cell carcinoma (BCC), EGFR inhibitors and ICi for cutaneous squamous cell carcinoma (cSCC), HER2 antagonists for extramammary Paget's disease (EMPD), ICi for Merkel cell carcinoma (MCC), and experimental approaches for skin adnexal carcinomas. They conclude that, emerging molecular targeting therapies are not necessarily effective for all NMSC patients. Development of further treatment options for NMSC is required, especially for rare forms of NMSC, such as skin adnexal carcinomas (Tanese et al.). A retrospective case series by Hiura et al. of 13 patients with unresectable cSCC demonstrates the potential advantage of continued chemotherapy after concurrent chemoradiotherapy (CCRT), which will be validated in a future study. Hidaka et al. discuss in a thought-provoking review the role that Aryl Hydrocarbon Receptor (AHR) plays in carcinogenesis and maintenance of skin cancers. AHR is a key modulator of UVR- and carcinogenic chemical-induced skin carcinogenesis and is also associated with the efficacy of MAPKi and ICi in melanoma (Hidaka et al.). Thus, the authors propose that the AHR system may provide a putative target for prevention and therapy of skin cancer (Hidaka et al.). Oka and Miyagaki review novel and future therapies for advanced Mycosis fungoides and Sézary syndrome covering a wide range of different drug classes. Most approaches showed limited efficacy. Thus, the authors recommend personalized therapy and call for creatively designed international clinical trials (Oka and Miyagaki).

UPDATES ON DIAGNOSTIC TOOLS FOR MELANOMA AND NON-MELANOMA SKIN CANCER

Dermoscopy has become an indispensable diagnostic tool for pigmented and unpigmented cutaneous lesions (7). Kato et al. review the role of dermoscopy in the diagnosis of melanoma and non-melanoma skin cancers including BCC, sebaceous carcinoma, actinic keratosis, Bowen's disease, cSCC, MCC, EMPD, and angiosarcoma. Oh et al. take skin imaging further by discussing the utility of ultrasound imaging, optical coherence tomography, confocal microscopy, and two-photon microscopy as diagnostic tools. Especially a combination of tools is advised to allow for highest resolution and highest imaging depth, which are usually reciprocal (Oh et al.). The principle of these devices is to analyze signals reflected or scattered from the skin. Indeed, the fact that autofluorescent structures within the skin (e.g., elastic fibers) can be co-imaged with highly crystalline triple-helix structures (e.g., collagen) utilizing the second harmonic generation phenomenon, which then can be quantified (8). Oh et al. suggest that the development of fluorescent probes will further improve the utility of these tools for the diagnosis and treatment of skin lesions. Fujisawa et al. take this further and discuss the strengths and limitations of a deep-learning technology using a convolutional neural network (CNN) for skin tumor diagnosis. They conclude that AI classifiers have dramatically improved over the last years and still keep improving and thus may gain sufficient sensitivity and specificity to bear the screening burden for detecting malignant skin tumors (Fujisawa et al.). Importantly, they emphasize that the advent of AI-based skin cancer diagnostic should be considered a useful assistance, rather than a threat to dermatologists (Fujisawa et al.).

Nakamura (b) discusses the new role of sentinel lymph node biopsy (SLNB) for invasive melanoma post DeCOG-SLT and MSLT–II, in the context of modern adjuvant and neoadjuvant therapy approaches.

Fujii and Kanekura review the methods for diagnosis of early stage T-Cell Lymphoma. They demonstrate that next-generation sequencing not only detects TCR clonality with superior sensitivity over conventional methods and is therefore better to diagnose early Mycosis fungoides, but also allows for temporal tracking of specific TCR clones and therefore better for assessment of progression or recurrence (Fujii and Kanekura).

AUTHOR CONTRIBUTIONS

TF and NH wrote the manuscript. All authors provided intellectual input to the editorial.

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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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ORIGINAL RESEARCH
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Immune Cell Infiltration of the Primary Tumor, Not PD-L1 Status, Is Associated With Improved Response to Checkpoint Inhibition in Metastatic Melanoma

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Immune checkpoint inhibition has resulted in dramatic improvements in overall and relapse-free survival in patients with metastatic melanoma. The most commonly used immune checkpoint inhibitors are monoclonal antibodies targeting programmed cell death protein 1 and cytotoxic T-lymphocyte-associated protein 4. Unfortunately, a significant subset of patients fail to respond to these therapies, which has resulted in intense research efforts to identify the factors which are associated with treatment response. To this end, we investigated immune cell infiltration in primary melanomas and melanoma metastases, in addition to tumor cell PD-L1 expression, to determine whether these factors are associated with an improved outcome after immune checkpoint inhibition. Indeed, the extent of the immune cell infiltration in the primary melanoma, measured by the Immunoscore, was associated with a significantly improved response to immune checkpoint inhibition in terms of increased overall survival. However, the Immunoscore did not predict which patients would respond to treatment. The Immunoscore was significantly reduced in metastases when compared to primary melanomas. In contrast, PD-L1 expression, exhaustively tested using four commercially available anti-PD-L1 clones, did not differ significantly between primary tumors and melanoma metastases and was not associated treatment response. Whilst replication in larger, prospective studies is required, our data demonstrates the relevance of immune cell infiltration in the primary melanoma as a novel marker of improved overall survival in response to immune checkpoint inhibition.

Keywords: melanoma, PD-L1, immunoscore, checkpoint inhibition, lymphocyte, metastases, checkpoint inhibitor therapy

INTRODUCTION

Although melanoma is highly refractory to treatment with conventional chemotherapy, the advent of immune checkpoint inhibition has dramatically improved the clinical outcome in metastatic disease (1). Immune checkpoint inhibition in melanoma relies on the use of antibodies blocking either the programmed cell death protein 1 (PD-1), for example Nivolumab and Pembrolizumab,

preventing melanoma tumor cells from escaping toxic T-cell action, or antibodies targeting the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), namely Ipilimumab, leading to prolonged T-cell activation and resulting in clonal expansion and enlarged T-cell repertoire. Whilst immune checkpoint inhibition has been associated with impressive long-term response rates, there remains a subset of patients who either fail to respond to therapy (primary resistance), or lose the initial response (secondary resistance) during treatment (2).

Therefore, current research efforts are focused on identifying factors associated with treatment response in order to individually tailor treatment (3). For example, an increased tumor mutational load is associated with improved outcome under checkpoint inhibition, potentially via the induction of immune cells which differentially recognize tumor- from normal cells (4, 5). On the other hand, melanoma can express a specific mutational profile which is able to induce an innate anti-PD1 resistance (IPRES) phenotype, rendering the melanoma effectively unresponsive to immune checkpoint inhibition (6).

The expression of programmed cell death ligand 1 (PD-L1) in melanoma is perhaps the most intensively studied marker of response to treatment with checkpoint inhibition (7, 8). In a comprehensive review of biomarkers for response of melanoma to checkpoint inhibition, Jessurun et al. found a significant correlation between tumor PD-1 and PD-L1 expression and response to checkpoint inhibition in five out of eight analyses. Interestingly, there was no significant correlation with progression-free survival. Whilst divergent methodology may make comparison of these studies difficult, it is clear that overall response, progression-free survival and overall survival are not synonymous, and were correctly reported separately. Moreover, prognostic markers are not necessarily predictive markers of response to treatment (8).

Ultimately, whilst PD-L1 status has been shown to correlate with response to treatment with anti-PD-1 antibodies in metastatic melanoma in some studies (9, 10), the expression of PD-L1 *per se* has not emerged as a predictive marker for treatment response, potentially due to its crucial role in engaging PD-1, a dominant negative regulator of anti-tumor T cell effector function (1, 9, 11). In the clinical setting, PD-L1 expression cannot be relied upon as a predictive marker of treatment response, given that not all tumors expressing PD-L1 respond to PD- inhibitors (12) and melanomas with little or no PD-L1 expression may still respond to checkpoint inhibition.

In contrast, pre-existing tumor immune cell infiltration is considered to be an important factor determining successful immune checkpoint inhibition and consequently treatment response (13). Melanoma is recognized as a tumor that is often infiltrated with immune cells; the grade of tumor-infiltrating lymphocytes being an independent predictor of survival irrespective of the treatment type (14–17). Given the immunogenic nature of melanoma (18), as well as the poor prognosis associated with metastatic disease, we sought to objectively determine the immune cell infiltration (Immunoscore) and PD-L1 status of both primary tumors and metastases in a retrospective cohort based study of patients with metastatic melanoma, treated with anti-CTLA-4 and/or anti-PD-1 antibodies. The Immunoscore captures the number und distribution of tumor-infiltrating lymphocytes and was first described by Clark et al. (19) The grade of tumor-infiltrating lymphocytes is defined as either brisk, nonbrisk or absent. Given the range of commercially available anti-PD-L1 antibodies, we also investigated antibody specificity before utilizing the optimal antibody for the immunohistochemical staining. Finally, we addressed the question of whether immune cell infiltration and/or PD-L1 status of primary melanomas and metastases were associated with the clinical response, specifically in terms of overall survival, to immune checkpoint inhibition.

MATERIALS AND METHODS

Study Population/Case Selection

The patient cohort comprised 32 patients (25 male, 7 female), who were diagnosed with metastatic melanoma and treated with checkpoint inhibitors at the Department of Dermatology, University of Luebeck. Patients underwent treatment with CTLA-4-inhibition (Ipilimumab) and/or anti-PD1-therapy (Nivolumab or (Pembrolizumab). 2 Patients were treated with Ipilimumab monotherapy. 12 patients were treated with Nivolumab ($n = 6$) or Pembrolizumab ($n = 6$). 11 patients received Ipilimumab prior to anti-PD-1-therapy, 4 patients received Ipilimumab prior to combined therapy with Ipilimumab and a PD-1-inhibitor and 3 patients initially received combination therapy with Ipilimumab and a PD1-inhibitor followed by a PD-1-inhibitor (**Table 1**).

The median age at time of diagnosis was 64 years. Nine patients remained alive at the last follow up point. Tissue blocks were retrieved from the archive, having been initially obtained between 2006 and 2016.

Out of the 32 patients, we retrieved primary tumor tissue from 22 patients, while from 10 patients only metastatic tissue was available. From a total of 22 patients for whom primary tumor samples were available, corresponding metastatic tissue was available from 19 cases. Out of the 19 patients with primary and metastatic lesions, 15 had metastatic lesions obtained prior to initiation of anti-PD-1-therapy (matched pairs). Up to 9 metastases (distant and/or lymph node) were available per patient.

Primary tumors, as well as lymph node and distant metastases, obtained before and after immune checkpoint inhibitor therapy were analyzed separately. The "tumor groups" were classified as follows (i) primary tumors (22 patients), (ii) distant metastases obtained pre-treatment (15 patients), (iii) lymph node metastases obtained pre-treatment (12 patients), (iv) distant metastases obtained during treatment (7 patients) and (v) lymph node metastases obtained during treatment (1 patient).

Baseline characteristics of the cohort including sex, age at diagnose, vital status at last follow up, treatment, overall survival, progression free survival, interval between diagnose and first dose checkpoint inhibitor, composition of FFPE material and the Immunoscore of primary tumors and metastases were recorded (**Table 1**). Observation time was the interval from the date of diagnosis to the date of last follow-up or death. Overall survival and progression-free survival ranged from 31 to 3,527

TABLE 1 | Patients' baseline characteristics.

SEX	
male	25
female	7
AGE AT DIAGNOSIS (YEARS)	
mean	64
range	32-91
VITAL STATUS AT LAST FOLLOW UP	
alive	9
dead	23
IMMUNE CHECKPOINT INHIBITOR THERAPY	
Ipilimumab mono	2
Nivolumab mono	6
Pembrolizumab mono	6
first Ipilimumab, afterwards PD-1-Inhibitor	11
first Ipilimumab, afterwards combinated therapy	4
first combinated therapy, afterwards PD-1-Inhibitor	3
OVERALL SURVIVAL (DAYS)	
mean	1272
range	31-3527
PROGRESSION FREE SURVIVAL	
mean	194
range	3-1310
INTERVAL BETWEEN DIAGNOSE AND FIRST DOSE OF PD-1-INHIBITOR (DAYS)	
mean	862
range	14-3425
BRAF-MUTATION STATUS	
wildtype	20
mutation	12
COMPOSITION OF FFPE MATERIAL	
cases with tissue from primary tumor and metastases	19
cases with tissue solely from primary tumors	3
cases with tissue solely from metastases	10
number of all metastases samples	88
number of naive metastases	54
number of metastasespost anti-PD1-therapy	20
number of metastases post Ipilimumab	14
TIL GRADE IN PRIMARY TUMORS	
non-brisk	9 (41%)
brisk	13 (59%)
TIL GRADE IN PRIMARY METASTASES	
non-brisk	37 (68,5%)
brisk	17 (31,5%)
TIL GRADE IN RELAPSED METASTASES (AFTER ANTI-PD1-THERAPY)	
non-brisk	16 (80%)
brisk	4 (20%)

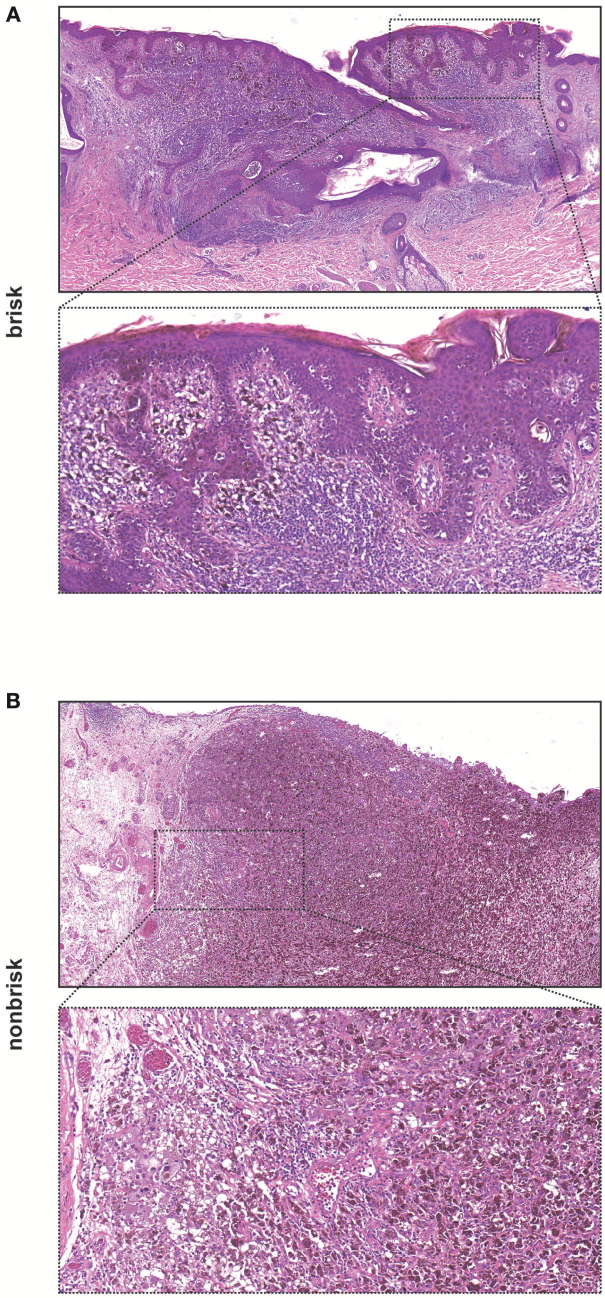

FIGURE 1 | Images of a melanoma with brisk and nonbrisk lymphocytic infiltration. **(A)** Brisk melanoma with entire base of the tumor surrounded by a dense band-like lymphocytic infiltration [H&E, original magnification x40 and x130 (insert)]. **(B)** Nonbrisk melanoma with only focal lymphocytic infiltration [H&E, original magnification x40 and x130 (insert)].

days (mean 1272 days) and from 3 to 1,310 days (mean 194 days), respectively.

Ethical approval for using human material in this study was obtained from the Internal Review Board of University of Luebeck (17–186). All data were anonymized before included to this retrospective study cohort.

Histopathological Analysis

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were retrieved from the archives of the Department of Pathology of the University Hospital Schleswig-Holstein, Campus Luebeck and Research Center Borstel, Leibniz Lung Center, Site Luebeck, the Clinic for Dermatology of the University Hospital Schleswig-Holstein, Campus Luebeck. Tissue microarrays (TMA) were

constructed from metastatic samples in triplicates of 0.6 mm diameter cores. A tumor sample was included for further investigation if at least two cores were evaluable. Values of protein expression generated by Immunohistochemistry (IHC) for all examined cores of a patient sample were recorded as a mean value. The TMA included 74 samples of metastatic lesions from 24 patients. Tissue from 14 metastases (from 9 patients) was too small for TMA and therefore investigated as a whole section. All primary tumors were investigated as a whole section due to the small tumor size in most cases. Evaluation of protein expression by IHC was performed by two independent pathologists (CK, SP) who were blinded to the clinico-pathological data.

Immunohistochemical Analysis

Immunohistochemical (IHC) staining was performed using the Ventana Discovery (Ventana Medical System) automated staining system. In brief, slides were incubated at room temperature with the following primary antibodies (dilution, clone, company): anti-PD-L1 (1: 50, E1L3N, Cell Signaling), anti-PD-L1 (RTU, SP263, Roche), anti-PD-L1 (RTU, SP 142, Roche), anti-PD-L1 (1:100, 28.8, Abcam), anti-PD-L2 (1:100, OTI6C3, Acris), anti-PD-1 (RTU, NAT105, Roche), anti-CD8 (RTU, SP57, Roche), anti-CD4 (RTU, SP35, Roche), anti-CD56 (RTU, MRQ-42, Roche), anti-FoxP3 (1:100, 236A/E7, Thermo Fisher) and anti-CTLA4 (1:100, BNI3, Abcam). Expression of PD-L1 and PD-L2 was investigated on tumor and immune cells. CD8, CD4, CD56, FoxP3, CTLA-4, and PD1 staining were used to further characterize the lymphocytes.

Scoring of Tumor Infiltrating Lymphocytes

The Immunoscore was investigated according to criteria formulated by Clark et al (19). In brief, lymphocytes were classified as brisk if they diffusely infiltrated the entire invasive component and were interposed between melanoma cells or if they were present alongside the entire base of tumor. Lymphocytes were classified as nonbrisk if they focally infiltrated the tumor and were not present along the entire tumor base. If no lymphocytes were present or if lymphocytes did not infiltrate the tumor, they were classified as absent.

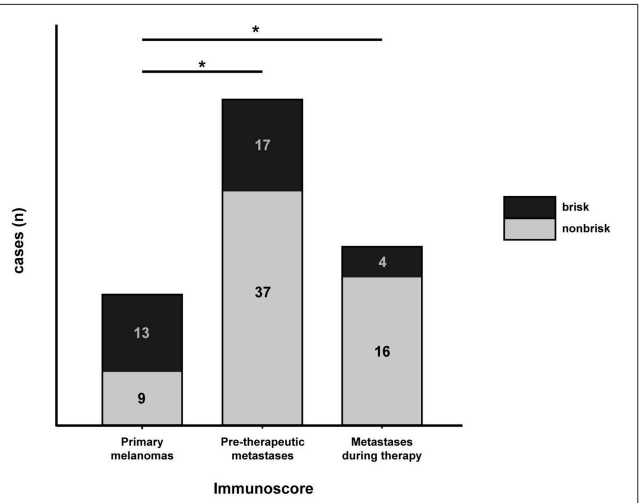

FIGURE 2 | Distribution of brisk vs. nonbrisk infiltration in primary melanomas, pre-therapeutic metastases and metastases which developed during anti-PD-1-therapy. Number of brisk cases is indicated in black and of nonbrisk cases in gray fields. Statistical significance between investigated groups was determined by Fischer's exact test (*$p < 0.05$).

Immunoscore dynamics

	Primary tumors	Pre-therapeutic metastases
Patient ID 1	nonbrisk	nonbrisk
Patient ID 20	nonbrisk	nonbrisk
Patient ID 24	brisk	brisk
Patient ID 26	brisk	brisk
Patient ID 28	brisk	brisk
Patient ID 30	brisk	brisk
Patient ID 16	nonbrisk	brisk
Patient ID 23	nonbrisk	brisk
Patient ID 39	nonbrisk	brisk
Patient ID 2	brisk	nonbrisk
Patient ID 9	brisk	nonbrisk
Patient ID 15	brisk	nonbrisk
Patient ID 31	brisk	nonbrisk
Patient ID 33	brisk	nonbrisk
Patient ID 34	brisk	nonbrisk

FIGURE 3 | Immunoscore dynamics between primary melanomas and pre-therapeutic metastases in the same patient. Top down: No change in Immunoscore was seen in 6 patients, increased Immunoscore in metastases was seen in 3 patients, and a decreased Immunoscore in metastases was seen in 6 patients. Patient identification numbers are denoted besides.

Lymphocytes were morphologically identified by H&E while subtyping of the lymphocytic infiltrate was performed by staining for CD8, CD4, CD56, FoxP, CTLA-4, PD1, PD-L1, and PD-L2.

Quantification of Lymphocytic Subtypes

Percentage of lymphocytes positive for CD8, CD4, CD56, Fox P3, CTLA-4, and PD-1 was calculated according to the total number of tumor infiltrating lymphocytes in a sample. Additionally, we determined ratios of CD4- and CD8-positive lymphocytes. Geographical associations of lymphocytic subtypes and tumor cells could not be investigated due to TMA used for the majority of samples. PD-L2 was evaluated as described below for PD-L1 immunohistochemistry.

Quantification of PD-L1 Expression

In order to determine the most specific PD-L1 expression pattern, we evaluated IHC obtained using four well-established anti-PD-L1 clones (E1L3N, cell signaling; SP263, Roche; SP142, Roche; 28.8, Abcam). Thereafter, PD-L1 staining in tumor cells was considered positive if staining was membranous, regardless of intensity. Tumors were defined as positive if they contained $\geq$5% PD-L1 positive tumor cells. Expression of PD-L1 in tumor infiltrating lymphocytes was evaluated by measuring the area of PD-L1 positive lymphocytes from the whole tumor area (20).

Statistical Analysis

Fisher's exact test was used to assess the differences in the distribution of brisk vs. nonbrisk lymphocytes between primary melanomas and metastases (including those present prior to initiation of treatment and those which developed during treatment.

Kaplan-Meier curves were used to determine overall survival and progression-free survival depending on the Immunoscore, PD-L1/PD-L2 expression of tumor and immune cells, the different lymphocytic subtypes and CD4/CD8-ratio. Data were statistically proved by log-rank tests.

T-tests were used to compare the mean expression between patients with or without progression during anti-PD1-therapy. Statistical tests were performed within the same tumor groups (primary tumors, lymph node metastases and distant metastases before and after checkpoint-inhibitor therapy).

All statistical analyses were performed using SPSS 2.0. p levels <0.05 were considered significant.

RESULTS

Immune Infiltration Is Significantly Increased in Primary Melanoma When Compared to That Seen in Metastases

We first determined the Immunoscore based on lymphocytic infiltration, classifying the tumors into absent, brisk and nonbrisk groups (**Figure 1**). We assessed the Immunoscore in a total of 22 samples of primary melanomas; 13 (59.1%) were classified as brisk and 9 (40.9%) samples were classified as nonbrisk. We additionally analyzed 88 metastases out of

TABLE 2 | Immunscore and PD-L1 expression before and after anti-PD1 therapy.

Patient ID	Pre-therapeutic tissue	n	PD-L1 expression (mean%)	Immunoscore	Metastases during therapy	n	PD-L1 expression (mean %)	Immunoscore
6	primary tumor	1	5	2	distant metastases	1	0	2
8	primary tumor	1	20	2	lymphe node metastases	3	60	2
9	distant metastases	1	<1	1	distant metastases	6	<1	1
11	satellite metastases	2	2	2	distant metastases	1	10	1
13	not available	X	X	X	distant metastases	1	15	1
14	distant metastases	1	20	1	distant metastases	1	30	1
21	lymphe node metastases	1	25	2	lymphe node- and distant metastases	7	0	1

TABLE 3 | Immunscore and PD-L1 expression before and after anti CTLA-4 therapy.

Patient ID	Pre-therapeutic tissue	n	PD-L1 expression (mean%)	Immunoscore	Metastases during therapy	n	PD-L1 expression (mean %)	Immunoscore
2	lymph node- and distant metastases	3	15	1	distant metastases	2	35	1
3	primary tumor	1	0	1	distant metastases	1	<1	1
4	lymphe node metastases	2	30	1	distant metastases	1	0	1
9	distant metastases	1	<1	1	distant metastases	1	2	1
12	not available	X	X	X	distant metastases	8	<1	1
21	lymphe node metastases	1	25	2	distant metastases	1	15	1

which 54 were obtained before treatment and 20 were obtained post treatment with anti-PD-1-therapy. Seventeen (31.5%) pre-therapeutic metastases (metastases present before any treatment) were classified as brisk and 37 (68.5%) as nonbrisk. In the cohort of metastases which developed during treatment, 4 (20%) were classified as brisk while 16 (80%) were classified as nonbrisk (**Figure 2**). The remaining metastases (n = 14) that were obtained after initial Ipilimumab therapy in patients that had not undergone Nivolumab/Pembrolizumab therapy were not included in the Immunoscoring.

In order to investigate the differential distribution of lymphocytic infiltration we compared brisk status in primary melanomas (prior to anti-tumor therapy) vs. pre-therapeutic metastases as well as between primary melanomas and metastases which developed during anti-PD-1-therapy. Immune infiltration was not only significantly increased in primary melanomas when compared to pre-therapeutic metastases ($p = 0.0381$), but also increased when immune infiltration in the primary melanomas was compared to that in metastases developed during treatment ($p = 0.0135$; **Figure 2**).

Next, we compared the Immunoscore in primary melanomas to that in pre-therapeutic metastases in the same patient (intra-individual immune cell infiltration). In 40% of cases there was no difference in the Immunoscore (6/15 patients). Whilst there was an increased metastatic Immunoscore in 20% of cases (3/15), in the remaining 40% (6/15) there

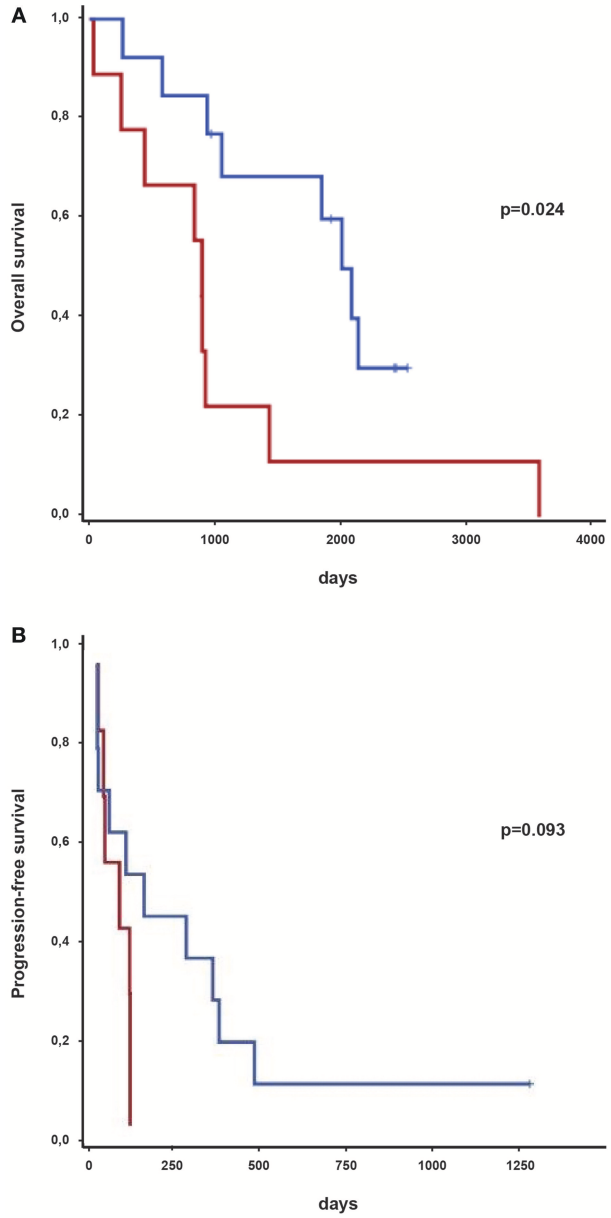

FIGURE 4 | Survival of melanoma patients treated with immune-checkpoint inhibitors depending on immune infiltration of primary tumors classified as brisk or nonbrisk. **(A)** Kaplan-Meier curves indicating overall survival of brisk (in blue) and nonbrisk (in red) primary tumors from melanoma patients. Survival groups were compared by log-rank test. *p*-values are indicated. **(B)** Kaplan-Meier curves indicating progression-free survival of brisk (in blue) and nonbrisk (in red) primary tumors from melanoma patients. Survival groups were compared by log-rank test. p-values are indicated.

TABLE 4 | Association between BRAF status und immunoscore.

		Immunoscore		
BRAF-status		**Nonbrisk**	**Brisk**	**Total**
Wildtype	n	4	10	14
Mutation	n	5	3	8
Total	n	9	13	22
				$p = 0.187$

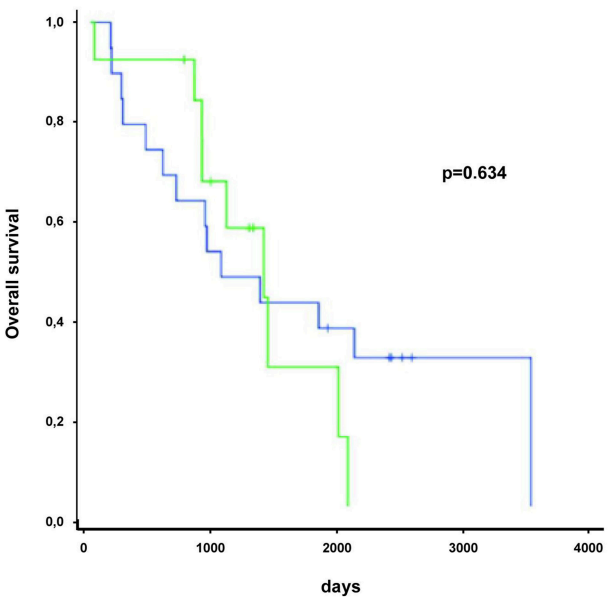

FIGURE 5 | BRAF mutation status in patients undergoing immune checkpoint therapy was not significantly associated with overall survival. Green line represents patients with BRAF mutation, blue line indicates patients without BRAF mutation.

was increased Immunoscore in the primary melanoma when compared to that in the pre-therapeutic metastases (**Figure 3** and **Tables 2**, **3**). Due to low number of metastases which developed during checkpoint therapy, we were not able compare Immunoscores from pre-therapeutic metastases to the Immunscore in metastases which developed during checkpoint therapy in the same patient.

The Immunoscore Is Associated With Improved Overall Survival During Checkpoint Therapy

Next, we aimed to determine whether the Immunoscore was associated with overall survival in melanoma. This was chosen as the most clinically significant parameter. We observed a statistically significant increase in overall survival in patients with a brisk lymphocytic infiltrate compared to patients with a nonbrisk infiltrate of their primary tumors ($p = 0.024$; **Figure 4A**). 5 year-survival rate for patients with a brisk tumor infiltrate and a nonbrisk infiltrate was 59.8 and 11.1%, respectively. Concordantly, we observed a trend in increased progression-free survival progression free survival of patients with a brisk lymphocytic tumor-infiltrate compared to patients with a nonbrisk infiltrate ($p = 0.093$; **Figure 4B**).

An association between the Immunoscore and survival rates could be demonstrated when evaluating primary melanomas, but there was no association between the Immunoscore in metastases and survival. Moreover, subtyping lymphocytic infiltrate using CD8, CD4, CD56, FoxP3, CTLA-4, PD-1, PD-L1, or PD-L2 expression did not lead to significant associations with overall survival (data not shown).

The Impact of BRAF Mutation Status on Clinical Outcome

We further evaluated the association of BRAF mutations with clinical outcome. BRAF mutation status was investigated in context of diagnostic work-up and not specifically for the current study. 20 (62.5%) out of 32 patients showed wt BRAF and 12 (37.5%) harbored mutations in the BRAF gene. Out of these 12 cases, 10 had the V600E mutation, 1 exhibited the D594V mutation and a further patient had the L597Q mutation. There was no association between BRAF status and either overall survival or progression-free in our melanoma cohort. We also observed no association between BRAF status and Immunoscore and/or PD-L1 status (**Table 4** and **Figure 5**).

The PD-L1 Antibody Clone SP263 Demonstrated the Highest Immunohistochemical Specificity

In order to determine the optimal protocol for determining PD-L1 expression in melanoma, we tested four distinct anti-PD-L1 clones, namely SP263, 28.8, E1L3N, and SP142 in 22 primary melanomas and 88 metastases. Two metastases were excluded from the results due to exhaustion of tissue material during the immunohistochemical staining. We observed strikingly different staining patterns as representatively shown in **Figure 6**. The percentage of PD-L1 positive tumor cells in the same investigated sample varied from 100% (clone SP263) to 0% (clone SP142). Using clone 28.8 and E1L3N, 60 and 20% respectively of tumor cells were PD-L1 positive. When comparing PD-L1 expression in primary tumors vs. metastases using the four antibody clones the results were also divergent. Specifically, in 22 cases of primary tumors, half ($n = 11$) were interpreted as PD-L1 positive by using clone SP263 (**Table 5**). On the other hand, by using clones 28.8, E1L3N and SP142, we observed 3 (13.6%), 3 (13.6%), and 1 (4.5%) positive cases, respectively. Mean PD-L1 expression value of positive tumor cells for clone SP263 was 11.5%, for clone 28.8 was 3.63%, for clone E1L3N was 3.75% and for SP142 was 2.27%. Expression range reached from 0 to 100 positive tumor cells for clone SP263, from 0 to 40 for clone 28.8, from 0 to 70 for clone E1L3N und 0-50 for clone SP142. When investigating metastases for PD-L1 expression, we observed a similar pattern. By using clone SP263, we observed 27 (31.4%) positive cases while for clones 28.8, E1L3N and SP142, we observed 11 (12.8%), 4 (4.7%), and 4 (4.7%), respectively. Mean PD-L1 expression value of positive tumor cells for clone SP263 was 12.9%, for clone 28.8 was 5.3%, for clone E1L3N was 1.7% and for SP142 was 1.1%. Expression range reached from 0 to 100 positive tumor cells for clone SP263, from 0 to 95 for clone 28.8, from 0-80 for clone E1L3N und 0-40 for clone SP142.

Overall, clone SP263 showed highest specificity and the strongest staining intensity of PD-L1 in both primary tumors and metastases. Conversely, clones 28.8 and E1L3N showed weaker staining intensity with a discrete staining of the cell membranes while clone 28.8 showed additional granular background staining. Clone SP142 showed the weakest staining intensity as well as the lowest frequency of positive tumor cells. These observations supported the use of clone SP263 for further investigations.

PD-L1 Expression Is Not Associated With Overall Survival

Given that the immune infiltration was significantly higher in primary melanomas when compared to pre-therapeutic metastases, we next sought to determine intra-individual PD-L1 expression in primary melanomas and untreated metastases. We were able to evaluate PD-L1 expression in primary melanomas and untreated metastases in 13 out of 15 patients. There was no difference in PD-L1 expression in 3 (23.1%) cases, PD-L1 was upregulated in metastases in 7 (53.8%) cases, and higher PD-L1 expression in primary tumors, when compared to metastases obtained before immunotherapy, was present in 3 cases (23.1%) (**Figure 7**).

There was no correlation between PD-L1 expression and overall survival of melanoma patients treated with immune checkpoint inhibitors.

We also investigated any possible association between PD-L1 expression and the Immunoscore but observed no statistically significant difference in PD-L1 status between the brisk and nonbrisk groups.

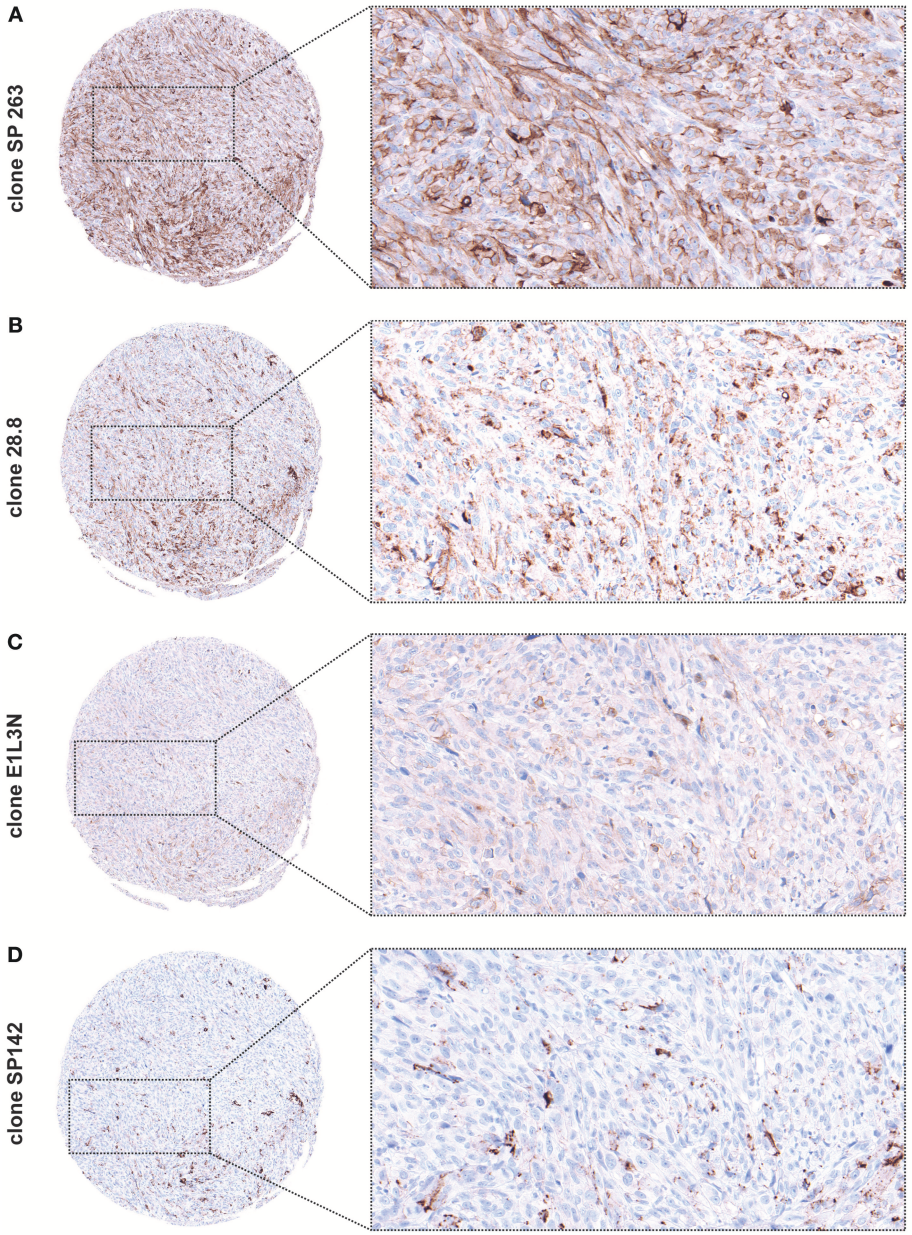

FIGURE 6 | PD-L1 expression using different anti-PD-L1 clones demonstrated on the same tumor core [original magnification x84 and x300 (insert)]. **(A)** Clone SP263 stains the highest proportion of tumor cells and shows the strongest expression. **(B)** Clone 28.8. shows weaker expression and additionally a granular background. **(C)** Clone E1L3N shows weak expression with a discreet staining of cell membranes. **(D)** Clone SP142 shows the weakest expression (black pigment accord to melanin pigment).

DISCUSSION

The treatment of metastatic melanoma continues to represent a major clinical challenge, not only due to the aggressive nature of the disease, but also due to the potentially life-threatening side-effects associated with immunotherapy. However, the development of immune checkpoint inhibitors has markedly increased our therapeutic armamentarium and translated into impressive improvements in overall survival. Unfortunately, a significant proportion of patients still fail to respond to treatment. In order to determine which patients may respond best to checkpoint inhibition we retrospectively analyzed immune cell infiltration and PD-L1 status in a cohort of melanoma treated with CTLA-4 and/or anti-PD-1 inhibitors. Increased tumor immune infiltration in primary melanomas (measured by the Immunoscore) prior to immune checkpoint inhibition was associated with

TABLE 5 | PD-L1 expression using different anti-PD-L1 clones.

	Mean PD-L1 expression	Range	Number of positive cases	Number of negative cases	Positive cases (%)	Negative cases (%)
Primary tumors ($n = 22$)						
SP263	11.5	0–100	11	11	50	50
Abcam 28.8	3.63	0–40	3	19	13.6	86.4
E1L3N	3.75	0–70	3	19	13.6	86.4
SP142	2.27	0–50	1	21	4.5	95.5
Metastases ($n = 86$)						
SP263	12.9	0–100	27	59	31.4	68.6
Abcam 28.8	5.3	0–95	11	75	12.8	87.2
E1L3N	1.7	0–80	4	82	4.7	95.3
SP142	1.1	0–40	4	82	4.7	95.3

improved overall patient survival (**Figure 4**). Interestingly, increased recruitment of cytotoxic CD8+ lymphocytes alone was not observed in the favorable "brisk" setting (data not shown). Furthermore, no significant difference was observed in the number of CD4+ helper cells between the brisk and nonbrisk groups and consequently no difference was observed in CD4+/CD8+ ratio between two settings (data not shown).

However, the long-term benefit of immune checkpoint inhibition was evidenced by increased overall survival in patients that harbored highly infiltrated primary melanomas. It is important to bear in mind that response, overall survival and progression-free survival are independent parameters. For example, response may vary over time, especially in the context of acquired resistance and progression-free survival does not necessarily equate with improved overall survival (see **Supplementary Table 1**). Furthermore, there is an important and recognized difference between predictors of treatment response and prognostic markers (8). In this context, the Immunoscore represents a novel prognostic marker of treatment response, but is not a suitable stand-alone parameter to predict which patients will clinically benefit from checkpoint inhibition.

In contrast to previous studies (1, 17), we did not find an association between CD8+ cell infiltration with response to checkpoint inhibition in melanoma patients. In fact, Madonna et al. (21) reported low densities of CD8+ lymphocytes at the tumor periphery and an association with response to Ipilimumab. PD-L1 status was not a predictive marker for survival or treatment response.

Whilst the reason for the divergent results in terms of CD8+ infiltration is unclear, it is important to draw attention to the methodological differences between the studies. For example, Tumeh et al. (13) investigated patients who underwent PD-1 (Pembrolizumab) monotherapy, albeit in three difference dosing schedules, and Madonna et al examined patients treated with Ipilimumab. Our "real world" cohort was more heterogeneous in terms of treatment modality (PD-1/CTLA4 monotherapy vs. PD-1/CTLA4 combined therapy) which may have influenced CD8+ cell infiltration. Large, prospective studies would be required to determine the extent to which CD8+ cell infiltration in treatment type dependent. Moreover, it would be interesting to determine the extent to which PD-L1 expression on peripheral T cells correlates with intratumoural T cell PD-L1 expression, given the association between circulating T cell PD-L1 expression and response to checkpoint inhibition (22, 23). We cannot exclude that the Immunoscore reflects a pre-existing anti-melanoma T cell response. However, this would be difficult to experimentally and/or clinically confirm or refute given that we do not have a patient cohort remain untreated. In any case, given that the Immunoscore in metastases was *not* associated with improved overall survival in our study, such pre-existing anti-melanoma T cell responses would have been limited to the primary tumor.

Next, we investigated BRAF mutation status in the context of absent, brisk and non-brisk Immunoscores in primary tumors and overall survival. BRAF status was not predictive for overall survival of melanoma patients during immune checkpoint therapy. The frequency of BRAF mutation in our cohort was similar to previous melanoma cohorts (24). Whilst there is conflicting data regarding the effect of BRAF status on treatment outcome during checkpoint immunotherapy, our study is in line with studies showing that Nivolumab treatment efficacy irrespective of BRAF status (25). It is currently unclear whether initial therapy with BRAF/MEK inhibitors followed by immune checkpoint therapy, or vice versa, translates to improved overall rates of survival for patients with the BRAF mutation.

Nevertheless, our study highlights the utility of the Immunoscore, a robust and readily available scoring tool, which is associated with overall survival in patients with metastatic melanoma undergoing immune checkpoint therapy.

We also aimed to clarify whether the pattern of tumor immune cell infiltration differs between primary melanoma, untreated metastases and metastases which developed during treatment with immune checkpoint inhibition. Due to more aggressive nature of metastatic melanoma, we expected to observe an increased immune-cell infiltration in primary melanomas when compared to that in metastases. Indeed, the pattern of immune-cell infiltration was dramatically different between primary

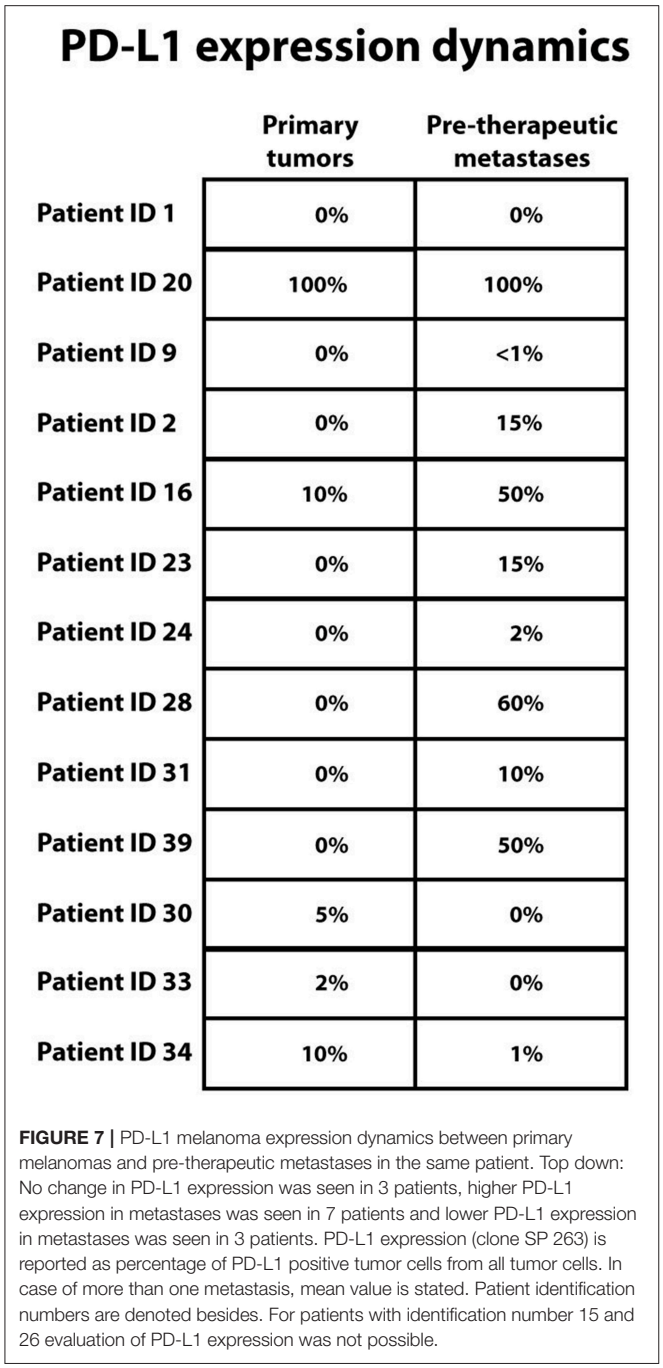

FIGURE 7 | PD-L1 melanoma expression dynamics between primary melanomas and pre-therapeutic metastases in the same patient. Top down: No change in PD-L1 expression was seen in 3 patients, higher PD-L1 expression in metastases was seen in 7 patients and lower PD-L1 expression in metastases was seen in 3 patients. PD-L1 expression (clone SP 263) is reported as percentage of PD-L1 positive tumor cells from all tumor cells. In case of more than one metastasis, mean value is stated. Patient identification numbers are denoted besides. For patients with identification number 15 and 26 evaluation of PD-L1 expression was not possible.

melanomas and both metastases subgroups, in line with our hypothesis (**Figure 2**). We then sought to compare the Immunoscore from primary tumors and metastases in individual patients. Although we observed generally a lower Immunoscore in untreated metastases, there was no reduction in tumor immune cell infiltration when comparing the primary melanoma to the metastases in every patient, probably due to the heterogenous nature of these tumors (**Figure 3**).

Theoretically, both immune cell tumor infiltration and expression of PD-L1 on tumor cells are required for successful anti-PD-1 and anti-PD-L1 checkpoint therapy. Therefore, we further investigated the correlation of tumor PD-L1 status in the context of the brisk and nonbrisk group as well as alone on the survival of melanoma patients after anti-PD-1 immunotherapy. We found no significant correlations in either setting (9). We also observed no correlation of PD-L1 expression dynamics in matching primary melanomas and corresponding metastases of the same patient. Again, it is important to note (i) the variations in immune-checkpoint inhibitor treatment regiments (single vs. combined anti-CTLA-4 and anti-PD-1/PD-L1 vs. sequential combined anti-CTLA-4 and anti-PD-1/PD-L1 immuno-checkpoint inhibition), (ii) the various antibodies used to detect tumor PD-L1 status, (iii) the tumor type (predominantly cutaneous melanoma as opposed to mucosal and/or acral) and (iv) the small size of metastases when taking our data into account. We could demonstrate that PD-L1 expression was heavily dependent on the PD-L1 antibody clone which was used, perhaps partially explaining the, at times, confounding effect of PD-L1 expression reported in the literature (**Figure 6** and **Table 5**). Based on our data, we selected and employed the most specific clone (263) and the overall level of melanoma PD-L1 expression in our study was similar to that reported in the literature (26, 27).

In conclusion, the results of our study suggest that total tumor immune infiltration, not PD-L1 status, is important for predicting the survival of melanoma patients undergoing checkpoint inhibitor therapy. However, this may be specific to our cohort where many melanoma patients were pretreated with Ipilimumab prior to administering Nivolumab/Pembrolizumab (see **Supplementary Table 1**). Whilst our results require replication in a large, prospective study, they provide evidence that the Immunoscore, a validated and easy to use tool, which does not require laborious and potentially erroneous cell counting, is a novel marker for survival in melanoma patients treated with immune checkpoint therapy. Provided that our findings can be replicated in larger, prospective studies, the Immunscore may represent an inexpensive, simple and robust tool which can be rapidly incorporated into routine clinico-pathological practice.

AUTHOR CONTRIBUTIONS

PT, SP, and CK: planned the research project. CK, MJ, AO, and WV: performed the pathological staining and data analysis. CK, MJ, EL, OH, VG, SP, and PT: wrote and/or revised the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00027/full#supplementary-material

Supplementary Table 1 | Immunoscore, treatment history and best response rate in the cohort.

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The remaining 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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Association of Baseline Serum Levels of CXCL5 With the Efficacy of Nivolumab in Advanced Melanoma

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Anti-programmed cell death protein 1 (PD1) antibodies are in wide use for the treatment of various cancers. PD1 antibody-based immunotherapy, co-administration of nivolumab and ipilimumab, is one of the optimal immunotherapies, especially in advanced melanoma with high tumor mutation burden. Since this combined therapy leads to a high frequency of serious immune-related adverse events (irAEs) in patients with advanced melanoma, biomarkers are needed to evaluate nivolumab efficacy to avoid serious irAEs caused by ipilimumab. This study analyzed baseline serum levels of CXCL5, CXCL10, and CCL22 in 46 cases of advanced cutaneous melanoma treated with nivolumab. Baseline serum levels of CXCL5 were significantly higher in responders than in non-responders. In contrast, there were no significant differences in baseline serum levels of CXCL10 and CCL22 between responders and non-responders. These results suggest that baseline serum levels of CXCL5 may be useful as a biomarker for identifying patients with advanced cutaneous melanoma most likely to benefit from anti-melanoma immunotherapy.

Keywords: baseline levels of CXCL5, melanoma, nivolumab, prediction of efficacy, nivolumab and ipilimumab combined therapy

INTRODUCTION

Anti-programmed cell death protein 1 (PD-1) antibodies such as nivolumab and pembrolizumab are in wide use for the treatment of various cancers, including advanced melanoma (1, 2), but cost-effective analyses of their use are sometimes controversial (3). Therefore, biomarkers for the evaluation of the efficacy of anti-PD1 antibody therapy are needed. Previous clinical studies suggested that the efficacy of nivolumab monotherapy is ∼40% in the Caucasian population (2, 4), which contains a high ratio of superficial spreading melanoma (SSM) with high levels of tumor mutation burden (TMB) (5). In contrast, in the Japanese population, there is a high ratio of acral

lentiginous melanoma (ALM) and mucosal melanoma (6), which have low levels of TMB (5). The efficacies of nivolumab and pembrolizumab in Japan have been reported to be 34.1% and 24.1%, respectively (7, 8), suggesting that another drug that could enhance the anti-tumor immune response in melanoma is needed.

Ipilimumab is a fully humanized immunoglobulin (Ig)G1 monoclonal antibody that blocks cytotoxic T-lymphocyte antigen (CTLA-4) and is one of the promising drugs that enhance the anti-tumor immune response for patients with advanced melanoma with or without BRAF gene mutation in combination with nivolumab (1, 4, 9). Indeed, the efficacy of this combination therapy for advanced melanoma has been reported to be 57.8% (4), and, therefore, combination therapy with nivolumab and ipilimumab is recommended by the NCCN guideline for cutaneous melanoma as a first-line therapy (10). This combination therapy achieves a high efficacy rate even for the treatment of brain metastases of melanoma (11). In addition, Blank et al. reported that the efficacy of co-administration of nivolumab and ipilimumab does not parallel TMB (12). In addition to co-administration of nivolumab and ipilimumab, sequential administration of nivolumab and ipilimumab with a planned switch leads to high efficacy in the treatment of advanced melanoma (4, 9). On the other hand, both co-administration of nivolumab and ipilimumab and sequential administration of nivolumab and ipilimumab with a planned switch lead to a high frequency of serious immune-related adverse events (irAEs), such as hepatitis, colitis, polyneuropathy, etc., in patients with advanced melanoma (1, 4, 11). Therefore, determining the efficacy of nivolumab monotherapy before starting first-line immune therapy for melanoma is important.

CXCL5 is a chemokine that can recruit not only neutrophils, but also CXCR2+ myeloid-derived suppressor cells (MDSCs) and CXCR2+ monocytes that can be precursors of tumor-associated macrophages (TAMs) (13–15). Indeed, Soler-Cardona et al. reported that CXCL5-overexpressing melanomas had significantly increased lymph node metastases of melanoma (15) caused by the recruitment of immunosuppressive PD-L1-expressing neutrophils, leading to interference with systemic activation of the anti-tumor immune system using poly (I: C) (14). In another report, the recruitment of CXCR2-expressing MDSCs played significant roles in the development of colitis-associated colon cancer (13). These reports suggested the production of CXCL5 in the cancer stroma of melanoma.

In addition to autoimmune-related chemokines, chronic inflammatory chemotactic factors such as CXCL10 are also important for the recruitment of immunosuppressive cells such as regulatory T cells (Tregs) and MDSCs. Jiang et al. reported that, compared to patients with stable disease, advanced melanoma patients had increased levels of IL-1β and CXCL10 in the serum associated with accumulation of monocytic (Mo)-MDSCs and Tregs in peripheral blood, which correlated with the progression-free survival of these patients (16). In addition, other reports also suggested that serum CXCL10 levels were correlated with disease activity in advanced melanomas (17) and angiosarcomas (18). These reports suggested that serum CXCL10 levels may represent disease activity in advanced melanoma.

Not only MDSCs, but Tregs are also important for tumor progression in melanoma (19, 20). Indeed, Johansenn et al. previously reported that Tregs at the tumor sites were correlated with tumor progression in melanoma (20). More recently, Ha et al. reported the significance of high CTLA4 expression for Tregs, leading to selective depletion of Tregs in melanoma, which might be an important tool in designing cancer immunotherapy (21). In addition, as described above, the reduction of CCL22 by TAMs decreases Tregs in the tumor site, which enhances the therapeutic effects of immune therapy in the mouse melanoma model (22). Taken together, these reports suggest that serum CCL22 may be correlated with the efficacy of immune therapy.

From the above findings, in this report, the baseline serum levels of TAM-associated chemokines were investigated in 46 advanced melanoma patients treated with nivolumab.

PATIENTS AND METHODS

Ethics Statement for Human Experiments

The protocol for this human study was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan (Permit No: 2017-1-064). All methods were performed in accordance with the relevant guidelines and regulations. All patients provided their written, informed consent.

Patients

Data from patients treated with nivolumab were collected from eight institutes in Japan. Patients were eligible if they had unresectable stage III melanoma, or if the patients had stage IV melanoma with accessible cutaneous, subcutaneous, and/or nodal lesions (staging was performed according to the AJCC Staging Manual, 7th edition, 2011). All patients received 2 mg/kg of nivolumab followed by a 3-week rest period or 3 mg/kg of nivolumab followed by 2 weeks of rest, both of which are approved dosing schedules in Japan. Serum was obtained from patients before the administration of nivolumab. The response to nivolumab was assessed according to Response Evaluation Criteria In Solid Tumors.

Baseline Serum Levels of CXCL5 and CXCL10

Before nivolumab administration, the serum was stored, and serum levels of CXCL5, CXCL10, and CCL22 were then analyzed by enzyme-linked immunoassay (ELISA) according to the protocol provided by the manufacturer (R&D Systems, Minneapolis, MN).

Statistical Methods

Receiver operating characteristic (ROC) curves were used to calculate cut-off values for serum levels of CXCL5, CXCL10, and CCL22 and areas under the curves (AUCs). Cut-offs were determined using Youden's index 12 (sensitivity + specificity −1) to determine the point of the maximum index value (23). ROC curves were established to evaluate serum levels of CXCL5 and CXCL10 in patients administered nivolumab. All statistical analyses were performed using JMP version 14.1 software (SAS

TABLE 1 | Characteristics and serum levels of CXCL5, CXCL10, and CCL22 in patients with cutaneous melanoma.

	Age (y)	Sex	Location	Efficacy	CXCL5 (pg/ml)	CXCL10 (pg/ml)	CCL22 (pg/ml)
1	51–60	M	Trunk	SD	226.9	69.31	290.9
2	31–40	F	Extremities	PD	307.7	212.8	814.6
3	61–70	F	Vagina	PD	237.6	117.9	611.2
4	61–70	M	Extremities	PR	497.5	144.4	314.6
5	61–70	M	Extremities	PR	332.6	72.13	401.5
6	81–90	F	Extremities	PR	434.8	355.5	977.3
7	61–70	M	Trunk	PD	862.1	113.0	891.5
8	81–90	F	Extremities	PD	433.9	186.1	1340
9	71–80	M	Head and neck	SD	461.3	97.21	615.7
10	81–90	F	Trunk	PD	314.9	74.89	637.2
11	91–100	M	Extremities	PD	423.4	122.2	582.6
12	71–80	M	Extremities	SD	471.9	84.24	1031
13	61–70	M	Extremities	PD	222.6	202.5	448.7
14	61–70	F	Vagina	SD	667.8	322.7	548.3
15	71–80	M	Trunk	PR	502.8	358.7	603.8
16	71–80	F	Extremities	PR	408.6	550.1	523.0
17	81–90	F	Unknown	SD	940	266.1	701.2
18	71–90	M	Nasal cavity	SD	332.5	188.1	840.2
19	61–70	M	Nasal cavity	PD	162.9	1001	788.0
20	61–70	M	Paranasal	PD	292.1	247.1	678.2
21	61–70	F	Vagina	PD	292.4	368.2	497.1
22	61–70	F	Vagina	PD	271.6	386.9	475.7
23	51–60	F	Conjunctiva	SD	380.9	336.7	355.5
24	81–90	M	Digestive duct	PD	237.4	208.1	438.3
25	61–70	F	Digestive duct	SD	5026	336.8	987.9
26	61–70	F	Trunk	PD	474.3	245.3	84.71
27	71–80	M	Extremities	PD	494.1	116.7	630.4
28	51–60	F	Head and neck	PD	370.5	93.53	983.2
29	31–40	M	Trunk	SD	501.8	97.59	857.8
30	31–40	F	Extremities	PR	407	138.7	963.1
31	71–80	M	Extremities	SD	529.6	108.4	637.7
32	31–40	M	Extremities	PD	687.1	147.1	845.1
33	71–80	F	Extremities	SD	544.7	104.6	935.6
34	71–80	M	Head and neck	PD	701.3	77.79	918.5
35	41–50	M	Extremities	PD	655.4	432.3	617.5
36	71–80	F	Extremities	PR	465.3	184.8	1065
37	61–70	M	Trunk	PR	555.2	105.7	915.5
38	41–50	M	Head and neck	SD	740.9	65.85	830.3
39	41–50	M	Extremities	PD	723.8	54.08	944.6
40	61–70	F	Head and neck	SD	410.7	62.46	470.7
41	71–80	F	Extremities	PR	1196	71.03	606.1
42	61–70	M	Digestive duct	PR	564	190.4	498.5
43	61–70	F	Palate	PR	1600	51.84	619.5
44	51–60	F	Extremities	CR	687.3	80.09	701.3
45	61–70	M	Paranasal	CR	1142	192	211.3
46	61–70	F	Vagina	CR	4939	68.73	573

Changes of CXCL5, CXCL10, and CCL22 serum levels in each patient (n = 46) before the administration of nivolumab were examined by ELISA. Data for each donor represent the means of duplicate assays.
CR, complete response.
PR, partial response.
SD, stable disease.
PD, progress disease.

Institute, Tokyo, Japan). For a single comparison of two groups, the Mann-Whitney *U*-test was used. The level of significance was set at $p < 0.05$.

RESULTS

Patients

Data were collected from 46 melanoma patients treated with nivolumab (**Table 1**). The mean patient age was 67 years (range, 33–93 years). Of the patients with melanoma, 58.7% were males, and 41.3% were females. The most common primary tumor site was the extremities (41.3%), followed by mucosal origin (30.4%), trunk (15.2%), head and neck (10.9%), and unknown origin (2.2%).

Efficacy and Adverse Events (AEs) of Nivolumab 3 Months After First Administration

In patients with advanced melanoma, complete response (CR) was seen in 3 patients (6.5%; 95% confidence interval [CI], 0–13.0%), partial response (PR) was seen in 11 patients (23.9%; 95%CI, 0–47.8%), stable disease (SD) was seen in 13 patients (28.3%; 95%CI, 0–56.6%), and progressive disease (PD) was seen in 25 patients (41.3%; 95%CI, 0–82.6%). The objective response rate 3 months after first administration was thus 30.4% (95%CI, 0–60.8%). Tumor responses of individual patients are listed in **Table 1**. The incidence of AEs was 41.3% (Grade 4: 2.2%, Grade 3: 19.6%, Grade 2: 17.4%, Grade 1: 2.2%) (**Table 2**).

Serum Levels of CXCL5, CXCL10, and CCL22

To determine whether baseline serum levels of CXCL5, CXCL10, and CCL22 may be associated with early response in melanoma patients treated with nivolumab, their levels were evaluated in 46 patients with advanced melanoma treated using nivolumab. Increases in baseline serum CXCL5 and efficacy 3 months after the first administration of nivolumab in each patient are shown in **Table 1**. The threshold value of CXCL5 at baseline to distinguish responders from non-responders was 497.5 pg/ml. The sensitivity and specificity of the baseline serum CXCL5 in advanced melanoma were 70.6 and 69.0%, respectively ($p = 0.0016$; **Figure 1A**). High baseline serum levels of CXCL5 were correlated with objective response to nivolumab in patients with advanced melanoma (**Figure 1B**). On the other hand, there were no significant relationships between serum levels of CXCL10 (**Figure 2A**) and CCL22 (**Figure 3A**) and the objective response to nivolumab in patients with advanced melanoma (CXCL10: $p = 0.674$, CCL22: $p = 0.360$). The threshold values of CXCL10 and CCL22 at baseline to distinguish responders from non-responders were 336.8 and 619.5 pg/ml, respectively. There were no significant differences in serum CXCL10 and CCL22 levels in patients with objective response and non-responding patients (**Figures 2B**, **3B**). Baseline serum CXCL5, CXCL10, and CCL22 levels in each patient are shown in **Table 1**. There were no significant relationships between serum levels of CXCL5 ($p = 0.0703$), CXCL10 ($p = 0.1748$), and CCL22 ($p = 0.2207$) and irAEs in patients with nivolumab-treated advanced melanoma.

TABLE 2 | Immune-related adverse events in patients with cutaneous melanoma.

	Adverse events	Grade
1	N.A.	N.A.
2	N.A.	N.A.
3	N.A.	N.A.
4	Bursitis	3
5	Hypophisitis	4
6	Radiation dermatitis	3
7	N.A.	N.A.
8	Thyroid dysfunction	2
9	N.A.	N.A.
10	N.A.	N.A.
11	N.A.	N.A.
12	N.A.	N.A.
13	Thyroid dysfunction	2
14	Thyroid dysfunction	2
15	Psoriasiform dermatitis	3
16	N.A.	N.A.
17	CIDP	3
18	N.A.	N.A.
19	Psoriasiform dermatitis	3
20	N.A.	N.A.
21	N.A.	N.A.
22	N.A.	N.A.
23	N.A.	N.A.
24	N.A.	N.A.
25	Rheumarthritis	3
26	Hypophisitis	2
27	N.A.	N.A.
28	Diarrhea	2
29	Abdominal pain	2
30	Hypophisitis	1
31	N.A.	N.A.
32	Diarrhea	2
33	N.A.	N.A.
34	N.A.	N.A.
35	N.A.	N.A.
36	N.A.	N.A.
37	N.A.	N.A.
38	N.A.	N.A.
39	Diarrhea	2
40	N.A.	N.A.
41	N.A.	N.A.
42	N.A.	N.A.
43	Hypophisitis	3
44	IDDM	3
45	N.A.	N.A.
46	IDDM	3

CIDP, chronic inflammatory demyelinating polyneuropathy.
IDDM, insulin dependent diabetes mellitus.

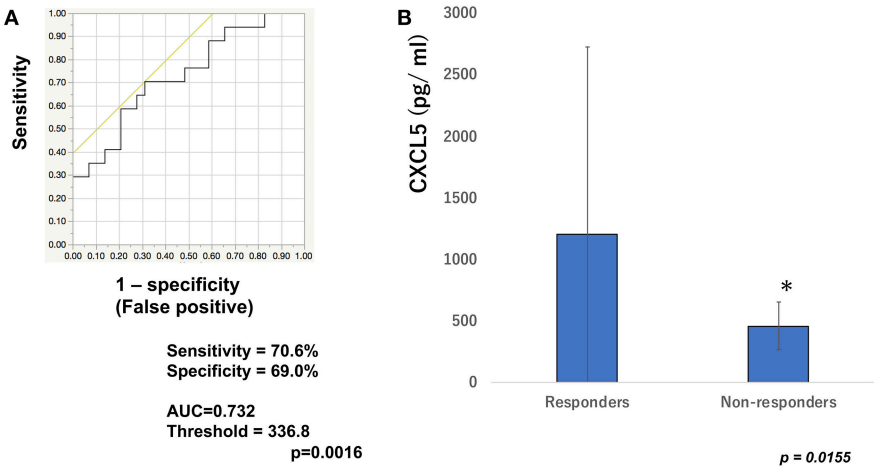

FIGURE 1 | Serum levels of CXCL5 and the ROC curve in melanoma. The ROC curve was used to calculate cut-offs for CXCL5 serum levels and the AUC. Cut-offs were determined to distinguish responders from non-responders using Youden's index **(A)**. Mean serum levels of CXCL5 in responders ($n = 16$) and non-responders ($n = 30$) at day 0 **(B)**. *$p < 0.05$ (n.s, not significant).

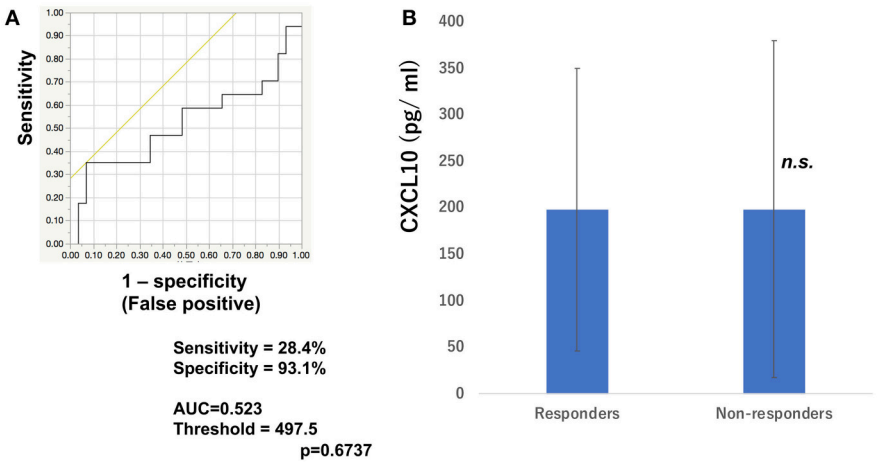

FIGURE 2 | Serum levels of CXCL10 and the ROC curve in melanoma. The ROC curve was used to calculate cut-offs for CXCL10 serum levels and the AUC. Cut-offs were determined to distinguish responders from non-responders using Youden's index **(A)**. Mean serum levels of CXCL10 in responders ($n = 16$) and non-responders ($n = 30$) at day 0 **(B)**. (n.s, not significant).

DISCUSSION

As previously reported, increased levels of soluble(s) CD163 at 6 weeks could predict the efficacy of nivolumab monotherapy 2–3 months after its first administration for the treatment of advanced cutaneous melanoma (24). Indeed, the sensitivity and specificity of serum sCD163 for the prediction of efficacy of nivolumab in cutaneous melanoma were 84.6 and 87.0%, respectively ($p = 0.0030$). Moreover, the absolute serum levels of sCD163 (baseline levels of sCD163 compared with day 42) were significantly increased in advanced melanoma patients who developed irAEs (24). This report concludes that the absolute serum levels of sCD163 are useful for the prediction of irAEs in melanoma patients, especially in combination with the absolute value of CXCL5 (25). Since serum sCD163 and CXCL5 are, at least in part, derived from CD163+ TAMs that are activated by periostin (24, 26), and chemokine profiles from TAMs are determined by the stimulation of stromal factors (27), spontaneously produced TAM-related factors could be detected in serum from melanoma patients (17, 25, 27). Notably, CD163+ M2 macrophages could be activated by periostin, leading to the production of characteristic chemokines, such as CXCL5, CXCL10, and CCL22, (28) that are correlated with recruitment of both immunosuppressive cells and immune-reactive anti-tumor cells (25). On the other hand, PD-1 expression is a key factor in maintaining TAMs as M2-polarized, and blockade of PD-1/PD-L1 leads to conversion of TAMs into

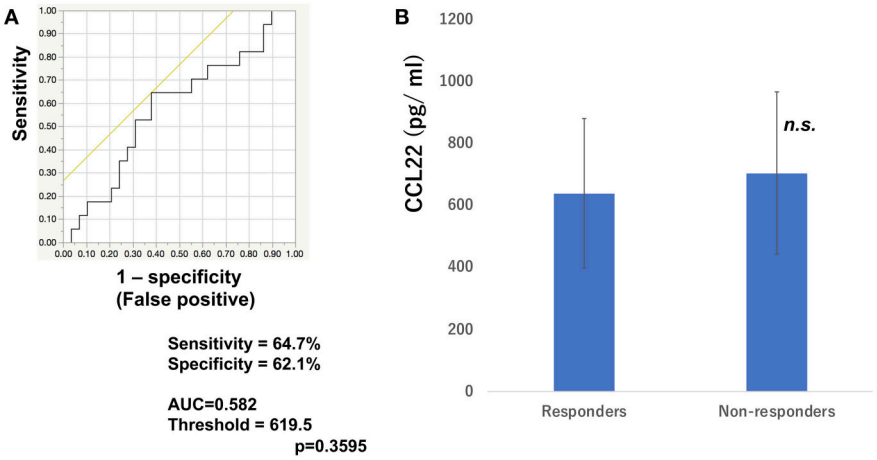

FIGURE 3 | Serum levels of CCL22 and the ROC curve in melanoma. The ROC curve was used to calculate cut-offs for CCL22 serum levels and the AUC. Cut-offs were determined to distinguish responders from non-responders using Youden's index **(A)**. Mean serum levels of CCL22 in responders ($n = 16$) and non-responders ($n = 30$) at day 0 **(B)**. (n.s, not significant).

M1-polarized activated macrophages (29). Since CD163+ TAMs are activated by anti-PD1 antibody (29), the TAM-related chemokines such as sCD163 and CXCL5 are important to evaluate the recruitment of anti-PD1 antibody in the tumor microenvironment.

From the above findings, in this report, we hypothesized that baseline serum levels of TAM-related chemokines, CXCL5, CXCL10, and CCL22, might be correlated with the efficacy of nivolumab in patients with advanced melanomas. To prove this hypothesis, serum levels of CXCL5, CXCL10, and CCL22 were analyzed in 46 cases of advanced melanoma treated with nivolumab. Baseline serum levels of CXCL5 were significantly increased in the response group compared to the non-response group in melanoma. In contrast, no significant differences in baseline serum levels of CXCL10 and CCL22 were seen between the nivolumab response and non-response groups. This discrepancy might be caused by the different sources of CXCL10 and CCL22, such as dendritic cells and endothelial cells that express lower levels of PD1 (29), leading to no effect of anti-PD1 antibody on the production of these chemokines in melanoma patients. Since CXCL5 is also reported as a biomarker for various T helper 17 cell-mediated autoimmune disorders (30–32), the high serum levels of CXCL5 might be correlated with the anti-tumor immune response of anti-PD1 antibody that could also induce autoimmune-like responses such as interstitial pneumonia, autoimmune-like colitis, and hepatitis (33). Taken together, CXCL5 may represent a predictive biomarker for evaluating the efficacy of nivolumab 3 months after its first administration for advanced melanoma. The present study suggested that CXCL5 may be a useful biomarker for the selection of those melanoma patients most likely to benefit from anti-melanoma immunotherapy using nivolumab and ipilimumab combined therapy. Because this was a pilot study, future independent studies with larger patient cohorts are needed to confirm the present findings.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The protocol for this human study was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan (Permit No: 2017-1-064). All methods were performed in accordance with the relevant guidelines and regulations. All patients provided their written, informed consent.

AUTHOR CONTRIBUTIONS

TFuj designed the research study. YS, TFuj, KT, CL, and YK gathered and analyzed the ELISA data. TFuj, YK, RA, AO, YF, KY, SM, HU, YY, HH, TFun, YN, RT, HO, NW and AH treated the patients and acquired the clinical data and samples. TFuj wrote the manuscript. TFuj and SA supervised the study.

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REVIEW
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Biomarkers for Immune Checkpoint Inhibitor-Mediated Tumor Response and Adverse Events

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In the last decade, inhibitors targeting immune checkpoint molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PD-1), and programmed cell death-ligand 1 (PD-L1) brought about a major paradigm shift in cancer treatment. These immune checkpoint inhibitors (ICIs) improved the overall survival of a variety of cancer such as malignant melanoma and non-small lung cancer. In addition, numerous clinical trials for additional indication of ICIs including adjuvant and neo-adjuvant therapies are also currently ongoing. Therefore, more and more patients will receive ICIs in the future. However, despite the improved outcome of the cancer treatment by ICIs, the efficacy remains still limited and tumor regression have not been obtained in many cancer patients. In addition, treatment with ICIs is also associated with substantial toxicities, described as immune-related adverse events (irAEs). Therefore, biomarkers to predict tumor response and occurrence of irAEs by the treatment with ICIs are required to avoid overtreatment of ICIs and minimize irAEs development. Whereas, numerous factors have been reported as potential biomarkers for tumor response to ICIs, factors for predicting irAE have been less reported. In this review, we show recent advances in the understanding of biomarkers for tumor response and occurrence of irAEs in cancer patients treated with ICIs.

Keywords: immune check point inhibitor, adverse event (AE), PD-1, CTLA- 4, tumor response

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INTRODUCTION

The recent development of immune checkpoint inhibitors (ICIs) has led to dramatic advances in cancer therapy. Ipilimumab is a monoclonal antibody to cytotoxic T-lymphocyte antigen 4 (CTLA-4), an inhibitory receptor expressed by both conventional and regulatory T cells (Tregs) and suppresses T cell activation by competing with CD28 to bind CD80/86. Ipilimumab not only activates conventional T cells at the initial stage of maturation but also may show antibody-dependent cell-mediated lysis of the Tregs that play a vital role in suppressing the antitumor immune response (1, 2). Programmed cell death 1 (PD-1) is an inhibitory receptor expressed mainly by activated T cells and its ligand, PD-L1, is widely expressed in cell types as diverse as epithelial cells, immune cells, and cancer cells. Both anti-PD-1 antibodies (nivolumab and pembrolizumab) and anti-PD-L1 antibodies (atezolizumab, durvalumab, and avelumab) exert antitumor effects by activating previously primed T cells which have lost effector and proliferative functions (3). ICIs firstly demonstrated efficacy for patients with advanced melanoma (4–6) and subsequently in other cancers, such as non-small cell lung cancer (NSCLC) and renal cell carcinoma (7–9). A recent clinical trial revealed that adjuvant therapies with anti-PD-1 antibodies prolonged

recurrence-free survival in resected high-risk melanoma (10–12). Moreover, there are currently ongoing trials for neoadjuvant therapies with anti-PD-1 antibodies in high risk resectable melanoma (11, 13). Numerous clinical trials testing additional indications of ICIs for other cancers are also ongoing (14, 15). Therefore, an ever-increasing number of patients will receive ICIs in the near future.

However, despite an improved overall survival (OS) with ICIs, the efficacy remains limited and tumor regression has not been universally achieved (16). In addition, use of ICIs may induce unique side effects, described as immune-related adverse events (irAEs). In a previous melanoma phase III clinical trial, patients who received nivolumab alone ($n = 313$), ipilimumab alone ($n = 311$) or nivolumab plus ipilimumab ($n = 313$) saw irAEs of grade 3 or 4 occurring at a rate of 21, 28, and 59%, respectively, and four patients died due to severe irAEs (16). Therefore, biomarkers to predict tumor response and irAE occurrence due to ICIs are necessary to gauge the benefits that each patient will obtain for avoiding overtreatment and minimizing irAEs. Here, we review recent advances in the understanding of biomarkers for tumor response and irAE occurrences.

Biomarkers for Tumor Response (Table 1)

Numerous factors have been reported as potential biomarkers for objective response rate (ORR), progression free survival (PFS) or OS. However, non-specific factors, which are associated with tumor responses to not only ICIs but also other therapies (such as traditional chemotherapies), can confound the use of these biomarkers. Therefore, specificity as well as correlative strength should be considered in choosing ICIs over other therapies.

Sex

Several studies have demonstrated that sex differences are associated with anti-tumor immune responses (70, 71). Although many clinical studies did not show a correlation between sex and tumor response to ICIs, meta-analyses with larger numbers of melanoma and NSCLC patients who were treated with ICIs revealed that both the PFS and OS of male patients were significantly longer than those of female patients (17). Based on a subtype analysis, sex differences in OS were greater in melanoma patients than NSCLC patients. In addition, in the anti-CTLA-4 antibodies group, the OS difference between male and female was greater than in the anti-PD-1 antibodies group. In line with this result, another study demonstrated that males were significantly associated with better ORR in melanoma patients treated with anti-PD-1 antibodies (18). Therefore, males seem to benefit more from ICIs than females do although the mechanism behind this effect has yet to be clarified.

Age

A recent preclinical study demonstrated that tumor response to anti-PD-1 antibodies in aged mice was significantly increased compared to younger mice, an effect attributed to the lower proportion of Tregs in aged mice (72). Consistent with these results, the tumor response to pembrolizumab in melanoma patients over age 60 was significantly higher than those under 60 years and the likelihood of response increased with age (72). Similarly, Nosrati et al showed that ages older than 65 years correlated with better ORR in melanoma patients treated with anti-PD-1 antibodies (18). However, opposite results have also been reported and a meta-analysis by Nishijima et al revealed a correlation between ages younger than 75 years with better ORR in patients treated with ICIs (19). Therefore, further studies are needed to evaluate the usefulness of age as a biomarker for ICI response.

Tumor Size

Huang et al. reported that reinvigoration of exhausted CD8 T cells (T_{ex}-cell) positively correlated with tumor size and the ratio of T_{ex}-cell reinvigoration to tumor size was significantly associated with better ORR and longer OS in melanoma patients treated with pembrolizumab (73), indicating that tumor size is a predictive factor for poor response to ICI treatments. Indeed, another study demonstrated that tumor size was independently associated with OS in melanoma patients treated with pembrolizumab although it was associated with many other clinical factors (20). Therefore, early detection of metastatic lesions may be important for better response to ICIs.

Immune Cell Infiltration

Because ICIs activate the immune response to cancer, infiltration of immune cells, including T cells, into tumors may induce tumor regression following treatment. Generally, higher numbers of tumor infiltrating lymphocytes (TILs) have been a favorable prognostic factor in many types of cancers, such as melanoma and colorectal cancer (74, 75). Similarly, Tumeh et al revealed that presence of $CD8^+$ TILs at the invasive margin, which was associated with higher PD-1/PD-L1 expression, correlated with better tumor response in melanoma patients treated with pembrolizumab (21). An increase in $CD8^+$ TILs from baseline to post-treatment biopsy, specifically at the tumor center and invasive margin, has been also significantly associated with tumor regression (21). Therefore, both baseline and post-treatment TIL numbers may be important biomarkers for predicting tumor response to ICIs.

Surface Molecules and Their Related Molecules

PD-L1

Since PD-L1 is a ligand of PD-1 and serves an inhibitory signal in PD-1 expressing cells, the expression of PD-L1 in tumor environments is speculated to correlate with better response in patients treated with anti-PD-1 antibodies. Indeed, in melanoma clinical trials with anti-PD-1 antibodies, better outcomes were observed in patients with positive PD-L1 expression in tumors although the definition of positive or negative expression differed across studies (22, 23). Higher PD-L1 expression has also been associated with better outcomes in NSCLC patients treated with anti-PD-1 antibodies (24). In addition, a recent clinical trial demonstrated that combinations of nivolumab with ipilimumab showed a better OS than nivolumab monotherapy in melanoma patients with PD-L1<1%, whereas the OS was comparable between the 2 treatment groups in patients with PD-L1≥1%, suggesting that anti-PD-1 antibody efficacy is largely dependent

TABLE 1 | Biomarkers for tumor responses.

Biomarkers	Cancer type	Patient number	Treatment	Key data and clinical significance	References	Evidence level
Sex	Melanoma, NSCLC	6,096	Ipilimumab, anti-PD-1 antibodies	PFS and OS of male patients were significantly longer than those of female patients.	Wu et al. (17)	1a
Age	Melanoma	315	Anti-PD-1 antibodies	Males were significantly associated with better ORR. Ages older than 65 years correlated with better ORR.	Nosrati et al. (18)	2b
	Melanoma, prostate cancer, NSCLC, RCC	5,265	Anti-CTLA-4 antibodies, anti-PD-1 antibodies	Ages younger than 75 years correlated with better ORR.	Nishijima et al. (19)	1a
Tumor size	Melanoma	459	Pembrolizumab	Tumor size was independently associated with OS, suggesting that early detection of metastatic lesions may be important for better response to ICIs.	Joseph et al. (20)	2b
TILs	Melanoma	46	Pembrolizumab	High density of $CD8^+$ TILs at the invasive margin correlated with better tumor response. An increase in $CD8^+$ TILs from baseline to post-treatment was associated with tumor regression.	Tumeh et al. (21)	2b
PD-L1 expression in tumors	Melanoma	277	Nivolumab after treatment with anti-CTLA-4 antibodies	Better ORR were observed in patients with positive PD-L1 expression in tumors.	Weber et al. (22)	1b
	Melanoma	451	Pembrolizumab	Better PFS and OS were observed in patients with positive PD-L1 expression in tumors.	Daud et al. (23)	2b
	NSCLC	410	Pembrolizumab + chemotherapy	Higher PD-L1 expression was associated with better PFS and OS.	Gandhi et al. (24)	1b
ICOS	Melanoma	14	Ipilimumab	Increased expression of ICOS on $CD4^+$ T cells that is sustained for more than 12 weeks correlated with improved OS.	Carthon et al. (25)	4
TIM-3	Melanoma	67	Ipilimumab	Increased TIM-3 expression on circulating T and NK cells prior to and during treatment was associated with shorter OS.	Tallerico et al. (26)	2b
IDO	Melanoma	82	Ipilimumab	Baseline IDO expression in tumor tissue assessed by IHC correlated with better ORR.	Hamid et al. (27)	1b
	NSCLC	26	Nivolumab	IDO activity as assessed by serum kynurenine/tryptophan ratio was negatively associated with longer PFS and OS.	Botticelli et al. (28)	2b
Soluble CTLA-4	Melanoma	113	Ipilimumab	Higher serum levels of soluble CTLA-4 at baseline had both better ORR and OS.	Pistillo et al. (29)	2b
Soluble PD-L1	Melanoma	446	Ipilimumab, anti-PD-1 antibodies	Higher levels of baseline soluble PD-L1 were associated with worse response. Increases in soluble PD-1 after treatment was associated with favorable clinical responses.	Zhou et al. (30)	2b
	NSCLC	39	Nivolumab	Higher levels of baseline soluble PD-L1 were associated with shorter OS.	Okuma et al. (31)	2b
Soluble CD163	Melanoma	59	Nivolumab	Serum levels of soluble CD163 were increased after 6 weeks in responders compared to non-responders after initial treatment for cutaneous melanoma.	Fujimura et al. (32)	2b
Soluble NKG2D	Melanoma	194	Anti-CTLA-4 antibodies, anti-PD-1 antibodies	Higher levels of circulating soluble ULBP-1, soluble ULBP-2 and LDH at baseline were independent factors of shorter OS.	Maccalli et al. (33)	2b

(Continued)

TABLE 1 | Continued

Biomarkers	Cancer type	Patient number	Treatment	Key data and clinical significance	References	Evidence level
IFN-γ	Melanoma	45	Ipilimumab	The post-treatment expression levels of IFN-γ responsive genes in tumor tissues were associated with longer OS.	Ji et al. (34)	2b
	NSCLC	97	Durvalumab	High levels of pre-treatment IFN-γ expression and its related genes in tumor tissues were associated with longer OS.	Higgs et al. (35)	2b
	Melanoma	43	Atezolizumab	High expression of IFN-γ and CXCL-9 was associated with better ORR.	Herbst et al. (36)	2b
TNF-α	Melanoma	15	Nivolumab	Patients who showed complete remission, partial remission or long-term stable disease due to nivolumab response had lower serum levels of TNF-α compared to non-responders.	Tanaka et al (37)	4
Lymphocyte counts	Melanoma	209	Ipilimumab	Higher levels of relative lymphocyte counts at baseline were associated with longer OS.	Martens et al. (38)	2b
	Melanoma	50	Ipilimumab	Absolute lymphocyte counts after treatment were associated with longer OS.	Wilgenhof et al. (39)	2b
	Melanoma	98	Nivolumab	Absolute lymphocyte counts after treatment correlated with better OS.	Nakamura et al. (40)	2b
Eosinophil counts	Melanoma	209	Ipilimumab	High absolute and relative eosinophil counts at baseline were associated with a longer OS.	Martens et al. (38)	2b
	Melanoma	616	Pembrolizumab	Relative eosinophil counts at baseline were an independent factor for longer OS and better ORR.	Weide et al. (41)	2b
	Melanoma	59	Ipilimumab	Early increases in absolute eosinophil counts from baseline during treatment were an independent factor for better responses.	Gebhardt et al. (42)	2b
NLR	Melanoma	90	Nivolumab	NLR was associated with poor tumor response.	Fujisawa et al. (43)	2b
	NSCLC	175	Nivolumab	NLR was associated with poor tumor response.	Bagley et al. (44)	2b
	Melanoma	44	Anti-PD-1 antibodies	NLR was the only factor associated with both poor ORR and shorter PFS.	Nakmaura et al. (45)	2b
Tregs	Melanoma	209	Ipilimumab	High levels of circulating Tregs at baseline were associated with longer OS.	Martens et al. (38)	2b
	Melanoma	95	Ipilimumab	Decreasing levels of circulating Tregs were associated with better responses.	Simeone et al. (46)	2b
MDSC	Melanoma	92	Ipilimumab	The baseline frequency of MDSCs in blood correlated with shorter OS.	Weber et al. (47)	2b
	Melanoma	83	Nivolumab	The baseline frequency of MDSCs in blood correlated with shorter OS.	Kitano et al. (48)	2b
	Prostate cancer	28	Ipilimumab plus a cancer vaccine	The baseline frequency of circulating MDSCs correlated with shorter OS.	Santegoets et al. (49)	2b
LDH	Melanoma	73	Ipilimumab	High baseline LDH was associated with poor anti-tumor response.	Delyon et al. (50)	2b
CRP	Melanoma	95	Ipilimumab	A decrease or no change in serum levels of CRP from baseline was associated with longer OS.	Simeone et al. (46)	2b
Mutation burden	Melanoma	64	Ipilimumab	High mutation burden was associated with a longer OS.	Synder et al. (51)	2b
	Melanoma	150	Ipilimumab	High mutation burden was associated with tumor responses.	Allen et al. (52)	2b
MSI	Colorectal cancer	74	Nivolumab	A high response to anti-PD-1 antibodies in colorectal cancer with high levels of MSI compared to traditional treatments was observed.	Overman et al. (53)	2b
HLA	Melanoma	13	Nivolumab	HLA-A expression in pre-treatment was elevated in responders compared to non-responders.	Inoue et al. (54)	4

(Continued)

TABLE 1 | Continued

Biomarkers	Cancer type	Patient number	Treatment	Key data and clinical significance	References	Evidence level
	Melanoma	69	Nivolumab	HLA-A26 correlated with tumor response to nivolumab in Japanese melanoma patients.	Ishida et al. (55)	2b
T cell repertoire	Melanoma	12	Ipilimumab	Both higher richness and evenness in pre-treatment peripheral blood were associated with a better response.	Postow et al. (56)	4
	Melanoma	46	Pembrolizumab	TILs with less diversity were associated with clinical response.	Tumeh et al. (21)	2b
Gut microbiome	Melanoma	26	Ipilimumab	Patients whose baseline microbiota was enriched with *Faecalibacterium* genus and other Firmicutes showed a longer PFS and OS than those whose baseline microbiota was enriched with *Bacteroides*.	Chaput et al. (57)	2b
	Melanoma	43	Anti-PD-1 antibodies	A higher diversity of gut microbiome and relative abundance of *Ruminococcaceae* family bacteria correlated with better ORR and longer PFS.	Gopalakrishnan et al. (58)	2b
	NSCLC, RCC	100	Anti-PD-1 antibodies	The relative abundance of *Akkermansia muciniphilia* was associated with better responses.	Routy et al. (59)	2b
ctDNA	Melanoma	76	Anti-PD-1 antibodies	Patients with a persistently elevated cDNA during the treatment showed a worse response and shorter PFS and OS. ctDNA may be a useful marker for differentiating pseudoprogression from true progression during immune checkpoint inhibitor treatment.	Lee et al. (60, 61)	2b
Exosomal molecules	Melanoma	44	Pembrolizumab	Lower baseline levels and increases during the treatment in exosomal PD-L1 protein correlated with tumor response.	Chen et al. (62)	2b
	Melanoma, NSCLC	26	Anti-PD-1 antibodies	Baseline exosomal PD-L1 mRNA expression was higher in responders, and exosomal PD-L1 mRNA expression in responders was decreased after treatment whereas it was stable in stabilized patients and increased in progressive disease cases.	Re et al. (63)	2b
	Melanoma	59	Ipilimumab	Increased exosomal PD-1 and CD28 levels in T cells were associated with longer PFS and OS while increased exosomal CD80 and CD86 in dendritic cells correlated with longer PFS.	Tucci et al. (64)	2b
irAE development	RCC	40	Ipilimumab	Overall irAEs were associated with tumor responses.	Yang et al. (65)	2b
	NSCLC	43	Nivolumab	Early development of all irAEs was associated with better ORR and longer PFS.	Teraoka et al. (66)	2b
	NSCLC, RCC, HNSCC, urothelial carcinoma	142	Anti-PD-1 antibodies	Only low grade irAEs were associated with better responses.	Judo et al. (67)	2b
	Melanoma	60	Ipilimumab after nivolumab	Occurrences of endocrine irAEs were associated with longer OS.	Fujisawa et al. (68)	2b
	Melanoma	5,737	Anti-CTLA-4 antibodies, anti-PD-1 antibodies	Development of vitiligo correlated with better responses.	Teuling et al. (69)	2a

NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; HNSCC, head and neck squamous cell carcinoma; ORR, overall response rate; PFS, progression free survival; OS, overall survival; TILs, tumor infiltrating lymphocytes; ICOS, inducible T cell costimulatory; IDO, indoleamine 2,3-dioxygenase; NLR, neutrophil-to-lymphocyte ratio; Tregs, regulatory T cells; MDSC, myeloid-derived suppressor cells; MSI, microsatellite instability; HLA, human leukocyte antigen; ctDNA, circulating tumor DNA; irAE, immune-related adverse event. Evidence level was evaluated based on the following criteria; 1a, systematic review/ meta-analysis of randomized controlled trials; 1b, individual randomized controlled trials; 2a, systematic review/ meta-analysis of cohort studies; 2b, individual cohort study; 3a, systematic review/meta-analysis of case-control studies; 3b, individual case-control studies; 4, case series; 5, expert opinions.

on PD-L1 expression (16). Therefore, PD-L1 expression may be a vital factor to predict tumor response to anti-PD-1 antibodies although tumor responses can be also observed in PD-L1 negative tumors. However, issues remain for accurately assessing PD-L1 expression, including different antibodies used in each study, and the low reproducibility of pathologist evaluations (76). In addition, PD-L1 expression has been reported to vary between primary tumors and metastatic sites (77). Therefore, establishing evaluative standards for tumor PD-L1 expression will enhance its usefulness as a predictive factor.

Inducible T Cell Co-stimulator (ICOS)

ICOS is a co-stimulating molecule expressed by activated conventional T cells and regulatory T cells. A previous report demonstrated that ipilimumab treatment increases expression of ICOS on conventional $CD4^+$ T cells in both blood and tumor tissue in patients with bladder cancer (78). These $CD4^+$ $ICOS^+$ T cells produced IFNγ and could recognize tumor antigens (78). In addition, increased expression of ICOS on $CD4^+$ T cells that is sustained for more than 12 weeks has been reported to correlate with improved survival in melanoma patients treated with ipilimumab (25). Thus, ICOS expression is a potential biomarker for tumor response to ICIs although further studies are needed to establish its utility.

Other Cell Surface Molecules

Pre-clinical studies using mouse models have indicated that upregulation of alternative inhibitory molecules causes resistance to anti-PD-1 antibody therapy (79). These molecules include TIM-3, LAG-3, and VISTA are therefore suggested to serve as potential target molecules for alternative checkpoint inhibitors. They could also serve as potential biomarkers for ICI response and, indeed, increased TIM-3 expression on circulating T and NK cells prior to and during treatment has been significantly associated with shorter OS in melanoma patients treated with ipilimumab (26).

Enzymes Related to Immune Response

Indoleamine 2,3-dioxygenase (IDO)

IDO is an enzyme that converts the essential amino acid l-tryptophan into kynurenine and carries an immunosuppressive effect through multiple mechanisms (80). Kynurenine, mediated by IDO, has been shown to induce T cell apoptosis (81), and IDO-induced starvation of tryptophan mediates the conversion of naïve $CD4^+$ T cells into Tregs through GCN2 kinase activation (82). A recent study demonstrated that IDO expression levels in melanoma cells were independently associated with tumor stage (83). IDO has been also reported as a predictor of anti-tumor response by ICIs. Hamid et al showed that baseline IDO expression, as well as baseline FoxP3 expression, in tumor tissue assessed by IHC significantly correlated with better ORR in melanoma patients treated with ipilimumab (27). However, on the contrary, IDO activity as assessed by serum kynurenine/tryptophan ratio has been negatively associated with longer PFS and OS in NSCLC patients treated with nivolumab (28). Therefore, through as-yet unknown mechanisms, IDO activity may serve as a predictive marker for outcomes, which are different dependent on the assessment.

Soluble Isoform of Surface Molecules

Soluble CTLA-4 (sCTLA-4)

Soluble CTLA-4 originates from a spliced variant of an alternative transcript that lacks the transmembrane sequence (84). It can be detected in normal human serum and higher levels of sCTLA-4 have been observed in autoimmune diseases and many types of cancers (84, 85). It can bind to CD80/86 on antigen-presenting cells and block the binding of membrane-bound CTLA-4 or CD28 on T cells, thus avoiding the downregulation of the immune activation cascade (86, 87). Pistill et al. demonstrated that higher serum levels of sCTLA-4 (>200 pg/ml) at baseline had both better ORR and OS than lower sCTLA-4 serum levels (≤200 pg/ml) in melanoma patients treated with ipilimumab (29), suggesting that serum sCTLA-4 could be a biomarker for better response to ipilimumab. It is speculated that sCTLA-4 might block the binding of membrane-bound CTLA-4 to its ligand and thus result in enhanced tumor immunity in synergy with ipilimumab.

Soluble PD-L1 (sPD-L1)

Soluble PD-L1 may result from alternative variants of the PD-L1 transcripts and cytokine treatment with IFN-α, IFN-γ, or TNF-α has been shown to increase secretion of sPD-L1 as well as expression of cell surface PD-L1 in melanoma cell lines (30). It can be detected in blood and elevated levels of circulating sPD-L1 have been associated with poor prognoses in many types of cancer (88–90). Consistent with these results, higher levels of baseline sPD-L1 have been significantly associated with worse response and shorter OS in melanoma patients treated with ICIs (30, 31). Therefore, baseline sPD-L1 could represent an immune suppressive state and poor response to ICIs although the function of sPD-L1 is not fully understood. In contrast, increases in sPD-1 after treatment with ICIs have been associated with favorable clinical responses (30). Secretion of sPD-1 after ICI treatment may be caused, at least partially, by enhanced production of cytokines such as IFN-α, IFN-γ, or TNF-α due to ICI-mediated anti-tumor response because altered levels of sPD-1 after treatment of ipilimumab corresponded to changes in the circulating cytokines (30).

Soluble CD163 (sCD163)

CD163 is a member of the scavenger receptor family and is mainly expressed by macrophages/monocytes (91). Several reports have shown that $CD163^+$ M2 macrophages comprised the main population of the tumor-associated macrophages (TAMs) that play important roles for suppressing anti-tumor immune responses and serum levels of sCD163, generated by proteolytic shedding, is thought to be a marker for TAMs (91, 92). Fujimura et al. reported that serum levels of sCD163 were significantly increased after 6 weeks in responders compared to non-responders after initial treatment with nivolumab for cutaneous melanoma (32). Interestingly, such an increase was not observed in patients with mucosal melanoma although the mechanism for this phenomenon remains unclear. These results

suggest that sCD163 may serve as a biomarker for patients with specific types of cancer treated with ICIs.

Soluble NKG2D Ligands (sNKG2DLs)

NKG2D is a member of the C-type lectin-like receptors and is expressed on T, NK, and NKT cells (93, 94). The binding of NKG2D with its ligands [MHC class I chain-related gene [MIC] and UL-16-binding protein [ULBP]] elicits activation signals to NK and T cells (93–95). NKG2DLs are usually absent on the surface of normal cells but are induced by various stressors (such as DNA damage) and are often overexpressed by cancer cells (93, 95, 96). Soluble NKG2DLs, generated as result of proteolytic shedding by tumor cells, can be detected in serum and their levels have been reported to correlate with tumor progression (94, 95, 97, 98). Soluble NKG2DLs suppress anti-tumor immune responses through multiple mechanisms that include the binding and subsequent endocytosis and degradation of NKG2D on NK and T cells (97, 99, 100). A multivariate analysis conducted by Maccalli et al. showed that higher levels of circulating sULBP-1, sULBP-2, and LDH at baseline were independent factors of shorter OS in melanoma patients treated with ICIs (33). Interestingly, only LDH, but not sNKG2DLs, significantly correlated with outcomes in patients treated with other therapies, such as chemotherapies and BRAF inhibitors (33), suggesting that soluble circulating sULBP-1 and sULBP-2 may be indicators that use of ICIs is more suitable than other therapies.

Cytokines and Chemokines

IFN-γ

IFN-γ is a functionally pleiotropic cytokine that modulates the expression of numerous proteins in exposed cells (101). PD-L1 expression is also upregulated mainly controlled by IFN-γ (102). IFN-γ and its targets play crucial roles in eliminating tumor cells through direct induction of cytotoxic activities as well as enhancing the Th1-related immune response (101). The post-treatment expression levels of IFN-γ responsive genes in tumor tissues were associated with better outcomes in patients treated with ipilimumab (34). Similarly, high levels of pre-treatment IFN-γ expression and its related genes in tumor tissues are associated with longer OS in NSCLC patients treated with durvalumab (35). Similar associations between high expression of IFN-γ and CXCL-9, an IFN-γ related chemokine, with better ORR was observed in melanoma patients treated with atezolizumab (36). Therefore, high expression of IFN-γ and its associated molecules in tumor tissues may be useful biomarkers that are indicative of a better anti-tumor response to ICIs.

TNF-α

TNF-α is an inflammatory cytokine produced by various cells, including immune cells and epithelial cells. It promotes tumor growth and higher serum levels of TNF-α have been reported to be associated with poor prognoses in cancer patients (103, 104). Tanaka et al. reported that melanoma patients who showed complete remission, partial remission or long-term stable disease due to nivolumab response had significantly lower serum levels of TNF-α compared to non-responders (37).

IL-6

IL-6 is produced by a broad variety of cells, including immune cell and tumor cells. It promotes tumor progression via inhibition of cancer cell apoptosis as well as promotion of angiogenesis (105). In a previous study, higher serum IL-6 was associated with shorter OS in melanoma patients treated with IL-2-based immunotherapy (106). Although association of serum IL-6 with response to ICIs has yet to be shown, CRP, whose production is mainly controlled by IL-6 (107), has been reported to be predictive of outcomes in patients treated with ICIs, which bolsters the argument of IL-6 as a potential biomarker of anti-tumor response during ICI treatment.

Blood Cell Counts

Lymphocyte Counts

Because both CTLA-4 and PD-1 are expressed mainly on lymphocytes, several reports have pointed out the association between blood lymphocyte count and tumor response to ICIs (38–40). Martens et al showed that higher levels of relative lymphocyte counts at baseline were significantly associated with longer OS in melanoma patients treated with ipilimumab (38). In another study, absolute lymphocyte counts after 2 doses of ipilimumab were associated with longer OS in melanoma patients (39). Similarly, Nakamura et al showed that absolute lymphocyte counts at week 3 and 6 after the initial administration of nivolumab significantly correlated with better OS in melanoma patients (40). These results suggest that lymphocyte counts both at baseline and after treatment with ICIs may be useful for predicting better outcomes.

Eosinophil Counts

Eosinophils also play a crucial role in tumor destruction and recruitment of T cells into the tumor environment (108). Indeed, mice with peripheral blood eosinophilia showed substantial tumor suppression (109). In addition, multiple studies have revealed a positive correlation between increased eosinophil infiltration into tumor tissues and a favorable prognosis in many cancers (110, 111). Consistent with this idea, numerous previous studies have reported that higher blood eosinophil counts correlate with favorable outcomes in patients treated with ICIs. Marten et al. demonstrated that in melanoma patients treated with ipilimumab, high absolute, and relative eosinophil counts at baseline were associated with a longer OS (38). Similarly, a multivariate study by Weide et al. demonstrated that relative eosinophil counts at baseline were an independent factor for longer OS and better ORR in melanoma patients treated with pembrolizumab (41). In addition, Gebhardt et al. reported that early increases in absolute eosinophil counts from baseline during ipilimumab treatment were an independent factor for better responses in melanoma patients (42). Therefore, eosinophil counts at both baseline and after ICI treatment may serve as biomarkers for better tumor response.

Neutrophil-to-Lymphocyte Ratio (NLR)

Fujisawa et al. showed that baseline NLR was associated with poor tumor response in melanoma patients treated with nivolumab (43). Similar findings have also been reported in

melanoma patients treated with ipilimumab and NSLC patients treated with nivolumab (41, 44). In a previously published study, our multivariate analysis revealed that NLR was the only factor associated with both poor ORR and shorter PFS in melanoma patients treated with anti-PD-1 antibodies, suggesting that NLR is a strong predictive factor for poor outcome in patients treated with ICIs (45). Given that lymphocytes play vital roles in the ICI-induced immune response to tumors while neutrophilia represents the response to systemic inflammation (112), a high NLR might represent an impaired specific immune response to tumors. However, increased turnover of tumor cells causes the release of large amounts of damage-associated molecular patterns (DAMPs) from tumor debris, leading to recruitment and activation of neutrophils (113, 114). Moreover, numerous reports have also shown that NLR serves as a biomarker for poor response to other treatments, such as chemotherapies and radiation (115, 116). Therefore, the NLR might simply represent rapidly expanding tumor cell populations rather than any potential immune response mediated by ICIs.

Tregs

Tregs, a population characterized by $FoxP3^+$ $CD25^+$ $CD4^+$ T cells, significantly suppress immune responses (117), and it has been shown that their depletion effectively eradicates tumor cells via an enhanced anti-tumor immune response (118, 119). In addition to their immune suppressive function, they may be a target for antibody dependent cellular cytotoxicity (ADCC) by ipilimumab due to their high expression levels of CTLA-4 that make Tregs sentinels for ICI-mediated anti-tumor responses. Indeed, high levels of circulating Tregs at baseline have been associated with longer OS in melanoma patients treated with ipilimumab (38). In addition, decreasing or stable levels of circulating Tregs 12 weeks after initial administration of ipilimumab significantly correlated with better disease control and longer OS than increasing Treg levels. Furthermore, similar results have been obtained in another study, with decreasing levels of circulating Tregs significantly associated with better responses to ipilimumab (46). Therefore, circulating Tregs both at baseline and after treatment with ipilimumab may be useful biomarkers for anti-tumor response.

Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are a heterogeneous population of myeloid origin characterized by a failure to differentiate into granulocytes, macrophages or dendritic cells (120). They expand in tumor environments and strongly suppress the activity of immune cells, including T cells, through a variety of mechanisms such as NO production and arginase-1 overexpression. Both of these processes lead to cell cycle arrest and downregulation of the T cell receptor (120). MDSCs are defined as $Lin^-CD14^+HLA^-DR^{-/low}$ (120) and clinical and experimental studies have shown that high infiltration of these cells into tumor tissues are associated with poor prognosis and resistance to therapies (121, 122). MDSCs can also be detected in the blood and several studies have demonstrated that the baseline frequency of MDSCs in blood significantly correlates with shorter OS in melanoma patients treated with ipilimumab or nivolumab (47, 48). Furthermore, in prostate cancer patients treated with ipilimumab plus a cancer vaccine, the baseline frequency of circulating MDSCs correlated with a shorter OS (49). These results suggest that the frequency of blood MDSCs also serves as a useful biomarker for ICI response.

Serum Markers

Lactate Dehydrogenase (LDH)

Generally, baseline serum LDH is an independent factor for poor prognosis in patients with advanced melanoma (123). The same applies to cases of ICI treatment and numerous reports have demonstrated that high baseline LDH was associated with poor anti-tumor response in various cancer patients who received ICI treatment (50, 124, 125). This poor outcome may simply be caused by increased turnover of tumor cells which enhances LDH release in similar fashion to a high NLR.

CRP

CRP is produced by hepatocytes and serum levels of it elevate quickly in response to most inflammation (such as bacterial infections). However, CRP does not usually increase during ICI-mediated tumor regression. Simeone et al. reported that a decrease or no change in serum levels of CRP from baseline were significantly associated with longer OS in melanoma patients treated with ipilimumab (46). Therefore, elevated CRP from baseline may indicate inflammation by tumor progression or irAE rather than an antitumor immune response from ICI treatment.

Genomic Mutations

Mutation Burden

Mutation burden, the number of mutations within a tumor genome, is different among and within the cancer types (126). Overall, multiple studies have shown that a high mutation burden was associated with a better response to ICIs (51, 52). This mechanism is not fully understood but an increased number of neoantigens (potential tumor-specific T cell targets) generated by a high mutation burden is thought to cause an enhanced response to ICIs (127). As for melanoma, our study demonstrated that acral lentiginous melanoma (ALM) and mucosal melanoma (MCM), both common types of melanoma in Asians, were less susceptible to immune checkpoint inhibitors than superficial spreading melanoma (SSM) and lentigo maligna melanoma, both major types of Caucasian melanoma (128). This may be explained, at least in part, by the lower mutation burden in ALM and MCM (129). Despite the poor ICI-mediated antitumor response in ALM patients, our retrospective study demonstrated that use of ICIs significantly improved OS in not only SSM but also ALM patients (128).

Microsatellite Instability

Mutation or silencing of mismatch repair genes, which causes deficient mismatch repair (dMMR), leads to accumulation of multiple mutations and microsatellite instability (MSI). Zhang et al. reported that the immune microenvironment in colorectal cancer differs between dMMR tumors and proficient mismatch

repair (pMMR) tumors (130). The number of $CD8^+$ TIL, PD-1^+ TIL and IDO^+ tumor cells was increased in tumors with dMMR compared to those with pMMR, suggesting that dMMR is indicative of exhausted T-cell-rich environments (130). It has been reported that colon cancer with dMMR frequently shows larger tumors with poorer differentiation (131). In addition, previous studies revealed that patients with dMMR had both a poorer response to conventional chemotherapies and shorter OS than patients with pMMR in many types of cancer (132, 133). However, due to the high mutation burden, several clinical trials revealed a high response to anti-PD-1 antibodies in colorectal cancer with dMMR or high levels of MSI (MSI-H) compared to traditional treatments (53), suggesting that dMMR serves as useful indicator for choosing ICIs over other therapies. Recently, a durable response was observed in patients with dMMR or MSI-H across five clinical trials treated with pembrolizmab (KEYNOTE-016, 164, 012, 028, 158). The cancer types included colorectal, endometrial, biliary, gastric, esophageal, pancreatic and breast cancers. Based on these results, the United States Food and Drug Administration approved pembrolizumab for the treatment of any unresetable or metastatic solid tumors that display dMMR or MSI-H. A combination of nivolumab with ipilimumab was also shown to effect a promising response to dMMR/ MSI-H colorectal cancer (134).

Human Leukocyte Antigen (HLA)

HLA encodes cell surface molecules which present antigenic peptides to the T-cell receptor (TCR) on T cells. Inoue et al reported that mRNA expression of HLA-A in pre-treatment melanoma was elevated in responders to nivolumab compared to non-responders (54). There are numerous variant alleles at the HLA loci which differ in each individual and Ishida et al reported that HLA-A26, which is relatively common in Japanese but rare in Caucasians, correlated with tumor response to nivolumab in Japanese melanoma patients (55).

T Cell Receptor (TCR) Repertoire

Since the TCR determines T cell specificity with respect to tumor cells, the TCR repertoire may be predictive of the ICI-induced anti-tumor immune response. As diversity of the repertoire is increased, the likelihood of a specific immune response to tumor cells is speculated to be elevated (56). A previous study showed that both higher richness and evenness in pre-treatment peripheral blood are associated with a better response to ipilimumab in melanoma patients (56). On the other hand, Tumeh et al. showed that TILs with less diversity were significantly associated with clinical response to pembrolizumab in melanoma patients (21). It is speculated that TILs with less diversity contain a higher proportion of tumor-specific T cells, and therefore, the anti-tumor response was enhanced by ICIs. In this study, a TIL clone population expanded more than 10 times in responders than non-responders after treatment with pembrolizumab (21), revealing that both diversity and clonal expansion of T cells may predict ICI response although this indication may differ between blood and tumor tissues.

Gut Microbiome

Emergent evidence has suggested that the gut microbiome plays crucial roles for the immune response of not only intestinal diseases but also other disorders, including various type of cancers (135). Sivan et al. reported that, in mice, commensal Bifidobacterium enhanced the response to anti-PD-1 antibodies through an augmented dendritic cell function (136). Several studies have also demonstrated that distinct gut microbiota were associated with ICI response in humans. Melanoma patients whose baseline microbiota was enriched with *Faecalibacterium* genus and other Firmicutes showed a longer PFS and OS than those whose baseline microbiota was enriched with *Bacteroides* upon ipilimumab treatment (57). In addition, Gopalakrishnan et al. reported that a higher diversity of gut microbiome and relative abundance of *Ruminococcaceae* family bacteria before starting anti-PD-1 antibodies in melanoma patients correlated with better ORR and longer PFS (58). Moreover, Routy et al. showed that dysbiosis by administration of antibiotics inhibited ICI response in both mice and humans (59). This study also revealed a correlation between clinical responses and the relative abundance of *Akkermansia muciniphilia*. They also showed that transplantation of *Akkermansia muciniphilia* into mice enhanced the efficacy of PD-1 antibodies in an IL-12 dependent manner (59). Therefore, gut microbiota may have important implications for the immune response to ICIs.

Liquid Biopsy

Circulating Tumor DNA (ctDNA)

Tumor-derived, fragmented DNA in blood is known as ctDNA, and its precise mechanism of release remains unclear but it has been postulated that it involves a passive release from dying cells and active release from living cells (137–139). It is associated with tumor burden (140), and high levels of ctDNA are an indicator of poor prognoses in patients with various types of cancer (141). Lee et al. demonstrated that melanoma patients with a persistently elevated cDNA during the treatment of anti-PD-1 antibodies show a worse response and shorter PFS and OS (60). In addition, it has been reported that, of nine melanoma patients treated with anti-PD-1 antibodies who showed pseudoprogression (defined as a tumor size increase prior to response often seen in ICI treatment), all patients had a favorable ctDNA profile defined by undetectable ctDNA at baseline or detactable ctDNA at baseline followed by >10-fold decreases (61). In contrast, in 20 patients with true progression, all but two had an unfavorable ctDNA profile defined by detectable ctDNA at baseline that remained stable or increased. These results indicate that ctDNA is a useful marker for differentiating pseudoprogression from true progression during ICI treatment.

In addition, the mutation burden of ctDNA has been also assessed and, in line with the correlation of a high mutation burden in tumor tissues, hyper-mutated ctDNA has also been associated with improved OS in patients with diverse cancers who received ICIs (142).

Exosomes

Exosomes are microvesicles actively released from various cells, including cancer cells, and contain proteins, RNA and DNA

(63). Exosomes isolated from the plasma of cancer patients contains various immune-related proteins, including PD-1, PD-L1, and CTLA-4, with PD-L1 in exosomes showing a suppressive effect on T cell activities by signaling via PD-1 (62, 143). Similar to the correlation between circulating sPD-L1 and response to ICIs, lower baseline levels, as well as increases, in exosomal PD-L1 protein have been correlated with response to pembrolizumab in melanoma patients (62). However, opposite results were observed in the association of exosomal PD-L1 mRNA expression with response to anti-PD-1 antibodies in patients with melanoma or NSCLS (63). Baseline exosomal PD-L1 mRNA expression was higher in responders compared to non-responders and exosomal PD-L1 mRNA expression in responders was significantly decreased after treatment whereas it was stable in stabilized patients and significantly increased in progressive disease cases (63). Therefore, although the mechanism is unknown, PD-L1 proteins and transcript in the exosome may provide conflicting information on ICI response.

As for other molecules, Tucci et al. recently evaluated the circulating exosomal proteins in T cells and dendritic cells in melanoma patients treated with ipilimumab (64). They demonstrated that increased exosomal PD-1 and CD28 levels in T cells were significantly associated with longer PFS and OS while increased exosomal CD80 and CD86 in dendritic cells correlated with longer PFS (64). Such exosomal proteins may reflect potential T cell/dendritic cell activities and thus lead to predictions of ICI response.

irAE Development

Since ICIs may cause both irAEs and tumor regression through an augmented immune response, several reports have shown associations between the two events. Overall irAEs have been associated with regression of metastatic renal cell carcinoma or melanoma treated with ipilimumab (65, 144). In addition, the presence of overall irAEs was significantly associated with longer OS in melanoma patients treated with nivolumab (145). And moreover, early development of all irAEs has been associated with better ORR and longer PFS in NSCLC patients treated with nivolumab (66). However, other studies failed to show such correlations (67, 68, 146). A multivariate analysis conducted by Judo et al showed that only low grade irAEs, but not high grade irAEs, are associated with better responses to anti-PD-1 antibodies in non-melanoma patients (67). Therefore, only certain irAEs might be associated with tumor regression by ICIs. As for irAEs in each organ, several reports showed correlations between endocrine irAEs and better prognoses. Fujisawa et al. demonstrated that occurrences of endocrine irAEs were associated with longer OS in melanoma patients treated with ipilimumab after nivolumab (68). Similarly, an adjusted analysis by Kim et al. showed that development of thyroid dysfunction was significantly associated with longer PFS and OS in NSCLC patients treated with anti-PD-1 antibodies (147), suggesting that endocrine irAEs may be representative of the potential immune reaction to tumor cells. In a similar fashion, multiple studies showed that development of vitiligo correlated with better responses to ICIs in melanoma patients; this may represent a common immune response against antigens shared by melanocytes and melanomas (69, 148). Although ICIs may cause vitiligo in patients with other cancer such as NSCLC and renal cell carcinoma (149, 150), associations with outcomes in such cases remain unclear. Several studies showed that skin irAEs, except for vitiligo, were also associated with better outcome in various types of cancer (145, 148). However, Fujisawa et al. reported conflicted findings that occurrences of skin irAEs, excluding vitiligo, correlate with a shorter OS in melanoma patients treated with ipilimumab after nivolumab (68). Since skin irAEs include various types of skin disorders, such as prurigo-like eruptions, psoriasiform dermatitis and lichenoid reactions, associations with outcomes may be different for each skin irAE.

Biomarkers of irAEs (Table 2)

The aforementioned irAEs can be induced by all ICIs. However, among the ICIs, both the frequency and the severity are highest in treatment with ipilimumab (161). Severe irAEs (grade $\geq$3) have occurred in 28–56% and 21–32% in patients treated with ipilimumab or anti-PD-1/anti-PD-L1 antibodies, respectively (10, 12, 16, 162, 163). In the combined treatment of ipilimumab plus nivolumab, much higher rates of severe irAEs are observed (16, 164). The organ most affected by irAEs is the skin followed by the gastrointestinal tract, respiratory tract and endocrine organs. A recent meta-analysis revealed that colitis, hypophysitis and rash were more frequent with anti-CTLA-4 antibodies whereas pneumonitis, hypothyroidism, arthralgia, and vitiligo were more common with anti-PD-1 antibodies (165). Most of these irAEs occur within 3–6 months from the initiation of ICI treatment (166–168). Given that most are mild and reversible if they are detected early and properly managed, biomarkers for predicting the occurrence of irAEs are essential. Compared with biomarkers for tumor response, those for irAEs have been less thoroughly investigated and some of the reported biomarkers for irAE overlap with those for tumor responses.

Body Composition Parameters

Previous reports revealed that sarcopenia was associated with poorer treatment tolerance and increased likelihood of adverse events by various chemotherapies (169, 170). In addition, low muscle attenuation (MA), which refers to increased intramuscular adipose tissue, has been associated with shorter survival in a wide variety of cancers such as melanoma and renal cell carcinoma (171, 172). Daly et al evaluated association of these body composition parameters by computer tomography with occurrences of irAEs in melanoma patients treated with ipilimumab. The multivariate analysis in this study showed that both sarcopenia and low MA were independent factors significantly associated with high-grade irAEs (151). Although the exact mechanism is unknown, many studies suggest that sarcopenia and low MA increase susceptibility to systemic inflammation (173, 174), and this may play a role in the higher frequency of severe irAEs.

Sex

Although males have been associated with a more favorable response to ICIs, a study in melanoma patients treated with ipilimumab by Valpione et al reported that females

TABLE 2 | Biomarkers for irAEs.

Biomarkers	Cancer type	Patient number	Treatment	Key data and clinical significance	References	Evidence level
Body composition parameters	Melanoma	84	Ipilimumab	Both sarcopenia and low MA were independent factors associated with high-grade irAEs.	Daly et al. (151)	2b
Sex	Melanoma	140	Ipilimumab	Females were associated with higher rates of irAEs.	Valpoine et al. (152)	2b
IL-6				IL-6 at baseline was negatively associated with irAE.		
	Melanoma	26	Ipilimumab	Lower circulating IL-6 was significantly correlated with higher incidences of colitis-related irAEs.	Chaput et al.(57)	2b
	Melanoma	15	Nivolumab	Increases in circulating IL-6 after treatment were significantly associated with development of irAEs.	Tanaka et al. (37)	4
IL-17	Melanoma	35	Ipilimumab	Circulating IL-17 levels at baseline correlated with the incidence of grade 3 irAEs of diarrhea/colitis, indicating that increased levels of circulating IL-17 may be reflective of patients with subclinical colitis.	Tarhini et al. (153)	2b
Soluble CD163, CXCL5	Melanoma	46	Nivolumab	The absolute change rate of soluble CD163 and CXCL5 after initial treatment was increased in patients with irAEs compared to those without irAEs.	Fujimura et al. (154)	2b
Blood cell counts	Melanoma, RCC, urothelial carcinom	167	Anti-PD-1 antibodies	Absolute lymphocyte and eosinophil numbers at baseline and 1 month after initial treatment were independent factors associated with a higher incidence of irAEs of grade $\geq$2.	Diehi et al. (155)	2b
	Melanoma	44	Anti-PD-1 antibodies	Both baseline absolute eosinophil count and relative eosinophil count at 1 month significantly correlate with the occurrence of endocrine irAEs.	Nakamura et al. (45)	2b
	Melanoma	101	Nivolumab	An increase in total WBC count and a decrease in relative lymphocyte count plus increase in relative neutrophil count on the same day of, or just prior to irAE occurrence were associated with development of lung or gastrointestinal irAEs.	Fujisawa et al. (156)	2b
autoantibodies	Melanoma, NSCLC	168	Nivolumab	TSH and TPOAb were associated with higher incidence of thyroid irAEs.	Kimbara et al. (157)	2b
	Solid cancer including melanoma, NSCLC, RCC	27	Anti-PD-1 antibodies, atezolizumab	Patients positive for type 1 diabetes antibodies at the time of presentation developed diabetes-related irAEs after fewer cycles than those without autoantibodies.	Stamatouli et al. (158)	2b
T cell repertoire	Prostate cancer	42	Ipilimumab plus granulocyte-monocyte colony-stimulating factor	An early increase in diversity and the generation of new T- cell clones correlated with the development of irAEs.	Oh et al. (159)	2b
Gut microbiome	Melanoma	26	Ipilimumab	Patients whose baseline microbiota was enriched with the *Faecalibaterium* genus and other Firmicutes showed a higher incidence of colitis-related irAEs.	Chaput et al. (57)	2b
	Melanoma	34	Ipilimumab	Increased representation of bacteria belonging to the *Bacteroidetes phylum* was associated with resistance to development of ipilimumab-induced colitis.	Dubin et al. (160)	2b

NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; irAE, immune-related adverse event; WBC, white blood cell; TPOAb, antithyroid peroxidase antibodies (TPOAb). Evidence level was evaluated based on the following criteria; 1a, systematic review/ meta-analysis of randomized controlled trials; 1b, individual randomized controlled trials; 2a, systematic review/meta-analysis of cohort studies; 2b, individual cohort study; 3a, systematic review/meta-analysis of case-control studies; 3b, individual case-control studies; 4, case series; 5, expert opinions.

were associated with higher rates of irAEs (152). Sex-specific factors, including hormones, play important roles in the immune response, and it is well-known that females are at a higher risk of several autoimmune diseases (175). Therefore, immune reactions to self-tissues mediated by female-specific factors may lead to an increased likelihood of irAEs.

Serum Factors

IL-6

Similar to the correlation with poor tumor response, it has been reported that circulating IL-6 at baseline was negatively associated with irAE occurrence in melanoma patients treated with ipilimumab (152). Another study showed that lower circulating IL-6, as well as IL-8, was significantly correlated with higher incidences of colitis-related irAEs (57). This may be explained by the immunosuppressive effects of IL-6 in certain conditions, including the induction of MDSC (176–178). In contrast, Tanaka et al. assessed the fluctuation of multiple cytokines in melanoma patients treated with nivolumab and showed that increases in circulating IL-6 after treatment were significantly associated with development of irAEs (37). These results indicate that both lower baseline IL-6 and increase after ICI treatment may serve as predictive markers for irAE occurrence.

IL-17

IL-17 is a cytokine with a variety of inflammatory effects, including the recruitment of neutrophils, and it is well-known that circulating IL-17 levels are increased in patients with inflammatory bowel disease (179). Tarhini et al. assessed candidate circulating factors which were associated with irAEs in melanoma patients treated with ipilimumab as a neoadjuvant therapy and revealed that circulating IL-17 levels at baseline significantly correlated with the incidence of grade 3 irAEs of diarrhea/colitis (153). This indicates that increased levels of circulating IL-17 may be reflective of patients with subclinical colitis, the development of which would be normally inhibited by CTLA-4.

Soluble CD163 (sCD163) and CXCL5

Circulating levels of sCD163, which is derived from macrophages, increase in various autoimmune disorders, including rheumatoid arthritis and pemphigus vulgaris, and are reflective of their activities (176, 180). CXCL5 is a chemokine which can attract $CXCR2^+$ myeloid cells and can be produced by $CD163^+$ macrophages. It is also known to be a biomarker for several autoimmune disorders (176, 181). Fujimura et al evaluated circulating sCD163 and CXCL5 levels at baseline and day 42 after initial treatment with nivolumab in melanoma patients (154), showing that the sCD163 absolute change rate was significantly increased in patients with irAEs compared to those without irAEs. Although there were no significant differences, the absolute change rate of CXCL5 also tended to be higher in patients with irAEs, suggesting that absolute changes within sCD163 and CXCL5 levels after ICI treatment could serve as possible biomarkers for irAE development.

Blood Cells

Since both T cells and eosinophils are crucial for cellular immunity, blood cell counts of these cells may also be correlated with irAE development. A multivariate analysis conducted by Diehi et al. demonstrated that, in solid tumor patients (including melanoma, renal cell carcinoma, and urothelial carcinoma) treated with anti-PD-1 antibodies, absolute lymphocyte and eosinophil numbers at baseline and 1 month after initial treatment were independent factors that were significantly associated with a higher incidence of irAEs of grade $\geq$2 (155). In addition, our study demonstrated that both baseline absolute eosinophil count and relative eosinophil count at 1 month significantly correlate with the occurrence of endocrine irAEs in melanoma patients treated with anti-PD-1 antibodies (45). Therefore, circulating lymphocyte and eosinophil numbers may predict not only tumor responses but also the occurrence of ICI-mediated irAEs.

In contrast, Fujisawa et al. investigated fluctuations in blood cell count on the same day of, or just prior to irAE occurrence, in melanoma patients treated with nivolumab (156). Univariate analyses revealed that increases in total white blood cell (WBC) count and decreases in relative lymphocyte count from baseline were associated with severe irAEs of grade $\geq$3 although multivariate analyses failed to show independence. They also analyzed the correlation with irAEs of each organ and found that the same factors, namely an increase in total WBC count and a decrease in relative lymphocyte count plus increase in relative neutrophil count, were significantly associated with development of lung or gastrointestinal irAEs. This could be caused by neutrophil-dominant infiltration into the affected organs since DAMPs from severely damaged cells promote neutrophil recruitment (182). Indeed, active colitis in patients treated with ipilimumab saw severe neutrophil infiltration into the lamina propria (183), indicating that these factors may be useful for predicting irAEs that are currently developing or may soon develop.

Autoantibodies

Detection of autoantibodies is speculated to predict development of irAEs related to the autoantibodies (184). Kimbara et al. assessed TSH, free T3, free T4, antithyroid peroxidase antibodies (TPOAb) and antithyroglobulin antibodies at baseline in patients with solid tumors treated with nivolumab and multivariate analyses revealed that TSH and TPOAb were significantly associated with higher incidence of thyroid irAEs (157). Stamatouli et al. measured diabetes autoantibodies (glutamic and decarboxylase 65 antibodies, islet antigen 2 antibodies, and insulin autoantibodies) in solid cancer patients treated with anti-PD-1 or anti-PD-L1 antibodies, and found that patients positive for type 1 diabetes antibodies at the time of presentation developed diabetes-related irAEs after fewer cycles than those without autoantibodies (158). They also measured autoantibodies prior to treatment in three patients, and one was already positive,

indicating that autoantibodies may be useful to predict their related irAEs.

T Cell Repertoire

The T cell repertoire has been reported to correlate with irAEs as well as tumor response. Oh et al assessed the repertoire of circulating T cells in patients with metastatic castration-resistant prostate cancer treated with a combination of ipilimumab and granulocyte-monocyte colony-stimulating factor (159). They found that initial broadening in the repertoire occurred within 2 weeks of treatment, which significantly preceded irAEs onset, and an early increase in diversity and the generation of new clones were correlated with the development of irAEs. These results suggest that increased T cell diversity in response to ICI treatment could be a sign of immune response to normal tissues as well as tumor tissues.

Gut Microbiome

It is suggested that inflammatory bowel diseases (IBD) may result from a loss of tolerance to commensal bacteria and dysbiosis is a well-known factor that is significantly involved in the pathogenesis of IBD (185). Gut microbiota have been also reported as predictive of colitis-related irAEs. Melanoma patients treated with ipilimumab whose baseline microbiota was enriched with the *Faecalibaterium* genus and other Firmicutes showed a higher incidence of colitis-related irAEs although they were also associated with better outcomes (57). In contrast, this study showed no occurrences of colitis irAEs in any patients with *Bacteroidetes* (57). Similarly, Dubin et al. demonstrated that increased representation of bacteria belonging to the *Bacteroidetes phylum* was associated with resistance to development of ipilimumab-induced colitis (160).

Tumor Type

A recent meta-analysis demonstrated that the frequency of each type of irAE depends on cancer type (165). Melanoma patients had a higher frequency of skin and gastrointestinal irAEs but a lower frequency of pneumonia compared with NSCLC patients (165). In addition, dermatitis, arthritis and myalgia were more frequent in melanoma patients than in renal cell carcinoma patients whereas pneumonitis and dyspnea were found to be less common in melanoma cases (165). Although the precise mechanism remains unclear, induced immune responses to antigens of normal tissue shared with or cross-reactive with those of each cancer may be an explanation.

CONCLUSION

Although numerous predictive biomarkers for tumor response and irAEs during ICI treatment have been identified, there are no absolutely predictive biomarkers as yet. Therefore, multiple biomarkers should be taken into consideration in choosing or quitting ICI treatments. Because immune reactions induced by ICIs are quite complex and many factors are involved, identifying new biomarkers will provide mechanistic insights into the ways how ICIs modulate the anti-tumor response and irAEs in specific patients, as well as lead to the development of novel treatments to target the identified biomarkers.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Novel and Future Therapeutic Drugs for Advanced Mycosis Fungoides and Sézary Syndrome

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Mycosis fungoides (MF) and Sézary syndrome (SS) are the most common subtypes of cutaneous T-cell lymphoma. The majority of MF cases present with only patches and plaques and the lesions are usually limited to the skin. On the other hand, in some cases, patients show skin tumors or erythroderma followed by lymph node involvement and rarely visceral organ involvement. SS is a rare, aggressive cutaneous T-cell lymphoma marked by exfoliative erythroderma, lymphadenopathy, and leukemic blood involvement. Because patients with relapsed or refractory MF/SS display a poor prognosis and the current treatment options are characterized by high rates of relapse, there is unmet need for the efficient treatment. This review provides a discussion of the recent and future promising therapeutic approaches in the management of advanced MF/SS. These include mogamulizumab, brentuximab vedotin, alemtuzumab, immune checkpoint inhibitors, IPH4102 (anti-KIR3DL2 antibody), histone deacetylase inhibitors (vorinostat, romidepsin, panobinostat, belinostat, and resminostat), pralatrexate, forodesine, denileukin diftitox, duvelisib, lenalidomide, and everolimus.

Keywords: mycosis fungoides, Sézary syndrome, peripheral T-cell lymphoma, clinical trial, novel therapeutic agents

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INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) comprises a clinically/pathologically heterogeneous group of uncommon non-Hodgkin's lymphomas that manifest primarily in the skin. Mycosis fungoides (MF) is the most common CTCL subtype that accounts for around 60% of CTCL (1). MF is generally an indolent lymphoma with slow progression over years or even decades. Typically, the initial lesions in MF are flat and erythematous skin patches, which evolve over a variable period of time into palpable plaques characterized by well-demarcated edges. In limited cases, plaques can be followed by tumors and those patients have patch, plaque, and tumor lesions simultaneously on different parts of the body. In some cases, skin lesions develop into erythroderma similar to Sézary syndrome (SS). In MF cases with tumors or erythroderma (advanced MF), lymph node or visceral involvement is sometimes observed and such cases present a poor prognosis. SS is a much rarer variant, accounting for only 3% of CTCL (1). Characteristics of SS are generalized erythroderma (defined as affecting > 80% of total body surface area), lymphadenopathy, and presence of circulating tumor cells in the blood. Progression of SS is usually more rapid compared to that of MF.

Although MF and SS are classified as distinct, separate entities, the same clinical staging system and therapeutic approaches have been used (1, 2). Patients with MF having limited T1 stage (limited patches, papules, and/or plaques covering < 10% of the skin surface) have a similar life expectancy to that of control populations (3). In addition, patients with early stage MF (stage I and IIA) have a good prognosis (a median survival: 15.8 years or more), while patients with advanced stage MF/SS (stage IIB or more) have a poor prognosis (a median survival: 4.7 years or less) (3). Current treatment consists of skin-directed therapies, such as topical corticosteroid, topical mechlorethamine, topical bexarotene, ultraviolet phototherapy, total skin electron beam therapy, and localized radiotherapy (2), for early stage disease and systemic therapies for advanced stage. For early stage MF confined to the skin, therapeutic concept is to control symptoms by skin-directed therapies with the lowest possible therapy-related side effects, as durable remissions cannot be achieved by early aggressive chemotherapy (4). For advanced stages of MF and SS, there is a variety of systemic therapies available, some of which are used from decades ago and some recently. However, currently available drug therapies are not curative treatment and the only option for curing MF/SS is stem cell transplantation (5).

As MS/SS have the chronic and recurrent nature, repeated treatment courses and maintenance regimens are necessary for disease control. Although there are available active systemic therapeutic strategies, including cytotoxic chemotherapy and biological therapy, better treatments of advanced stage and refractory MF/SS are desired by both patients and physicians. Purpose of the present paper is to review the clinical results obtained in clinical trials of novel currently used and future promising therapies for advanced MF/SS patients (**Table 1**).

MOGAMULIZUMAB

C-C chemokine receptor 4 (CCR4) is the receptor for thymus and activation-regulated chemokine and macrophage-derived chemokine and is involved in skin trafficking of type 2 helper T cells and regulatory T cells. CCR4 is also consistently expressed on the surface of tumor cells in T-cell malignancies, such as CTCL, including MF and SS, adult T-cell leukemia-lymphoma, and peripheral T-cell lymphoma (PTCL) (30–33). Mogamulizumab is a humanized IgG1 κ monoclonal antibody with a defucosylated Fc region, which selectively binds to CCR4. The antibody exerts its antitumor activity by antibody-dependent cellular cytotoxicity (34). First, mogamulizumab has been approved in Japan for relapsed or refractory $CCR4^+$ adult T-cell leukemia-lymphoma (2012), PTCL (2014), and CTCL (2014) (35).

Before the approval of mogamulizumab in Japan, seven patients with MF had been enrolled in a multicenter phase 2 study for patients with relapsed PTCL and CTCL in Japan (6). Intravenous infusions of 1.0 mg/kg mogamulizumab were administered to patients once per week for 8 weeks. The overall response rate (ORR) for MF patients was 28.6% [all partial response (PR) with no complete response (CR)]. A phase 1/2 study was also conducted for 38 patients with pretreated CTCL (MF and SS) in USA. Mogamulizumab was administered once weekly for 4 weeks using an escalation scheme (0.1 mg/kg and subsequent doses of 0.3 and 1.0 mg/kg) followed by 1.0 mg/kg every 2 weeks until disease progression or withdrawal. The ORR was 36.8% (CR 7.9% and PR 28.9%). Mogamulizumab was more effective for patients with SS than those with MF; ORR was 47.1% in SS ($n = 17$) and 28.6% in MF ($n = 21$). Eighteen of 19 (94.7%) patients with blood involvement had a response in blood, including 11 CRs (7). In an international, open-label, randomized, controlled phase 3 trial in patients with relapsed or refractory MF/SS (MAVORIC study), mogamulizumab (1.0 mg/kg once weekly for 4 weeks followed by every 2 weeks) significantly showed the high ORR and prolonged progression free survival (PFS) compared with 400 mg/day vorinostat (8). The ORR of mogamulizumab was 28% (21% in MF and 37% in SS), while the ORR of vorinostat was 4% (8). The median PFS was 7.7 months for the mogamulizumab group, compared with 3.1 months for vorinostat. Compartment response rates were 78/186 (42%) in skin, 83/122 (68%) in blood, 21/124 (17%) in lymph nodes, and 0/3 (0%) in viscera, suggesting that mogamulizumab is effective especially for blood involvement. In all studies, mogamulizumab showed an acceptable safety profile and common toxicities included nausea, chills, headache, fever, diarrhea, pruritus, and infusion reactions. Based on these results, mogamulizumab was approved for the treatment of patients with CTCL who have received at least 1 prior systemic therapy by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2018.

BRENTUXIMAB VEDOTIN

CD30 is a cell membrane protein that belongs to the tumor necrosis factor receptor family. CD30 was originally discovered on Reed-Sternberg cells of Hodgkin's lymphoma, and its expression was subsequently demonstrated on subsets of non-Hodgkin lymphoproliferative disorders, notably systemic, and primary cutaneous anaplastic large T-cell lymphoma (ALCL) and lymphomatoid papulosis. CD30 is also expressed on tumor cells of some MF/SS cases at various levels, and cases with large cell transformation frequently show higher expression. Brentuximab vedotin (BV) is an antibody-drug conjugate composed of the cytotoxic antitubulin agent monomethyl auristatin E (MMAE) and a chimeric monoclonal anti-CD30 antibody (36). After BV binds to CD30, the antibody-drug conjugate is internalized, and the antibody is cleaved by the lysosome, leading to the intracellular release of MMAE (37). MMAE inhibits tubulin

Abbreviations: CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma; MF, mycosis fungoides; SS, Sézary syndrome; ALCL, anaplastic large cell lymphoma; AE, adverse event; ORR, overall response rate; CR, complete response; PR, partial response; SD, stable disease; PFS, progression free survival; OS, overall survival; DOR, duration of response; CCR4, C-C chemokine receptor 4; HDAC, histone deacetylase; PNP, purine nucleoside phosphorylase; MMAE, monomethyl auristatin E; IL-2R, IL-2 receptor; PI3K, Phosphoinositide-3-kinase; FDA, Food and Drug Administration; EMA, European Medicines Agency; CTLA-4, cytotoxic T lymphocyte-associated protein 4; PD-1, programmed cell death protein 1; mTOR, mammalian target of rapamycin.

TABLE 1 | Summary of the results of clinical trials of single-agents in cutaneous T-cell lymphoma or peripheral T-cell lymphoma including a given number of mycosis fungoides or Sézary syndrome patients.

	Ref	Phase	Subtypes†	Number of patients‡	ORR, %	CRR, %	Median DOR	PFS	Approval year§		
									FDA	EMA	PMDA (Japan)
Mogamulizumab	(6)	2	MF/pcALCL	8(7)	37.5	0	ND	ND	2018	2018	2014
	(7)	1/2	MF/SS	38	36.8	7.9	10.4 months	50% at 11.4 months			
	(8)	3	MF/SS	186	28	3	14.1 months	50% at 7.7 months			
Brentuximab vedotin	(9)	2	MF/SS	30	70	3	ND	54% at 12 months	2017	2017	-
	(10)	3	$CD30^+$ MF	28	54	7	8 months	ND			
	(11)	3	$CD30^+$ MF	48	65	10	15.1 months	50% at 16.7 months			
Alemtuzumab	(12)	2	MF/SS	22	55	32	ND	ND	-	-	-
Nivolumab	(13)	2	MF				Ongoing		-	-	-
Pembrolizumab	(14)	2	MF/SS				Ongoing		-	-	-
IPH4102	(15)	1	MF/SS				Ongoing		-	-	-
	(16)	1	SS				Ongoing				
Vorinostat	(17)	2	MF/SS	74	29.7	0	6 months or more	ND	2007	2004 (orphan), 2009 withdrawn	2011
	(18)	2	MF/SS	33	24.2	0	3.8 months	50% at 3 months			
Romidepsin	(19)	2	MF/SS	71	33	7	13.7 months	ND	2009	2005 (orphan), 2012 refused (PTCL)	2018 (PTCL)
	(20)	2	MF/SS	96	34	6	15 months	ND			
Panobinostat	(21)	2	MF/SS	139	17.3	1.4	ND	ND	-	-	-
Belinostat	(22)	2	MF/SS/other CTCL#	29 (23)	13.8	10.3	3 months	ND	2014 (PTCL)	2012 (orphan, PTCL)	-
Pralatrexate	(24)	2	MF	109	58	16.7	4.4 months	50% at 5.3 months	2009 (PTCL)	2007 (orphan), 2012 refused (PTCL)	2018 (PTCL)
Forodesine	(25)	2	MF/SS	144	16	1	8.7 months	ND	-	2007 (orphan), 2012 refused (PTCL)	2018 (PTCL)
Denileukin diftitox	(26)	3	MF/SS/other CTCL#	100 (91)	44	10	7.8 months	50% at 26.5 months	1999	2001	-
Duvelisib	(27)	1	MF/SS/pcALCL	19 (9)	31.6	0	ND	50% at 4.5 months	-	-	-
Lenalidomide	(28)	2	MF/SS				Ongoing		-	-	-
Everolimus	(29)	2	MF				Ongoing		-	-	-

† *When data regarding patients with MF/SS is separable in the original paper, data on MF/SS patients is shown. When inseparable, data on CTCL patients is shown.*

‡ *When data regarding patients with MF/SS is inseparable in the original paper, the number of patients with MF/SS is shown in parentheses.*

§ *When the drug was approved or refused not for CTCL but for PTCL, the comment "(PTCL)" is added. When the drug was approved as orphan drug from EMA, the comment "(orphan)" is added.*

Other CTCL includes pcALCL, peripheral T-cell lymphoma, not otherwise specified, and subcutaneous panniculitis-like T-cell lymphoma.

Ref, reference; ORR, overall response rate; CRR, complete response rate; DOR, duration of response; PFS, progression free survival; FDA, food and drug administration; EMA, European medicines agency; PMDA, pharmaceuticals and medical devices agency; MF, mycosis fungoides; pcALCL, primary cutaneous anaplastic large cell lymphoma; ND, not described; SS, Sézary syndrome; PTCL, peripheral T-cell lymphoma; CTCL, cutaneous T-cell lymphoma.

polymerization and consequently disrupts the microtubule network within the cells causing cell cycle arrest and apoptosis. In addition, a small fraction of MMAE is released from $CD30^+$ cells, killing neighboring cells in the tumor microenvironment in a CD30-independent manner (36, 37). BV has received regulatory approval in more than 65 countries for the treatment of relapsed or refractory Hodgkin's lymphoma and systemic ALCL (38).

The results of two phase 2 studies of BV for $CD30^+$ CTCL including MF/SS were reported in 2015. In one phase 2 trial of 30 evaluable patients with pretreated $CD30^+$ MF/SS by Kim et al, the patients received up to 16 cycles of BV (1.8 mg/kg) every 3 weeks. The ORR was observed in 21 (70%) of 30 patients (CR in one patient and PR in 20 patients), and patients with CD30 expression <5% exhibited a decreased probability of response compared with patients with CD30 expression >5%. (9). In the other trial of BV for 48 pretreated patients with primary cutaneous $CD30^+$ lymphoproliferative disorders, 28 patients with $CD30^+$ MF were included (10). BV was administered intravenously at 1.8 mg/kg every 3 weeks for a maximum of eight doses. The ORR in MF patients was 54% with CR in two cases and the response was independent of CD30 expression. Based on these promising results, the international randomized phase 3 trial (ALCANZA study) for pretreated $CD30^+$ CTCL (MF or primary cutaneous ALCL) had been conducted recently to compare BV against the chosen standard therapy by physicians (methotrexate or bexarotene). In this clinical trial, included cases expressed the CD30 molecule on at least 10% of the skin infiltrate BV (1.8 mg/kg every 3 weeks) and methotrexate (5–50 mg weekly) or bexarotene (300 mg/m^2 daily) were administered until disease progression or the development of major toxicity. Among the enrolled patients, 97 patients with MF were included. Forty-eight patients were treated with BV and the remaining 49 patients were treated with methotrexate or bexarotene. The ORR lasting at least 4 months was increased in the BV cohort compared with the physician's choice cohort (50 vs. 10%). Five patients achieved CR with BV, while methotrexate or bexarotene failed to achieve CR in any patient. After a median follow-up time of 17.5 months, the median PFS was 15.9 months for patients in the BV cohort and 3.5 months for patients in the methotrexate or bexarotene cohort (11). Peripheral neuropathy was the most frequent adverse event (AE) and was observed in 67% of patients undergoing treatment with BV. After a median 22.9 months of follow-up, 82% of patients with peripheral neuropathy experienced improvement or resolution. Other common side effects reported during the study included nausea, diarrhea, vomiting, alopecia, itching, fever, and loss of appetite. These data suggested that BV can be a preferable treatment option for the treatment of MF when biopsy samples have 10% or more $CD30^+$ malignant cells. In 2017, FDA and EMA approved BV for the treatment of adult patients with $CD30^+$ MF who have received prior systemic therapy.

ALEMTUZUMAB

CD52 is a small glycopeptide composed of 12 aminoacids expressed on the cell surface of several different types of leukocytes, including normal and malignant T lymphocytes. Alemtuzumab is a humanized IgG1 antibody that targets the CD52 antigen. The phase 2 study of alemtuzumab in patients with advanced MF/SS who did not respond adequately to treatment with at least PUVA, radiotherapy, or chemotherapy, showed that the ORR was 55% with 32% CR and 23% PR (12). The effect was better on erythrodermic patients (69% ORR with 38% CR) than on patients with plaques or tumors (40% ORR with 30% CR). In that study, alemtuzumab was administered using escalating doses (5, 10, 30 mg intravenously on days 1–3) and then 30 mg/day three times a week for up to 12 weeks. Because AEs of alemtuzumab such as infusion reaction, hematologic toxicity, and infectious complications were severe, clinical trials of low-dose alemtuzumab were performed for CTCL. In 14 patients with SS treated with subcutaneous low-dose alemtuzumab (3 mg on day 1, 10 mg on day 3, then 15 mg on alternating days or 3 mg on day 1, then 10 mg on alternating days), the ORR was 85.7% with 21.4% CR and 64.3% PR (39). Infectious episodes were observed only in patients treated with 15 mg alemtuzumab. These studies suggest that low-dose alemtuzumab can be an effective treatment for erythrodermic MF/SS with acceptable safety. Consistently, a recent report on 23 patients with leukemic involvement treated with low-dose alemtuzumab (10 mg subcutaneously, three times a week) described that 13 of 17 patients presented with erythroderma showed CR and that the remaining 4 patients could be controlled by following skin-directed therapy alone. In contrast, CR was not achieved in any patient with discrete patches, plaques, or tumors (40).

IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint molecules, such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), act as negative regulators that inhibit normal T-cell responses to avoid the emergence of pathological self-reactivity. On the other hand, cancers occasionally have the capacity to avoid anti-tumor immunity by abusing such immune checkpoint molecules. Thus, immune checkpoint inhibitors can antagonize the immunosuppressive interaction between the tumor cells and T cells and improve antitumor immune T-cell responses. In recent years, the efficacy of immune checkpoint inhibitors blocking the CTLA-4 and PD-1 pathways has been confirmed by several clinical trials in a variety of cancers. PD-1-blocking antibodies (nivolumab and pembrolizumab) and CTLA-4-blocking antibody (ipilimumab) achieved durable objective responses and improved OS in patients with solid tumors (23, 41–44) and hematologic malignancies, including Hodgkin's lymphoma (45). Concerning hematological malignancies, in 2016, nivolumab was approved for the treatment of patients with classical Hodgkin lymphoma that has relapsed or progressed after autologous hematopoietic stem cell transplantation and the following post-transplantation BV by FDA. Subsequently, FDA approved pembrolizumab for the treatment of refractory primary mediastinal large B-cell lymphoma patients in 2018.

Current data suggest that the PD-1, PD-L1/PD-L2 pathway may play a significant role in preventing immune-driven eradication of MF/SS tumor cells. Expression of PD-1 and PD-L1 has been detected in tumor cells of various morphological subsets of MF (46) as well as tumor cells circulating in the peripheral blood of SS (47). A recent phase 1b study of nivolumab in 81 patients with relapsed or refractory hematologic malignancy included 13 patients with MF. The ORR in MF patients was

15% (all PR) with 59% stable disease (SD) and the median PFS was 10 weeks (13). Khodadoust et al. presented preliminary data from a multicenter phase 2 open label study of pembrolizumab in 24 advanced and refractory CTCL patients (9 MF, 15 SS) (14). The ORR was 37.5% with 1 CR, 8 PR, and 9 SD, and the median PFS has not yet been reached. Of the 9 responding patients, 6 patients had 90% or greater decrease in modified Severity Weighted Assessment Tool score. Treatment was well-tolerated with a toxicity profile which was consistent with prior studies (48), although a notable skin flare reaction was developed in 40% of SS patients. Although it is necessary to wait for the results of several ongoing clinical trials using immune checkpoint inhibitors such as nivolumab, ipilimumab, and durvalumab (anti-PD-L1 antibody), immune checkpoint inhibition can be a novel strategy to treat advanced MF/SS.

IPH4102 (ANTI-KIR3DL2 ANTIBODY)

KIR3DL2 (CD158k), a member of the highly polymorphic killer-cell immunoglobulin-like receptor family, has the capacity to bind to MHC class I and transduce an inhibitory signal. KIR3DL2 is expressed on subsets of normal $CD8^+$ T cells and NK cells, but not on normal $CD4^+$ cells (49). On the other hand, several studies demonstrated that KIR3DL is expressed by neoplastic $CD4^+$ T cells in SS, advanced MF, and primary cutaneous ALCL (50–54). The relative specific expression of KIR3DL2 on the malignant CTCL cells makes it an ideal therapeutic target. IPH4102 is a humanized, monoclonal antibody specific toward KIR3DL2 which lacks cross-reactivity with other members of the human killer-cell immunoglobulin-like receptor family. IPH4102 selectively and efficiently can deplete $KIR3DL2^+$ cells including primary Sézary cells through antibody-dependent cell cytotoxicity and phagocytosis (55).

Preliminary results from the phase 1 study were presented at the 2017 European Organization for Research and Treatment of Cancer: Cutaneous Lymphoma Task Force in London (15). The aim of the trial is to characterize IPH4102 safety profile and identify the maximum tolerated dose and recommended phase 2 dose. A total of 25 patients, including 20 patients with SS, four patients with MF, and one patient with $CD4^+$ CTCL (neither MF nor SS), have been treated at the 10 preplanned ascending dose levels (0.0001–10 mg/kg). All patients had relapsed after or had been refractory to at least two prior systemic therapies. The ORR was 44% (1 CR and 10 PR). Two patients achieved a near CR (>90% reduction in skin involvement). The median duration of response (DOR) was 8.2 months, and the median PFS was 9.8 months. As IPH4102 was safe and well-tolerated in those dose-escalation cohorts, expansion cohorts started at the flat dose of 750 mg in 2017. Preliminary results of expansion cohorts were presented at the 60th American Society of Hematology annual meeting in 2018 (16). The study included 35 SS patients with at least two prior systemic therapies. The ORR was 42.9% (5.7% CR and 37.2% PR) with a favorable safety profile. The median DOR was 13.8 months and the median PFS was 11.7 months. Preliminary phase 1 data suggest that IPH4102 is both efficacious and well-tolerated. A global, multi-cohort, phase 2 study evaluating the potential of IPH4102 in different subtypes of T-cell lymphoma will be initiated this year (NCT03902184).

HDAC INHIBITORS

Histone deacetylase (HDAC) inhibitors have the capacity to increase acetylation of histones and other proteins, which exerts chromatin remodeling, promotion of tumor suppressor gene transcription, and apoptosis, resulting in antitumor activity. Its clinical activity is largely confined to hematologic malignancies, particularly CTCL (56). HDAC inhibitors have the prevalent AEs of fatigue, thrombocytopenia, diarrhea, and nausea in common (57).

Although vorinostat is not a novel drug, we referred to the drug in this paragraph, because it is the first approved HDAC inhibitor. Vorinostat is an oral competitive inhibitor of class I/II HDAC enzymes. In the pivotal phase 2B multicenter trial, 400 mg of vorinostat was administered daily to 74 stage IB-IVA MF/SS patients, who were previously treated with two or more prior systemic therapies, until disease progression or intolerable toxicity (17). The ORR was 29.7% (22/74) and all initial responses were confirmed PR. The other phase 2 clinical trial showed similar results (18). Eight of 33 patients (24.2%) with refractory MF/SS who had received a median of 5 prior therapies achieved PR. In 2006, FDA approved vorinostat for the treatment of CTCL patients who have progressive, persistent or recurrent disease on or following two systemic therapies. Also in Japan, the drug was approved in 2011 based on the phase 1 clinical trial conducted in Japan (58). In a recent phase 3 randomized study, vorinostat was compared with mogamulizumab in patients with stage IB-IV MF/SS (8). The ORR for the vorinostat was significantly lower than that of mogamulizumab (5 vs. 28%).

Romidepsin is a bicyclic peptide that inhibits class I HDAC selectively. Preclinical studies suggest that romidepsin is among the most potent HDAC inhibitors. Two multicenter phase 2 clinical trials of romidepsin for CTCL were conducted before 2010. In one clinical trial, 71 refractory IA-IVB MF/SS patients with a median of four prior treatments were enrolled (19). Some patients received 18 mg/m^2 romidepsin on days 1 and 5 of a 21-day cycle and to other patients romidepsin was administered at 14 mg/m^2 on days 1, 8, and 15 every 28 days. CR was observed in four patients (5.6%) and 20 patients achieved PR (28.2%). The median DOR was 13.7 months. In the other international single-arm, open-label, phase 2 study, 96 patients with IB-IVA MF/SS who had received one or more prior systemic therapies (median three), received romidepsin intravenously 14 mg/m^2 on days 1, 8, and 15 every 28 days (20). The ORR was 34% (33/96), including 6% (6/96) CRs and the median DOR was 15.0 months, which were similar to the previous study. Interestingly, in the clinical trial, romidepsin is active in subtypes of CTCL with less favorable outcomes, such as tumor stage and folliculotropic MF. The ORR was 45% (9/20) in patients with cutaneous tumors and 60% (6/10) in patients with folliculotropic disease involvement (59). Of note, Kim et al. reported that a clinically significant effect on pruritus was confirmed in a large number of patients, even in patients without any objective clinical response (60). In 2009,

romidepsin was approved for the treatment of CTCL patients by FDA.

Panobinostat is an orally bioavailable pan HDAC inhibitor approved for the treatment of multiple myeloma by FDA in 2015. In a phase 2 study, 139 patients with stage IB-IVA MF/SS who had been pretreated with two or more prior systemic therapies, received 20 mg of oral panobinostat three times every week (21). The 139 patients included 79 bexarotene-exposed patients and 60 bexarotene-naïve patients. The ORR was 17.3% in all patients (15.2% in the bexarotene-exposed group and 20.0% in the bexarotene-naïve group). One CR was observed in each group. The median PFS was 4.2 months in the bexarotene-exposed group and 3.7 months in the bexarotene-naïve group. The median DOR was 5.6 months in the bexarotene-exposed group and was not reached at data cutoff in the bexarotene-naïve group.

Belinostat is an intravenous inhibitor of pan HDAC, which was approved for the treatment of relapsed or refractory PTCL by FDA in 2014. In the phase 2 clinical trial of belinostat in patients with relapsed or refractory PTCL and CTCL, 29 patients with CTCL including 17 MF patients and seven SS patients were enrolled. Patients with CTCL had received a median of four prior systemic therapies. Belinostat was administered at 1,000 mg/m^2 intravenously for consecutive 5 days of a 21-day cycle (22). The ORR was 13.8% (10.3% CR and 3.4% PR), and the median DOR was 83 days.

Resminostat is an oral drug which selectively inhibits class I, IIB, and IV HDAC enzymes. A phase 2, multicenter, double-blind, randomized, placebo-controlled trial is currently ongoing to evaluate whether resminostat can be used as maintenance treatment for MF/SS patients after disease control with other systemic therapies (NCT02953301). Patients will receive either placebo or 600 mg resminostat for consequent 5 days followed by 9 days of rest in a 14-day cycle. This clinical trial will be completed in 2020.

PRALATREXATE

Pralatrexate, an anti-neoplastic folate analog, inhibits dihydrofolate reductase, targeting DNA synthesis and resulting in tumor cell death. Pralatrexate has the improved anti-tumor activity compared to methotrexate due to higher affinity for the reduced folate carrier-1 and more selective accumulation in tumor cells.

A phase 2 study of pralatrexate in 109 patients with PTCL including 12 transformed MF patients who progressed following one or more prior systemic therapy (PROPEL study) showed that the ORR was 29% (32 of 109), including 11% CR and 18% PR, with the median DOR of 10.1 months. The median PFS and overall survival (OS) were 3.5 and 14.5 months, respectively (61). Subgroup analysis patients with transformed MF revealed that the ORR was 58% with the median DOR and PFS were 4.4 and 5.3 months, respectively per investigator assessment (24). Pralatrexate was administered at 30 mg/m^2/week for 6 weeks followed by one week of rest (7-week cycle) in this study. FDA approved pralatrexate for the treatment of PTCL in 2009. In Japan, after phase 1/2 clinical study was conducted, pralatrexate was approved in 2018 (62).

As for CTCL, a dose de-escalation study of pralatrexate showed that the recommended regimen was identified as 15 mg/m^2/week for 3 weeks followed by 1 week of rest (4-week cycle) (63). Twenty-nine patients with refractory MF/SS and primary cutaneous ALCL with at least one prior systemic therapy received recommended dosing regimen. The ORR was 45% with 1 CR and 12 PR. In any study, the most observed toxicity is mucositis. To reduce this risk, patients received supplementation of vitamin B12 and folate, and leucovorin (folinic acid) during pralatrexate treatment. Pralatrexate can be a promising treatment with the potential to provide lasting benefit for advanced CTCL patients with the relative low toxicity. Recently, a phase 1/2 study suggested that combination therapy of 150 mg/m^2 daily bexarotene plus 15 mg/m^2/week for 3/4 weeks pralatrexate is active with high ORR (60%) and minimal toxicity for CTCL (64). A phase 1 study of pralatrexate (10 to 25 mg/m^2) and romidepsin (12 to 14 mg/m^2) on 1 of 3 schedules: every week × 3 every 28 days, every week × 2 every 21 days, and every other week every 28 days, for patients with PTCL also showed high ORR (57%) (65). These combination therapies with pralatrexate plus bexarotene or romidepsin can be an efficient and tolerated treatment option.

FORODESINE

Purine nucleoside phosphorylase (PNP) is an important enzyme for the phosphorolysis of purine nucleosides. Severe immunodeficiency syndromes are caused by congenital defects in this enzyme through selective depletion of T cells but not of B cells (66, 67). Based on increased nucleoside metabolism of malignant T cells, T-cell tumor cells can be highly sensitive to the inhibition of PNP (68). Forodesine is a potent inhibitor of PNP that causes apoptosis in both neoplastic T cells and normal T cells.

In a multicenter phase 2 open-label study, 144 patients with MF/SS who had been treated with three or more systemic therapies were enrolled. The patients received oral forodesine 200 mg daily. The drug showed limited clinical activity in this study. No CRs were observed, and only 11% of the patients achieved PR and 50% maintained SD. The median DOR was 191 days (25). Although almost all patients (96%) experienced at least one AE, most AEs were grade 1/2. Common AEs were peripheral edema, fatigue, insomnia, pruritus, diarrhea, headache, and nausea.

Forodesine was approved in Japan for the treatment of PTCL at the dose of 600 mg daily based on efficacy and safety results of the phase 1/2 clinical trial in patients with 48 relapsed PTCL including one transformed MF patient (65). In 41 evaluable patients, the ORR was 25% including 4 CRs. The most common grade 3/4 AEs were lymphopenia (96%), leukopenia (42%), and neutropenia (35%). Dose reduction and discontinuation due to AEs were uncommon. There is a possibility that such high-dose can be an effective and acceptable treatment for advanced MF/SS.

DENILEUKIN DIFTITOX

Denileukin diftitox is a genetically engineered fusion protein combining the full-length sequence of human IL-2 with the cytotoxic and membrane-translocating domains of the diphtheria toxin. After binding to the IL-2 receptor (IL-2R) on neoplastic T cells, the drug is internalized. The diphtheria toxin results in the production of a single polypeptide chain that is capable of inhibiting protein synthesis in the cells, leading to cell death (69). The human IL-2R consisted of three forms: low, intermediate, and high affinity. The high affinity IL-2R is a complex of distinct proteins of α chain (CD25), β chain (CD122), and γ chain (CD132). The intermediate one is composed of CD122 and CD132, and CD25 alone defines the low affinity one. Although denileukin diftitox can bind to all forms of the IL-2R, internalization is caused by only intermediate or high affinity receptors (70). In addition, it is known that the baseline expression level of CD25, which is not included in the intermediate affinity IL-2R, on CTCL cell in lesional skin correlated with their clinical response to denileukin diftitox (71), suggesting that the high affinity IL-2R is the most important receptor to elicit an effect.

The largest study of denileukin diftitox was a multicenter, randomized, double-blind placebo-controlled phase 3 trial that evaluated denileukin diftitox (9 or 18 μg/kg/day) vs. placebo in 144 stage IA-III MF/SS patients who had been treated with at most three prior therapies (26). The trial excluded patients with low CD25 expression disease (defined as detectable CD25 on <20% of T cells in lesional skin). The drugs were administered for consequent 5 days every 3 weeks for up to eight cycles. The ORR for the denileukin diftitox 18 μg/kg/day group was 49.1% with 9.1% CR (n = 55), compared with 15.9% with

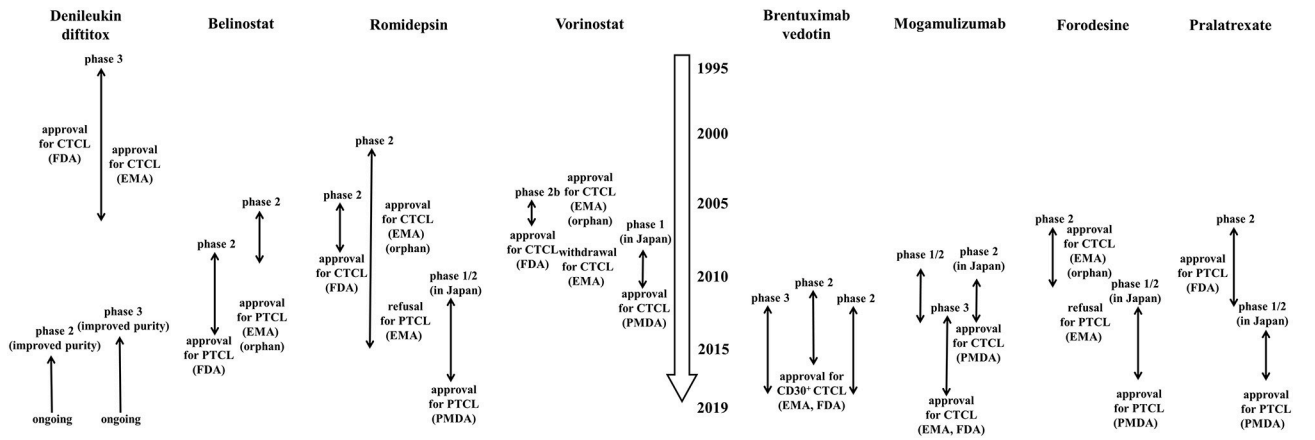

FIGURE 1 | History of clinical trials of single-agents which have been approved for cutaneous T-cell lymphoma or peripheral T-cell lymphoma by FDA, EMA, or PMDA. The data were collected on March 31, 2019. When the drug was approved as orphan drug from EMA, the comment "orphan" is added. CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma; FDA, food and drug administration; EMA, European medicines agency; PMDA, pharmaceuticals and medical devices agency.

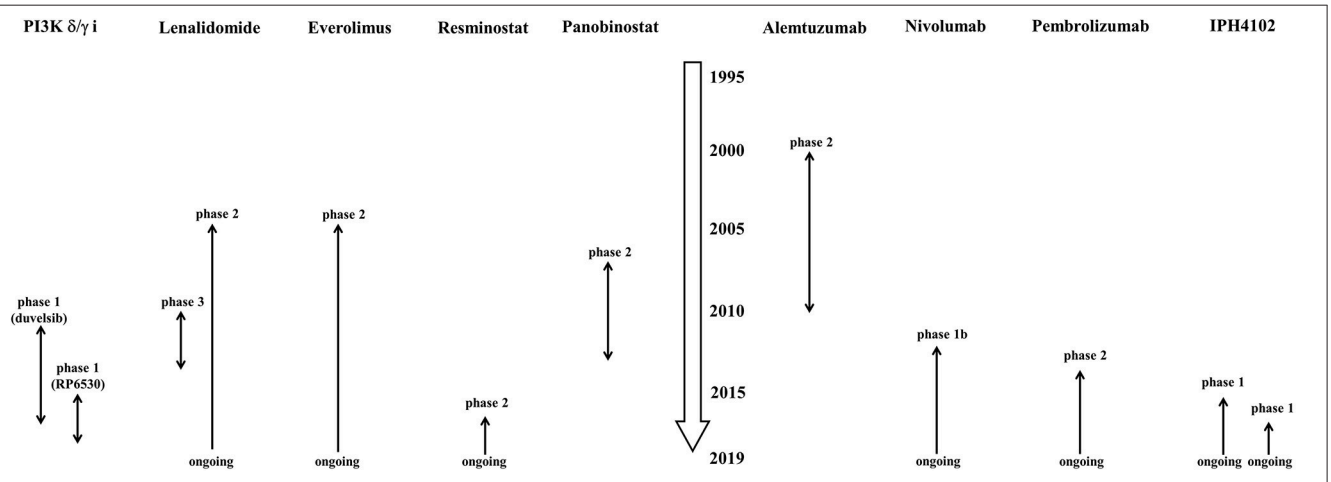

FIGURE 2 | History of clinical trials of single-agents which have not been approved for cutaneous T-cell lymphoma or peripheral T-cell lymphoma by FDA, EMA, or PMDA. The data were collected on March 31, 2019. PI3K δ/γ I, phosphoinositide-3-kinase δ/γ inhibitor.

2.3% CR for placebo ($n = 44$). For the denileukin diftitox 9 μg/kg/day group, the ORR was 37.8% ($n = 45$; 11.1% CR and 26.7% PR). The PFS was significantly prolonged for denileukin diftitox-treated patients compared to patients treated with placebo. Estimated median PFS was at least 971 days for the denileukin diftitox 18 μg/kg/day cohort, 794 days for the denileukin diftitox 9 μg/kg/day cohort, and only 124 days for placebo cohort. The drug-related severe AEs occurred in 25% of the participants receiving denileukin diftitox with premedication of acetaminophen and antihistamine. The most common drug-related severe AEs were dehydration (2%) and capillary leak syndrome (2%). To assess the denileukin diftitox effect on patients with low CD25 expression, 36 patients with MF/SS who had been excluded from the placebo-controlled trial due to low CD25 expression were enrolled in another clinical trial. In the clinical trial, patients were treated with denileukin diftitox 18 μg/kg/day for 5 consecutive days every 3 weeks for up to eight courses. The ORR was 30.6% (8.3% CR and 22.2% PR) (72). This study suggests that low CD25 expression does not necessarily preclude a meaningful clinical response to denileukin diftitox in patients with CTCL.

Denileukin diftitox had been approved by the FDA in 1999 for the treatment of patients with CTCL refractory to standard treatment options. However, denileukin diftitox is unavailable on the global market at this time. Currently, the related agent E7777 which shares an amino acid sequence with denileukin diftitox but has improved its purity and an increased percentage of active protein monomer species is being evaluated. A phase 1 study for 13 patients with PTCL conducted in Japan showed that E7777 is well-tolerated and has antitumor activity with 38% ORR (73). A phase 2 clinical trial of E7777 for relapsed or refractory PTCL and CTCL (NCT02676778) and a phase 3 clinical trial for persistent and recurrent CTCL (NCT01871727) are ongoing.

DUVELISIB

Phosphoinositide-3-kinase (PI3K) is a lipid kinase involved in intracellular signal transduction and regulates multiple cellular functions relevant to oncogenesis. The PI3K-δ and PI3K-γ isoforms, which are preferentially expressed in leukocytes, can modulate both innate and adaptive immune response (74–77). PI3K-δ and PI3K-γ mediate multiple pathways contributing to survival, proliferation, and differentiation in malignant hematopoietic cells. Moreover, PI3K signaling is involved in development of tumor microenvironment through juxta-, para-, and endocrine effects on stromal and immune cells (78–80). Additionally, PI3K-γ may also suppress antitumor immune response by inhibiting phagocytosis by tumor-associated macrophages (81). Thus, there are at least three different mechanisms via which PI3K-δ and PI3K-γ inhibitors could be effective for hematopoietic malignancies.

Duvelisib (also known as IPI-145) is an oral, dual inhibitor of PI3K-δ and PI3K-γ. In a recent phase 1 open-label trial, clinical activity of duvelisib was promising and the toxicity was acceptable in relapsed or refractory PTCL and CTCL (27). Thirty-five patients (16 PTCL, 19 CTCL) were enrolled in this study and 27 (77%) were treated at the maximum tolerated of oral duvelisib 75 mg twice daily on a 28-day cycle. The 19 patients with CTCL had received a median of six prior therapies. The CTCL population was composed of 13 patients with MF, five patients with SS, and one primary cutaneous ALCL patient. In the CTCL population, the ORR was 31.6% (all PR) and the median PFS was 4.5 months. The most common grade 3/4 AEs were increase of liver enzymes (40%), neutropenia (17%), maculopapular rash (17%), and pneumonia (17%). Thus, this study suggests that duvelisib has clinical activity with an acceptable toxicity, while further studies are needed to determine the optimal dose and identify an appropriate combination therapy. A phase 1/1b clinical trial of the other dual inhibitor of PI3K-δ and PI3K-γ, RP6530, in relapsed and refractory T-cell Lymphoma has been finished, but data analysis is incomplete (NCT02567656).

LENALIDOMIDE

Lenalidomide, a derivative of thalidomide, is an oral immunomodulatory drug with direct immune-mediated mechanism (82). Lenalidomide has been shown to induce growth arrest and apoptosis in lymphoma cell lines and FDA approved the drug for the treatment of myelodysplastic syndrome, refractory/relapsed multiple myeloma, and mantle cell lymphoma (83, 84). In addition, lenalidomide is currently being used in clinical trials to treat other various hematopoietic malignancies.

A multicenter phase 2 study of lenalidomide in 32 patients with MF/SS who progressed following a median of 4 systemic therapies was conducted between 2005 and 2010 (28). The first 19 patients received lenalidomide at a daily dose of 25 mg orally for 21 days of a 28-day cycle. The remaining 13 patients initiated treatment at a dose of 10 mg daily and the dose was then increased by 5 mg every 28 days to a maximum of 25 mg daily, based on patient safety and response. The ORR was 28% (all PR) with the median PFS of 8 months. The most frequent AEs were lower leg edema, anemia, fatigue, and transient flare reaction that mimic worsening of the patient's disease. Patients with a 25-mg starting dose showed AEs more frequently than those with a 10-mg starting dose. In a phase 3 randomized study of lenalidomide maintenance vs. observation alone after disease control with other therapies in 21 advanced CTCL patients, the median PFS was 5.3 months in the maintenance lenalidomide group ($n = 9$) and 2 months in the observation alone group ($n = 12$) (85). Because lenalidomide was used as a maintenance therapy, ORR was not evaluated. The main AEs noted in the lenalidomide arm were neutropenia, erythema multiforme, periorbital edema, hypothyroidism, and pruritus. Although statistical comparison in this study was severely underpowered, lenalidomide may be used as a maintenance therapy after debulking therapy.

EVEROLIMUS

Everolimus is an oral agent that targets the mammalian target of rapamycin (mTOR) pathway. The mTOR regulates several survival and growth pathways in a variety of cancers, which was

also shown for T-cell non-Hodgkin lymphoma. In addition, an immunohistochemical study revealed that activation of mTOR pathway in MF is associated with the acquisition of a more aggressive phenotype (86). In the recent phase 2 clinical trial, 16 patients with relapsed or refractory T-cell lymphoma including 7 patients with MF were enrolled and received oral everolimus 10 mg daily. The ORR was 44% and the median PFS was 4.1 months (29). Regarding MF, three of seven patients showed PR and none reached CR. The most frequent AEs were hematologic toxicity and skin rash.

CONCLUSION

Although many patients with early CTCL have slow-progressing disease with a normal life expectancy, prognosis of patients with advanced stages of CTCL is poor. Generally speaking, CTCL is incurable without allogeneic stem cell transplantation. Current treatment outcome is characterized by high relapse rates and low durable remission rates. As treatment of advanced-stage CTCL is mostly palliative and not curable, a stage-based approach utilizing sequential therapies in an escalated manner is currently favorable. Existing clinical practice guidelines are quite heterogeneous. Consequently, therapeutic decisions should be individualized to each patient by means of a risk-proportionate approach. Although many novel therapeutic agents have been developed and clinical trials for CTCL and PTCL had or have been implemented (**Figures 1**, **2**), such drugs also showed limited efficacy as reviewed in this paper. Thus, it is necessary to know which therapy is preferable for each patient with MF/SS. Creatively designed international clinical trials, such as MAVORIC study and ALCANZA study, should be encouraged.

AUTHOR CONTRIBUTIONS

TO conceived the concept and wrote the manuscript. TM co-conceived the concept, edited and improved the manuscript, and drafted the table.

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CASE REPORT
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Successful Treatment of Unresectable Advanced Melanoma by Administration of Nivolumab With Ipilimumab Before Primary Tumor Resection

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Ipilimumab, in combination with nivolumab, is one of the promising drugs that enhance the anti-tumor immune response of patients with advanced melanoma. Since the co-administration of nivolumab with ipilimumab in the neoadjuvant setting expands melanoma-reactive T cells at the primary site of melanoma and has a high rate of histological complete response, the pre-surgical administration of this combination could be the optimal therapy for unresectable advanced melanoma. In this report, a case of unresectable advanced melanoma treated successfully with administration of nivolumab with ipilimumab before primary tumor resection is presented. In addition, CD8+ T cells increased among the tumor-infiltrating lymphocytes that were surrounding melanoma cells and caspase 3+ cells. The present case suggests that pre-surgical administration of nivolumab with ipilimumab could be the optimal therapy for the treatment of unresectable advanced melanoma.

Keywords: nivolumab, ipilimumab, advanced melanoma, pre-surgical administration, T cell expansion

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BACKGROUND

Nivolumab, anti-PD1 antibody (Abs), monotherapy has been one of the first-line therapies for advanced melanoma, especially for BRAF mutation-negative melanoma, with a reported efficacy rate of ∼30–40% (1, 2). Ipilimumab, cytotoxic T-lymphocyte antigen (CTLA-4) Abs, is another immune checkpoint inhibitor (ICI) for the treatment of advanced melanoma that activates and increases T cells, and it expands effector T cells at the site especially when given with nivolumab (3). Therefore, pre-surgical treatment with the combination of nivolumab and ipilimumab could be optimal therapy for unresectable, advanced melanoma.

CASE PRESENTATION

A 64-year-old Japanese man visited our outpatient clinic with a 3-months history of an easily bleeding, black nodule on his back. At the initial physical examination, a black nodule (8 × 7 cm) with a dark-red nodule was seen on the back (**Figure 1a**). In addition, there were numerous subcutaneous nodules on the scalp, face, trunk, and extremities. Biopsy of the primary tumor showed markedly atypical melanocytes arranged in irregular nests and solitary units

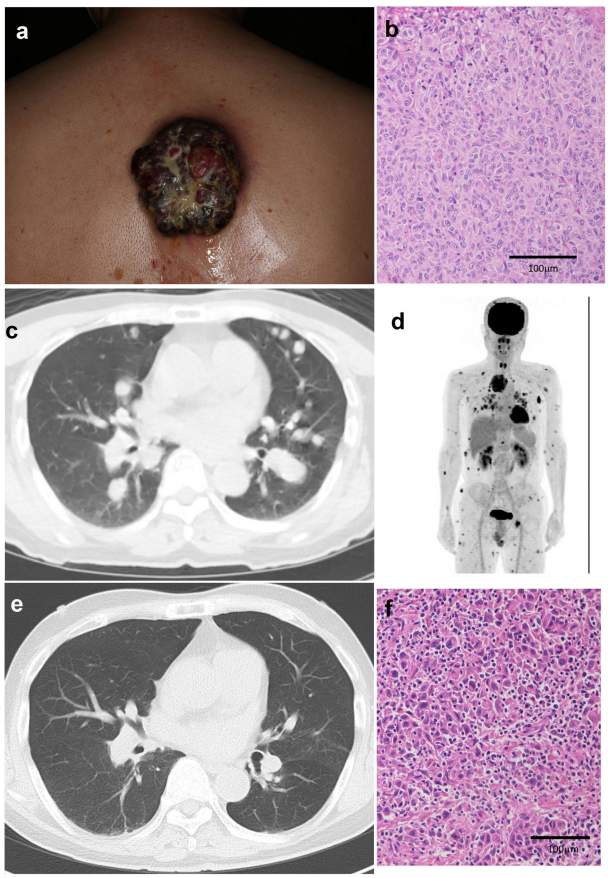

FIGURE 1 | A black nodule (8 × 7 cm) with a dark-red nodule on the back **(a)**. Histological findings of the primary tumor before treatment: markedly atypical melanocytes arranged in irregular nests and solitary units **(b)**. Multiple lung metastases **(c)**, cutaneous, pharyngeal, and peritoneal nodules, lymph node metastases, and bone metastases on PET-CT **(d)**. After treatment, multiple lung metastases are decreased **(e)**. Histological findings of the primary tumor after single administration of nivolumab with ipilimumab showing dense infiltration of lymphocytes in the melanoma lesion **(f)**.

(**Figure 1b**). The THxID kit revealed that the primary tumor possessed the $BRAF^{V600E}$ mutation. Immunohistochemical staining showed that these melanoma cells were positive for Melan A and HMB45. PET-CT showed multiple lung (**Figure 1c**), cutaneous, pharyngeal, and peritoneal nodules, as well as lymph node and bone metastases (**Figure 1d**). Biopsy from the pharyngeal wall showed dense infiltration of markedly atypical melanocytes. In addition, serum LDH levels were elevated (336 U/l). From the above findings, the diagnosis was malignant melanoma with multiple lung, peritoneal, pharyngeal, subcutaneous, lymph node, and bone metastases [pT4bN3cM1c(1) stage IV].

TREATMENT COURSE AND OUTCOME

Since the patient had metastases in 6 organs (>3 organs) and elevated serum LDH levels, suggesting that dabrafenib plus trametinib combined therapy might not be useful (4), nivolumab (80 mg/body/every 3 weeks) was given in combination with ipilimumab (3 mg/kg/every 3 weeks) before surgical treatment. Eighteen days after the administration of nivolumab and ipilimumab, the primary tumor was palliatively resected, and nivolumab (80 mg/body/every 3 weeks) in combination with ipilimumab (3 mg/kg/every 3 weeks) was continued for three more cycles (**Supplemental Figure 1**). The skin metastases regressed rapidly with scar formation, and follow-up CT 2 months after the combination therapy suggested significant regression of lung (**Figure 1e**), peritoneal, pharyngeal, subcutaneous, lymph node, and bone metastases. Histological findings of the resected primary tumor showed dense infiltration of lymphocytes in the melanoma lesion (**Figure 1f**). Four months have passed, and a grade 3 skin rash and grade 4 peripheral neuropathy, which is controlled by the intravenous administration of methylprednisolone sodium succinate at a starting dose of 2 mg/kg, were observed.

IMMUNOHISTOCHEMICAL INVESTIGATION OF TUMOR INFILTRATING LYMPHOCYTES (TILs)

Since a previous study suggested that combination therapy with nivolumab and ipilimumab significantly increased a neoantigen-specific melanoma-resident T cell clone, inducing a durable anti-immune response in melanoma patients (1), immunohistochemical staining for CD3 and CD8 was performed before and after the administration of combination therapy (**Figure 2A**). The ratios of CD3, CD8, PD1, and Foxp3+ cells among tumor infiltrating lymphocytes (TILs) in the primary tumor before the administration of nivolumab plus ipilimumab combination therapy and in the primary tumor 18 days after the administration of combined therapy were analyzed using the BZ-X800 (KEYENCE, Tokyo, Japan). The lymphocyte fractions, CD3+ cells, CD8+ cells, PD1+ cells, and Foxp3+ cells, were counted, and the ratios of cells staining positive on immunohistochemistry (CD3+ cells/total TILs, CD8+ cells/total TILs, PD1+ cells/total TILs, Foxp3+ cells/total TILs) were calculated in the full tumor areas of low magnification fields. These data showed a marked increase of CD8+ TILs in the post-treatment specimen (**Figure 3**). In addition, immunohistochemical staining for PD-L1 was performed, showing no difference between before and after the administration of combination therapy (**Figure 2B**). Moreover, immunofluorescence staining for caspase 3, CD8, and tyrosinase showed the induction of apoptotic cells in the melanoma lesion (**Figure 2C**).

Ethics Statement

The patient gave written informed consent for the publication of this case report.

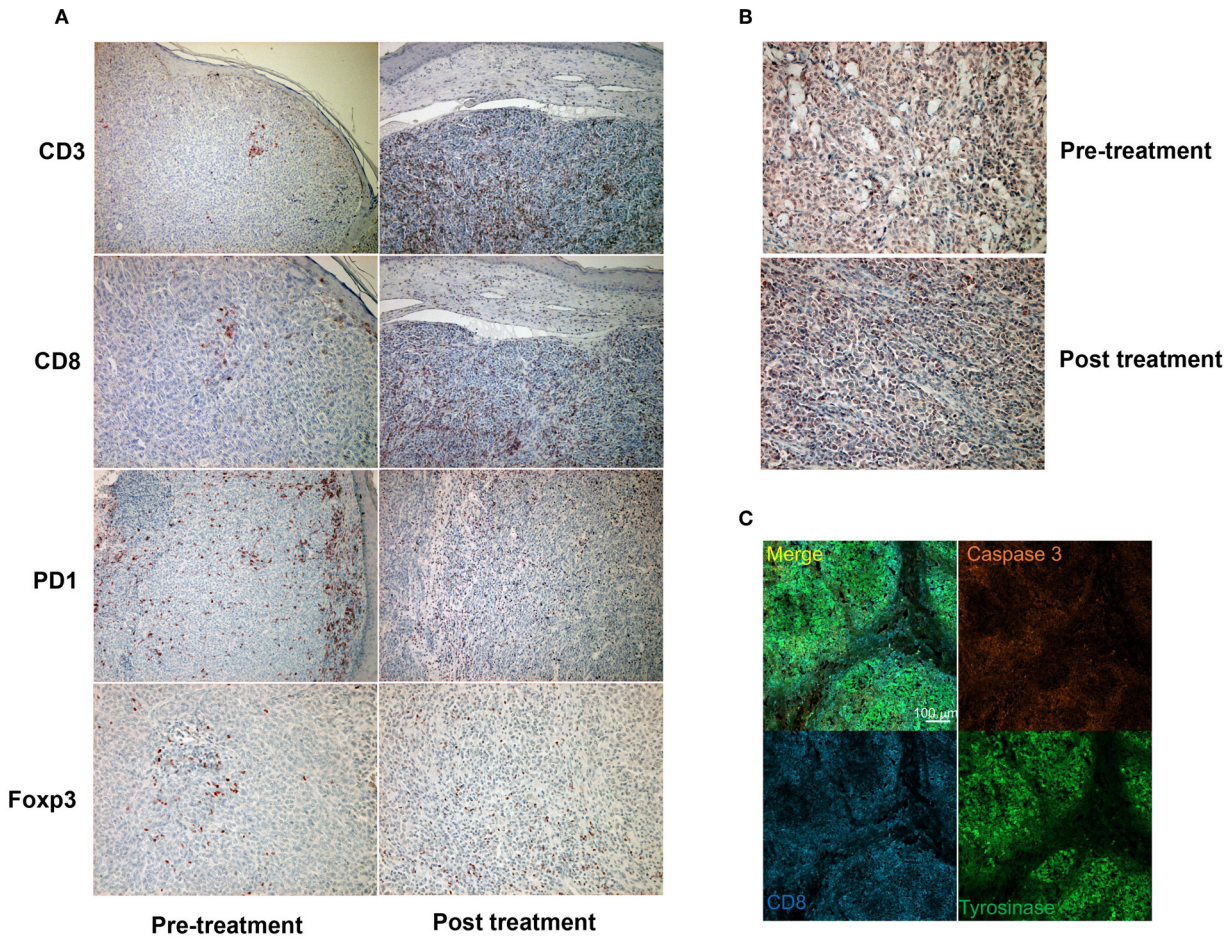

FIGURE 2 | Immunohistochemical staining for CD3, CD8, PD1, and Foxp3 before and after a single administration of nivolumab with ipilimumab **(A)**. Immunohistochemical staining for PD-L1 before and after a single administration of nivolumab with ipilimumab **(B)**. Immunofluorescence staining of CD8 (cytotoxic T cells: blue), caspase 3 (apoptotic cells: orange), and tyrosinase (melanoma cells: green) **(C)**.

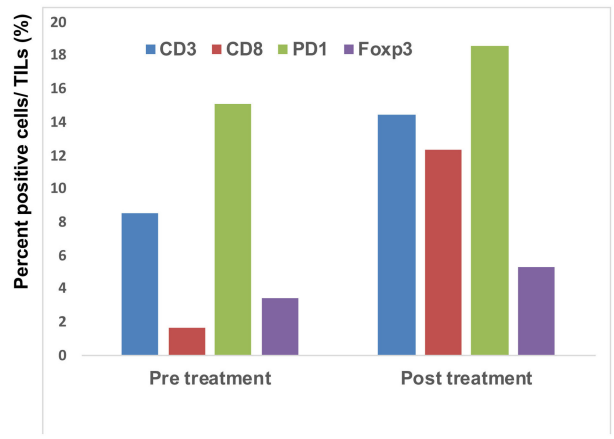

FIGURE 3 | Quantitative analysis of CD3+ T cells, CD8+ T cells, PD1-expressing cells, and Foxp3+ cells: the IHC-positive cells within the lymphocyte fraction and the percentage of IHC-positive cells per all tumor-infiltrating cells were automatically counted using a BZ-X800.

DISCUSSION

Ipilimumab, in combination with nivolumab, is one of the promising drugs that enhance the anti-immune response of patients with advanced melanoma with or without BRAF gene mutation (5–7). Indeed, the response rate to this combination therapy for advanced melanoma has been reported to be 57.8% (7), and it is recommended by the NCCN guideline for cutaneous melanoma as a first-line therapy (8), despite its high toxicity. In addition, this combination therapy has a high efficacy rate even for the treatment of brain metastases of melanoma (5). Notably, Blank et al. reported that the efficacy of the combination of nivolumab and ipilimumab in the neoadjuvant setting does not parallel tumor mutation burden (TMB) and achieves a high histological complete response (CR) rate (3), suggesting that pre-surgical administration of nivolumab with ipilimumab could be the optimal treatment for unresectable advanced melanoma in real-world practice. In addition, the efficacy of a BRAF inhibitor combo, such as dabrafenib and trametinib combination therapy or encorafenib and binimetinib combination therapy, is limited

in advanced melanoma with multiple organ metastases (4, 9). Although there is still insufficient evidence for the efficacy of pre-operative treatment by nivolumab plus ipilimumab, these reports suggested that pre-operative treatment by nivolumab plus ipilimumab might induce a stronger and broader tumor-specific T cell response, as in a pre-clinical study (10). In addition, Navarrete-Dechent reported one case of stage III, unresectable melanoma treated with nivolumab plus ipilimumab combined therapy, and evaluated its efficacy using reflectance confocal microscopy (11). Their report suggested that ipilimumab plus nivolumab combined immune therapy is useful for the treatment of unresectable melanoma (11).

Concerning the present case, although the patient had at least 6 organ metastases, pre-surgical administration of nivolumab with ipilimumab dramatically reduced tumor masses in all organs. Interestingly, a single administration of this combination therapy increased the ratio of CD8+ T cells among total TILs from 1.7 to 12.3% (**Figure 3**). Moreover, immunofluorescence staining showed that caspase 3+ apoptotic cells were surrounded by CD8+ T cells in the melanoma area, suggesting that increased CD8+ T cells might directly induce apoptosis of melanoma cells. Taken together, administration of nivolumab with ipilimumab before primary tumor resection increased CD8+ T cells in the primary tumor, probably a melanoma-specific T cell clone, inducing a systemic anti-melanoma immune response in advanced melanoma. To prove this hypothesis, another clinical study to evaluate nivolumab plus ipilimumab combination therapy prior to surgery is needed.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This patient gave written informed consent.

AUTHOR CONTRIBUTIONS

TF designed the research study and wrote the manuscript. TF, YK, YS, KT, and TH performed and analyze the IHC staining. TF, YK, RA, and AH treated the patients and acquired the clinical data and samples. TF and SA supervised the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00140/full#supplementary-material

Supplemental Figure 1 | Primary tumor on the back before and after the administration of nivolumab plus ipilimumab.

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REVIEW
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Updates on the Systemic Treatment of Advanced Non-melanoma Skin Cancer

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Non-melanoma skin cancers (NMSCs), which represent a diverse group of cutaneous malignancies, are the most common forms of human neoplasia. The incidence of these diseases is increasing due to a number of factors, including that of increasing human lifespans. The majority of NMSCs are basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (cSCC), with the remainder being various rare skin cancers, including extramammary Paget's disease (EMPD), Merkel cell carcinoma (MCC), and several skin adnexal carcinomas. Of these, MCC usually shows aggressive behavior with a high mortality rate. On the other hand, BCC, cSCC, EMPD, and skin adnexal tumors usually show an indolent clinical course and metastasize only rarely. Nevertheless, the metastatic forms of these tumors commonly lead to poor patient outcome. A definitive management strategy for the treatment of advanced NMSC has not been established, mainly due to their rarity and lack of reliable information based on well-controlled randomized trials. Chemotherapeutic regimens for treatment of these diseases have been mainly based on the observations of isolated, small case series or clinical trials with a limited numbers of patients. However, accumulating evidence regarding their pathobiological backgrounds as well as recent advances in molecular biotechnology have facilitated the development of novel drugs for treatment of these diseases. Over the past decade, the U.S. Food and Drug Administration has approved several molecular targeting therapies, including Hedgehog inhibitors for BCC, monoclonal antibodies targeting anti-programmed death ligand-1 and anti- programmed cell death 1 (PD-1) for MCC, and anti-PD-1 for cSCC. Here, we review their clinical utility and discuss updated systemic treatment strategies for advanced NMSC.

Keywords: squamous cell carcinoma, basal cell carcinoma, extramammary Paget's disease, merkel cell carcinoma, adnexal carcinoma

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INTRODUCTION

Non-melanoma skin cancers (NMSCs) are the most common forms of human neoplasia, with more than 3 million newly diagnosed cases estimated to occur in the USA every year (1). NMSCs represent a diverse group of skin tumors, including cutaneous squamous cell carcinoma (cSCC), basal cell carcinoma (BCC), extramammary Paget's disease (EMPD), Merkel cell carcinoma (MCC), and skin adnexal carcinomas. Of these, BCC and cSCC account for the majority of NMSCs (75–80% and 20–25% of all NMSC cases, respectively) in Australia (2, 3). In addition, the incidence of these disease is increasing. The incidence rates of BCC and cSCC increased by 145% and 263%, respectively, from 2000 to 2010 in the United States of America (USA) (4). These increases are associated with several factors, including

raised awareness of NMSC in the general population, increased number of patients undergoing surgical treatment with confirmed histopathology, improved registration, transition of patient population toward the elderly, and increased exposure to ultraviolent (UV) radiation (5–7). Therefore, NMSC has now become a substantial economic burden (8, 9).

NMSCs show differences in progression and metastatic behavior according to each cancer type. MCCs are highly aggressive malignancies with high mortality rates (10). The 5-year overall survival (OS) rate of MCC patients with localized disease in USA was reported to be 55.6%, with historical 5-year OS rates of 35.4 and 13.5% for patients with nodal and distant metastatic disease, respectively (11). On the other hand, BCC, cSCC, and EMPD generally have a favorable prognosis with surgical resection, especially when detected in the early stages. While its precise clinical behavior is unclear due to its rarity, skin adnexal carcinoma is also considered to have low metastatic potential (12). However, once metastasis occurs, the prognosis of these tumors becomes extremely poor. The median OS period of metastatic BCC was reported to be 10.0 months (range, 0.5–108.0 months) after the detection of metastasis (13). The median progression-free survival (PFS) and OS of stage IV cSCC patients were reported to be 0.67 and 2.19 years, respectively, and the 5-year survival rate was 26% in USA (14). The median OS of EMPD patients with distant metastasis was reported to be 1.5 years with a 5-year survival rate of 7% in Japan (15). While chemotherapeutic agents and treatment strategies for these patients were mainly based on the results of isolated small case series or clinical trials in limited numbers of patients, accumulation of pathobiological evidence as well as advances in molecular biotechnology are facilitating the development of novel drugs and therapeutic regimens. This review presents a summary of updates on the treatment of advanced NMSC based mainly on the results of clinical trials with high evidenced level.

BASAL CELL CARCINOMA

BCCs are common skin cancers arising mainly from the basal layer of the epidermis. Clinically, they tend to appear on sun-exposed skin, especially on the face and neck (16). Generally, BCCs are slow growing and have low metastatic potential. However, deeply invasive or large lesions >10 cm^2 in diameter may show metastasis (17). The estimated metastasis rate ranges from 0.0029 to 0.55%, and common metastatic sites are regional lymph nodes, lungs, bones, skin, and liver (13, 17). Pathobiologically, activation of the Hedgehog (HH) signaling pathway has been shown to play a critical role in the majority of cases and is recognized as a therapeutic target (18–20). It was first characterized by identification of a germ line mutation in the *patched homolog 1* (*PTCH1*) gene in basal cell nevus syndrome, which was then reinforced by the discovery of mutations of *PTCH1*, *smoothened homolog* (*SMO*), and other genes related to the HH signaling pathway in sporadic BCC (20, 21). In general, activation of HH signaling is initiated by the cell-surface protein, SMO, which is inhibited by another cell-surface protein, PTCH1 (20). Binding of the HH ligand to PTCH1 prevents this inhibition and thus activates signaling. Mutations in *PTCH1* cause loss of its inhibitory role, and mutations in *SMO* release the inhibition resulting in constitutive signaling activation (**Figure 1A**) (22–24).

TREATMENT OF ADVANCED BCC

Chemotherapeutic Agents

Before the emergence of molecular target therapies, metastatic BCC had been treated with several conventional cytotoxic chemotherapies. However, only a few small case series reported the efficacy of these treatments, due to the rarity of metastatic BCC. In a review of 12 reported cases treated with platinum-containing regimens, five showed complete response (CR) and four showed partial response (PR) (25).

Molecular Targeting Agents

Two molecular targeting agents are currently available for treatment of advanced BCC, i.e., vismodegib and sonidegib, which were approved by the U.S. Food and Drug Administration (FDA), USA in 2012 and 2015, respectively. Both are oral small molecule inhibitors of SMO, which block HH signaling activation (19) (**Figure 1A**). One open-label trial of vismodegib showed a response rate (RR) of 68.5% for 1,119 cases of locally advanced BCC (laBCC), including a CR rate of 33.4% and PR rate of 35.1%. The RR in 96 metastatic BCC (mBCC) cases was 36.9%, including 4.8% CR and 32.1% PR. The median PFS was 23.2 months in laBCC and 13.1 months in mBCC (26). In a randomized trial, 12-month administration of sonidegib at 200 mg/day showed RR of 57.6 and 7.7% in 66 laBCCs and 13 mBCCs, respectively. Disease control rates, including cases with stable disease, were 91.9% in laBCC and 92.3% in mBCC (27, 28). In a meta-analysis of 18 reports, the overall response rates (ORR) were similar for vismodegib and sonidegib in laBCC (68.8 vs. 56.6%, respectively), but the complete RRs were markedly different (30.9 vs. 3.0%, respectively). In mBCC, the ORR of vismodegib was 2.7-fold higher than that of sonidegib (39.7 vs. 14.7%, respectively). With regard to side effects, muscle spasms, dysgeusia, and alopecia were noted at similar frequencies for both agents, and their combined prevalence rates were 67.1, 54.1, and 57.7%, respectively (29).

The antifungal drug, itraconazole, has also been reported to inhibit HH signaling activity by acting on SMO (30). In an open-label exploratory phase II trial, 42.1% of cases showed reduction of tumor size and re-epithelization (8/19 cases) (31). Tumor area was reduced by a mean of 24%. Further trials are required to determine its clinical utility.

CUTANEOUS SQUAMOUS CELL CARCINOMA

Cutaneous squamous cell carcinomas (cSCCs) are the second most common skin cancer in NMSCs. In addition to UV irradiation, several risk factors may cause cSCC, including

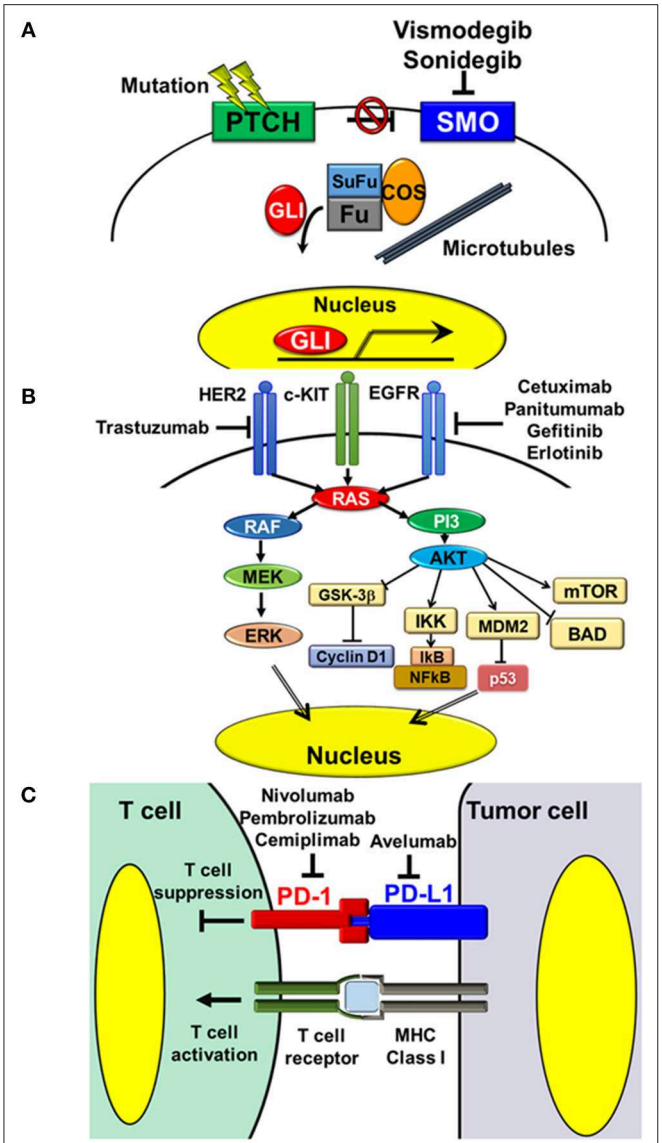

FIGURE 1 | Targeting pathways and molecules in the treatment of NMSC. **(A)** Hedgehog signaling pathway. Activation of Hedgehog signaling is initiated by the cell-surface protein, SMO, which is inhibited by another cell-surface protein, PTCH1. Binding of the Hedgehog ligand to PTCH1 releases this inhibition and thereby activates the pathway. Mutations in *PTCH1* result in loss of its inhibitory function, while mutations in *SMO* lead to constitutive signaling activation. Vismodegib and sonidegib are oral small molecule inhibitors of SMO, which block HH signaling activation. **(B)** Receptor tyrosine kinases and downstream MAPK and PI3-AKT signaling pathways. Aberrant overexpression or mutations of receptor tyrosine kinases, such as EGFR and HER2, cause activation of downstream signaling pathways, thus triggering several tumorigenic processes, including cell proliferation, cell survival, and resistance to apoptosis. The monoclonal antibodies, cetuximab and panitumumab, and the oral small molecules, gefitinib and erlotinib, inhibit the activity of EGFR. The monoclonal antibody, trastuzumab, inhibits the activity of HER2. **(C)** Interaction between T cells and tumor cells via the PD-1/PD-L1 axis. PD-1/PD-L1 interaction inhibits activation of T cell functions, including Th1 cytokine secretion, T cell proliferation, and cytotoxicity. Inhibition of PD-1/PD-L1 interaction with the anti-human PD-L1 antibody, avelumab, and anti-human PD-1 antibodies, nivolumab, pembrolizumab, and cemiplimab, releases these inhibitions and thereby activates the cytotoxic effects of T cells on tumor cells.

ionizing radiation, chemical carcinogen exposure, chronic wounds or scars, immunosuppression, infection with certain genotypes of human papillomavirus (HPV), and genetic abnormalities of genes involved in DNA repair (32–34). While the majority of these lesions can be treated by surgical resection, a small proportion will develop metastasis with significant morbidity and mortality (35). Of all metastatic cases, around 80% are localized in the regional lymph nodes, with the remainder showing distant metastasis in multiple organs, including the lungs, liver, brain, bones, and skin (32).

Pathobiologically, cSCCs are characterized as tumors with high mutational burdens. Exome-level sequencing of eight primary cSCCs revealed approximately 1,300 somatic single-nucleotide variations per cSCC exome (1/30,000 bp) (36). Among them, several genomic alterations were reported to show strong associations with the development and progression of cSCC. One study regarding single nucleotide and copy number variations in DNA samples extracted from metastatic cSCCs indicated that TP53 (79% of cases), NOTCH-1/2/4 (69%), and CDKN2A (48%) genes were frequently altered and several signaling pathways, including the RAS/RAF/MEK/ERK1/2 MAPK pathway (MAPK pathway) and phosphatidylinositol 3/AKT pathway (PI3-AKT pathway), are frequently activated (45% of cases) (37). Inactivating mutation of TP53 due to UV irradiation has been reported to be a cause of cutaneous malignancy with loss of programmed cell death in abnormal squamous cells (38). Loss of NOTCH-1 was reported to be associated with disease progression (39). CDKN2A encodes two cell cycle regulatory proteins, p16 and p14, loss of which causes aberrant mitosis (40). In addition, several reports indicated aberrant overexpression or mutations of epidermal growth factor receptor (EGFR) in cSCC samples. EGFR belongs to the receptor tyrosine kinase (RTK) family that activates several downstream signaling pathways, including MAPK and PI3-AKT pathways, and is involved in various cellular processes, including cell proliferation and survival (41, 42) (**Figure 1B**).

TREATMENT OF ADVANCED CSCC

Chemotherapeutic Agents

Chemotherapeutic agents commonly utilized in treatment of advanced cSCC include bleomycin, platinum, anthracycline, taxanes, interferon alpha, 5-fluorouracil, and its precursor drug capecitabine (43–49). While there have been a number of reports of the efficacies of chemotherapeutic regimens combining these agents, the level of evidence was generally poor as the trials had several limitations, such as small sample size, heterogeneous patient populations, and lack of randomization.

Molecular Targeting Agents

Based on the frequent aberrant overexpression or mutations of EGFR in cSCC, the utility of agents targeting EGFR has been suggested (**Figure 1B**). The efficacy and safety of the monoclonal antibodies, cetuximab, and panitumumab, and oral small molecules, gefitinib and erlotinib, have been reported in several clinical trials and retrospective studies in small patient

populations. Cetuximab was evaluated prospectively in a phase II study in 36 cases of advanced cSCC, including three metastatic and 33 locally advanced cSCCs in France. The ORR was 27.8%, including two cases of CR and eight cases of PR, the rate of stable disease was 41.7% (15/36 cases), and the overall disease control rate was 69.4% (25/36 cases). The most common grade 3 and 4 toxicities were infection (22.2%) and bleeding from the tumor (11.1%) (50). In a retrospective study in 34 locally advanced cSCC patients in France treated with cetuximab alone or in combination with platinum and 5-fluorouracil as neoadjuvant therapy, the RR of cetuximab alone patients was 55.5% (5/9 cases), including three cases of complete histological response, while that of combination therapy patients was 92% (23/25 cases), including 15 cases of complete histological response (51). The utility of panitumumab was evaluated in a small phase II study in 16 patients with advanced cSCC in Australia. The ORR was 31.3% (5/16 cases), including two cases of CR (52). While monoclonal antibodies showed certain RRs, oral small molecules showed limited efficacy for advanced cSCC. In phase II studies conducted in USA, gefitinib and erlotinib only showed PR rates of 15% (5/40 cases) and 10.3% (3/29 cases), respectively (53, 54).

Immune Checkpoint Inhibitors

Recent advances in cancer immunotherapy have provided new therapeutic approaches involving blocking of immune checkpoints. In particular, antibodies targeting PD-1 and its ligand, programmed cell death ligand 1 (PD-L1), have been reported to have prospective efficacy in various cancer types (55) (**Figure 1C**). The same applies to advanced cSCC; administration of the anti-PD-1 monoclonal antibody, cemiplimab, showed impressive efficacy. Utility of cemiplimab in a phase I expansion cohort study including 16 metastatic and 10 locally advanced cSCCs showed ORR of 50% (13/26 cases) and a durable disease control rate of 65.4% (17/26 cases). Duration of response exceeded 6 months in 53.8% (7/13 cases) of patients who showed a response. In a phase II study in 59 cases of metastatic cSCC, the ORR was 47.5% (28/59 cases), including four cases of CR, the durable disease control rate was 61.0% (36/59 cases), and the duration of response exceeded 6 months in 57.1% (16/28 cases) of patients who showed a response. By integrating the results of these two studies, the RR of cemiplimab for metastatic cSCC was 46.7% (35/75 cases). As high mutational and neoantigenic burdens have been reported to be strong predictors of responsiveness to immunotherapy, cSCC is expected to be responsive to immune checkpoint inhibitors. Safety assessment evaluation indicated that treatment was well-tolerated. The most common adverse events in the above phase II study were diarrhea (27.1%), fatigue (23.7%), nausea (16.9%), constipation (15.3%), and rash (15.3%). The observed grade 3 and 4 toxicities were infection (22.2%) and tumor bleeding (11.1%). The most common grade 3 and 4 toxicity was pneumonitis (3.4%), and no severe toxicities were present in more than 5% of patients (56). Based on these observations, cemiplimab was approved by the FDA, USA in 2018 for treatment of advanced cSCC.

EXTRAMAMMARY PAGET'S DISEASE

EMPD is a rare intraepithelial adenocarcinoma that principally affects the genital and axillary regions. EMPD typically grows slowly and is diagnosed as an *in situ* lesion, and it generally shows favorable prognosis with surgical resection. However, once it invades the dermis, EMPD easily causes metastasis and the prognosis becomes poor (57). Although its pathogenesis remains to be clarified, it has been reported to resemble breast cancer in its immunohistochemical and molecular profiles (58).

Pathobiologically, human epidermal growth factor receptor 2 (HER2) has been reported be overexpressed in 15–60% of EMPD cases, mainly based on amplification of the *HER2* gene (59–61). HER2 is a member of the RTKs, which regulate several downstream pathways, including MAPK and PI3-AKT pathways (42, 62) (**Figure 1B**). *HER2* gene amplification causes HER2 protein overexpression, resulting in ligand-independent homodimerization and aberrant activation of downstream signaling pathways (63). Of note, immunohistochemical staining for phosphorylated ERK and phosphorylated AKT, which are signatures reflecting activation of MAPK and PI3-AKT pathways, respectively, are positive even in HER2-negative cases (59, 61). A recent report indicated that 81.3% (13/16 cases) of Japanese metastatic EMPDs are positive for phosphorylated ERK and phosphorylated AKT regardless of the HER2 expression status, and 68.8% (11/16 cases) were positive for both signatures (61). Furthermore, DNA sequence analysis of EMPD revealed that 19% of cases had mutant *RAS* or *RAF* genes and 35% of cases had mutations in *PIK3CA*, which encodes the catalytic subunit of PI3K, or in *AKT1* that activates these pathways, suggesting that these two signaling pathways play critical roles in the pathogenesis of EMPD (64).

Signaling through androgen hormone receptor (AR) was also reported to be associated with the development of EMPD. The AR-positive rate in EMPD has been reported to be 54–90%, and its level of expression was significantly higher in invasive EMPD than non-invasive EMPD (65, 66). Moreover, the expression levels of the androgen-producing enzymes, 5α-reductase and 17β-hydroxysteroid dehydrogenase type 5, were shown to be higher in invasive EMPD than non-invasive EMPD (66, 67). These results suggested an association between androgen–AR signaling and the progression of EMPD.

TREATMENT OF METASTATIC EMPD

Chemotherapeutic Agents

At present, no chemotherapeutic agents have yet been approved for the treatment of metastatic EMPD. Several cytotoxic chemotherapeutic regimens have been used to treat metastatic EMPD, such as combination of low-dose 5-fluorouracil and cisplatin (FP therapy), combination of 5-fluorouracil, epirubicin, carboplatin, vincristine, and mitomycin C (FECOM therapy), combination of cisplatin, epirubicin and paclitaxel (PET therapy), combination of docetaxel and S-1, docetaxel monotherapy, and S-1 monotherapy (68–76). In general, certain patient populations initially respond well to these therapies.

However, the efficacies are usually temporary, and tumors soon recur due to acquisition of resistance. Low-dose FP and FECOM regimens showed RR of 59 and 57% with median PFS of 5.2 and 6.5 months as first-line treatment in Japanese metastatic EMPD patients, respectively. However, the median OS rate was <1 year (68, 69). The DTX monotherapy for Japanese metastatic EMPD patients was reported to show an RR of 58%, but its median PFS and OS were 7.1 and 16.6 months, respectively (70). Nevertheless, modification of the treatment schedule or improvement of the chemotherapeutic regimen may enhance the efficacy. In PET therapy, adjustment of the dosing interval enabled Japanese metastatic EMPD patients to continue treatment by reducing its severe toxicity, while maintaining its high efficacy (76).

Molecular Targeting Agents

HER2 is recognized as a prospective therapeutic target in cases of advanced HER2-positive EMPD based on the pathobiological similarities to breast cancer and its frequent overexpression in EMPD (**Figure 1B**). The HER2-specific humanized monoclonal antibody, trastuzumab, is an established treatment option for metastatic HER2-positive breast cancers (77). Several case reports have indicated the effectiveness of trastuzumab in combination with cytotoxic chemotherapeutic agents in EMPD (78–81). Based on these findings, a phase II study of trastuzumab with docetaxel for HER2-positive unresectable or metastatic EMPD (jRCTs031180073) is currently underway in Japan.

For therapy targeting androgen–AR signaling, one case report showed the utility of combined androgen blockade (CAB) therapy consisting of bicalutamide (anti-androgen drug) and leuprolide acetate (LH–RH agonist), which is used in the treatment of prostate cancer. While its efficacy lasted for only 6 months, it significantly reduced multiple bone metastases of EMPD (82).

MERKEL CELL CARCINOMA

Merkel cell carcinoma (MCC) is a rare but highly aggressive cutaneous neuroendocrine carcinoma (10). While they are named after sensory Merkel cells in the skin based on their ultrastructural and immunophenotypic resemblance, the true origin of MCC tumor cells remains unknown (83). Risk factors for MCC include age ≥65 years, immunosuppression, previous sun exposure, and Merkel cell polyomavirus (MCPyV) infection (84). The majority of MCCs are caused by the integration of MCPyV into the genome, and the remainder are associated with exposure to UV irradiation (84, 85).

MCPyV is a non-enveloped double-stranded DNA virus that belongs to the polyomavirus family and is highly prevalent in the general population. Primary MCPyV infection usually occurs during childhood and can be detected in the skin of nearly all healthy individuals. Despite its widespread infection, MCC develops in only a small percentage of the population. The viral oncogenes, LT and sT antigens, have been suggested to play important roles in MCPyV-induced tumorigenesis. LT antigen has been shown to bind to retinoblastoma (RB) protein, inactivating its tumor suppressive function (86). sT is the major viral oncogene that contributes to virally induced cellular transformation (87). It can maintain the hyperphosphorylated and inactivated state of the eukaryotic translational initiation factor 4E-binding protein 1 (4E-BP1), ultimately leading to acceleration of cell proliferation and malignant transformation (87). sT also inhibits the cellular ubiquitin ligase, SCFFbw7, and suppresses proteasomal degradation of MCPyV LT and other cell cycle regulators, thus contributing to viral replication and host cell transformation (88). The viral DNA was shown to be clonally integrated into the genome of MCC cells (84).

Integration of MCPyV has also been reported to be associated with immunogenicity of MCC (84, 89). Several reports indicated that MCPyV-positive tumor cells express viral antigens at high levels, which are recognized by the innate and adaptive immune systems (90). In particular, patients with higher immune responses to MCPyV show better disease outcomes than those with modest responses (91, 92). MCPyV-negative MCCs may also have immunogenicity, based on their high mutational burden and neoantigens generated by exposure to UV irradiation. In fact, MCPyV-negative MCCs have more gene mutations than cells in most other cancer types (85). Furthermore, about 50% of MCCs have been reported to express PD-1 on tumor-infiltrating lymphocytes and PD-L1 on tumor cells or infiltrating macrophages (93).

TREATMENT OF ADVANCED MCC

Chemotherapeutic Agents

Several cytotoxic chemotherapies have been preferred as treatment options for advanced MCC. Chemotherapeutic regimens were designed based on those used in small cell lung cancer (94). These include combination of platinum and etoposide, combination of cyclophosphamide, doxorubicin (or epirubicin) and vincristine, and monotherapies of topotecan and etoposide (95). Although MCC shows a relatively high RR to first-line chemotherapy, the response is rarely durable, and resistance develops quickly. One retrospective study has reported RRs of 69 and 57% to first-line chemotherapy for locally advanced and metastatic MCC, respectively, but survival was limited to averages of 24 and 9 months, respectively (96).

Immune Checkpoint Inhibitors

Based on its high immunogenicity, the application of immune checkpoint inhibitors for MCC has been examined in several clinical trials with promising results. In particular, blockade of the PD-1/PD-L1 interaction prolonged the responses and improved OS of advanced MCC. Avelumab is a monoclonal anti-human PD-L1 antibody, which activates antibody-dependent cell-mediated cytotoxicity as well as blocking PD-1/PD-L1 interactions (**Figure 1C**). In an international multicenter phase II trial in 88 cytotoxic chemotherapy-refractory metastatic MCC patients, avelumab treatment (10 mg/kg every 2 weeks) showed ORR of 33% (29/88 cases) over the minimum follow-up period of 2 years (median 29.2 months), including a CR rate of 11% (10/88 cases) (97, 98). With regard to safety concerns, only 5% (4/88 cases) of patients had grade 3 adverse events and there were no

grade 4 or 5 treatment-related adverse events. In an international, multicenter, single-arm, open-label clinical trial in 39 treatment-naive patients, avelumab showed ORR of 62.1% (29/39 cases) at the minimum follow-up period of 3 months, including CR of 13.8% (4/39 cases) and PR of 48.3% (14/39 cases) (99). Pembrolizumab is a monoclonal anti-human PD-1 antibody, which also blocks PD-1/PD-L1 interactions (**Figure 1C**). In a phase II study in 26 systemic therapy-naive stage 3 or 4 MCC patients, administration of pembrolizumab (2 mg/kg every 3 weeks) showed ORR of 56% (14/25 cases) including CR in four cases and PR in 10 cases. With regard to safety concerns, grade 3 or 4 adverse events occurred in only 15% (4/26) of patients and these were manageable (93). Based on these reports, avelumab and pembrolizumab have now been approved by the FDA, USA for treatment of metastatic MCC. With regard to other agents targeting PD-1/PD-L1 interaction, the anti-human PD-1 antibody, nivolumab, showed efficacy for MCC. In a phase I/II study in 15 treatment-naive and 10 previously treated metastatic MCC patients, administration of nivolumab (240 mg every 2 weeks) showed ORR of 68% (15/22 cases) including CR and PR in three and 12 of 22 evaluable cases, respectively. PFS and OS rates at 3 months were 82 and 92%, respectively (100).

SKIN ADNEXAL CARCINOMA

Skin adnexal carcinomas are a group of malignancies exhibiting histopathological features of follicular, sebaceous, apocrine, or eccrine differentiation. Pilomatrix carcinoma, trichilemmal carcinoma, and trichoblastic carcinoma are categorized as those showing follicular differentiation, while sebaceous carcinoma is categorized as those showing sebaceous differentiation. Malignant tumors showing sweat gland differentiation include porocarcinoma, spiradenocarcinoma, hidradenocarcinoma, apocrine adenocarcinoma, microcystic adnexal carcinoma, adenoid cystic carcinoma, malignant mixed tumor, malignant cylindroma, digital papillary carcinoma, syringoid eccrine carcinoma, and mucinous carcinoma of the skin (101). The precise molecular pathogenesis of skin adnexal carcinomas is still under investigation. Whole-exome sequencing of sebaceous carcinomas indicated that they can be divided into three clinically distinct classes: pauci-mutational type harboring fewer mutations, UV damage type with a high mutational burden due to UV damage, and microsatellite instability (MSI) type with microsatellite repeat sequence replication errors. Ocular sebaceous carcinoma belongs to the pauci-mutational type, sebaceous carcinomas associated with Muir-Torre syndrome, a hereditary cancer syndrome associated with germline mutations in mismatch repair pathway components, belongs to the MSI type, and UV damage type skin adnexal carcinomas tend to show poorly differentiated histological features (102). HRAS and EGFR have been suggested to play pathobiological roles in some cases of porocarcinoma (103). Expression of EGFR has also been reported in several sweat gland carcinomas (104). In addition, expression of HER2 (105–107) and c-KIT (108, 109) and the activation of MAPK or PI3-AKT signaling pathways have been described in single case reports of several skin adnexal carcinoma types (108–110).

TREATMENT OF SKIN ADNEXAL CARCINOMA

At present, there are neither approved chemotherapeutic agents nor uniform guidelines for the treatment of skin adnexal carcinoma. Therefore, information regarding the utility of systemic treatments for these diseases is available only in published case reports. In general, skin adnexal carcinomas are considered to be relatively chemoresistant, and the prognosis of their metastatic forms is considered to be poor. Although further studies are needed to clarify the usefulness of proposed treatment options, several reports indicated the efficacy of various chemotherapeutic agents used in combination therapy.

Chemotherapeutic Agents

With regard to metastatic skin adnexal carcinomas with sweat gland differentiation, one report indicated CR for 16 months in one case with a combination of doxorubicin, mitomycin, vincristine, and 5-fluorouracil followed by maintenance combination therapy consisting of cyclophosphamide, vincristine, and 5-fluorouracil (111). Other reports have also shown the efficacy of various other combinations of drugs, including 5-fluorouracil, thiotepa, and cyclophosphamide (112, 113), anthracycline, cyclophosphamide, vincristine, and bleomycin (114), interferon-alpha and weekly paclitaxel (115), and doxorubicin, mitomycin C, vincristine, and cisplatin (116). Notably, the combination of carboplatin and epirubicin maintained CR for 4 years in a case of porocarcinoma (49).

Molecular Targeting Agents

With regard to targeted therapy, administration of sunitinib, an oral small molecule, multi-targeted RTK inhibitor, stabilized disease progression over 8 months in a patient with metastatic clear cell hidradenocarcinoma and achieved PR for 10 months in a patient with metastatic trichoblastic carcinoma (117). Administration of trastuzumab showed CR for 7 months in a patient with HER2-positive apocrine carcinoma. Moreover, combination of capecitabine and lapatinib, an oral anti-HER2 targeted therapy, in the same patient achieved CR for 6 months after the metastatic lesion developed resistance to trastuzumab (118).

Immune Checkpoint Inhibitors

The usefulness of targeting PD-1/PD-L1 interaction has also been reported. The results of immunohistochemical analysis indicated that PD-L1 was expressed in 50% (12/24) of ocular sebaceous carcinoma cases (119). Administration of anti-human PD-1 antibodies showed efficacy in patients with metastatic sebaceous carcinoma (120, 121).

CONCLUSION

While a standard management strategy for treatment of advanced NMSC has not been established, advances in our molecular biological understanding of NMSC and improvement of drug discovery techniques over the past several decades have facilitated the establishment of novel treatment strategies. Nevertheless, emerging molecular targeting therapies are not

necessarily effective for all NMSC patients. Development of further treatment options for NMSC is required, especially for rare forms of NMSC, such as skin adnexal carcinomas.

AUTHOR CONTRIBUTIONS

TF wrote the part of squamous cell carcinoma. IH wrote the part of extramammary Paget's disease. YN wrote the part of basal cell carcinoma. KT wrote the part of Merkel cell carcinoma and adnexal carcinomas. KT also unified the format of entire manuscript.

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Biomarkers for Predicting Efficacies of Anti-PD1 Antibodies

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Therapeutic options for treating advanced melanoma are progressing rapidly. Although anti-programmed cell death 1 (PD1) antibodies (e.g., nivolumab, pembrolizumab) have been approved as first-line and anchor drugs, respectively, for treating advanced melanoma, the efficacy appears limited as we expected, especially in Asian populations. Biomarkers to predict or evaluate the efficacy of anti-PD1 antibodies are needed to avoid subjecting patients to potentially severe adverse events associated with switching to other anti-melanoma drugs. This review focuses on the recent development of biomarkers for assessing the efficacy of anti-PD1 antibodies using routine blood tests such as the neutrophil-to-lymphocyte ratio, eosinophil ratio, serum markers such as lactate dehydrogenase, programmed cell death ligand 1 (PD-L1) expression on melanoma cells, microsatellite instability and mismatch repair deficiency assays, as well as soluble CD163, and tumor-associated macrophage-related chemokines (e.g., CXCL5, CXCL10).

Keywords: anti-PD1 antibodies, routine blood test, LDH, MSH, TMB, TAM-related factors

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INTRODUCTION

Anti-programmed cell death 1 (PD-1) antibodies are in wide use for the treatment of various cancers, particularly cancers with a high tumor mutation burden (TMB) such as advanced cutaneous melanoma (1–4). Although BRAF inhibitors in combination with MEK inhibitors are useful for the treatment of $BRAF^{V600}$-mutant advanced melanoma, the population of $BRAF^{V600}$-mutant advanced melanoma is limited, particularly in the Japanese population, which contains large populations with acral lentiginous melanoma and mucosal melanoma (5, 6). Most patients with advanced melanoma are therefore administered nivolumab with or without ipilimumab, or pembrolizumab as a first-line therapy.

Ipilimumab is a fully humanized immunoglobulin (Ig)G1 monoclonal antibody that blocks cytotoxic T-lymphocyte antigen (CTLA-4) to activate and increase T cells, particularly the tumor-recognized T-cell clones that reside in primary tumors (7, 8). Combination therapy comprising nivolumab and ipilimumab or sequential administration of nivolumab and ipilimumab with a planned switch are among the most effective chemotherapies against advanced melanoma (9–11), and even increase the response rate (RR) for untreated metastasis of melanoma to the brain compared to nivolumab monotherapy (12). On the other hand, the efficacy of ipilimumab in patients with nivolumab-resistant melanoma is low after objective tumor progression compared to planned-switched patients (13). In addition, ipilimumab leads to a high frequency of immune-related adverse events (irAEs) among patients with advanced melanoma, particularly combination therapy with nivolumab (9, 11). Taken together, evaluation of the efficacy of these treatments in advance is important.

This review focuses on the recent development of biomarkers for assessing the efficacy of anti-PD1 antibodies using routine blood tests such as the neutrophil-to-lymphocyte ratio, eosinophil ratio, serum markers such as lactate dehydrogenase (LDH), PD-L1 expression on melanoma cells, microsatellite instability (MSI) and mismatch repair deficiency assays, as well as soluble CD163, and tumor-associated macrophage (TAM)-related chemokines (e.g., CXCL5, CXCL10) (**Table 1**).

TABLE 1 | Highlighted papers in each chapter.

	Interest	Considerable interest
Routine blood test	14, 21, 25	23
PD-L1 expression	31	32
MSI and TMB	40, 43	3, 39
TAMs related factors	24, 50	48

SIGNIFICANCE OF ROUTINE BLOOD TESTS FOR PREDICTING THE EFFICACY OF ANTI-PD1 ANTIBODY

Leukocyte-to-Lymphocyte Ratio (LLR), Neutrophil-to-Lymphocyte Ratio (NLR), Monocyte Count, and Absolute Lymphocyte Count (ALC)

Recent reports have suggested the significance of routine blood tests, such as cell counts and cell ratios, for predicting the efficacy of anti-PD1 antibodies against advanced melanomas (14–18). Indeed, Fujisawa et al. reported that increased baseline NLR combined with serum LDH was significantly correlated with the efficacy rate of nivolumab according to multivariate analysis, and negatively correlated with efficacy of nivolumab for advanced melanoma (14). In another report, Chasseuil et al. found that increased monocyte count was significantly associated with decreased overall survival (OS) and progression-free survival (PFS) in patients with advanced melanoma according to multivariate analysis. In addition, they also reported that LLR was significantly associated with decreased OS (15). In addition, Rosner et al. reported that not only a low NLR, but also high proportion of eosinophils, high proportion of basophils, low absolute monocyte count and low LDH might be independently associated with favorable OS (16). Since several previous reports have also suggested that NLR is significantly correlated with the efficacy of ipilimumab in the treatment of melanoma patients (17, 18), baseline NLR could be one possible predictive marker for immune checkpoint inhibitor (ICI)-treated patients with advanced melanoma.

Lower ALC shows significantly less clinical benefit from anti-PD1 antibody (19), which is associated with pretreatment NLR in patients with head and neck squamous cell carcinoma. They concluded that patients with pretreatment ALC <600 cells/μl had shorter PFS than patients with pretreatment ALC ≥ 600 cells/μl. In another report, Soyano et al. retrospectively analyzed 157 patients with advanced non-small cell lung cancer (NSCLC) treated with anti-PD1 antibodies using logistic regression analysis, suggesting that a high baseline NLR correlated significantly with increased risks of death and disease progression (20). In addition, they also reported that a high baseline myeloid-to-lymphoid cell ratio significantly increased the risk of death, even after multivariate analysis [hazard ratio (HR) $= 2.31$, $p = 0.002$]. Indeed, a meta-analysis of 14 retrospective studies that had examined the benefits of nivolumab in patients with NSCLC suggested an association of high NLR with poor PFS and OS after nivolumab treatment (21). Moreover, they also reported that post-treatment NLR acted as a predictor of PFS and OS. Overall, these reports have suggested that baseline routine blood tests are important for predicting the efficacy of ICI (**Table 2**).

Clinical Use of LDH

Generally, large baseline tumor size in parallel with increased levels of LDH correlates with poor prognosis in advanced melanoma patients (25). Diem et al. first reported the benefit of measuring serum LDH in 66 patients with advanced melanoma treated using anti-PD1 antibody (22). Indeed, patients with elevated baseline LDH showed significantly shorter OS compared to patients with normal LDH. Moreover, they suggested serum LDH as a useful marker during treatment for predicting both early response of anti-PD1 antibody and progressive disease (22).

ECOG performance status (PS) and elevated LDH were reported as independent variables significantly associated with poor OS (26). More recently, Wagner et al. reported serum LDH levels and S100B among the early prognostic markers for response and OS in advanced melanoma patients treated with ICI (27). They concluded that, compared with patients showing normal LDH, increased serum LDH ($>25\%$) was significantly associated with impaired OS when co-existing with increased serum levels of S100B (27). Increased LDH correlated with the poor prognostic factors of not only cutaneous melanoma, but also uveal melanoma, which possesses a high potential for rapid metastasis (28), and NSCLC (29). Since anti-PD1 antibody applies to various cancers, including gastric cancer, renal cell carcinoma, and Hodgkin lymphoma, measurement of LDH might offer a useful, standard marker for patients treated using ICI.

EXPRESSION LEVELS OF PD-L1

In cutaneous melanoma, both tumor cells and TAMs express PD-L1, leading to the maintenance of an immunosuppressive microenvironment at tumor sites (30, 31). Hino et al. first reported PD-L1 expression on melanoma cells as an independent prognostic factor that correlates with vertical invasion of melanoma cells (31). Accordingly, many studies have suggested that PD-L1 expression on melanoma cells can represent a biomarker for predicting the efficacy of anti-PD1 antibodies (32, 33), and even other ICIs (34). For example, PD-L1 expression on melanoma cells in pretreatment tumor biopsy samples correlated

TABLE 2 | Summary of biomarkers and their efficacy.

Treatment	Patients number	Subpopulation	Outcome	Marker	Result (95% CI) RR = 14%	*p*-value	References
Nivolumab	*n* = 90	LDH (upper normal limit)	RR	NLR > 2.2 NLR < 2.2	RR=14% RR=57%	<0.05	(14)
		LDH normal	RR	NLR > 2.2 NLR < 2.2	RR=37% RR=67%	<0.001	
Nivolumab	*n* = 87		OS	Monocyte count (upper normal limit)	HR = 4.31 (1.46–12.74)	0.01	(15)
			PFS	Monocyte count (upper normal limit)	HR = 3.5 (1.01–12.1)	0.04	
Nivolumab + Ipllimumab	*n* = 209		OS	NLR Eosinophils Basophils Absolute monocyte LDH (246<)	HR = 1.95 (111–3.47) HR = 2.38 (1.27–4.46) HR = 1.86 (0.94–3.66) HR = 2.75 (1.30–5.80) HR = 3.71(2.08–6.61)	0.02 0.007 0.08 0.01 <0.0001	(16)
Lpllimumab	*n* = 183		OS	Baseline NLR NLR (end of treatement)	HR = 1.06 (1.01–1.10) HR = 1.06 (1.02–1.09)	0.016 <0.001	(17)
Lpllimumab	*n* = 720		OS PFS	ANC ANC	HR = 3.38 (2.62–4.36) HR = 2.52 (1.97–3.21)	<0.0001 <0.0001	(18)
Anti-PD1 antibody	*n* = 66		OS	LDH elevated LDH normal	4.3 months 15,7 months	<0.00623	(22)
Nivolumab	*n* = 210		RR	PD-L1 positive PD-L1 negative	52.7% (40.8–64.3) 33.1% (25.2–41.7)		(23)
Nivolumab	*n* = 59		RR	Increased Scd163	Sensitivity 84.6% Specificity 87.0%	<0.0030	(24)

ANC, absolute neutrophilcount; HR, hazzard ratio; NLR, neutrophilto lymphocyte ratio; OS, overall survival; PFS, progress free survival; RR, response rate; UNL, under normal limit.

with RR, PFS, and OS in advanced melanoma patients treated using anti-PD1 antibodies (33). In another report, expression of PD-L1 correlated with 24-month survival rate in patients with advanced melanoma treated with pembrolizumab (32). Indeed, median PFS in patients with PD-L1 positive melanoma cells was 6.6 months (95% confidence interval (CI), 4.2–9.7 months), while median PFS in patients with PD-L1-negative melanoma cells was 2.8 months (95%CI, 2.8-3.7 months) (32). On the other hand, Hodi et al. reported that assessment of the expression of PD-L1 alone offers a poor predictor of OS in patients treated with nivolumab or nivolumab in combination with ipilimumab (CheckMate 067) (34). Notably, even in PD-L1-negative or -intermediate expressing groups, the RR is still high (33.1%; 95%CI, 25.2-41.7%), suggesting that PD-L1 expression might represent an independent prognostic factor (35). Although those reports suggested the clinical benefits of assessing PD-L1 expression on melanoma cells in predicting the clinical outcomes of ICI treatment, the clinical utility in the real world is limited because of the low sensitivity of immunohistochemical (IHC) assays using different antibody clones, staining platforms and scoring systems in each institute (32–36). To avoid misprediction by IHC staining, more recently, Conroy et al. tried to assess the expression of PD-L1 using next-generation RNA sequencing, but the sensitivity of their system resembles that of IHC assay systems (36). In future, additional assays will be needed to improve the sensitivity of PD-L1 analysis in the prediction of clinical outcomes for ICI treatment of melanoma.

MSI AND TMB

The high RR to anti-PD1 antibodies for cancers with high frequency of MSI has been highlighted in many recent clinical studies (37, 38). Among cancer species, colorectal cancer and endometrial cancer possess a high frequency of MSI (approx. 20∼33%) (38, 39), leading to the results of clinical studies that have presented significantly improved RR, PFS and OS in patients with mismatch-repair deficient colorectal cancers compared to those of mismatch repair-proficient colorectal cancers (37–39). Recent reports have also suggested that high infiltration of T-helper 1 (Th1) cells and cytotoxic T lymphocytes (CTLs) produce substantial amounts of interferon gamma (IFNγ), leading to increased expression of PD-L1 in tumors with a high frequency of MSI (37, 40). As described above, since high expression of PD-L1 can provide a biomarker for predicting the efficacy of anti-PD1 antibodies, a high frequency of MSI could correlate with RR, PFS and OS following use of anti-PD1 antibodies.

High TMB correlated with increased neoantigens in various cancers, and could provide predictors for the efficacy of ICI treatment (1, 2, 41). For example, cutaneous squamous cell carcinoma (cSCC) possesses a high TMB (50 mutations per megabase DNA pairs) (42), leading to a high RR for cemiplimab [47% (95%CI, 34–61%)] (43). In addition, since an ultraviolet (UV) damage subclass of SCC and sebaceous carcinoma harbors a high somatic mutation burden with >50 mutations per megabase, UV damage signatures in TMB in these skin cancers

could be predictive biomarkers for ICI treatment (44, 45). In melanoma, Madore et al. reported that a lower non-synonymous mutation burden correlated with negative results for PD-L1 expression on melanoma cells, and significantly worse melanoma-specific survival in stage III melanoma (HR = 0.28; 95%CI, 0.12-0.66; P = 0.002) (3). In addition, significant increases in the gene expression signatures of cytotoxic T-cell (CTL) and macrophage-specific genes were seen in PD-L1-positive melanomas, correlating with better melanoma-specific survival (HR = 0.2; 95%CI, 0.05-0.87; $P = 0.017$). Taken together, those reports might suggest the significance of assessing TMB before the administration of ICIs, especially anti-PD1 antibodies, although further studies are needed to confirm its effectiveness.

PILOT STUDY FOR PREDICTABLE BIOMARKERS: TAM-RELATED FACTORS (SCD163, CXCL5)

TAMs are functionally reprogrammed to polarized phenotypes by exposure to various factors, leading to the maintenance of a tumor microenvironment (30). Expression of PD-L1 on TAMs is modified by both stromal factors such as regulatory T cells (Tregs) and exogenous factors including immune therapies (46). For example, in a mouse melanoma model (ret, B16 melanoma), depletion of Tregs decreased PD-L1, B7H3, and B7H4 expression on TAMs *in vivo* (46). In patients with esophageal carcinoma, a high density of CD163+ TAMs, which is also associated with significantly increased PD-L1 expression (47), was associated with significantly worse OS than a low density (log-rank $P = 0.0025$) (47). That report suggested that a high density of PD-L1-expressing CD163+ TAMs could offer a prognostic biomarker for esophageal carcinoma (47). Since PD-L1 on tumor cells could be one prognostic factor for melanoma patients treated with ICIs (as described in Chapter 3), TAM-related factors could offer biomarkers for predicting the efficacy of ICI.

TAMs in melanoma patients express not only PD-L1, but also PD-1 (48). Because PD-1 expression in TAMs is one of the key factors in M2 macrophage polarization (49), administration of an anti-PD1 antibody might repolarize TAMs, leading to TAM activation in melanoma patients. Notably, the main population of TAMs in skin cancer is CD163+ M2 macrophages, with soluble (s)CD163 as the activation marker (14). This means that CD163 activated with PD1 antibody should release sCD163, suggesting its utility as a prognostic marker for anti-PD1 antibody treatment. Indeed, serum levels of sCD163 were significantly increased in responders compared to non-responders 6 weeks after initial administration of nivolumab for cutaneous melanoma (84.6% sensitivity, 87.0% specificity; $p = 0.0030$) (24). Moreover, absolute serum levels of sCD163 after 6 weeks were significantly increased in patients treated with nivolumab who developed irAEs ($p = 0.0018$) (49). Those reports suggested that serum sCD163 could offer a predictive marker for the efficacy and irAEs of anti-PD1 antibodies.

In addition to sCD163, TAM-related chemokines could provide another group of prognostic markers for the outcomes of anti-PD1 antibody treatment (50). For example, CXCL5 is a chemokine that can recruit neutrophils, CXCR2+ myeloid-derived suppressor cells (MDSCs) and CXCR2+ monocytes. As we previously reported, production of CXCL5 from TAMs is increased by stimulation with periostin (51), which is detected in the stroma of cutaneous melanomas (23, 49). As we previously reported, baseline serum CXCL5 is associated with the efficacy of nivolumab in advanced melanoma (50) and increased serum levels of CXCL5 correlated significantly with irAEs from nivolumab (49). Unlike CXCL5, baseline serum concentrations of CXCL10 and CCL22 have not shown any correlations with the efficacy of nivolumab against advanced melanoma (50). These data suggested that TAM-related chemokines could further improve the predictive value of sCD163 systems in the future.

Although combination therapy with nivolumab and ipilimumab is recommended by the NCCN guideline for cutaneous melanoma as a first-line therapy (52), as described above, this combination therapy leads to a high frequency of SAEs among patients with advanced melanoma (9, 11). In the future, the evaluation of serum sCD163 as well as several TAM-related chemokines will undoubtedly play an important role in avoiding the administration of ipilimumab for patients who respond to anti-PD1 antibodies.

CONCLUDING REMARKS

Although several studies have suggested useful predictive markers for the efficacy and irAEs of ICIs, exact methods to determine predictive markers remain under investigation. Further studies are needed to improve the systems for predicting the efficacy of ICI treatment.

AUTHOR CONTRIBUTIONS

YK, TF, and TH wrote manuscript. SA supervise the manuscript.

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Next-Generation Sequencing Technologies for Early-Stage Cutaneous T-Cell Lymphoma

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The diagnosis of early stage cutaneous T-cell lymphoma is often difficult, particularly in mycosis fungoides (MF), because the clinical presentation, histological findings, and laboratory findings of MF resemble those of inflammatory skin diseases such as atopic dermatitis, psoriasis, and parapsoriasis en plaque. Furthermore, MF sometimes occurs with or after these inflammatory skin diseases. The current diagnostic criteria heavily rely on clinical impressions along with assessments of T cell clonality. To make a diagnosis of early-stage MF, the detection of a malignant clone is critical. T cell receptor (TCR) gene rearrangements have been detected by southern blotting or polymerase chain reaction for this purpose, but the results of these methods are insufficient. High-throughput TCR sequencing has provided insights into the complexities of the immune repertoire. Accordingly, his technique is more sensitive and specific than current methods, making it useful for the detection of early lesions and monitoring responses to therapy.

Keywords: mycosis fungoides, early stage, T-cell receptor, rearrangement, next-generation sequencing

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INTRODUCTION

Cutaneous T cell lymphomas (CTCLs) comprise a heterogeneous class of non-Hodgkin lymphomas that are derived from skin-tropic T cells. Mycosis fungoides (MF), the most prevalent type of primary CTCL, accounts for almost half of all cases (1–3). MF is clinically characterized by erythematous patches, plaques, or skin tumors, and is can be associated with lymph node, blood, and internal organ involvement. More than two-thirds of MF patients are in early stage at first presentation (3–5). MF often starts as an unspecific erythema, similar to many inflammatory skin diseases.

Histopathologically, MF can be characterized by the epidermotropic proliferation of small- to medium-sized pleomorphic lymphocytes forming intraepidermal collections, also called Pautrier's microabscesses. This microabscess is considered the histopathological hallmark of disease, but it is only observed in <20% of early MF cases (6). These microabscesses are also usually recognized as epidermotropic atypical lymphocyte infiltration without spongiosis, although spongiotic variants of MF have been reported (7, 8). Morphologic characterization of early-stage MF might show non-specific findings (9), because skin lesions are infiltrated by large numbers of non-malignant memory T cells, often making it impossible to distinguish malignant T cell clones from activated benign infiltrating T cells based on histopathology (6). Clinical and histopathological algorithms have been developed to aid early diagnosis (10), but the specificity and sensitivity of these algorithms for early diagnosis in individual patients are by no means established. A definitive diagnosis can only be made based on careful clinicopathological correlations (9).

Early stages of MF can resemble benign inflammatory skin disorders (11, 12) like chronic dermatitis including atopic dermatitis (AD), psoriasis, and parapsoriasis en plaque (PEP), among others. AD is a common chronic inflammatory skin disorder that has a T-helper (Th) 2 type-dominant phenotype, skin-barrier dysfunction, and pruritus (13). In contrast AD is an inflammatory disorder, and its pathophysiology is similar to that of AD. Mycosis fungoides, characterized as a Th2-type disease (14, 15), is frequently linked to eosinophilia and high serum immunoglobulin E levels. Although affected skin and peripheral blood T-cells express a Th1 cytokine profile during early-stage MF (16, 17), chemokines expressed in MF lesional skin, such as CCL17, CCL11, and CCL26, are supposed to induce a Th2 milieu in MF (18).

Barrier dysfunction is also observed in MF (19). Lower levels of skin moisture, with increased transepidermal water loss, have been observed in the lesional skin of CTCL, compared to that in normal skin. CTCL lesional skin also displays decreased levels of *filaggrin* and *loricrin* mRNAs compared to those in normal skin, which has also been demonstrated for AD. Pruritus is often present in MF patients (20) and constitutes one of the most disturbing symptoms for patients (21). Therefore, it is occasionally difficult to clinically differentiate MF from AD. The coexistence of MF and AD in patients was also reported in several studies (22, 23).

Psoriasis is a common, chronic inflammatory skin disorder defined by thickened, red, scaly plaques with systemic inflammation. The relationship between MF and psoriasis is sometimes difficult to determine, as there is often significant overlap in terms of pathological and clinical observations, particularly in early stages (24). Psoriasis and MF have the abnormal function of T cells as a common symptom. Psoriasis was recognized as a Th1 disease, although recent data suggest that it might be a Th17 disease (25). MF during preliminary stages also exhibits a Th1 phenotype (26); moreover, Krejsgaard et al. (27) reported that malignant T cells from CTCL lesions produce IL-17. Therefore, many early MF cases are misdiagnosed as psoriasis, whereas another group of cases occur in which the two diseases coexist and/or psoriasis develops into MF. The prevalence of psoriasis among patients with MF was found to be higher than that estimated for the general population (24, 28) and patients with psoriasis have an elevated risk of lymphomas including CTCL (29, 30). In addition to the common symptoms, immunosuppressive agents might also promote MF development in patients with psoriasis (31).

PEP is a chronic, inflammatory skin disorder, closely resembling early-stage MF. Clinically, PEP consists of persistent, scaly, well-demarcated erythematous lesions. Pathologically, it is associated with superficial lymphocyte infiltration with various degree of epidermotropism (32). More than 30% patients with large plaque parapsoriasis develop pathologically-confirmed MF (32), and therefore, PEP is often an early manifestation of MF. However, the individual clinical course might determine the difference between early-stage MF and PEP.

Because of difficulties associated with differential diagnosis, MF often remains undiagnosed for years. Accordingly, the average time from the appearance of lesions to a definitive diagnosis was to be estimated 3–6 years (33, 34).

T-CELL RECEPTOR CLONALITY IS AN IMPORTANT CRITERION IN MF DIAGNOSIS

Diagnosing T-cell malignancies is often hampered by difficulties in distinguishing neoplastic T cells from reactive T cells based on conventional morphological and immunopathological criteria (35). Skin lesions of MF patients are infiltrated by many non-malignant memory T cells, and thus, it is often impossible to distinguish a malignant T cell clone from activated, benign, infiltrating T cells by histopathology, particularly for early-stage lesions (10).

T-cell receptor (TCR) gene configurations are thought to be the most promising marker to identify malignant T-cell proliferation (36). TCRs are cell-surface protein heterodimers that are expressed on T cells and comprise either α and β chains or γ and δ chains. Immature T-cells rearrange their TCR genes during maturation in the thymus, then mature as either αβ or γδ lineage T-cells (37). TCRs are unique to individual T cell clones. Malignant cells in MF express αβ TCRs. The gene segments that encode variable (V), diversity (D) (β and δ chains only), joining (J), and constant (C) domains of the TCR protein exist as multiple unique sets (38). Since TCR genes are rearranged during thymic T-cell development (**Figure 1**) but not in mature T-cells, a peripheral T-cell lymphoma clone of malignant cells should only have a single TCR gene sequence. TCR genes are rearranged polyclonally in normal and reactively proliferating T cells as rearrangements are random, whereas neoplastic cells contain identically-rearranged TCR genes. Similarly, molecular analysis of such rearrangements can be useful to differentiate MF from benign skin conditions (39). Half of patients with large plaque parapsoriasis were reported to have TCR monoclonality in the lesional skin (40), and thus, the detection of monoclonality in a skin lesion generally suggests CTCL including MF, rather than inflammatory skin disorders.

CONVENTIONAL METHODS FOR DETECTING T-CELL CLONALITY

Initially, Southern blotting was used to determine TCR gene rearrangement clonality (41). This technique can detect clonal T-cell populations in most T-cell lymphomas without prior amplification, but has several disadvantages including low sensitivity and the requirement for large amounts of fresh frozen tissue. Therefore, since the effectiveness of Southern blotting in the diagnostic work-up of MF was reported (42–44) in the early 1990s, more sensitive polymerase chain reaction (PCR)-based methods have been developed. PCR amplification of rearranged TCR gamma genes using genomic DNA as the template was reported to permit the detection of clonal T cells with a sensitivity of 0.1–1% from a background population of polyclonal T-cells (45). Conventional agarose gel electrophoresis of the PCR products often fails to reliably differentiate polyclonal from

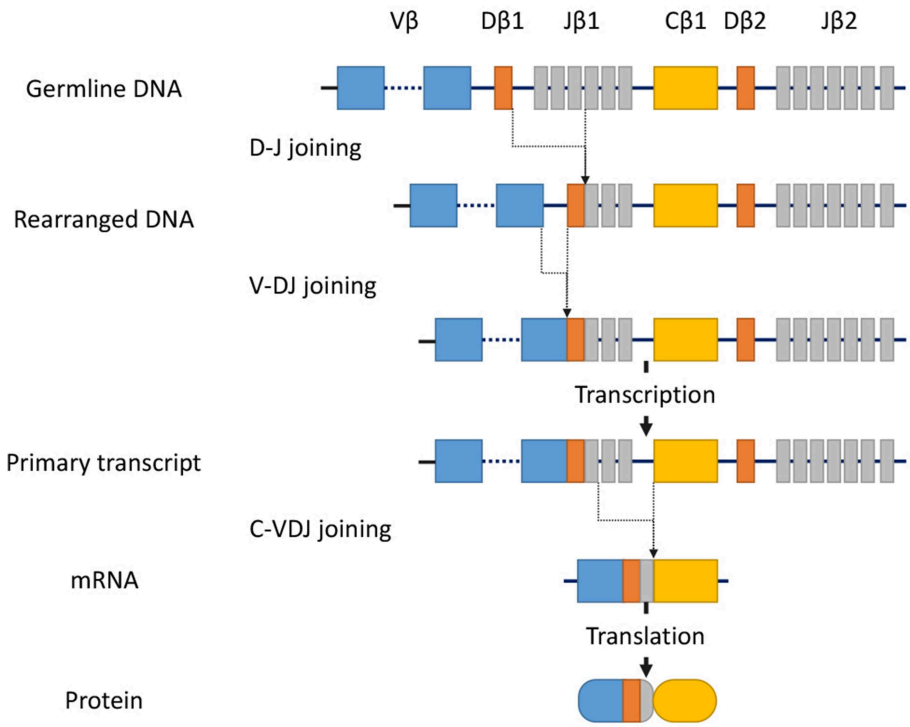

FIGURE 1 | Somatic recombination of the *TCRβ* gene. Rearrangement begins with D-J recombination followed by subsequent V-DJ recombination. The segments recombine randomly to generate TCR diversity. After transcription, intervening sequences are spliced, generating the *TCRβ* chain transcript with V, D, J, and C region segments. Finally, transcripts are translated into protein. In contrast, the *TCRα* gene lacks the D-segment, and its rearrangement starts with V-J recombination.

monoclonal TCR junctions (46), because the narrow size range of the PCR products makes multiple bands appear as single bands. Therefore, PCR amplification with subsequent denaturing gradient polyacrylamide gel electrophoresis and gel scanning (47, 48) or the Biomed GeneScan analysis of flat or capillary polyacrylamide gels (49, 50) has been used as a diagnostic assay for clonality in CTCL patients.

Despite these technical advances, current methods for TCR clonality are still sometimes insufficient for a definitive diagnosis, particularly at early stages, because early lesions often do not contain sufficient numbers of clonal T-cells (51–53). These non-quantitative tests have significant false negative and positive rates for MF (50, 54) and are particularly unreliable for early-stage MF (55).

HIGH-THROUGHPUT SEQUENCING TECHNOLOGIES FOR DIAGNOSING MF

Recent improvements in assays to assess T cell clonality have been achieved based on next-generation high-throughput sequencing (NGS) technologies. By sequencing the third complementarity-determining regions (CDR3s) of genes encoding TCRβ and TCRγ, the number of individual T cell clones present in a sample, the relative proportions of specific clones, and the CDR3 region sequences of each clone can be quantified (56, 57). Likewise, NGS represents a superior method to diagnose CTCL through the precise identification of malignant T cell clones (58–60). This technique is more sensitive than previous techniques for the detection of clonality (59, 60). Further, NGS-based methods allow the clinician to follow specific clones when monitoring disease recurrence and progression (60). Furthermore, TCR sequencing has clarified that neoplastic cells in some MF lesions might be as few as 1% of the total population of T cells (59). These data clearly explain the difficulties encountered in the histopathological assessment of early-stage MF. In contrast, the frequencies of the most dominant T cell clones range from 1 to 10% in most cases of inflammatory skin disorders such as psoriasis and eczematous dermatitis, among others, whereas the frequency of the most dominant T cell clones adjusted by total nucleated cells could distinguish MF from inflammatory skin disorders (59). Therefore, PEP often demonstrates TCR rearrangement, and NGS-based TCR gene analysis might overcome difficulties in distinguishing PEP and early-stage MF T-cell repertoires.

Most MF cases present as early-stage, typically with a chronic, indolent clinical course. Greater than 80% of patients with early-stage disease will present with an indolent life-long course, free of disease progression, independent of the treatment modality (5). For many years, most patients will also exhibit short-term clinical response associated with recurrent disease, as well as a normal life expectancy in the majority of cases. Furthermore, the limited efficacy associated with chemotherapy has been discussed in retrospective studies (61, 62), making it clear that potentially toxic and aggressive therapies should

be avoided (63, 64). However, a small number of early-stage cases will progress. Since advanced-stage patients have poor prognoses, the early identification of high-risk subpopulations is important.

Using NGS technologies, de Masson et al. (55) demonstrated that an enhanced proportion of a malignant T cell clone in the skin is strongly correlated with reductions in progression-free and overall survival for patients with CTCL, and particularly for patients with early-stage MF with a T2 distribution. Further, based on high throughput DNA sequencing of the TCRβ gene, a tumor clone frequency of >25% was found to be a strong predictor of disease progression and poor survival for MF patients with disease limited to the skin.

In summary, evidence for TCR clonality from any method is strong evidence for malignancy. However, it is not conclusive, because benign conditions have also been associated with clonal T-cell populations, such as reactive or autoimmune conditions (65, 66).

CONCLUSION

NGS can be used to assess TCR clonality with superior sensitivity compared to current methods and is useful to diagnose early stage MF. Moreover, this technique permits the tracking of specific clones across different time points or in multiple lesions for a more accurate diagnosis of MF recurrence or progression (55, 59, 60).

AUTHOR CONTRIBUTIONS

KF conceived the concept and wrote the manuscript. TK edited and improved the manuscript.

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Dermoscopy of Melanoma and Non-melanoma Skin Cancers

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Dermoscopy is a widely used non-invasive technique for diagnosing skin tumors. In melanocytic tumors, e.g., melanoma and basal cell carcinoma (BCC), the effectiveness of dermoscopic examination has been fully established over the past two decades. Moreover, dermoscopy has been used to diagnose non-melanocytic tumors. Here, we review novel findings from recent reports concerning dermoscopy of melanoma and non-melanoma skin cancers including BCC, sebaceous carcinoma, actinic keratosis, Bowen's disease, squamous cell carcinoma (SCC), Merkel cell carcinoma (MCC), extramammary Paget's disease (EMPD), and angiosarcoma.

Keywords: dermoscopy, melanoma, basal cell carcinoma, sebaceous carcinoma, actinic keratosis, Bowen's disease, squamous cell carcinoma, Merkel cell carcinoma

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INTRODUCTION

Dermoscopy is a non-invasive technique for diagnosing skin lesions, which aids in the differentiation between benign and malignant alterations. Dermoscopy has been used to diagnose non-melanocytic tumors. Recently, vascular morphology has been identified as an important criteria in dermoscopic diagnosis when assessing non-melanocytic tumors. Here, we review recent reports of novel findings related to dermoscopy of melanoma and non-melanoma skin cancers.

Melanoma

Dermoscopy has been shown to improve significantly the diagnosis of melanocytic lesions in the clinical practice. Following the Consensus Net Meeting on Dermoscopy in 2000, a two-step algorithm for a method of dermoscopic diagnostic was established, especially for pigmented skin lesions (1). Marghoob and Braun subsequently devised a revised two-step algorithm including polarized dermoscopy and blood vessel morphology (2). In the second step of the revised algorithm, pattern analysis, the ABCD rule, the Menzies method (3), and the seven-point checklist are employed to diagnose melanoma. The seven-point checklist is based on seven melanoma-specific criteria; each is classified as major or minor. Major criteria consist of atypical networks, blue-whitish veils, and atypical vascular patterns while minor criteria are irregular dots/globules, irregular streaks, irregular blotches, and regression structures. A score of 2 is given to each of the major criteria, whereas a score of 1 is given to each of the minor criteria. Results yielding 3 points or more should be considered suspicious enough to justify exclusion.

Lentigo Maligna and Lentigo Maligna Melanoma

The differential diagnoses related to lentigo maligna (LM) and lentigo maligna melanoma (LMM) include a myriad of other pigmented skin lesions, including solar lentigo, seborrheic keratosis, and pigmented actinic keratosis. While the pigment network is the dermoscopic hallmark of superficial spreading melanoma (SSM) located on the trunk and extremities, a true pigment network is rarely found in LM. A pseudonetwork pattern refers to the common dermoscopic

finding of melanocytic and non-melanocytic pigmented macules of the face; it is a structureless diffuse brown pigmentation interrupted by numerous, variably broad, and hypopigmented holes, which correspond to hair follicles and sweat gland openings. Based on a report by Schiffner et al., the four most important features are asymmetric pigmented follicular openings, dark rhomboidal structures, slate-gray globules, and slate-gray dots. This analysis yields a sensitivity of 89% and a specificity of 96% (4).

Nodular Melanoma

Considering that most dermoscopic features have been described in the context of SMM, the dermoscopic recognition of nodular melanoma (NM) is also challenging since the tumor often lacks the well-known melanoma-specific criteria. Therefore, several accepted dermoscopic criteria of melanoma are not detected in purely nodular tumors.

Argenziano et al. described a new predictor of NM, namely the presence of blue-black color within the lesion (5). The blue-black color is thought to reflect the combination of pigments localized in the mid-deep dermis (blue) and the epidermis (black). The authors reported that all lesion surfaces that were comprised of at least 10% blue and black areas were significantly associated with pigmented NM. Moreover, Pizzichetta et al. reported NM was related to features such as ulceration, homogeneous disorganized patterns, homogeneous blue-pigmented structureless areas, multiple ($\geq$3) colors, the combination of polymorphous vessels and milky-red globules/areas, and symmetric shapes (6).

Acral Melanoma (Volar)

Saida et al. reported dermoscopic patterns of acral melanoma in the Japanese population (7). They found that the parallel ridge pattern (PRP) is the most specific dermoscopic finding for acral melanomas. PRP consists of a band-like pigmentation located on the ridges of the skin markings. In their report, the sensitivity and specificity of PRP for acral melanoma were 86.4 and 99%, respectively. In additional studies, Phan et al. and Ozdemir et al. reported that PRP was detectable in 53 and 60.8% of their cases (8, 9). Lallas et al. proposed the BRAAFF checklist, a score composed of four positive features, i.e., irregular blotches, PRP, asymmetry of structures, and asymmetry of colors, and two negative features such as the parallel furrow pattern (PFP) and fibrillar pattern. Based on the results of the BRAAFF checklist, they proposed a dermoscopic diagnostic algorithm that achieves 93.1% sensitivity and 86.7% specificity for acral melanoma diagnosis (10).

It is difficult to differentiate melanoma from melanocytic nevus if PRP or PFP is not observed. In melanoma on the volar skin without typical dermoscopic patterns, Mikoshiba et al. recently reported frequently observed features consisting of asymmetry, greater numbers of colors ($\geq$3), irregular distribution of blue-white structures, dots and globules, vascular structures including milky red areas, ulcers, diffuse pigmentation, and irregular streaks (11).

Nail Apparatus (Subungual) Melanoma

Important dermoscopic features of nail apparatus (so-called subungual) melanoma include irregular lines on a brown background, micro-Hutchinson's sign triangular pigmentation on the nail plate, and a wide pigmented band (12). The width of pigmentation is an important risk factor. Ohn et al. reported the following factors to be associated with adult-onset *in situ* lesions: width of pigmentation $\geq$3 mm, width of pigmentation $\geq$6 mm (the minimum 6-mm width was more strongly associated with melanoma), multicolor pigmentation, asymmetry, border fading, and the Hutchinson sign (13). Moreover, Benati et al. reported that a width more than 2/3 of the nail plate suggested melanoma (14). They also reported that the second most important predictor of nail apparatus melanoma was the presence of gray to black color. This gray to black color was associated with 12.5% of nevus and 76% of nail apparatus melanoma.

Clinically, age is important to differentiate melanoma from melanocytic nevus. Most cases of nail apparatus melanoma are diagnosed in adults. Caution must be observed before diagnosing nail apparatus melanoma in children because irregular findings are frequently observed in the benign nevus of nails in children.

Amelanotic Melanoma

Amelanotic melanomas are a relatively rare subtype of melanomas with little or no pigment at visual inspection. Since the majority of melanoma-associated dermoscopic structures are pigmented, amelanotic melanomas remain a demanding diagnostic. The atypical vascular structures are frequently the only clue for its diagnosis (15, 16). Therefore, diagnosis and treatment are often delayed. Menzies et al. reported that the dermoscopically diagnostic accuracy of hypomelanotic or amelanotic melanomas was inferior to that of pigmented melanoma (15). In a recent study of amelanotic melanomas, Lin et al. reported polymorphous vessels as common features in 20 of 27 melanomas. However, in truly amelanotic melanomas, the vascular pattern of polymorphous vessels may not suffice as an only feature utilized to diagnose amelanotic melanoma since it is also found in other skin cancers (16).

Basal Cell Carcinoma

The value of dermoscopy of basal cell carcinoma (BCC) has been extensively demonstrated over the past few decades. Menzies et al. reported the sensitivity of diagnostic criteria for pigmented BCC was 97% (17). The dermoscopic criteria associated with BCCs include the absence of a pigment network and the presence of specific features, e.g., arborizing vessels, large blue-gray ovoid nests, multiple blue-gray globules, leaf-like areas, spoke wheel areas, and ulceration. In reports from Menzies et al. and Altamura et al., arborizing vessels (52% in Menzies et al., 48.5% in Altamura et al.), large blue-gray ovoid nests (55%, 60.8%), multiple blue-gray globules (27%, 38.5%), leaf-like areas (17%, 22.7%), spoke wheel areas (10%, 13.1%), and ulceration (27%, 34.6%) were dermoscopically observed in pigmented BCCs (17, 18). Additional features have been reported recently, specifically multiple small erosions, shiny white streaks, and concentric structures (19–23). Altamura et al. reported that multiple small erosions were seen in 14.1% of non-pigmented BCCs (18), whereas shiny white streaks have been seen only in polarized dermoscopy (22, 24). Moreover, vascular patterns such as short fine telangiectasias (SFTs), arborizing microvessels,

and milky-pink backgrounds have been reported, and these patterns may be useful particularly for non-pigmented BCCs. SFTs are small vessels without branches. Micantonio et al. (20) and Emiroglu et al. (25) reported that SFTs were the second most common vascular pattern found in BCCs. These telangiectasias were significantly more common in superficial BCCs than in nodular BCCs (20). Additionally, Pan et al. reported that arborizing microvessels, short, bright red, sharply focused, fine-caliber branching vessels, were seen in 62% of superficial BCCs (26). Reports on milky-pink backgrounds indicated that they were more common in superficial BCCs (21, 25–27).

Dermoscopic characteristics for each BCC subtype have been described. Some reports differentiated between superficial BCC and other subtypes by dermoscopy (19, 24, 25). In superficial BCC, maple leaf-like areas, spoke wheel areas, SFTs, multiple small erosions, and concentric structures were frequently observed (17–19, 23). Ahnlide et al. (23) summarized dermoscopic features in the common subtype of BCC, including superficial BCC ($n = 202$), nodular BCC ($n = 76$), and infiltrated BCC ($n = 142$) (**Table 1**). Based on their reports, both in nodular and infiltrated BCCs, arborizing vessels were the most common, while ulcerations were the second most common findings. Moreover, Lallas et al. reported that blue-gray ovoid nests may be predictors for non-superficial BCCs. According to their findings, both arborizing vessels and ulcerations would exclude superficial BCC (19).

TABLE 1 | Dermoscopic features in subtype of BCC in the report of Ahnlide et al. (23).

	Nodular BCC (%)	Superficial BCC (%)	Infiltrated BCC (%)
Arborizing vessels	79.7	28.6	86.5
Blue-gray ovoid nests	28.9	21.4	18.3
Ulceration	35.3	20	58.7
Leaf-like areas/spoke wheel areas	16	37.1	50.8
Multiple small erosions	10.7	47.1	15.1
Shiny red-white, structureless areas	25.1	55.7	29.4

TABLE 2 | Dermoscopic features in sebaceous carcinoma.

References	Number of cases	Dermoscopic findings
Coates et al. (30)	2	Variably yellow background Polymorphous vessels Ulceration
Lallas et al. (31)	2	Yellowish structure White-yellowish well-circumscribed areas Polymorphous vascular pattern Ulceration
Manríquez et al. (32)	2	Yellow globules Multiple telangiectasia
Iikawa et al. (33)	1	Homogeneous yellow background Polymorphous vessels
Satomura et al. (34)	5	Yellow background Polymorphous vessels
Horimoto et al. (35)	3	Pink-whitish areas Yellow-whitish globules Polymorphous vessels Ulceration
Nair et al. (36)	1	Yellowish background with peripheral vessels Ulceration

Sebaceous Carcinoma

Although the common clinical presentation of sebaceous carcinoma (SC) is a yellow or red nodule, clinical diagnosis is challenging due to the absence of criteria for dermoscopic diagnosis (28, 29). The main dermoscopic features of SC are yellowish structures, polymorphous vessels, ulcerations, and whitish-pink areas (30–36) (**Table 2**). The most common findings are yellowish structures, i.e., background, globules and yellow/yellowish areas, which were described as the heterogeneously distributing yellowish objects with varying size, shape and number in the literatures. Polymorphous vessels and ulceration are the second most common appearances. Polymorphous vessels were reported as the combination of various types of vessels, i.e., linear irregular vessels, hairpin vessels, comma-like vessels, dotted vessels, arborizing vessels, and telangiectasias. Ulcerations or crusts on the surface of the tumor suggest the possibility of malignancy, which might help distinguish SC from the benign sebaceous tumors. Whitish-pink areas were described as the homogeneous white-to-pink background (35). Dermoscopically, benign sebaceous tumors should be differentiated from SC. Benign sebaceous tumors are classified as sebaceous hyperplasia, sebaceous adenoma, and sebaceoma (31). Sebaceous hyperplasia consists of a white-yellow, umbilicated, structureless center, radially arranged yellow globules, and peripheral telangiectasias (crown vessels). The absence of polymorphous vessels and ulcerations might be helpful to differentiate from SC.

Actinic Keratosis

A grading classification of actinic keratosis (AK) has been proposed based on clinical criteria (37). Grade I (mild) consists of slightly palpable AK, grade II (moderate) exhibits readily palpable and visible AK and grade III (severe) is characterized by very thick and hyperkeratotic AK. This clinical classification of AK corresponds to dermoscopic characteristics (38–42). In grade I, red pseudonetwork patterns and white scales are seen. In grade II, a strawberry pattern is typical. The strawberry pattern results from an erythematous background with white-to-yellow keratotic and dilated follicular openings. In addition, targetoid hair follicles are also seen in grade II (43). In grade III, either enlarged follicular plugs filled with keratotic plugs over a white to yellow background or marked hyperkeratosis are observed.

AKs cannot always be distinguished from SCC *in situ* or invasive SCC. Vascular patterns may be helpful concerning this. Casari et al. reported that the increasing atypia is usually associated with dotted vessels around follicles in severe AKs (44). Therefore, enlarging dotted vessels, forming glomerular vessels

were seen *in situ* SCC. With the progression to invasive SCC, hairpin and/or linear-irregular vessels will appear.

In pigmented AK, the most typical dermoscopic structure is the superficial brown network consisting of brown, curved double lines that surround enlarged, partially confluent, keratotic follicles of various sizes. Pigmented AK may also reveal an annular-granular pattern and pseudonetwork and rhomboidal structures, which are also seen in lentigo maligna (45). The annular-granular pattern in pigmented AK tends to be more prominent and keratin may be observed in the follicular openings. Moreover, the strawberry sign, rosette pattern, and scale may help to distinguish AK from LM when making a diagnosis (43).

Bowen's Disease

The dermoscopic characteristics of Bowen's disease (BD) were first described in 2004 (46).

Based on several studies, glomerular vessels (69–97%) and scaly white-to-yellow surfaces (64–96%) were commonly observed in non-pigmented BD (26, 46–50). Brown to gray globules/dots and structureless pigmentation were observed in 21–80% and 70–78% of pigmented BD, respectively (46, 48, 50, 51). These pigmented globules/dots are often distributed at the periphery, sometimes exhibiting a streak-like or leaf-like structure. However, these are not specific to pigmented BD. Chung et al. reported that streak-like structures did not converge toward the center of the lesion, nor did they connect to a common base and therefore may be distinguished from those seen in melanocytic lesions or in BCCs (52). Recently, Yang et al. found two new dermoscopic signs by analyzing 146 lesions of BD (49). One was parallel pigmented edges at the periphery of the lesion, named the double-edge sign. The other was several aggregated large pigmented massive structures, often distributed at the periphery of the lesion, named clusters of brown structureless areas. The former was observed in 44 of 146 lesions (30.1%), and the latter was in 56 (38.4%).

In rare occasions, BD develops in the nail, periungually, in the fingers and palm, often displaying the type of pigmented BD (53–55). Nakayama et al. reported four cases of periungual pigmented BD (53). Histologically, dilated vessels in the papillary dermis corresponding to a dermoscopic finding of glomerular vessels were observed, but none of the four cases presented glomerular vessels upon dermoscopic examination. They speculated that acral skin has a thicker stratum corneum than other skin, so papillary vessels might not be detectable by dermoscopy. In addition, the PFP was found in two of four cases, while the PRP was not found in any case. Similarly, Cavicchini et al. reported a case of pigmented BD developing in the palm that showed a PFP (54). The presence of the scaly surface and the lack of the PRP may distinguish acral BD from acral melanoma. Regarding BD developed in the fingernails, several case reports described subungual BD presented longitudinal melanonychia (55–57). Additionally, Hutchinson's sign was also observed in some reports (53, 58, 59).

Squamous Cell Carcinoma

Dermoscopic criteria for squamous cell carcinoma (SCC) include the presence of keratin/scales, blood spots, white circles, white structureless areas, hairpin vessels, linear-irregular vessels perivascular white halos, and ulceration (39, 60, 61). Keratin and scales are homogeneous opaque yellow to brown structures, corresponding to hyperkeratosis and parakeratosis (60, 62). Blood spots are the multiple red to black dots in the keratin mass, corresponding to small crusts or hemangiomas (60). White circles are the bright white circles surrounding a dilated infundibulum corresponding to acanthosis and hypergranulosis of the infundibular epidermis (62). White structureless areas are the whitish areas covering large areas of tumors, corresponding to large targetoid hair follicles (39, 63). Among the criteria discussed above, keratin and white circles reached the sensitivity and specificity for SCC diagnostic at a rate of 79 and 87%, respectively (60). The presence of vessels in more than half of the tumor's surface with a diffuse distribution of vessels and bleeding significantly increased the possibility of poorly differentiated SCC. Conversely, keratin/scales are a potent predictor of well- and moderately differentiated SCC (64). However, Pyne et al. reported that moderately and poorly differentiated SCC displayed more branched and serpentine vessels than well-differentiated SCC and that moderately and poorly differentiated SCC displayed larger numbers of vessel types than well-differentiated SCC (61). Regarding lip SCC, Benati et al. recently reported that scales, white structureless areas, and white halos were observed in the majority of the cases (100, 91, and 86%, respectively) (65).

Merkel Cell Carcinoma

Several recent studies have defined useful significant dermoscopic features for Merkel cell carcinoma (MCC). The most common dermoscopic finding is the milky-red areas that are usually associated with linear irregular vessels. Jalilian et al. reported dermoscopic features of 12 MCC cases (66). All cases presented polymorphous, linear irregular vessels, and structureless areas. Milky-pink areas were observed in 11 cases. Furthermore, Harting et al. reported that milky-red areas or linear irregular vessels were most commonly observed in 10 MCC cases studies (67). Additionally, Dalle et al. observed milky-red areas in 80% of their patients. While these patterns may be observed in amelanotic melanoma, the lack of findings of pigmentation or blue-gray veils could direct diagnosis against MCC (68).

Extramammary Paget's Disease

Dermoscopic features of extramammary Paget's disease (EMPD) have not yet been established due to its rarity. Recently, Mun et al. compared the dermoscopic appearance of 35 EMPD cases and EMPD-mimicking lesions, e.g., eczematous dermatitis (ED), fungal infections (FI), and Bowen's disease (BD). In EMDP, they observed milky-red areas (32/35), dotted vessels (18/35), glomerular vessels (7/35), polymorphous vessels (6/35), surface scales (7/35), linear irregular vessels (1/35), ulcers/erosions (15/35), pigmented structures (11/35), shiny white lines (4/35), and white structureless areas (3/35) (69). Milky red areas

were significantly more prevalent in EMPD than in ED, FI, or BD. Moreover, invasive EMPD correlated statistically with polymorphous vessels.

Angiosarcoma

Dermoscopic features of angiosarcoma (AS) have not yet been established due to its rarity. De Giorgi et al. described steam-like areas with a white or skin-colored central area as a characteristic dermoscopic feature of AS (70). Furthermore, Oiso et al. reported that a gradation of various colors within the lesion may be an important dermoscopic feature of AS since it is not present in common purpura or ecchymosis (71). Minagawa et al. reported that AS is characterized by the absence of well-defined vascular structures, e.g., lacunae/lagoons that are commonly found in other vascular lesions such as angioma and pyogenic granuloma. Those features might be useful toward differential diagnosis with amelanotic melanomas (72).

CONCLUSION

We summarized recent reports of novel findings related to dermoscopy of melanoma and non-melanoma skin cancers. Dermoscopy is presently thought to be effective and helpful for diagnosing melanoma and non-melanoma skin cancers. However, it is important to consider that dermoscopy is just one of several means, others being clinical history, age and gross appearance, that can be utilized in cancer diagnosis. Therefore, we should not hesitate to do a biopsy in cases in which a diagnosis cannot be reached clearly through dermoscopy.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Possibility of Deep Learning-Based, Computer-Aided Skin Tumor Classifiers

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The incidence of skin tumors has steadily increased. Although most are benign and do not affect survival, some of the more malignant skin tumors present a lethal threat if a delay in diagnosis permits them to become advanced. Ideally, an inspection by an expert dermatologist would accurately detect malignant skin tumors in the early stage; however, it is not practical for every single patient to receive intensive screening by dermatologists. To overcome this issue, many studies are ongoing to develop dermatologist-level, computer-aided diagnostics. Whereas, many systems that can classify dermoscopic images at this dermatologist-equivalent level have been reported, a much fewer number of systems that can classify conventional clinical images have been reported thus far. Recently, the introduction of deep-learning technology, a method that automatically extracts a set of representative features for further classification has dramatically improved classification efficacy. This new technology has the potential to improve the computer classification accuracy of conventional clinical images to the level of skilled dermatologists. In this review, this new technology and present development of computer-aided skin tumor classifiers will be summarized.

Keywords: artificial intelligence, deep learning, convolutional neural network, clinical image, dermoscopy, skin tumor classifier

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INTRODUCTION

The incidence of skin cancers, including melanoma and non-melanoma skin cancers (NMSC), is globally increasing. In the United States, the incidence of melanoma is reported to be 22.1 per 100,000 people, the number of new yearly melanoma patients is estimated to be more than 63,000, and melanoma is now rated as the 6th most common of all cancers (1). In spite of new therapeutic agents, such as checkpoint and BRAF inhibitors which improve survival of advanced cases, melanomas are still lethal (2–4). On the other hand, most NMSCs, which are responsible for 4.3–5.4 million new cases each year in the United States (5, 6), can be treated simply by surgical removal. Most of these (>90%) are comprised of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) (7) and skilled dermatologists can detect these by clinical appearance and a tumor magnifying dermatoscope (8). Consequently, most SCCs and BCCs are detected at an early stage and resolved by surgery alone. However, SCC can become lethal when it metastasizes, since few standardized and effective therapies for advanced SCC have been established. Although metastatic BCC is very rare, any delay in diagnosis may allow tumors to become unresectable. Therefore, early detection of all skin cancers, not limited to melanoma, is required to prevent progression of these cancers to advanced stages and reduce skin cancer-related deaths.

Skin tumor screening is one solution for the early detection of skin cancer, but it is not practical for dermatologists to check all patients for skin tumors. In most countries, primary vigilance is maintained through primary care clinics before being referred to dermatologists and, consequently, up to 20% of patients consult at primary care clinics complaining about skin-related symptoms (9–11). A study by Julian et al. reported that 16% of patients with dermatologically related diseases had benign skin tumors, 3.3% had actinic keratosis, and 3% had malignant skin tumors (10), meaning ∼20% of patients who consulted a primary care doctor with skin-related complaints received a tumor diagnosis. Similar results were reported by Kerr et al. (11), showing that 11.4% of studied patients had benign skin tumors with <5% having malignant skin tumors. Although the percentage of patients with malignant skin tumors among patients who consult at primary care clinics is not so high, primary care doctors are under a heavy burden to correctly screen patients who present with skin-related symptoms and determine which patients are to be transferred to the dermatologists. Thus, any device or service that can accurately give the probability of malignancy by analyzing a simple photograph of the tumor would be very helpful for both primary care doctors and their patients. In this context, the development of artificial intelligence (AI) that can classify skin tumor images within seconds, at a skill level similar to trained dermatologists, is an ideal solution for this problem.

MACHINE LEARNING: NECESSITY OF LABELED DATA

Artificial Intelligence (AI) is a term used to describe machine software that can mimic human cognitive functions, such as learning and problem solving (12). Machine learning achieves this via changes in the program algorithm that allow it to complete tasks more efficiently. These changes

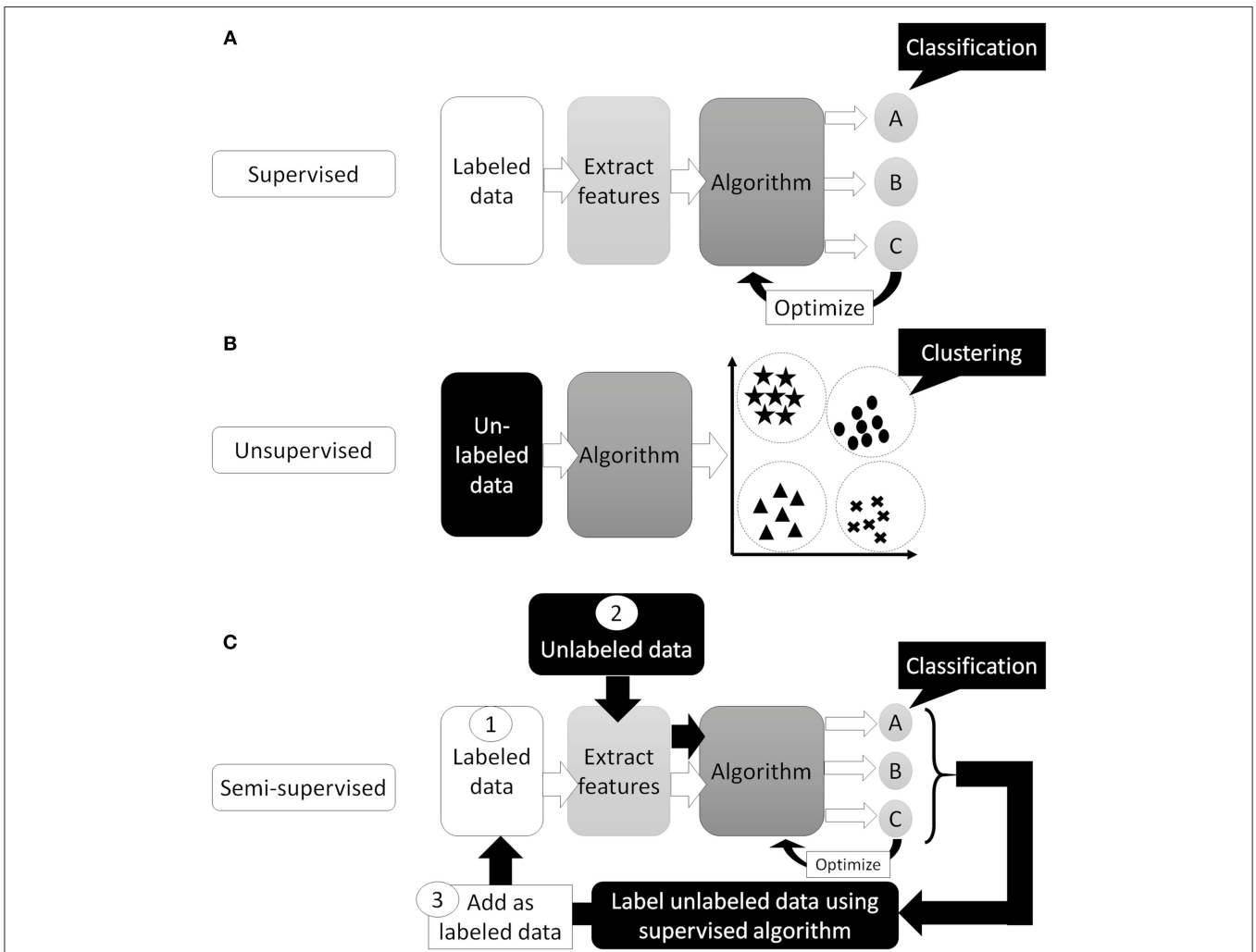

FIGURE 1 | Supervised or unsupervised training. **(A)** Supervised training which can predict classification or regression of the input data. **(B)** Unsupervised training which can cluster the data. **(C)** Semi-supervised training. First, train the algorithm by small number of labeled data. Then, use trained algorithm to "label" unlabeled data. Next, re-train algorithm using newly labeled data and originally labeled data. Finally, algorithm trained with all data and can predict classification or regression of the input data.

come from training using labeled data (supervised learning method, **Figure 1A**), data without labels (unsupervised learning method, **Figure 1B**), or both (semi-supervised learning method, **Figure 1C**) (13). For the supervised learning method, the program processes data and compares its output with the correct answer (label), adjusting its own parameters so that it can reach the correct result. This process should be repeated for as many training datasets as are available but, to achieve satisfactory efficiency, it requires a certain amount of labeled data to adjust the parameters. Thus, preparing a high enough number of datasets means that this supervised learning method could achieve a high classification efficacy (**Figure 2**). However, preparation of labeled data is often difficult, especially in the medical field. On the other hand, unsupervised learning does not require labeled data but instead uses a large amount of unlabeled data for learning. Where it differs from the supervised method is in the output of this algorithm, which clusters data instead of classifying it (**Figure 1B**). This method is useful for huge amounts of data without labels but, since this algorithm does not know the "answer," the meaning of each cluster in the output needs to be determined. The third method, semi-supervised learning, requires a small amount of labeled data with a large amount of unlabeled data. It functions on the principle that unlabeled data is classified using an algorithm trained with labeled data (**Figure 1C**). These unlabeled data are labeled with categories that were found to have high hit probability as calculated by the algorithm which was trained (supervised learning method) on the small amount of labeled data. These newly labeled data are added to the originally labeled data and supervised learning is then conducted again to re-train the algorithm. This method is useful for huge amounts of unlabeled data that would otherwise require a high cost to label. However, if a small error exists in the initial supervised learning algorithm, that error will be amplified at the end of the procedure. Collectively, the best way to train AI algorithms is to prepare large amounts of correctly labeled data for supervised learning but this is often difficult for the medical field, since the "gold standard" pathological confirmation of lesion labels requires the excision of "all" lesions, which is not ethical for confirmed, benign lesions.

METHOD OF MACHINE LEARNING FOR IMAGE CLASSIFICATION: BEFORE THE DEEP LEARNING ERA

Developing a computer-aided diagnostic support system for skin cancer diagnosis requires many steps, as reviewed by Masood and Al-Jumaily (14) (**Figure 3**). The first step of the classification process starts by removing irrelevant structures and artifacts in the image (14, 15), such as hair, air bubbles/gel (if dermoscopy images are used), ink markings or reflectance, by using general image filters (16). These irrelevant objects or artifacts may affect the efficacy of border detection and skew the final output. Next, to analyze the internal properties for further analysis, the lesion within the objective tumor area should be separated from the surrounding skin in a procedure called segmentation. As it is not practical to manually define areas for all images, many automatic lesion segmentation systems have been reported (17–19), but this step is still a challenging task for engineers (16).

Next, the important features need to be extracted from the segmented image. Border shapes [asymmetry indices, symmetry axes, or aspect ratios (20)] or color features [average values and standard deviation of the RGB or HSV color channels (21)] are calculated and these values are used for further classification. However, there is a cost for attempting to extract more features, namely more training time, more complex algorithms, less generalization behavior, and less prediction accuracy. Thus, it is important to select only the useful feature values for classification

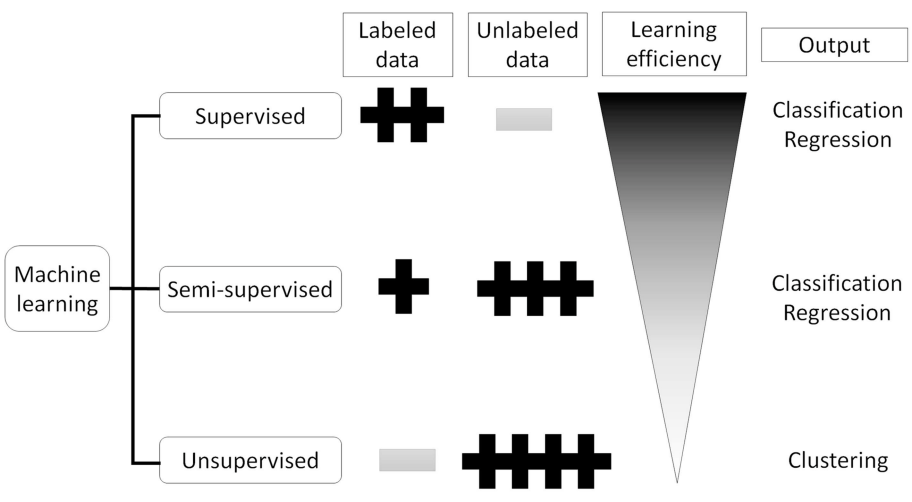

FIGURE 2 | Supervised, semi-supervised, and unsupervised training. Supervised training needs labeled data but can learn the most efficient. Unsupervised training does not need labeled data which sometimes difficult to prepare, but can only cluster the input data. Semi-supervised training can produce labeled data from unlabeled data using small number of labeled data.

while eliminating less useful ones (feature selection). There are diverse and numerous methods that have been proposed for this feature selection process (22–24).

Finally, the classification algorithm outputs the result using the selected feature values calculated in the previous phase. There are many different algorithms available for this classification task: support vector machine (25), decision tree (26), statistical [logistic regression (27)], or artificial neural network (ANN) (28). Of those, the performance of the support vector machine classifier is reported to be similar or better than other algorithms but, as it can only provide a dichotomous distinction between two classes (e.g., benign or malignant), this algorithm will not work for multi-class sorting with probabilities for each class. ANN, on the other hand, mimics the structure of biological neural networks in the human brain (**Figure 4A**) and can change connectivity between decision nodes (back propagation, **Figure 4B**) so that the network can achieve satisfactory results (28). Many ANN studies have reported on dermoscopic image analysis as (28) it has the ability to derive meaning from data which is too complex for humans to understand. The downside, however, is that ANN requires multiple repetitions of the training data to adjust network connections.

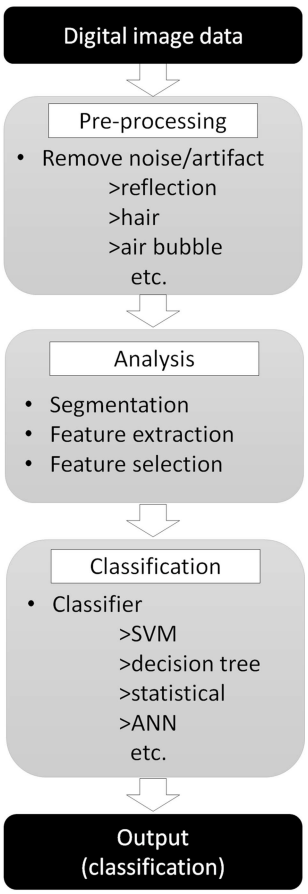

FIGURE 3 | Skin tumor classifier by "traditional" machine learning. Digital image data needs pre-processing to remove noise or artifact to improve the efficacy of the next step. Pre-processed images then analyzed to extract features required for classification step. Finally, classifier use extracted features to classify input images.

INTRODUCTION OF DEEP LEARNING TECHNOLOGY WITH CONVOLUTIONAL NEURAL NETWORKS

The image classification machine learning algorithms described above are very complex; they are based on hand-engineered features and are highly dependent on prior knowledge. For example, in some reports, more than 50 different feature values thought to be useful in the classification process, such as color, shape, or border information, were extracted from a single image for the training of the system (29, 30). In the annual ImageNet Large Scale Visual Recognition Challenge (ILSVR) computer vision competition, where 1 to 2 million images of objects are classified into 1,000 categories, a classifier using traditional machine learning had an error rate of 30% (31) compared to humans who logged an error rate of 5.1% (32). This striking gap in accuracy was dramatically reduced in the 2012 ILSVR competition; deep-learning technology using a convolutional neural network (CNN) achieved an error rate of 16.4% while other classifiers using traditional machine learning had an error rate of 26–30% (32). After the introduction of CNN, the error rates in the ILSVR competition dropped rapidly and the error rate in the 2017 competition was below 5%, indicating that CNN classified images more precisely than humans (http://image-net.org/challenges/LSVRC/2017/results).

This new CNN technology can learn and automatically determine what features are important for classification from the training image set. The extraction and selection of the features for classification was a key component of the traditional methods (33), and also the most difficult part. Thus, by using CNN, complicated image pre-processing is no longer necessary to obtain optimal feature values for the image classification. A schematic structure of CNN is shown in **Figure 5A**. In ANN, every node fully connects to the next layer (**Figure 4B**) but, in CNN, each node connects only to some nodes in the next layer (**Figure 5A**). This key feature of CNN can successfully capture the spatial and temporal dependencies in an image through the application of relevant filters (34). In this type of classifier, output values of the feature extractor usually input to a fully connected network and the softmax function finally converts input vectors to real numbers for normalization into a probability distribution (35). As an example, if the input images had 4 different classes, the final CNN layer would have 4 nodes as in **Figure 5A**. But, as the sum of the output of all 4 nodes would not be 1, it would be difficult to interpret the output. However, a softmax function that converts each node's output from 0 to 1 would allow for the components to add up to 1 and result in a final output that can be interpreted as a probability (0–100% probability).

There are many available CNN architectures used in the medical field such as LeNet (36), AlexNet (36), ZFNet (37), VGGNet (38), GoogLeNet (39, 40), ResNet (41), or SENet (42) (**Table 1**). Not only are these architectures free to use, pre-trained models are also available that are commonly trained by

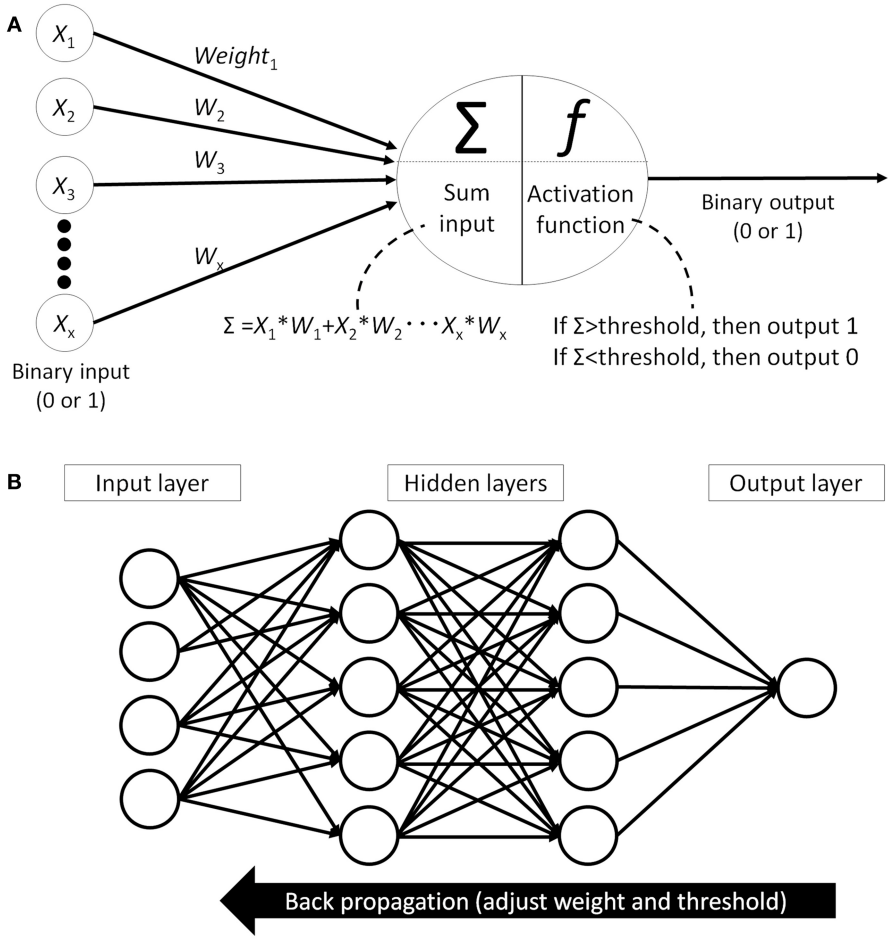

FIGURE 4 | Artificial neural network (ANN). **(A)** Single perceptron model which mimics the structure of biological neural networks in the human brain. Each node receives signal from other nodes (X1, X2… Xx). Add the multiplied values of input and weight (W) and when this sum(Σ) cross the threshold, then this node outputs signal. **(B)** An example of artificial neural network model which has hidden layer between input layer and output layer. All the nodes between the layers are fully connected and each connection has weight. Machine learning is adjusting each weights and thresholds in the network to reach the correct output (back propagation).

the previously mentioned ILSVR2012 dataset, which contains 1.2 million images within 1,000 classes (available at http://image-net.org/download-imageurls). Since the ability to extract image features by pre-trained models is very high, we can use these pre-trained models as a "feature extractor" in a technique called transfer learning (43). One example of transfer learning is shown in **Figure 5B**. Basically, the classifier part is replaced with an untrained classifier appropriate to the new task and the system is trained using a new training image dataset. This method is useful when large numbers of datasets cannot be prepared due to rarity, expense in collection/labeling, or inaccessibility (43). Therefore, transfer learning would be useful for the medical field since it is often difficult to collect images of rare diseases.

Collectively, the introduction of CNN has not only dramatically improved image classification efficacy, it has also made adoption of machine learning and image classification easier and cheaper since most of the initially needed resources are easily accessible.

SKIN TUMOR CLASSIFICATION BY USING DERMOSCOPIC IMAGES

The clinical diagnosis of melanoma is difficult since the morphological characteristics of other pigmented skin lesions may sometimes mimic it. Dermoscopy can magnify the skin and enables clinicians to better evaluate morphological features which are difficult to see with the naked eye. The introduction of dermoscopy has been reported to improve diagnostic sensitivity by 10–30% (44–46). Physicians are usually taught the ABCD-rule (47), Menzie's method (48), 7-point checklist (48), or some other pattern classification methods (49) to distinguish between melanoma and non-melanoma pigmented skin lesions. However, becoming an experienced dermoscopic reader (50, 51) who can score 90% diagnostic sensitivity (proportion of images correctly detected as malignant within all malignant images) and 59% diagnostic specificity (proportion of images correctly detected as benign within all benign images) requires a significant time and training investment

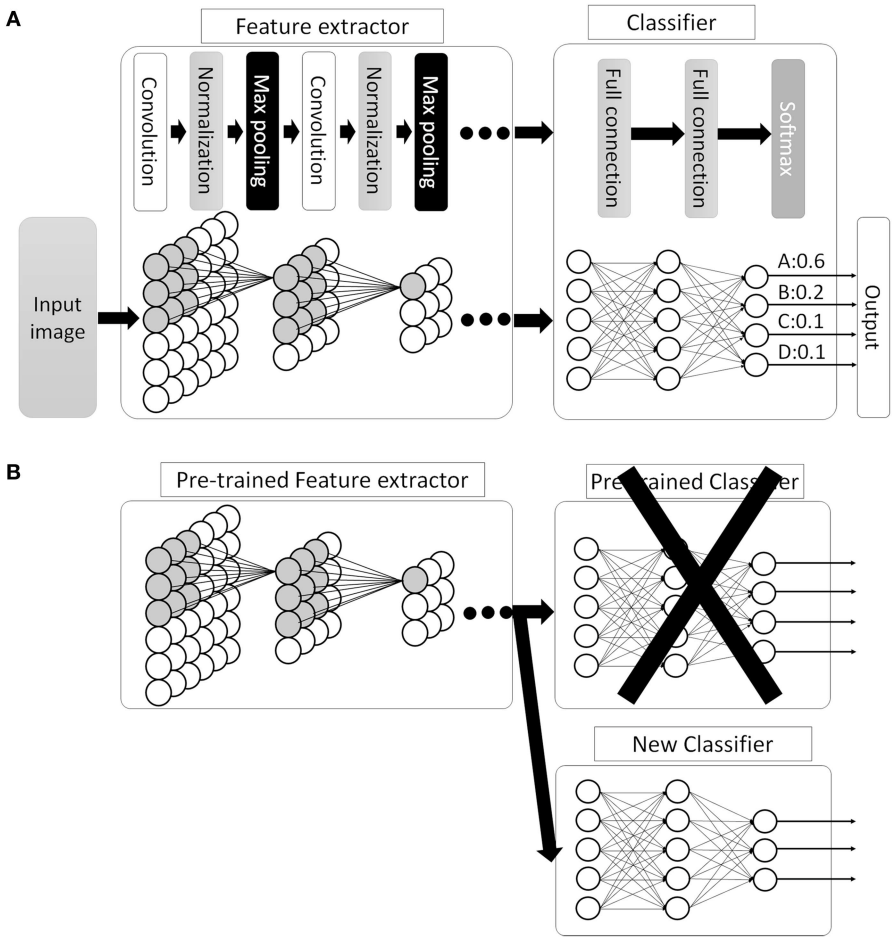

FIGURE 5 | Convolutional neural network (CNN). **(A)** Schematic image of CNN. Between the convolutional layers, each nodes connect to distinct nodes of the previous layer, which is different compared with ANN (as in **Figure 4B**, all the nodes between the layers are fully connected). By this feature, CNN can successfully capture the spatial and temporal dependencies in an image. Then, the fully connected classifier output the result as a probability distribution. **(B)** An example of transfer learning in CNN. In this example, replace classifier and use pre-trained CNN layers as feature extractor. Then, train the system to fit the new task.

(52). Moreover, even after such training, the readings are often complex and subjective.

To make readings more objective and qualitative, as well as support physicians using dermoscopy, many computer-based analyses of dermoscopic images to classify melanomas have been conducted. In a 2009 review by Rajpara et al. (53) that reviewed studies of AI classifiers (12 studies) and dermoscopy (23 studies) published between 1991 and 2002, the melanoma detection sensitivity and specificity of AI classifiers were already similar to that of physicians using dermoscopy; pooled sensitivity, and specificity for AI classifiers and physicians were 91 vs. 88% and 86 vs. 79%, respectively. In a 2013 review (14), 15 new studies on AI classifiers were included and showed sensitivities ranging from 60.7 to 98% and specificities ranging from 72 to 100%, which were similar to the previous 2009 report. Haenssle et al. reported on an AI classifier using the CNN deep learning-based algorithm and compared the classification efficiency against 58 dermatologists (54). Their CNN showed higher accuracy than most dermatologists (sensitivity 88.9 vs. 86.6% and specificity 82.5 vs. 71.3%, respectively) (54). Similar results were recently reported by Tschandl et al. (55) which compared 511 human readers, including 283 board-certified dermatologists, and 139 machine learning algorithms on a classification task consisting of 30 image batches from the test image set. When comparing 37 human experts (>10 years of experience) with the top three machine learning algorithms, the mean number of correctly classified images by humans was 18.78 images per 30 test images, whereas machine learning algorithms scored 25.43, which was statistically higher than human expert readers.

Although AI classifiers seemed to score similar marks as physicians, it is difficult to judge whether AI classifiers have already surpassed physicians or not, since most of these reports were unable to verify results by outside data and biases, such as selection bias of study training/testing data or publication bias (53). However, in spite of such possible biases, development of AI classifiers using dermoscopic images is still an attractive research area.

TABLE 1 | List of CNN architectures.

Architecture	Year	Top-5 error rate at ILSVRC*	References
LeNet	1998	NA	(36)
AlexNet	2012	15.3%	(36)
ZFNet	2013	14.8%	(37)
GoogLeNet	2014	6.67%	(39, 40)
VGG Net	2014	7.3%	(38)
ResNet-50	2015	3.6%	(41)
SENet	2017	2.3%	(42)

ILSVRC: ImageNet Large Scale Visual Recognition Challenge.

CLASSIFICATION OF SKIN TUMOR USING CLINICAL IMAGES

As described above, many highly accurate AI classifiers focusing on melanoma detection using dermoscopic images have been developed. Although conventional machine learning algorithms that require human intervention to extract and select features have the capacity to output a binary result (benign or malignant), accurate diagnoses of multi-class skin diseases are difficult (56). Moreover, even if clinical images are cheap and easy to collect, these clinical photographs are believed to have limited morphologic information that is useful for classification (14). Collectively, outside of a binary "benign or malignant" output, AI classifiers using conventional machine learning algorithms are considered to be inferior at handling clinical images for sorting into multiple classes.

To overcome these issues, deep learning-based CNN classifiers, which surpass the general object classification capability of humans (32), became popular for use in these tasks. Several studies have been published, including from our group (39, 57, 58), since Esteva et al. (40) first reported a dermatologist-equivalent classifier of skin cancers using CNN in 2017. They used 129,450 skin lesion images for the training of a Google Inception v3 CNN architecture, which was pre-trained on the Image Net dataset consisting of 1.28 million images over 1,000 generic object classes. The CNN was fine-tuned to classify skin lesion images by the transfer learning method and was validated on its efficiency of binary classification (benign or malignant). Although the study did not reveal its overall accuracy at skin tumor classification, the CNN surpassed average-level dermatologists in the sensitivity/specificity of classifying epidermal tumors (epidermal cancers and seborrheic keratosis) and melanocytic tumors (melanoma and benign nevi). Han et al. (57) used 15,408 skin lesion images from 12 benign and malignant skin tumors to train a Microsoft ResNet-152 CNN architecture (pre-trained on the same Image Net dataset as above). Similarly, to the Esteva et al. report, they used skin tumor images to fine-tune their CNN which subsequently outperformed 16 dermatologists. They also opened their CNN to the public and it could be externally validated, which was noteworthy. In our study (39), we used only 4,800 skin lesion images from 14 benign and malignant skin tumors to train a GoogLeNet CNN architecture (pre-trained on the same Image Net dataset as above). However, even with less images in the training set, our CNN was more accurate at image classification than 13 board-certified dermatologists and 8 dermatologist trainees (**Figure 6**), reaching a 96.3% sensitivity, and 89.5% specificity in the detection of skin cancer.

Brinker et al. (58) reported an interesting result showing that a CNN trained only with dermoscopic images could classify clinical melanoma images at a similar level to 145 dermatologists. They trained a Microsoft ResNet-50 CNN architecture (pre-trained on the same Image Net dataset as above) using 2,196 melanomas and 18,566 atypical nevi. This study is particularly interesting because this is the first report to show that dermatologist-equivalent tumor image classification was achieved by a CNN that was not trained by clinical images. This study indicates that CNN may benefit from training with dermoscopic images (that have a higher resolution than clinical images) even for low-resolution classification tasks. Another approach is to combine available data for classification as Yap et al. (59). They used 2,917 cases containing both clinical and dermoscopic images and trained a Microsoft ResNet-50 CNN architecture. They showed that a CNN trained with dermoscopic images had higher accuracy than a CNN trained with clinical images. However, when they trained their CNN on combined feature information from dermoscopic and clinical images, the accuracy outperformed single modal CNN, indicating that both clinical and dermoscopic images have distinct classification information. Collectively, the new machine learning algorithm CNN could be a "breakthrough" for developing a multi-class skin tumor classifier, which can accept clinical tumor images.

LIMITATIONS

Several issues remain for the CNN skin tumor classifier to overcome. First, there are no standardized evaluation test datasets to measure the efficacy of CNN classifiers. However, if the test dataset is known in advance, there is a risk of adapting the CNN classifier to the test dataset. Therefore, it might be better to conduct tests by a third-party organization to measure classification efficiency using closed datasets. Second, datasets used to train the CNN are comprised of regionally homogenous images, e.g., our dataset was composed of nearly 100% Asians. In a study by Han et al. (57), they tested using external tumor images (Edinburgh dataset; available from the Edinburgh Dermofit Image Library) to see if their CNN that was trained on Asian tumor images could also classify tumor images from Caucasian patients. As anticipated, both sensitivity and specificity dropped in this case. Third, although CNN requires an increased number of training datasets to improve classification efficiency, rare tumors and subtypes (such as amelanotic melanoma or pigmented basal cell carcinoma) will always mean a scarcity of available images for these diseases. Fourth, the clinical images were less standardized, with varying camera angles, orientations, multiple skin backgrounds, lighting, and even pen markings or rulers included in the photos (60). According to a study by Narla et al. (60), algorithms are more likely to classify images with rulers as malignant because images with rulers were more likely to be malignant. Fifth, the "black box" nature of CNN makes it impossible to interpret how and why CNN arrived

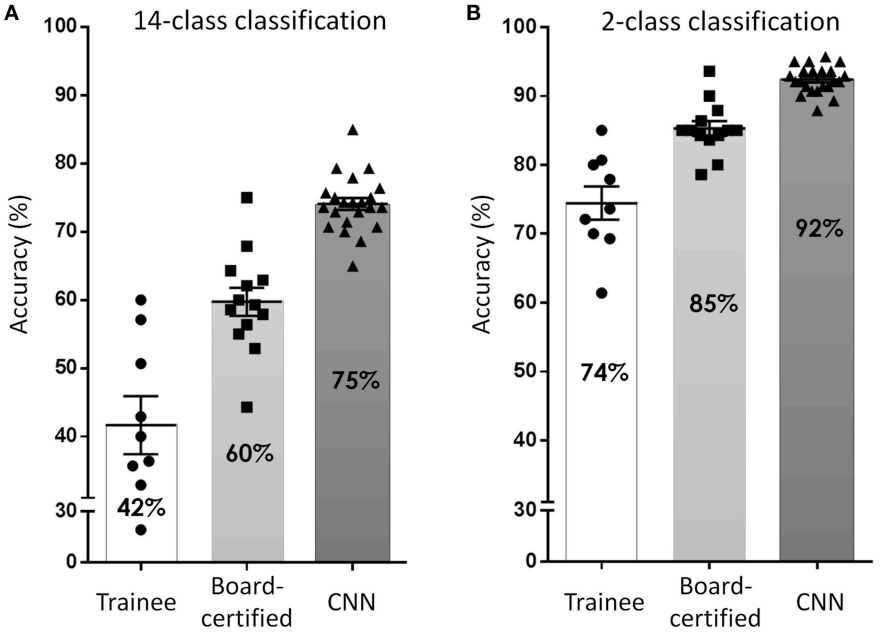

FIGURE 6 | Accuracy of skin tumor classification by our CNN classifier. **(A)** Result of 14-class classification by dermatology trainees, board-certified dermatologists, and CNN classifier. Adapted from Fujisawa et al. (39). **(B)** Result of 2-class classification (benign or malignant). In both classification level, the accuracy of CNN surpassed board-certified dermatologists.

at its output. As an example, Navarrete-Dochent et al. (61) reported that the output of their CNN was affected by the size, rotation, or color tone of images. A similar phenomenon was observed in our system; the output of our CNN was affected by changing the size parameters of the tumor (data not shown). To improve the robustness of the CNN classifier, establishment of an open-access, standardized, large skin tumor image dataset, which includes both rare tumors/subtypes and all ethnicities, is mandatory. Moreover, a robust, standardized measurement method for evaluation and comparison of systems should be established.

FUTURE PERSPECTIVE

AI classifiers for the image classification field have been dramatically improved and made more popular by the introduction of CNN. Many strategies, such as creating ensembles of multiple models (62, 63) or using additional information other than image labels, to improve the accuracy of the classifier outside of increasing the number of images for training have been reported (64). Some studies have reported that CNN algorithms have already surpassed the classification efficacy of dermatologists and, in the near future, AI classifiers may gain sufficient sensitivity and specificity to bear the screening burden for detecting malignant skin tumors. Therefore, some physicians may consider AI as a potential threat, but we believe it to be no more than a diagnostic assistance system due to many limitations detailed in previous studies and difficulty in performance comparisons within published results. Besides such limitations, AI classifiers still have important role in assisting non-dermatologist physicians, since most skin cancer patients will consult them before being transferred to dermatologists. Early detection and treatment are both still essential in the management of melanoma and, therefore, an efficient AI classifier would help to "detect" patients in the early stage of the disease. Further research is thus required to both improve classification efficacy and develop independent evaluation methodologies to accurately measure system efficacy. Moreover, integration of other medical information such as vital signs, routine blood testing, or even omics data, may give us new insights into the biology or pathology of the disease.

AUTHOR CONTRIBUTIONS

YF, SI, and YN contributed to the conception and design of the study. SI and YF wrote the first draft of the manuscript. All authors contributed to the revision of the manuscript, and read and approved the submitted version.

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Aryl Hydrocarbon Receptor Modulates Carcinogenesis and Maintenance of Skin Cancers

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The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that responds to a wide range of chemicals, including chemical carcinogens such as dioxins and carcinogenic polyaromatic hydrocarbons, and induces a battery of genes associated with detoxification, proliferation, and immune regulation. Recent reports suggest that AHR plays an important role in carcinogenesis and maintenance of various types of skin cancers. Indeed, AHR is a susceptibility gene for squamous cell carcinoma and a prognostic factor for melanoma and Merkel cell carcinoma. In addition, the carcinogenic effects of ultraviolet (UV) and chemical carcinogens, both of which are major environmental carcinogenetic factors of skin, are at least partly mediated by AHR, which regulates UV-induced inflammation and apoptosis, the DNA repair system, and metabolic activation of chemical carcinogens. Furthermore, AHR modulates the efficacy of key therapeutic agents in melanoma. AHR activation induces the expression of resistance genes against the inhibitors of V600E mutated B-Raf proto-oncogene, serine/threonine kinase (BRAF) in melanoma and upregulation of programmed cell death protein 1 (PD-1) in tumor-infiltrating T cells surrounding melanoma. Taken together, these findings underscore the importance of AHR in the biology of skin cancers. Development of therapeutic agents that modulate AHR activity is a promising strategy to advance chemoprevention and chemotherapy for skin cancers.

Keywords: aryl hydrocarbon (Ah) receptor, squamous cell carcinoma, melanoma, ultraviolet, air pollutant, BRAF inhibitor, PD-1

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INTRODUCTION

Recently, the incident rate of skin cancer has been greatly increasing. The number of patients treated for skin cancers has increased by 44% during the past 5 years (1), and skin cancer has become the most common cancer type in Caucasians (2). Although both genetic and environmental factors contribute to the carcinogenesis of skin cancer, this rapid increase suggests the relative importance of environmental factors. The skin is the outermost interface between the body and the environment and is ineluctably exposed to environmental insults such as ultraviolet radiation (UVR) or air pollutants (3). As UVR and air pollutants can induce carcinogenesis in the skin (4), the skin contains a system that recognizes and detoxifies these carcinogenic insults, the dysregulation of which leads to the initiation of skin cancer. In addition to the increase in carcinogenesis of skin cancer, recent therapeutic aspects of skin cancer have greatly changed. In particular, the emergence of molecular targeted therapies including inhibitors for V600E mutated B-Raf proto-oncogene, serine/threonine kinase (BRAF) and checkpoint inhibitors, which attenuate suppression of the anti-tumor immune response, have drastically improved the outcome of advanced melanoma. These drugs retrogradely elucidated the critical contribution of specific proliferative signals

and tumor immunity in the maintenance of melanoma. These recent changes in skin cancers imply the importance of identifying a key molecule that modulates carcinogenesis and maintains skin cancer to improve prevention of and therapy for skin cancers.

The aryl hydrocarbon receptor (AHR) is an evolutionarily conserved, ligand-activated transcription factor, which is a member of the basic helix-loop-helix/PER-ARNT-SIM family (5). Due to its broad capacity to recognize a wide range of chemicals in the environment, AHR is often described as an environmental sensor. Once activated by ligand binding, AHR translocates into the nucleus and dimerizes with ARNT (Ah receptor nuclear translocator). Then the AHR/ARNT heterodimer enhances the expression of its target genes that encode drug-metabolizing cytochrome P450s, including *CYP1A1*, *CYP1A2*, and *CYP1B1* (6) (**Figure 1**). These target molecules of AHR facilitate the metabolic degradation of its ligands. In addition to this role in detoxification, recent works have also revealed novel roles for AHR in tumor biology. In various tumors, differential expression of AHR is indeed observed compared to normal tissue. This different expression status of AHR plays a critical role in pro- or anti-tumor activity according to the cell state (7). Regarding skin cancer, a genome-wide association study of cutaneous squamous cell carcinoma (SCC) also identified *AHR* as a novel susceptibility locus (8). Furthermore, among various solid tumors, the expression level of *CYP1A1*, *CYP1A2*, and *CYP1B1* is associated with prognosis of melanoma (9). These findings imply that AHR also plays important roles in the biology of skin cancers. In support of this hypothesis, AHR has recently been found to be associated with UVR and air pollutant-induced carcinogenesis of skin cancer (10, 11). Furthermore, AHR may play a role in modulating the efficacy of BRAF inhibitors and checkpoint inhibitors (12, 13) (**Figure 2**). In the following sections, we introduce the function of AHR in the context of carcinogenesis and maintenance of skin cancer and mainly focus on environmental carcinogens and molecular targeted therapy.

ENVIRONMENTAL FACTOR-INDUCED SKIN CARCINOGENESIS VIA AHR ACTIVATION

Ultraviolet Radiation

As much as 90% of non-melanoma skin cancers are associated with exposure to UVR (14). UVR causes mutagenesis of DNA and inflammation, which may eventually lead to the formation of skin cancers. UVA radiation (400-320 nm wavelength) excites endogenous chromophores and generates reactive oxygen species, leading to modifications of oxidative bases and generation of 7,8-dihydro-8-oxoguanine at guanine bases (15). In contrast, UVB radiation (320-290 nm wavelength) activates a photochemical reaction and forms photoproducts, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidones, at adjacent pyrimidine nucleotides (15). To keep genomic integrity, these DNA photoproducts have to be removed by DNA repair system or apoptosis, depending on the extent of DNA damage (16). However, once the incorrect repair of these DNA modifications occurs, it may inhibit polymerases, lead to the arrest of replication or cause misreadings during transcription or replication, which results in the formation of mutations, initiation of carcinogenesis, and skin cancer (15). The importance of DNA repair enzymes is clearly evident as seen by the drastically increased risk of developing UV signature mutations and subsequent skin cancers in Xeroderma Pigmentosum patients who lack one of the DNA repair enzymes (17). In addition to mutagenesis, UVR causes the body a UV stress response, the inflammatory response at the exposed site. Increasing evidence suggests that sustained inflammation induced by UVR plays an important role in cancer initiation and progression (18).

AHR acts as a light sensor in keratinocytes following activation by UVR. UVR (in particular UVB) generates formylindolo (3,2-b)carbazole (FICZ), a tryptophan derivative, in epidermal keratinocytes (19). FICZ functions as a high-affinity ligand for AHR and induces UVR-mediated AHR activation, which is associated with UV-induced skin carcinogenesis. Pollet et al. reported that the chronic irradiation of UVB causes only a half numbers of cutaneous SCCs on $AHR^{-/-}$ mice compared to $AHR^{+/+}$ littermates, which implies a critical contribution of AHR in carcinogenesis of SCC. As a molecular mechanism, they revealed AHR activation attenuates the clearance of UVB-induced CPDs by repressing global genomic repair in a p27-dependent manner (10). In addition to the role of AHR in the attenuation of DNA repair systems, AHR works as a negative regulator of apoptosis in UVB damaged keratinocytes. Frauenstein et al. reported that chemical inhibition or knockdown of AHR sensitize keratinocytes to UVB-induced apoptosis by decreasing the expression of E2F1 and its target gene checkpoint kinase 1 (CHK1) (20). AHR also promotes the UV stress response (19). Although the concise molecular mechanism of the UV stress response remains largely unknown, the involvement of different tyrosine kinases including the epidermal growth factor receptor (EGFR) and pro-inflammatory molecules has been suggested (21). For instance, FICZ induces the internalization and subsequent activation of EGFR in an AHR-dependent manner, which is also induced by UVB radiation (19). Moreover, AHR activation in keratinocyte induces the expression of various pro-inflammatory molecules. Irradiation of UVR followed by topical application with FICZ on $Ahr^{+/+}$ mice induces cutaneous expression of a neutrophil directing chemokine (C-X-C motif) ligand 5 (Cxcl5) compared with UV alone, which cannot be observed in $Ahr^{-/-}$ mice (22). In addition, the exposure of FICZ to keratinocyte cell-line induces the activation of AHR and ROS production, which leads to the production of pro-inflammatory cytokine, IL-6 (23). Furthermore, AHR activation induced by UVB irradiation can activate the expression of cyclooxygenase-2, which is pro-inflammatory and associated with the development of skin cancer (19, 24).

These observations imply that AHR activation promotes UVR-induced skin carcinogenesis via attenuation of the DNA repair system and apoptosis and via enhancement of the UV response.

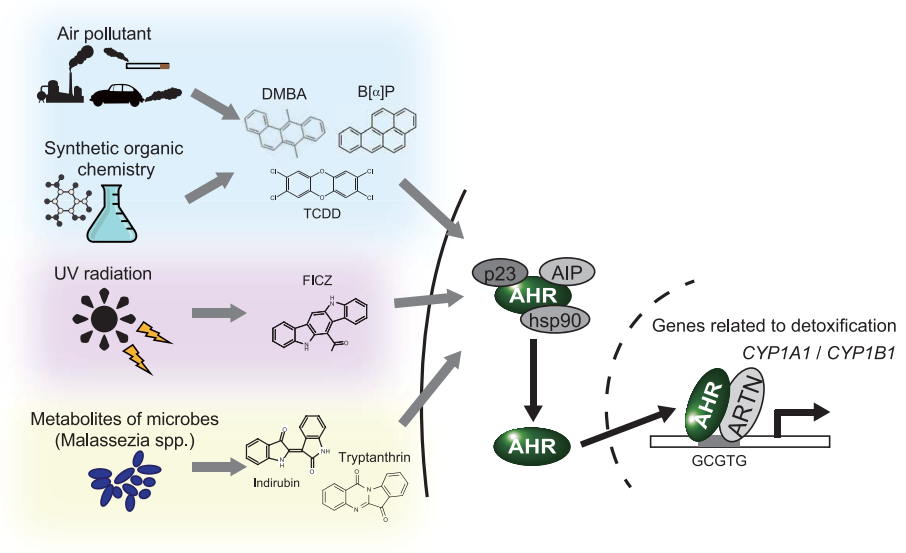

FIGURE 1 | AHR works as an environmental sensor. AHR binds to polycyclic aromatic hydrocarbons and their derivatives derived from environment. Once these ligands binded, AHR isolates from the complex in cytoplasm, translocates into nucleus and activates translation of the target genes, including CYP1A1 and CYP1B1. DMBA, 7,12-Dimethyl benz[*a*]anthracen; B[*a*]P, Benzo[*a*]pyrene; TCDD, 2,3,7,8-Tetrachloro dibenzo-*p*-dioxin; FICZ, 6-Formylindolo [3,2-*b*]carbazole.

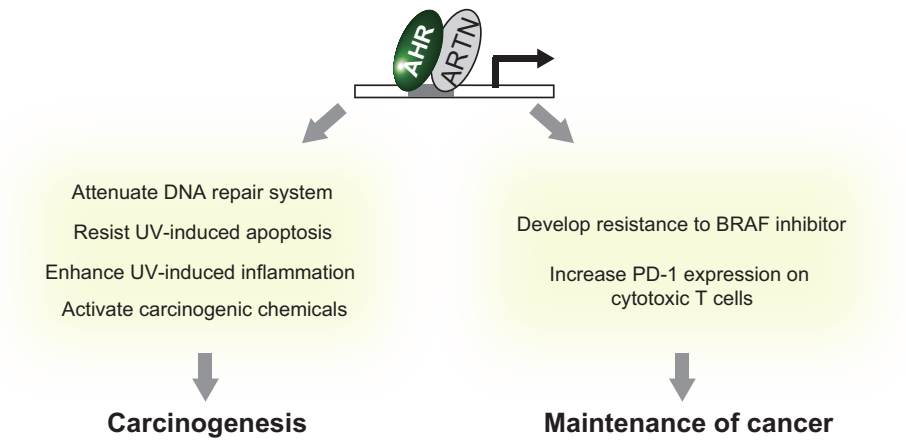

FIGURE 2 | Summary of the effect of AHR activation on skin cancer.

Carcinogenic Chemicals in Air Pollutants

Carcinogenic chemicals are another well-known type of environmental carcinogen that leads to skin cancer. Airborne particulate matter (PM) and ambient air pollution, which contain various carcinogenic chemicals, are considered group 1 human carcinogens by the International Agency for Research on Cancer (25, 26). As the skin is located at the outermost layer of the body, it is continuously exposed to air pollutants, which may increase the risk of skin cancer. Carcinogenic chemicals, including polycyclic aromatic hydrocarbons (PAHs) and dioxins, contained in PM are responsible for PM-induced carcinogenesis (27). Due to their lipophilicity, these chemicals easily penetrate through the skin (28, 29) and are retained in the skin for a long time (30). PAHs and dioxins exert their biological effects via binding to AHR. AHR activation by these chemicals has gained a lot of attention as a mechanism that contributes to skin carcinogenesis. In fact, PAHs and dioxins cause SCC in *in vivo* animal models. For instance, chronic subcutaneous injection of TCDD to hamster results in formation cutaneous SCC (31). In addition, application of 7,12-Dimethylbenz[*a*]anthracene (DMBA), a member of the PAH family that is typically found in cigarette smoke, to murine skin causes lesions that are histologically similar to benign papilloma to SCC (32, 33). Whole-exome sequencing analysis has been conducted in this murine model of DMBA-induced SCC to investigate its mutational landscape (34). As a result, the majority

of DMBA-induced SCC possesses mutations in oncogenes including *Hras*, *Kras*, and *Rras2*. These mutations in human SCCs are similar to those in head and neck, esophageal, lung, and cervical SCC (34–36). In addition to SCC, the development of melanoma is also accelerated by the application of DMBA in some genetically engineered mouse models of melanoma (37).

In these models of PAH-induced skin carcinogenesis, AHR plays a considerable role. Chronic topical application of organic extracts of airborne particulate matter causes SCCs in a half of AHR $^{+/+}$ mice but none of AHR$^{-/-}$ mice (11). Benzo[*a*]pyrene, another PAH contained in PM from cigarettes or air pollutants, can also induce SCC following subcutaneous or topical application to wild-type mice. This carcinogenic property of benzo[*a*]pyrene is attenuated when applied to *Ahr*-deficient mice (38). In the case of DMBA-induced carcinogenesis, the mice possessing the 375A allele of *Ahr*, encoding the high-affinity ligand-binding receptor, develop skin cancers, but the mice possessing the 375V allele, encoding the low-affinity one do not (39); in contrast, there is another report demonstrating no significant differences in carcinogenesis between $Ahr^{+/+}$ mice and $Ahr^{-/-}$mice by topical application of DMBA (40). Taken together, these results suggest that AHR activation promotes tumor induction of PAH-induced skin carcinogenesis.

Several studies investigated the mechanism of PAHs-induced carcinogenesis and revealed that AHR-dependent induction of CYP1A1/CYP1B1 expression likely plays a key role (38). In general, CYP1A1 and CYP1B1 enzymes facilitate removal of AHR ligands by degrading them to metabolites with decreased activity and increasing their water solubility (41). In contrast, in the case of carcinogenic PAHs, the same metabolic reaction results in the metabolic activation of PAHs. For instance, CYP1B1-mediated metabolism of DMBA results in the synthesis of DMBA-trans-3,4-diol, which is highly electrophilic and causes damages to DNA (42). Moreover, CYP1A1, CYP1B1 and epoxide hydrolase-mediated metabolism of benzo[*a*]pyrene results in the synthesis of highly electrophilic benzo[*a*]pyrene-7,8-diol-9,10-epoxide (43).

Regarding the mechanism of dioxins-induced carcinogenesis and its dependency of AHR, they remain largely unknown, as dioxins are not generally metabolized to be directly genotoxic and there is lack of related articles.

MAINTENANCE OF SKIN CANCER VIA AHR ACTIVATION

In addition to the carcinogenic role of AHR activation, AHR also greatly contributes to the maintenance of various skin cancers. In non-cutaneous tumors, AHR is an established factor that induces suppression of the anti-tumor immune response, resulting in the escape of tumor cells from immune-mediated cell death (44). Furthermore, AHR affects multiple aspects of cancer biology, including cell survival and proliferation (45). Recent findings show that AHR modulates anti-tumor immunity and proliferative signals in skin cancers. In the following sections, we introduce recent findings regarding how AHR contributes to the maintenance of skin cancers, mainly focusing on melanoma.

Melanoma

Melanoma is believed to be derived from malignant transformation of melanocytes, which are pigment-producing cells that generally reside in skin (46). Studies investigating the melanocytes of *Ahr*-deficient mice indicated that AHR is essential for proliferation of melanocytes (47). In addition, some reports using melanoma cell lines indicate that AHR activation attenuates tumorigenicity (48, 49); in contrast, others reported that AHR activation promotes tumorigenicity of melanoma (50, 51). These observations suggest the contribution of the AHR system to the biology of melanoma, the details of which have been revealed in recent reports.

In the clinical setting, therapy for melanoma is based on the staging system, which scores clinical and pathological risk factors, including tumor thickness, mitotic rate, and presence of ulceration and metastases (52). In the past, once a melanoma was scored as high grade, patients were considered to have an extremely high mortality rate due to resistance to chemotherapy (53). However, recent development of molecular targeted therapies against the oncogene or checkpoint inhibitors has drastically improved the prognosis of patients with advanced melanoma (54). This improvement indicates the critical importance of BRAF and checkpoint molecules in the maintenance of melanoma. Surprisingly, recent findings have revealed a significant role for AHR in modulating the effect of these critical molecules.

The BRAF V600E mutation is the most prevalent mutation and is present in approximately half of patients with advanced melanoma (55). Specific inhibitors of mutated BRAF have achieved high response rates and improved overall survival (56). Meanwhile, the efficacy of BRAF inhibitors is transient due to acquired resistance, which usually appears within a year after the time of response and results in relapse of melanoma (57, 58). One mechanism of the induction of resistance to BRAF inhibitors is upregulation of genes related to resistance to BRAF inhibitors, including AXL receptor tyrosine kinase (*AXL*), *EGFR*, and neuropilin 1 (*NRP1*) (59, 60). Recently, Corre et al. demonstrated that in a subset of melanoma cells, AHR is constitutively activated, which drives expression of these genes that are related to resistance to BRAF inhibitors (12). In addition, they also reported that co-administration of AHR antagonists with BRAF inhibitors maintains at least partial sensitivity to BRAF inhibitors in melanoma cells.

Melanoma is a solid tumor with a high mutational burden, which induces the generation of neo-antigens and the infiltration of cytotoxic T cells (CTLs) that recognize neo-antigens (61, 62). The level of mutational burden is correlated with that of transcripts related to cytolytic activity of local immune infiltrates (63). To evade the anti-tumor immune response, melanoma cells express molecules associated with checkpoint pathways. Approximately 40% of melanoma biospecimens express programmed death-ligand 1 (PD-L1), one of the molecules associated with the checkpoint pathway (64). When PD-L1 expressed on melanoma cells binds to the PD-1 receptor expressed on CTLs, CTLs become dysfunctional, and melanoma cells escape immune-mediated cell lysis (65, 66). As mentioned, PD-1 blockade by checkpoint inhibitors significantly improves

overall survival and progression-free survival compared with classical chemotherapy in patients with advanced melanoma (67). These findings imply the importance of elucidating how melanoma cells upregulate the expression of PD-1 on CTLs. Liu et al. found that tumor-repopulating cells, a subpopulation of cancer cells having stem cell-like property that are tumorigenic and can grow in soft 3D matrices, produce kynurenine, a known AHR ligand of tryptophan metabolism, by type I IFN-induced expression of indolamine 2,3-dioxygenase. Then kynurenine activates AHR in tumor-repopulating cells, which enters them into dormancy, the condition resistant to immune-therapies (68, 69). In addition, released kynurenine is taken up by surrounding CTLs and upregulates PD-1 expression on CTLs in an AHR-dependent manner (13). This finding tells us that the AHR system may be a significant modulator of PD-1-mediated suppression of the anti-melanoma immune response.

Other Cutaneous Carcinomas

Several reports have suggested possible links between the AHR system and tumor biology in Merkel cell carcinoma (MCC) and extramammary Paget's disease (EMPD).

MCC is a rare and aggressive neuroendocrine skin cancer, and ∼80% of patients are infected with merkel cell polyomavirus. Univariate analysis of clinical specimens revealed that a longer overall survival is achieved in the group with lower expression of tryptophan 2,3-dioxygenase 2 (TDO2) and AHR in cells surrounding the tumor (70). As TDO2 is an enzyme in the tryptophan-kynurenine metabolic pathway, the TDO2-AHR axis may play a significant role in the pathophysiology of MCC.

Another study of EMPD, an adenocarcinoma of apocrine origin, reported that the epidermis adjacent to EMPD lesions expresses CYP1A1 and CCL20, an interleukin-17-related chemokine. Malassezia yeast, which are often pathogenic in apocrine lesions, produce a metabolite that activates AHR and induces the Th17 immune response. Thus, a possible link may be present between Malassezia metabolite-induced AHR activation and the Th17-skewed tumor immune response in EMPD (71).

CONCLUDING REMARKS

As summarized above, AHR was recently found to be a key modulator of UVR- and carcinogenic chemical-induced skin carcinogenesis. In addition, this molecule is associated with the efficacy of BRAF inhibitors and checkpoint inhibitors, which are core therapeutic drugs in melanoma. Taken together, these data underscore the importance of the AHR system in carcinogenesis and maintenance of skin cancers, especially SCC and melanoma. This means that the AHR system is a putative target, particularly for chemoprevention and cancer chemotherapy of skin cancer. The emergence of research investigating the effect of AHR antagonists for various skin cancers is promising and eagerly awaited.

AUTHOR CONTRIBUTIONS

TH wrote the manuscript. TF and SA supervised and reviewed this work.

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BRIEF RESEARCH REPORT
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Continued Chemotherapy After Concurrent Chemoradiotherapy Improves Treatment Outcomes for Unresectable Cutaneous Squamous Cell Carcinoma: An Analysis of 13 Cases

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Background: There is no standard systemic therapy for unresectable cutaneous squamous cell carcinoma (ucSCC), although various chemotherapy regimens have been reported. In our department, concurrent chemoradiotherapy (CCRT) for ucSCC resulted in a 1-year survival rate similar to that of resectable cutaneous squamous cell carcinoma (cSCC). Treatment involves continued chemotherapy after CCRT. Here, we report the importance of continued chemotherapy after CCRT, based on treatment outcomes.

Patients and Methods: We retrospectively evaluated 13 patients with ucSCC, assessing the overall survival, overall response rate (ORR), and disease control rate (DCR).

Results: CCRT with continued chemotherapy resulted in an ORR of 84.6%, DCR of 92.3%, and 1-year survival rate of 75%. Of the 13 patients treated with CCRT with continued chemotherapy, 6 had no metastasis. The remaining 7 patients developed metastasis to other organs or lymph nodes beyond the regional lymph nodes, although most sites of metastasis were outside the irradiation area.

Conclusion: We conclude that CCRT with continued chemotherapy was effective in treating the irradiation site (primary lesion and regional lymph nodes) and any organ metastasis, although, it is unclear for how long the treatment remains effective.

Keywords: concurrent chemoradiotherapy (CCRT), low-dose cisplatin and 5-fluorouracil, overall response rate, OS, disease control rates, 1-year survival rate, continued chemotherapy, unresectable cutaneous squamous cell carcinoma (ucSCC)

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INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common type of non-melanoma skin cancer (1). We consider surgery as an option for treating cSCC during the early stages, but exclude surgical excision as a treatment option for unresectable cSCC (ucSCC) in advanced stages. We define ucSCC as an unresectable case of either the primary site and/or regional lymph

nodes (2–6). Currently, there is no standard treatment for ucSCC, although various chemotherapy regimens have been reported.

In our department, concurrent chemoradiotherapy (CCRT) is performed for ucSCC. Chemotherapy and radiotherapy (RT) begin after surgical excision. If either the primary site or regional lymph nodes are unresectable, RT is performed.

We mainly administer chemotherapy regimens of low-dose cisplatin and 5-fluorouracil (low-dose FP) or carboplatin and 5-fluorouracil (FP') (7). In addition, in our department, we continue chemotherapy if the tumor clearly remains at the primary site and/or regional lymph nodes after CCRT.

The treatment outcomes and 1-year survival rates of CCRT for stage IV cSCC in our department are not significantly different from the outcomes for surgical excision cases and unresectable cases with CCRT with continued chemotherapy (8). Here, we report the importance of continued chemotherapy after CCRT.

METHODS

Staging of cSCC was performed using the TNM classification (8th UICC) (9). The first-line treatment for cSCC was determined on the basis of whether the primary site and regional lymph nodes were resectable. If the primary site and regional lymph nodes were resectable, surgical excision was performed. If the primary site and/or regional lymph nodes were not resectable, CCRT was performed. If surgical excision of the regional lymph nodes was difficult, we surgically excised the primary site, and treated the regional lymph nodes with CCRT. If surgical excision of the primary site was difficult, we surgically excised the regional lymph nodes and treated the primary site by CCRT. Patients who had a performance status score ≥3 were not selected for CCRT, and instead underwent RT monotherapy and palliative treatment (**Figure 1**). No patients underwent chemotherapy monotherapy as a first-line therapy in our department.

The study included 13 patients who were diagnosed with ucSCC and who underwent CCRT with continued chemotherapy. RT irradiation was performed at the primary site and/or regional lymph nodes. The radiation dose was ≥50 Gy. In addition, for chemotherapy during CCRT, we used low-dose FP [(days 1–5) 15 mg/m2 cisplatin plus 800 mg/m2 5-fluorouracil; every 4 weeks] or FP' [(day 1) carboplatin area under the blood concentration-time curve (AUC): 5 (days 1–5) 600 mg/m2 5-fluorouracil; every 4 weeks]. We administered FP' in renal dysfunction cases.

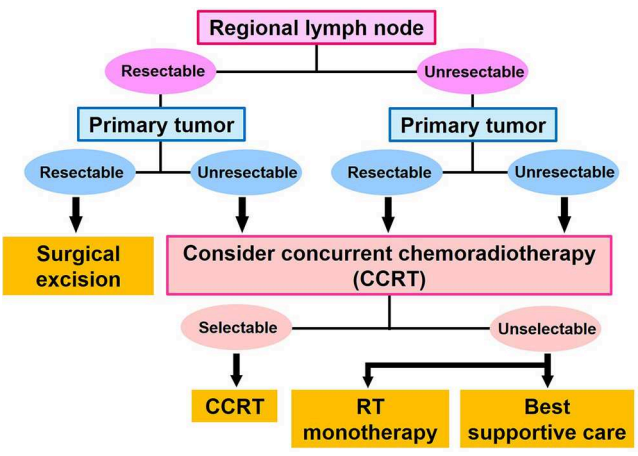

FIGURE 1 | Decision tree for first-line treatment of cutaneous squamous cell carcinoma.

Clinical data included age, sex, primary tumor site, metastasis site, N phase, M phase, histopathological differentiation type, irradiation dose, treatment effect [overall response rate (ORR), disease control rate (DCR)], progression-free survival (PFS), and overall survival (OS). The treatment effect was determined by using computed tomography (CT) every 1 to 3 months based on RECIST (version 1.1) (10) for solid tumors. The PFS and OS were analyzed retrospectively using the Kaplan-Meier method. All statistical analyses were conducted using Microsoft Excel 2016. This study was approved by the Ethics Committee of the Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, in accordance with the Declaration of Helsinki. We obtained informed consent from each patient before the treatment.

RESULTS

The patients' age ranged from 44 to 87 years (mean age, 72.1 years); the study included 8 men and 5 women. Primary lesions were present in the head and neck in 2 cases, in the lower limbs in 6 cases, and in the perineal region in 5 cases. The clinical stage was 4, and PS was 2 or less for all patients (**Figure 2**). Analysis for the 13 cases was performed retrospectively using the Kaplan-Meier method. **Figure 3** shows the survival curves in the 13 cases according to treatment with CCRT with continued chemotherapy as the first-line therapy for ucSCC. The patients treated with CCRT with continued chemotherapy showed an ORR of 84.6%, DCR of 92.3%, 1-year survival rate of 75 %, 2-year survival rate of 58.3 %, and a median survival time of 768 days.

Of the 13 patients treated with CCRT with continued chemotherapy, 5 patients had lymph node metastases beyond the regional lymph nodes without other organ metastasis before CCRT, and 8 patients had metastases only in the regional lymph nodes before CCRT. Three of 5 patients with lymph node metastases beyond the regional lymph nodes, had no progressive disease during continued chemotherapy, after CCRT. Three of 8 patients with metastases within the regional lymph nodes, had no progressive disease during continued chemotherapy, after CCRT. In 6 patients, who had no progressive disease, there was no difference in histopathological differentiation. Seven patients had lymph node metastasis beyond the regional lymph nodes or other organ metastases during continued chemotherapy (after CCRT), but most sites of metastasis were outside the irradiation area. Seven patients with metastasis during continued chemotherapy showed a 1-year PFS of 64.3 %, and median PFS of 262 days.

In our hospital, 3 patients requested to stop chemotherapy after CCRT, and 1 patient stopped chemotherapy during CCRT due to side effects of chemotherapy. Three patients who were administered low-dose FP stopped chemotherapy after 2, 6, and

No.		PS	Primary site	T	N	M	histopathological differentiation	RT (Gy)	Chemotherapy	Adverse event (>Grade3)	Vital status
1	44M	1	Lower limb	X	2	1	Moderately	50	Low-dose FP	G3 : Neutrophil count decreased	Dead on disease
2	74F	1	Lower limb	2	3	0	Well	50	TS-1		Dead on disease
3	78F	1	Perineal	3	2	0	Poorly	50.4	Low-dose FP		Alive with disease
4	75M	1	Lower limb	3	2	1	Poorly	60	Low-dose FP		Alive with disease
5	67M	1	Perineal	X	2	0	Poorly	59.4	Low-dose FP	G3 : Neutrophil count decreased	Dead on disease
6	74F	1	Perineal	2	2	1	Poorly	59.4	Low-dose FP	G3 : Neutrophil count decreased G3 : Anemia	Alive with disease
7	59M	1	Perineal	X	3	1	Poorly	60	Low-dose FP	G3 : Neutrophil count decreased G3 : Anemia	Dead on disease
8	78F	1	Perineal	3	2	0	Well	66	Low-dose FP	G3 : Neutrophil count decreased	Alive with disease
9	71M	1	Face	3	2	0	Well	60	Low-dose FP	G3 : Neutrophil count decreased	Alive with disease
10	77M	1	Lower limb	X	3	0	Well	60	Low-dose FP	G3 : Neutrophil count decreased	Alive with disease
11	87M	1	Face	3	2	0	Moderately	60	FP'		Dead on other disease
12	76M	1	Lower limb	X	2	1	Well	50	Low-dose FP		Dead on other disease
13	77F	2	Lower limb	3	2	0	Well	60	FP'		Alive with disease

FIGURE 2 | Patients characteristics.

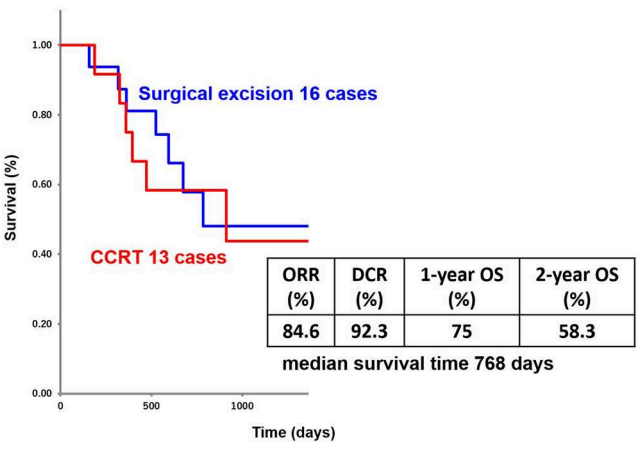

FIGURE 3 | Treatment outcomes of first-line CCRT with continued chemotherapy.

FIGURE 4 | A case of ucSCC with invasion into the right knee ligament.

8 times, but 6, 10, and 13 months later (respectively), they had recurrence within the irradiation area or other organ metastasis. One patient who stopped due to side effects from chemotherapy had organ metastasis during CCRT.

In our study, among the 13 patients treated with CCRT with continued chemotherapy, 10 patients received low-dose FP therapy, 2 patients received FP' therapy, and 1 patient received other chemotherapy regimens. The results showed low-dose FP and FP' therapy as effective with an ORR of 91.7%, DCR of 91.7%, 1-year survival rate of 72.7%, 2-year survival rate of 63.6%, and median survival time of 804 days. **Figure 4** shows an ucSCC with invasion into the right knee ligament and several regional lymph node metastases. It was successfully treated with CCRT with continued chemotherapy. Surgery in this case would have required the patient to undergo an amputation above the

knee. Currently, 2 years and 4 months since treatment, the primary lesion has not been observed. The only remnant of the lesion at that location is a scar, moreover, his leg remains and allows the patient to walk normally. Thus, CCRT with continued chemotherapy is a suitable treatment option for ucSCC, as it can improve the quality of life regarding appearance and function.

CONCLUSION

Surgical excision is the first choice for the treatment of resectable cSCC, but there is no established treatment for unresectable cases. However, the results of our study show that CCRT with continued chemotherapy was an effective treatment method for unresectable cases. While there are several effective chemotherapy regimens for cSCC, our department mainly uses low-dose FP therapy.

The administration of neoadjuvant chemotherapy with FP therapy significantly improves the overall survival of patients with resectable stage II/III esophageal cancer (11). Moreover, FP therapy is an effective treatment for squamous cell carcinoma.

The age at the onset of cSCC is high, and the mean age of patients in our department was 72.1 years. Cisplatin can cause kidney dysfunction owing to age-related decline in kidney function. Therefore, we believe it is better to use low-dose FP therapy, which enhances the action of 5-fluorouracil as a biochemical modulator with low doses of cisplatin. For patients with renal dysfunction, carboplatin should be used instead of cisplatin.

For most cases of ucSCC after CCRT, the tumor clearly remained at the primary site and/or regional lymph nodes. One patient who stopped chemotherapy during CCRT had an organ metastasis immediately. Three patients who stopped continued chemotherapy after CCRT had recurrence within the irradiation area or other organ metastasis.

Although cutaneous angiosarcoma is a type of skin cancer, patients who received CRT for maintenance chemotherapy showed a significant improvement in OS over patients who received CRT alone (12). From these results, we considered continuing treatment after CCRT using the same chemotherapy regimen.

During continued chemotherapy, 7 of 13 patients had lymph node metastasis beyond the regional lymph nodes or other organ metastasis. Metastasis occurred in 7 patients during continued chemotherapy. This group had a 1-year PFS of 64.3%, and a median PFS of 262 days. Because of these results, we believe that it is important to continue chemotherapy after CCRT. Seven patients who had progressive disease changed to other chemotherapy regimens. Six other patients who had no recurrence within the irradiation range or other organ metastasis continued receiving low-dose FP or FP' therapy.

We recognize it is difficult to control ucSCC with CCRT alone during long-term observation. Thus, although CCRT with continued chemotherapy is effective, metastasis may be observed later. By continuing chemotherapy after CCRT, recurrence within an irradiation area and other organ metastasis were suppressed. Therefore, the treatment outcome of CCRT with continued chemotherapy for ucSCC and that of surgical excision for resectable cSCC were similar (8). Additionally, we understood that continued chemotherapy after CCRT improved the treatment outcome of ucSCC.

In recent years, treatment with immune checkpoint inhibitors has become another option for advanced cSCC. Treatment response for SCC with metastases was 47 and 7% of the patients discontinued the treatment because of an immune-related adverse event (13). Our treatment of CCRT with continued chemotherapy is rarely discontinued due to side effects. We consider chemotherapy as a reasonable treatment option to administer to patients who are elderly. The response rate of CCRT with continued chemotherapy was 84.6%, suggesting that it is an effective treatment for ucSCC.

We evaluated the effectiveness of CCRT with continued chemotherapy for the treatment of ucSCC. We conclude CCRT with continued chemotherapy was effective for treating the irradiation site (primary lesion and regional lymph nodes) and the other organ metastasis.

At this time, due to the small number of cases in this study, the optimal duration of chemotherapy therapy is unknown for patients who receive CCRT with continued chemotherapy without progressive disease. We intend to investigate this in the future.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Tokyo Metropolitan Cancer and Infectious Disease Center Komagome Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH held primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. AH was also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. AH should also be available post-publication to respond to any queries or critiques. All authors contributed conception and design of the study. All authors contributed to manuscript revision, read and approved the submitted version.

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The Role and Necessity of Sentinel Lymph Node Biopsy for Invasive Melanoma

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Sentinel lymph node biopsy (SLNB) is a widely accepted procedure for melanoma staging and treatment. The development of lymphatic mapping and SLNB, which was first introduced in 1992, has enabled surgeons to detect microscopic nodal metastases and stage-negative regional nodal basins with low morbidity. SLNB has also facilitated the selective application of regional lymph node dissection for patients with microscopic nodal metastases, enabling unnecessary lymph node dissection. In contrast, recent major randomized phase III trials (DeCOG-SLT and MSLT–II trial) compared the clinical benefit of early completion lymph node dissection with observation after detecting microscopic nodal disease. The results of those studies indicated that there was no significant difference in the survival between the two groups, although regional control was superior after early completion lymph node dissection compared to that obtained after observation. Thus, the role and value of early completion lymph node dissection worldwide are currently very limited for patients with microscopic nodal disease. However, the use of SLNB is still controversial. In addition, the recent approval of adjuvant therapy using novel agents, such as anti-programmed death-1 antibodies, and molecular targeted therapeutics may influence the skipping of complete lymph node dissection in patients with micrometastatic nodal disease in a real-world setting. Furthermore, modern neoadjuvant therapy, which is now under investigation, may have the potential to change the surgical procedure used for nodal disease. Herein, we describe the current role and value of SLNB and completion lymph node dissection and discuss the major controversies as well as the favorable future outlook.

Keywords: melanoma, lymphatic metastasis, sentinel lymph node biopsy, completion lymph node dissection, observation, adjuvant therapy

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INTRODUCTION

Malignant melanoma is among the most common types of cancer, with an increasing incidence rate of 7.9 per 100,000 people in 1975 to 25.8 per 100,000 people in 2015 (1). Approximately 7% of patients are diagnosed with stage III disease, who have a 5 year survival rate was 60.8% (2). The treatment approach for stage III patients is crucial because cutaneous melanoma often metastasizes first to the regional lymph nodes and the sentinel lymph node (SLN) is the first lymph node to receive lymphatic drainage from the primary site.

The surgical approach for treating regional lymph node metastasis has continued to develop, particularly considering sentinel lymph node biopsy (SLNB). Although most patients with melanoma have no clinical nodal disease at the first visit, some patients have clinically undetectable micrometastasis in the regional lymph node. The main controversy is whether completion lymph node dissection (CLND) improves the overall or disease-specific survival of patients with SLN micrometastasis. Furthermore, the advent of promising systemic therapies, confirmed in recent clinical trials, and the results of several trials, confirming the efficacy of SLNB and immediate CLND in patients with positive SLN, may drastically change the conventional methods used for surgical control of the regional lymph nodes by using CLND for all patients with a positive SLNB.

APPLICATION OF SLNB

SLNB is the most appropriate technique for accurate staging of clinical stage I and II disease. The main risk variables associated with higher SLN metastasis are Breslow thickness (BT), ulceration, and a number of mitoses. Per the 8th edition cancer staging guidelines recommended by the American Joint Committee on Cancer (AJCC) (3), SLNB is generally not recommended for melanoma patients with a BT of <0.8 mm without ulceration because the probability of a positive SLN is <5%. However, SLNB should generally be considered for melanoma patients with clinical stage IB or II disease, with the following considerations:

1. T1b (BT of <0.8 mm with ulceration or BT of 0.8 mm^{-1} mm with or without ulceration) or T1a lesions with BT < 0.8 mm with other adverse features [e.g., very high mitotic index $\leq 2/mm^2$ (particularly in young patients), lymphovascular invasion, or a combination of these factors], because the probability of a positive SLN is 5–10%. SLNB should be considered for these patients after discussion.
2. Stage IB (T2a) or II (BT of >1 mm with any feature), because the probability of a positive SLN is >10%. SLNB should be offered SLNB for these patients after discussion.

No globally accepted protocols are available for processing SLNs. However, small metastases are overlooked in conventional processing, which involves the examination of a single routine hematoxylin-eosin (HE)-stained section obtained by bivalving the SLN along the long axis (4, 5). In another procedure, the SLN is sectioned serially along the short axis (breadloaf technique) to increase the amount of subcapsular tissue in the HE-stained sections (6). When routine H&E staining does not reveal SLN metastases, immunohistochemistry (IHC) for S100, HMB-45, and MART-1/Melan-A is useful for detecting additional SLN-positive patients (7, 8). Reverse transcription-polymerase chain reaction (RT-PCR) and cell culture can increase the detection rates of positive SLN even when there are only a few metastatic melanoma cells in the SLN (9); however, these molecular biology techniques are not widely used in most institutions.

MANAGEMENT OF PATIENTS WITH POSITIVE SLN: RESULTS OF RECENT STUDIES AND THE ROLE OF SLNB

When patients show positive results for SLN metastasis, CLND has traditionally been indicated. However, the findings of recent studies regarding the therapeutic value of SLNB and immediate CLND after positive SLNB have resulted in a change in this traditional strategy.

DERMATOLOGIC COOPERATIVE ONCOLOGY GROUP-SENTINEL LYMPH NODE TRIAL (DECOG-SLT)

The Dermatologic Cooperative Oncology Group-Sentinel Lymph node Trial (DeCOG-SLT), conducted in Germany, was the first phase III randomized clinical trial to evaluate the efficacy of immediate CLND in patients with melanoma on the trunk and limbs with BT of ≥ 1.0 mm and positive SLN (10). The patients were randomly assigned to the immediate CLND group ($n = 240$) or the observation group ($n = 233$; patients underwent delayed CLND only if regional metastasis was suspected on ultrasonography performed every 3 months). There were no significant differences in the distant metastasis-free survival, recurrence-free survival, and overall survival (OS) between the two groups. In this study, most patients ($n = 311$) had SLN tumor burdens of ≤ 1.0 mm. This high proportion of SLN micrometastasis leads to the high probability of negative non-SLN in both groups. There was no significant difference in distant metastasis-free survival between the two groups in this cohort. Therefore, distant metastasis-free survival in the cohort with SLN tumor burdens of >1.0 mm was also analyzed. There was no significant difference in the distant metastasis-free survival between the two groups, but the sample size was small in each group ($n = 62$ in the CLND group and $n = 59$ in the observation group). The authors concluded that immediate CLND was not associated with improved distant metastasis-free survival, recurrence-free survival, and OS after a median follow-up of 72 months, and no longer recommend CLND for patients with micrometastases.

MULTICENTER SELECTIVE LYMPHADENECTOMY TRIAL (MSLT-II)

MSLT–II enrolled a large number of patients with positive SLN (9). This was also a multicenter, phase III randomized trial that compared the immediate CLND group ($n = 824$) with the observation group ($n = 931$; patients underwent CLND only when regional metastasis was suspected on ultrasonography performed every 4 months). The mean 3 year melanoma-specific survival rate was statistically insignificant between the two groups after a median follow-up of 43 months. The disease-free survival (DFS) was slightly significantly better in the CLND group than in the observation group ($P = 0.05$). A positive non-SLN status was

a reliable, independent prognostic factor for recurrence [hazard ratio (HR), 1.78; $P = 0.005$]. The occurrence of post-operative lymphedema was higher in the CLND group (24.1%) than in the observation group (6.3%). Likewise, the authors concluded that immediate CLND was not associated with improved melanoma-specific survival, but improved the regional recurrence rate and provided prognostic information.

HOW ARE PATIENTS HARBORING POSITIVE SLN MANAGED?

The above-mentioned two randomized trials demonstrated no survival benefit even if patients received immediate CLND after positive SLNB, although the nodal recurrence rate decreased in the immediate CLND group. The results of these trials do not recommend routine CLND in most patients after positive SLNB. However, their conclusions are still limited, as most patients in these studies had lower tumor burdens in the SLN (>60%). Those populations have a low probability of positive non-SLN in both groups. The true efficacy of immediate CLND after positive SLNB in patients with a higher risk, with SLN tumor burdens of >1 mm, is still unknown because of the small sample size in these trials. Therefore, current NCCN guidelines still recommend CLND, along with careful observation in patients with positive SLN after appropriate risk stratification (11). Accordingly, some guides, such as nomograms, should be utilized for accurate prediction of non-SLN status, regional control, and prognosis. This will enable us to conduct clinical trials for confirming the survival advantage of CLND in a more homogenous cohort with positive non-SLNs. Previously published predictive models for positive non-SLNs (12–18) are shown in **Table 1**. Although several studies have suggested similar clinicopathological characteristics as predictive parameters, a recent study by Bertolli et al. proposes BT, the number of positive SLNs, and large tumor diameter as significant predictive parameters, using their nomogram (18). This model shows the best discriminatory power (AUC 0.752) and Brier score (0.085) among all published predictive models (18) (**Table 1**).

The racial difference in the proportion of clinical type is also crucial for considering the role of SLNB and immediate CLND. For example, acral melanoma (AM) shows drastic differences from other clinical types considering the biological, genetic, and clinicopathological aspects, although SLNB is also widely applied in clinical practice. The actual role of SLNB in this cohort remains unclear, as limited number of AM patients were included in the large trials of SLNB (i.e., DeCOG-SLT and MSLT-II) that mainly investigated Caucasian people (9, 10, 19). Ito et al. retrospectively investigated Japanese AM patients ($n = 116$) who received SLNB (20). Positive SLN was associated with significantly shorter melanoma-specific survival and DFS. The impact of positive SLNs on melanoma-specific survival was increased in AM patients with >1 mm thickness (5 year survival, 22.7 vs. 80.8%; $P = 0.0005$). Although the sample size was small in these studies, the trends of positive SLN status in association with more frequent recurrence and worsened survival in AM patients were similar to those trends in larger prospective randomized trials; however, there are no data regarding the efficacy of immediate CLND compared with observation.

ROLE OF ADJUVANT THERAPY FOR POSITIVE SLN PATIENTS WITHOUT IMMEDIATE CLND

The recent development of novel agents, including immune checkpoint inhibitors (ICIs) and molecular target agents, and their approval in many countries worldwide changed the treatment strategy for not only disease in the advanced stage but also treatment in the post-operative adjuvant setting. All these clinical trials mainly included stage III patients who underwent CLND and no patients skipped CLND after positive SLNB.

ANTI-CTLA-4 ANTIBODIES

A phase III randomized controlled trial (EORTC 18071) comparing ipilimumab with placebo for stage III melanoma patients indicated a significant improvement in the 3 year relapse-free survival (RFS), distant metastasis-free survival, and OS in the ipilimumab group (21). However, severe immune-related adverse events were observed in 41.6% of patients in the ipilimumab group, leading to discontinuation of ipilimumab in half of the patients.

ANTI-PD-1 ANTIBODIES

The clinical benefits of two anti-PD-1 agents as adjuvant therapy were reported recently. A phase III randomized controlled trial (Checkmate 238) comparing nivolumab with ipilimumab for stage IIIB to IV melanoma patients (22) demonstrated better 1 year RFS with lower toxicity in the nivolumab group than in the ipilimumab group. Likewise, a phase III randomized trial (KEYNOTE-054) comparing pembrolizumab with placebo for stage III patients, except for <1 mm of tumor burden in the SLN, also demonstrated improvement in the recurrence-free survival of patients receiving pembrolizumab compared to those receiving placebo after a median follow-up of 15 months (HR, 0.57; $P < 0.001$) (23).

BRAF INHIBITOR/MEK INHIBITOR

A phase III randomized trial (COMBI-AD) comparing dabrafenib plus trametinib with placebo for patients with stage III BRAF mutant melanoma, except for <1 mm of tumor burdens in the SLN, showed improved RFS in the dabrafenib/trametinib group after a median follow-up of 44 months in the dabrafenib/trametinib group and 42 months in the placebo group (24). There also was a trend of improvement in the OS [the 3-year OS rate was 86% in the dabrafenib/trametinib group and 77% in the placebo group (HR, 0.57; $P = 0.0006$)], although the data obtained on statistical analysis did not fulfill the pre-specified interim analysis boundary ($P = 0.000019$) (25).

Based on the above-mentioned clinical trials, the latest NCCN guidelines recommend adjuvant nivolumab for stage IIIB/C

TABLE 1 | The performance of published prediction models for non-sentinel lymph node positivity.

References	Patient no. for research	Significant clinicopathological parameters	Discrimination AUC (95% CI)	Calibration brier score (95% CI)
Lee et al. (12)	191	Breslow thickness SLN tumor burden diameter	0.65 (0.60–0.70)	0.19 (0.18–0.20)
Sabel et al. (13)	221	Sex Breslow thickness Extranodal extension in SLN No. of positive SLNs	0.67 (0.63–0.74)	0.18 (0.16–0.20)
Gershenwald et al. (14)	343	SLN tumor burden diameter Breslow thickness No. of SLNs harvested	0.65 (0.60–0.70)	0.18 (0.17–0.20)
Murali et al. (15)	309	Sex Primary tumor regression No. of positive SLNs SLN tumor burden diameter SLN metastasis site	0.65 (0.60–0.70)	0.18 (0.17–0.19)
Kibrite et al. (16)	171	Breslow thickness SLN tumor burden diameter	0.65 (0.60–0.70)	0.19 (0.18–0.20)
Rossi et al. (17)	1220	Breslow thickness Primary tumor site SLN tumor burden diameter SLN metastasis site No. of SLNs harvested No. of positive SLNs	0.74 (0.70–0.79)	0.16 (0.15–0.17)
Bertolli et al. (18)	1213	Breslow thickness No. of positive SLNs SLN tumor burden diameter	0.86 (0.73–0.99)	0.085 (N.A.)

SLN, sentinel lymph node; AUC, area under the curve; CI, confidence interval; N.A., not available.

and IV melanoma patients after complete tumor removal. Pembrolizumab was recommended for stage III melanoma patients with $\geq$1 mm tumor burden in the SLN. In patients with BRAF mutations, dabrafenib plus trametinib can also be alternatively recommended for stage III disease with $\geq$1 mm tumor burden in the SLN.

The result of SLNB can be used to classify patients without clinical nodal disease for undergoing adjuvant therapy. However, all the above-mentioned clinical trials required CLND before initiating adjuvant therapy. Conversely, in the real-world setting, patients who have positive SLN and do not undergo CLND will increase considering the results of the DeCOG-SLT and MSLT-II trials, even if the patients' tumor burdens exceed 1 mm. Currently, there are no data about the survival benefit of adjuvant therapy with the novel agents in patients who skipped CLND after positive SLNB. Therefore, further research is required to investigate the survival differences between the clinical trial populations and the more heterogeneous real-world population.

POSSIBLE ROLE OF NEOADJUVANT THERAPY

The reports of modern neoadjuvant clinical trials using ICIs or molecular targeted agents demonstrate promising efficacy, mainly for clinical stage III disease. All agents for neoadjuvant use have not yet been approved worldwide.

ANTI-PD-1 ANTIBODIES AND ANTI-PD-1/ANTI-CTLA-4 ANTIBODY

Huang et al. conducted a phase Ib trial investigating the safety of neoadjuvant/adjuvant pembrolizumab for resectable clinical stage III and IV melanoma (26). Enrolled patients received neoadjuvant/adjuvant pembrolizumab (1 cycle of neoadjuvant pembrolizumab 3 weeks before surgery and 17 cycles of adjuvant pembrolizumab). Eight of 27 patients (30%) achieved complete or major pathological response, and they remain free of disease.

Amaria et al. reported a randomized phase II trial comparing the efficacy and safety of neoadjuvant nivolumab (four cycles of neoadjuvant and 13 cycles of adjuvant nivolumab) to neoadjuvant nivolumab/ipilimumab (three cycles of neoadjuvant nivolumab/ipilimumab and 13 cycles of adjuvant nivolumab) for resectable clinical stage III and IV melanoma (27). Neoadjuvant nivolumab/ipilimumab demonstrated higher response rates (RRs) [objective RR, 73 vs. 25%; pathological complete response (pCR), 45 vs. 25%] but also showed higher toxicity (grade 3 treatment-related adverse events, 73 vs. 8%).

Blank et al. also reported a randomized phase II trial (OpACIN) comparing neoadjuvant nivolumab/ipilimumab (two cycles of neoadjuvant nivolumab/ipilimumab and two cycles of adjuvant nivolumab/ipilimumab) with adjuvant nivolumab/ipilimumab (four cycles of adjuvant nivolumab/ipilimumab) in patients with palpable stage III melanoma (28). The neoadjuvant arm achieved high pathological responses (78%), and no patients showing response developed recurrence during the median follow-up or 25.6 months. However, 9 of 10 patients experienced grade 3/4 adverse events in both treatment arms.

Rozeman et al. conducted a phase II randomized trial (OpACIN-neo) comparing three different doses and cycles of neoadjuvant nivolumab/ipilimumab for resectable clinical stage III melanoma (29). The following were three protocols: group A, two cycles of nivolumab (1 mg/kg) and ipilimumab (3 mg/kg); group B, two cycles of nivolumab (3 mg/kg) and ipilimumab (1 mg/kg); and group C, two cycles of ipilimumab (3 mg/kg) followed by two cycles of nivolumab (3 mg/kg). The objective radiological and pathological RRs were 63% (19/30) and 80% (24/30) in group A, 57% (17/30) and 77% (23/30) in group B, and 35% (9/26) and 65% (17/26) in group C, respectively. The rate of grade 3/4 immune-related adverse events was lower in group B than in groups A and C (group A, 40% [12/30]; group B, 20% [6/30]; group C, 50% [13/26]). One group A patient died of encephalitis.

BRAF INHIBITOR/MEK INHIBITOR

Amaria et al. reported a randomized phase II trial for patients with resectable clinical stage III or oligometastatic stage IV melanoma harboring BRAFV600E/K mutation (30). The patients were randomly assigned to either undergo surgery followed by adjuvant therapy without ICIs or targeted agents or to receive neoadjuvant/adjuvant dabrafenib/trametinib (8 weeks of neoadjuvant and 44 weeks of adjuvant dabrafenib/trametinib). The neoadjuvant group showed significantly long event-free survival (median event-free survival: 19.7 vs. 2.9 months; HR 0.016; $P < 0.0001$).

Long et al. also reported a single-arm phase II trial (NeoCombi) for patients with resectable clinical stage IIIB-C (AJCC 7th edition) melanoma harboring BRAFV600 mutation (31). The patients received neoadjuvant/adjuvant dabrafenib/trametinib (12 weeks of neoadjuvant and 40 weeks of adjuvant dabrafenib/trametinib). Thirty of 35 patients (86%) achieved a response (46%, complete response; 40%, partial response). All patients achieved pathological response, including 17 patients (49%) with pCR.

These novel neoadjuvant therapies, involving active regimens mainly for clinical stage III melanoma, showed high pathological RRs. Remarkably, no patients achieving pCR after treatment with ICIs developed recurrence during the follow-up periods. However, these esults must be interpreted with caution as these trials did not report OS after long-term follow-up.

IMMUNOLOGY OF SLNs

Immunohistological and molecular characteristics of SLNs may be useful in predicting the development of regional or distant metastasis, because the SLN represents the immunological site at which anti-tumor immune dysfunction is established and where potential prognostic immunologic markers can be found. Considering the immunologic microenvironment, the number of CD3+, CD4+, and CD8+ tumor-infiltrating lymphocytes in positive SLN is associated with better recurrence-free survival and OS (32). Elevated levels of regulatory T cell markers, such as FOXP3 and indoleamine 2,3-dioxygenase, correlate with increasing rates of local, regional, and distant metastases (33, 34). A study focused on regression of the primary tumor indicated that a regression of more than 10% was a reliable cutoff to divide different risk categories (35). Only a small number of CD4+/CD25+, FOXP3+/CD4+, or PD1+/CD4+ lymphocytes infiltrated the regressed areas. These lymphocytes were correlated with anergy and lower CD8+ lymphocyte immune response to melanoma cells. Thus, these findings may help in developing novel therapeutic strategies for selecting SLNB and immediate CLND for patients with stage III melanoma. As for molecular characteristics, Vallacchi et al. reported a pilot study involving integrated analysis of genome-wide transcriptional profiles and *in vitro* assessment of immune cells present in positive SLNs. This analysis identified microRNA, involved in the regulation of the TNF receptor superfamily member 8 gene that encodes the CD30 receptor, as a marker in the lymphocytes of melanoma patients with progressive disease. These findings demonstrate that microRNA is associated with the regulation of immune dysfunction in SLNs, providing a valuable prognostic molecular marker for identifying stage III melanoma patients at risk of recurrence.

CONCLUSIONS

SLNB has contributed to the selection of earlier CLND in patients without nodal disease by detecting microscopic positive SLN. Conversely, it is questionable whether CLND is required if SLN itself was therapeutic in patients with microscopic positive SLN alone. The results of two recent randomized clinical trials suggested that immediate CLND for positive SLN patients was not associated with DFS, OS, and metastasis-free survival, despite an increased risk of delayed non-SLN recurrence. Currently, SLNB provides prognostic information and has a therapeutic role in patients with a low tumor burden with intermediate-thickness melanoma. SLNB is also useful to select patients with the appropriate stage for undergoing post-operative adjuvant therapy. Immediate CLND is no longer routinely recommended for all patients with positive SLNB, particularly for patients without suspected non-SLN metastasis. At present, immediate CLND is ideal for patients at low risk of distant metastasis but at high risk of delayed regional metastasis.

The future of SLNB and CLND will depend on the development of promising neoadjuvant/adjuvant therapies and excellent biomarkers, which may drastically change the treatment strategies for stage III melanoma patients as well as the current

TNM classification. This may lead to the advent of a new era in which surgical procedures would not be required for high-risk patients, including those with stage III disease.

AUTHOR CONTRIBUTIONS

YN had full access to all of the data in the study and take responsibility for study concept and design, acquisition, analysis, interpretation of data, drafting of the manuscript, and critical revision of the manuscript for important intellectual content.

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REVIEW
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Skin Imaging Using Ultrasound Imaging, Optical Coherence Tomography, Confocal Microscopy, and Two-Photon Microscopy in Cutaneous Oncology

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With the recognition of dermoscopy as a new medical technology and its available fee assessment in Korea comes an increased interest in imaging-based dermatological diagnosis. For the dermatologist, who treats benign tumors and malignant skin cancers, imaging-based evaluations can assist with determining the surgical method and future follow-up plans. The identification of the tumor's location and the existence of blood vessels can guide safe treatment and enable the use of minimal incisions. The recent development of high-resolution microscopy based on laser reflection has enabled observation of the skin at the cellular level. Despite the limitation of a shallow imaging depth, non-invasive light-based histopathologic examinations are being investigated as a rapid and pain-free process that would be appreciated by patients and feature reduced time from consultation to treatment. In the United States, the current procedural terminology billing code was established for reflectance confocal microscopy in 2016 and has been used for the skin cancer diagnosis ever since. In this review, we introduce the basic concepts and images of ultrasound imaging, optical coherence tomography, confocal microscopy, and two-photon microscopy and discuss how they can be utilized in the field of dermatological oncology.

Keywords: skin imaging, skin cancer, benign skin tumor, ultrasound, optical coherence tomography, confocal microscopy, two-photon microscopy

INTRODUCTION

Efforts to diagnose skin cancer without skin biopsy are ongoing. The diagnoses of patients with suspected skin cancer are confirmed by punch biopsy followed by histopathological examination, which involve the collection of a small portion of the entire lesion to diagnose skin cancer (1). In this case, since only vertical information of a specific region is acquired, dermoscopy can supplement horizontal information of the entire lesion to identify the most suitable biopsy site. However, dermoscopy has an inherent depth limit confined to the upper dermis (**Table 1**).

TABLE 1 | Pros and cons of skin biopsy and dermoscopy.

	Skin biopsy	Dermoscopy
Advantages	1. Provide universal validity based on long-term accumulated histopathological criteria	1. Identify optimal biopsy sites 2. Reduce unnecessary biopsy 3. Determine horizontal extent of skin lesion 4. Continue to observe lesion treatment
Disadvantages	1. Limitation of evaluating whole lesion by vertical information of specific region 2. Limitations of repeated practice due to pain, bleeding, and infection risk	1. Inherent depth limitation (upper dermis) 2. Difficulty implementing 3D image 3. No reflection of functional and dynamic information (blood flow velocity, oxygen saturation, etc.) of the skin

To observe lesions deep to the upper dermis, the maximum depth that can be observed with dermoscopy, non-invasive techniques, such as confocal microscopy, multiphoton microscopy, optical coherence tomography, and ultrasound must be used. Although each operation principle is different, they all use the reflection characteristic as if it is mirrored, and the skin's depth and resolution differ among device types (**Table 2**). Here we briefly discuss each available device and its clinical use in the dermatology field.

ULTRASOUND IMAGING

Ultrasound imaging uses high-frequency sound waves that cannot be heard by the human ear. When it is sent inside the human body, the degree of absorption and reflection is cut off depending on the constituents and the reflected sound waves are sensed and imaged (2). Therefore, the probe that sends and detects the sound waves forms the core equipment for ultrasound technology. Higher-frequency (MHz) sound waves enable high-resolution observation of the skin surface, but the observable depth decreases. In the field of dermatology, ultrasound is mainly used to identify benign tumor type and extent (**Table 3**). Before surgery, it can provide information about tumor type and size, locate the existence of surrounding vessels, identify the best location for the incision, and set the range while viewing the ultrasound screen in real time with the patient. It can also help the clinician evaluate whether the tumor was completely removed after surgery (**Figure 1**).

In the case of epidermoid cysts, one of the most common benign tumors, it is often seen as a well-defined ovoid-shaped heterogeneous hypoechoic lesion in the subcutaneous layer with strong posterior acoustic enhancement (**Figure 2**). Ultrasonographic findings corresponding to epidermal cyst rupture include pericystic changes, increased vascularity, deep abscess formation, and others (9). Trichilemmal cyst, a benign

TABLE 2 | Device resolution and imaging depths[1].

	Resolution	Penetration depth
Confocal microscopy	1 μm	∼500 μm
Optical coherence tomography	2–10 μm	∼2 mm
Ultrasonography	150 μm	∼10 cm
High-resolution computed tomography	300 μm	Entire body
Magnetic resonance imaging	1 mm	Entire body

appendage lesion derived from the outer root sheath of the hair follicle, is often seen as a well-defined hypoechoic lesion with internal calcification and posterior sound enhancement (**Figure 3**) (8). Identifying these sites just prior to surgery and optimizing the incision site and approach can improve the success rate and reduce recurrence rates.

Pilomatricoma, a benign superficial tumor of the hair follicle, is often seen as a well-defined mass with inner echogenic foci and a peripheral hypoechoic rim or a completely echogenic mass with strong posterior acoustic shadowing in the subcutaneous layer on ultrasonography (**Figure 4**) (7). Pilomatricoma often shows angiographic findings and may be difficult to differentiate from hemangioma.

A lipoma appears as a well-defined hypoechoic mass with multiple echogenic strands on ultrasound (**Figure 5**). If the encapsulation is well-formed, it is easier to remove. Ultrasonography is especially useful for diagnosing and treating lipoma in the forehead. A lipoma occurring in the forehead is often located under the frontalis muscles, and it is important to confirm its precise position using preoperative ultrasonography. It typically has a semispherical shape when located under the muscles and an ovoid shape when it is located in the subcutaneous fat layer (**Figure 6**) (11). However, this is not always the case, so a comprehensive judgment should be made by checking whether it is close to the periosteum or using a special technique that uses the angulation of the probe to point out the lateral borders of the lesion (12).

There are no obvious criteria that can diagnose malignant cutaneous tumors using ultrasound imaging. However, tumor size >5 cm, infiltrated margins, rapid clinical growth, moderate to severe intratumoral hypervascularity (**Figure 7**), and an absence of the typical features of benign tumors are highly suggestive of malignancy (13, 14). High-definition ultrasound with transducers up to 70 MHz, which can observe more detail, has been used to diagnose cutaneous angiosarcoma of the breast and is expected to be useful for the identification of malignant skin cancers (15).

OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography (OCT), a three-dimensional (3D) imaging technique based on low coherence interferometry,

Abbreviations: OCT, optical coherence tomography; TPM, two-photon microscopy; EMPD, extramammary Paget's disease; SHG, second harmonic generation; MPM, multi-photon microscopy; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; AK, actinic keratosis.

[1]Available online at: http://obel.ee.uwa.edu.au/research/fundamentals/introduction-oct/

TABLE 3 | Key articles comparing ultrasound imaging and histopathology.

Tumor type	Year	Main findings	Correlation with histopathological findings	Probe frequency	Sample size
Basal cell carcinoma (3)	2008	1. BCC tumor ultrasound shows an oval and hypoechoic lesion 2. Compare tumor thickness measurements between ultrasound and histology	Good thickness correlation with histology (intraclass correlation coefficient, 0.9)	7–15 MHz probe	25 patients
Basal cell carcinoma (4)	2007	Lesions that may have a higher aggressive potential may also appear as hyperechoic spots	Hypersonographic spots in BCCs seemed to correspond to calcification, horn cysts, or clusters of apoptotic cells in the centers of nests of basal cell carcinoma	15 or 30 MHz	29 basal cell carcinomas
Invasive squamous cell carcinoma (5)	2009	SCC metastasized to lymph node showed asymmetrical cortical area with high elasticity	Presence of metastatic tumor cells located asymmetrically in a small section of the cortical area	Not mentioned	1 patient
Merkel cell carcinoma (6)	2017	1. Hypoechoic pattern with variable vascularization 2. Useful in the diagnostic work-up of MCC and can help more precisely delimit the tumor prior to complete surgical resection	Not mentioned	18 MHz	7 patients
Pilomatricoma (7)	2005	Well-defined mass with inner echogenic foci and a peripheral hypoechoic rim or a completely echogenic mass with strong posterior acoustic shadowing	Inner echogenic foci may relate with calcification or ossification	7–12 MHz	20 pilomatricomas from 19 patients
Trichilemmal cyst (TC) (8)	2019	Well-defined hypoechoic lesions with internal calcification and posterior sound enhancement	TC contains homogeneous eosinophilic keratinous materials Calcified foci within this keratin can be found	3–12 MHz 6–18 MHz	54 TCs from 50 patients
Ruptured epidermal cyst (REC) (9)	2008	RECs were classified into three types: with lobulations showing echogenic inner contents (type I), with protrusions (type II), and with abscess pocket formations showing poorly defined pericystic changes and increased vascularity around the abscess formation (type III)	Histopathology of the excised RECs also showed similar morphology	5–10 MHz 5–12 MHz	13 patients
Lipoma in the forehead (10)	2016	1. Hyperechoic striated septae parallel to the skin suggestive of lipoma 2. Ultrasonographic findings were accurate in 9 of 14 cases (64.3%).	Unlike the preoperative ultrasonographic findings, 13 of 14 cases were confirmed as frontalis-associated lipomas intraoperatively	12 or 15 MHz	14 patients with lipomas in the forehead

creates an image by detecting the interference phenomena from light scattering or reflection as it passes through different layers of skin via the time domain or Fourier-domain method. OCT non-invasively provides skin images similar to the B mode of ultrasound to a depth of 1–2 mm and a resolution of 2–10 μm with high imaging speed. Functional OCT techniques that can provide additional information, such as polarization and vasculature were recently developed and applied for the detection of abnormal vasculature of a port-wine stain or skin cancer (16–18). Our research group developed a device that matches an OCT image with that obtained by dermoscopic imaging and provides more information than dermoscopy alone (19). Through this, we expect to be able to assess the extent of scar treatment (**Figure 8**). It is expected that a stage of nevus flammeus will be established, and treatment feasibility and degree will be evaluated (**Figure 9**). The limitations of OCT are limited depth of examination and lack of resolution to observe cancer cell morphology. Line-field confocal OCT, which can reveal comprehensive structural mapping of the skin at the cellular level with an isotropic spatial resolution of ∼1 μm to a depth of ∼500 μm, was recently reported to correlate with conventional histopathological images of skin tumors (20). Key articles comparing OCT and histopathology are summarized in **Table 4**.

CONFOCAL MICROSCOPY

Confocal microscopy is based on the existence of one focal point when a laser, used as a light source, is reflected off a subject. The "out of focus" signal is blocked by a pinhole, and contrast is generated by reflections at the interfaces of tissue and cellular structures due to variations of the index of refraction. Since image acquisition is not possible with a single signal point, imaging occurs by scanning across several pinholes. Imaging up to a depth of 100–200 μm at a 1-μm resolution is possible. Confocal microscopy is capable of providing rapid bedside pathological analysis by producing images with subcellular resolution without skin biopsy and physical sectioning (24–26). There are two ways to use this approach for Mohs surgery. One is used *in vivo* and can help the identification of the surgical margins in a perioperative setting (27). It is also possible to check the remaining lesion using intraoperative images *in vivo* after removing the main skin cancer mass (28). The other is for *ex vivo* use, in which the surgical margins are removed and confocal microscopy is used to confirm whether the tumor remains within it (29). However, when used for detection in Mohs surgery, the grayscale confocal image was difficult to interpret by the surgeons. To improve this, each frozen specimen was stained with acridine orange

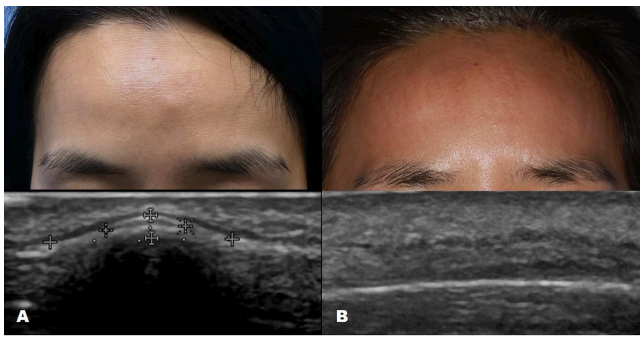

FIGURE 1 | Ultrasound images of forehead osteoma. **(A)** Before excision. **(B)** After excision performed through a remote incision above the hairline.

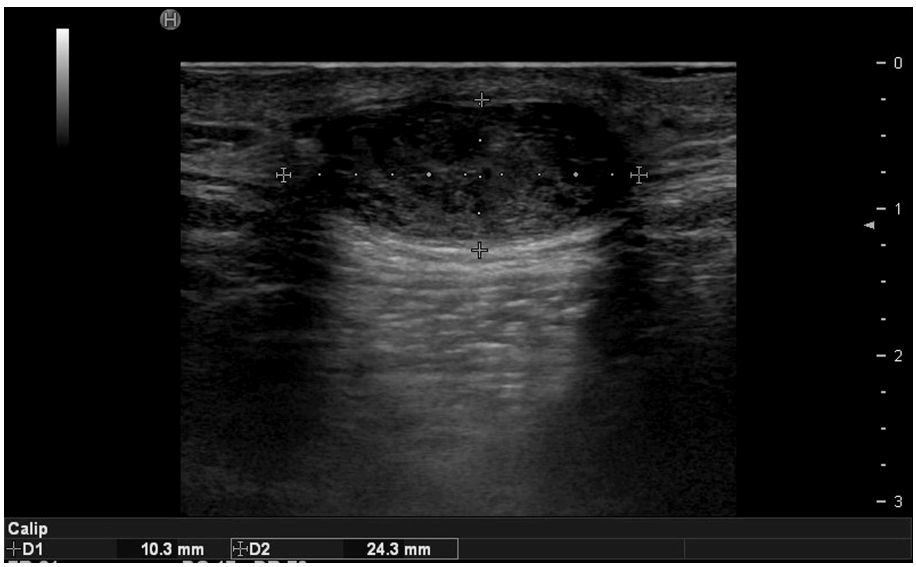

FIGURE 2 | Ultrasound image of epidermal cyst.

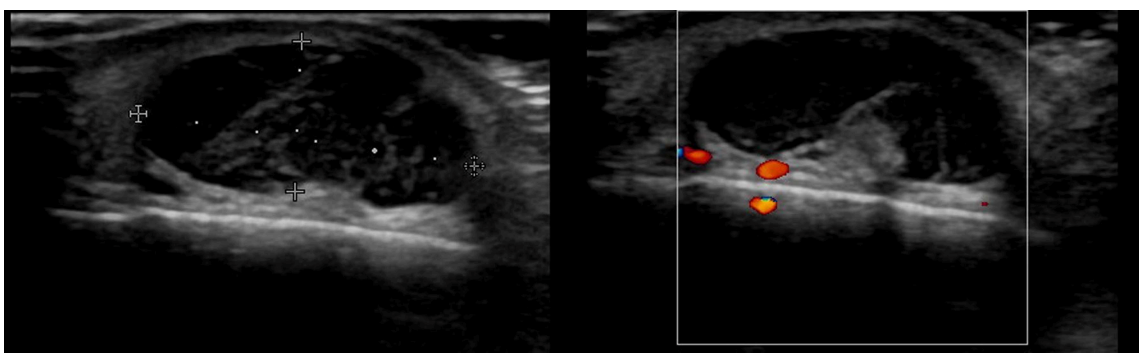

FIGURE 3 | Ultrasound image of trichilemmal cyst.

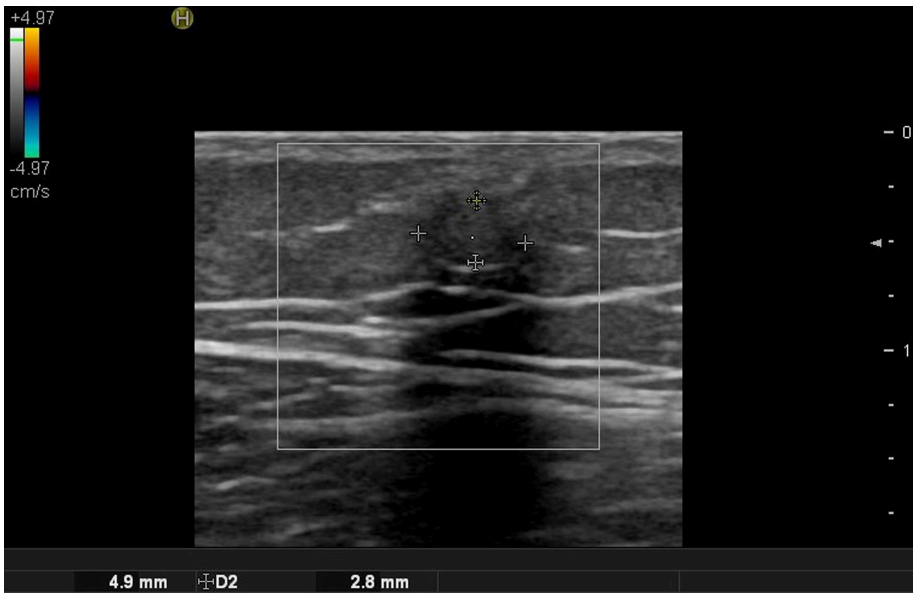

FIGURE 4 | Ultrasound image of pilomatricoma.

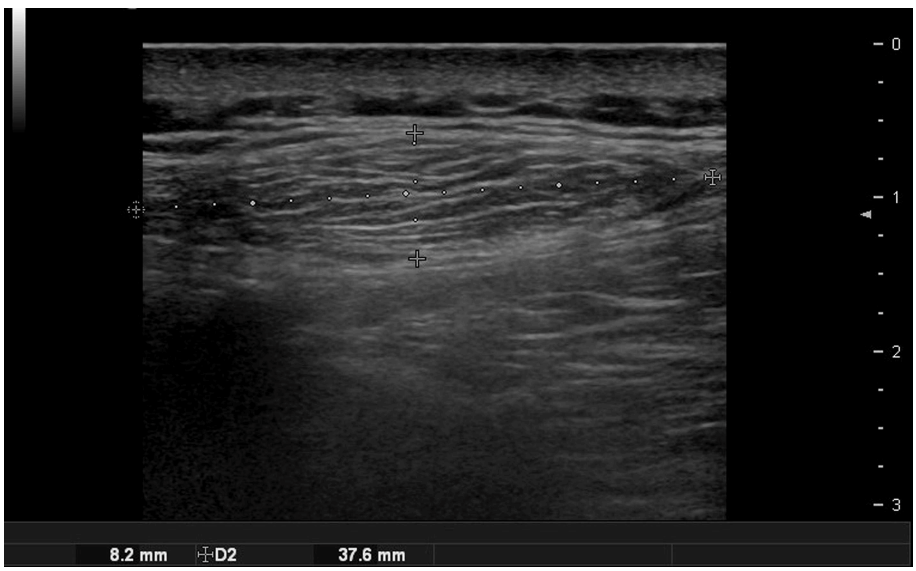

FIGURE 5 | Ultrasound image of lipoma.

(pH 6.0) and eosin (pH 6.0) and then scanned with confocal mosaicking microscopy to imitate hematoxylin and eosin-stained Mohs frozen sections. This approach and physician training can improve the accuracy of the non-melanoma skin cancer diagnosis (30). Key articles comparing confocal microscopy and histopathology are summarized in **Table 5**.

Confocal microscopy has also been applied to diagnose mammary and extramammary Paget's disease (EMPD) (37), frequently showing Paget cells predominantly within the epidermis (38). However, due to the limited depth of imaging (100–200 μm) when applied non-invasively, the invasion site is difficult to determine. A major limitation of this technique is that it can only provide morphological information and does not reflect the tissue's internal structure or functional state.

TWO-PHOTON MICROSCOPY

Two-photon microscopy (TPM) is a technique that uses the fluorescence released after excitation from simultaneously

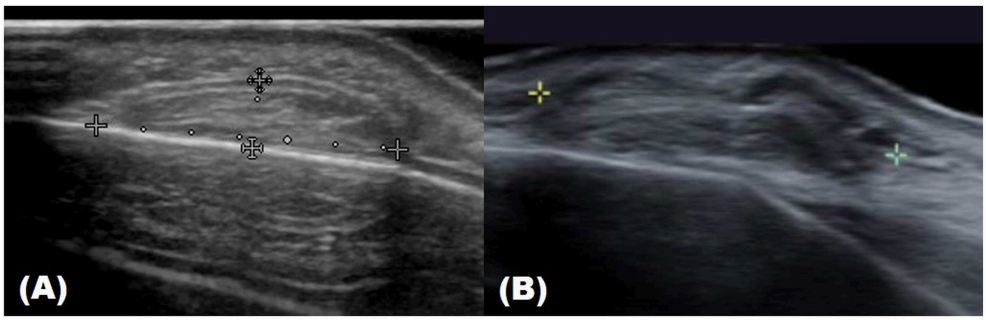

FIGURE 6 | Ultrasound image of forehead lipoma. **(A)** Submuscular layer. **(B)** Subcutaneous layer.

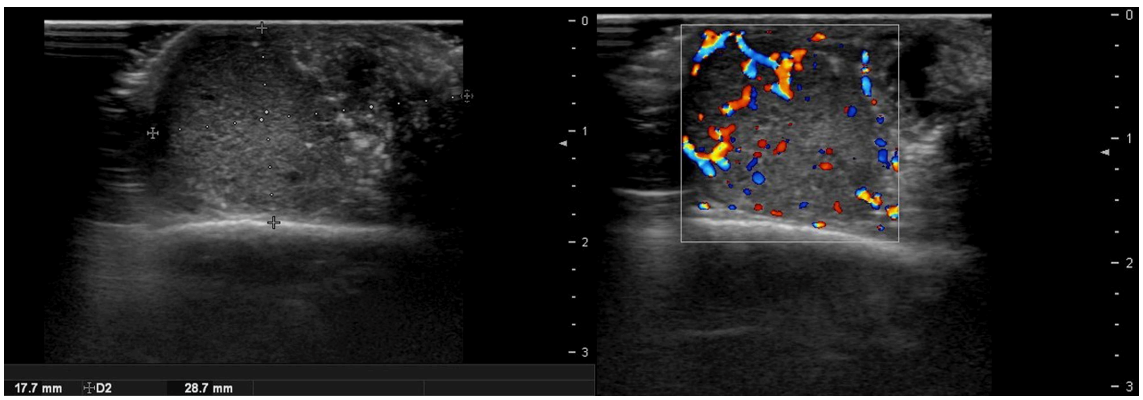

FIGURE 7 | Ultrasound image of malignant proliferating trichilemmal tumor.

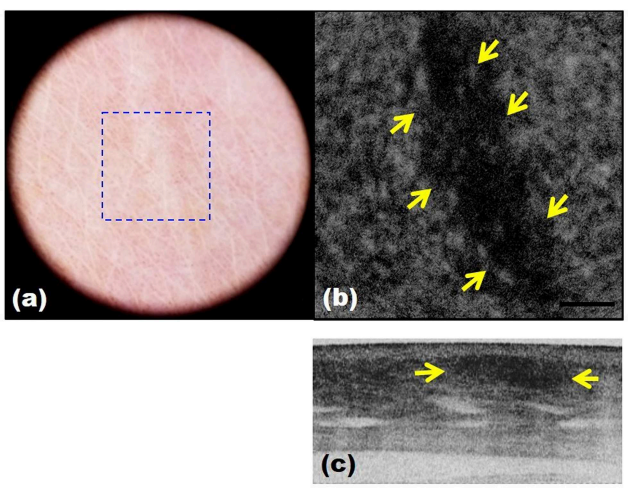

FIGURE 8 | Scar images by dermoscopy-guided multifunctional optical coherence tomography (OCT). **(a)** Dermoscopic image. **(b,c)** Intensity OCT showing a dark area and frequent banding pattern due to stronger light scattering and birefringence.

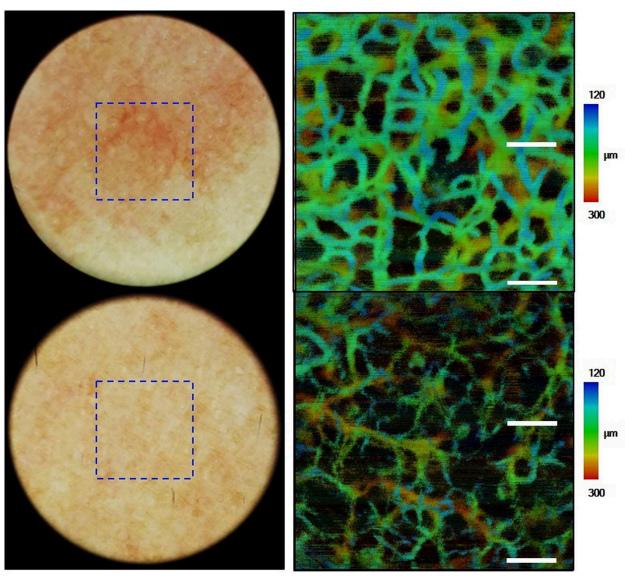

FIGURE 9 | Images of nevus flammeus and normal skin acquired by dermoscopy-guided angiographic optical coherence tomography.

absorbing two photons with long wavelengths and low energy. TPM allows observation of vital phenomena in cells and *in vivo* at the molecular level. In particular, it has the advantage of being able to identify the distribution of collagen within the dermis using the second harmonic generation (SHG) produced when two photons simultaneously interfere. Non-invasive *in vivo*

TABLE 4 | Key articles comparing optical coherence tomography and histopathology.

Tumor type	Year	Type	Main findings	Correlation with histopathology findings	Sample size
Basal cell carcinoma (BCC) (21)	2014	High-definition optical coherence tomography (HD-OCT)	Lobulated nodules, peripheral rimming, epidermal disarray	Peripheral rimming in HD-OCT correlates with peritumoral mucin deposition	25 cases of BCC
BCC (22)	2016	Dynamic OCT enables the detection of blood flow *in vivo* and visualization of the skin microvasculature	Blood vessels varied from dilated, larger-than normal vessels to the smallest detectable vessels	Loose and more vascularized dermis between tumor nests	1 patient with BCC on the cheek
BCC, Melanoma (20)	2018	Line-field confocal OCT	BCC: lobulated structures within the dermis, dark cleft due to mucin deposition; melanoma: general architectural disarrangement, disruption of the dermal-epidermal junction, pagetoid spread of atypical melanocytes	BCC and melanoma approximate shapes observed in OCT appeared similar histopathologically	2 patients with BCC 2 patients with melanoma
Actinic keratosis (AK), Squamous cell carcinoma (SCC) (23)	2015	HD-OCT	Absence of an outlined dermo-epidermal junction on cross-sectional images allowed discriminating SCC from AK and normal skin	It related to irregular budding of the epidermis outstanding into the upper dermis and/or presence of periadenexal collars penetrating through the dermo-epidermal junction	37 cases of AK 16 cases of SCC

TABLE 5 | Key articles comparing confocal microscopy and histopathology.

Tumor type	Year	Type	Main findings	Correlation with histopathological findings	Sample size
Basal cell carcinoma (BCC) (31)	2002	Real-time, confocal reflectance microscopy (*in vivo*)	Confocal features correlated very well with hematoxylin and eosin (H&E)-stained sections of the biopsy specimen	Features that were readily identified by both *in vivo* confocal microscopy and standard microscopy of H&E-stained sections included parakeratosis, actinic changes overlying the BCC, relative monomorphism of BCC cells, BCC nuclei exhibiting characteristic elongated or oval appearance, high nucleocytoplasmic ratios, and the presence of prominent nucleoli, increased vascularity, and prominent predominantly mononuclear inflammatory cell infiltrate	8 BCC lesions
Actinic keratosis (AK), squamous cell carcinoma (SCC), keratoacanthoma (32)	2009	Reflectance confocal microscopy (*in vivo*)	All 38 cases displayed an atypical honeycomb and/or disarranged pattern of the spinous-granular layer of the epidermis; round nucleated cells were seen in 20 SCCs (65%) and 1 AK (14%) Round blood vessels were seen in the superficial dermis in 28 SCCs (90%) and 5 AKs (72%)	Round nucleated cells at the spinous-granular layer correspond to atypical keratinocytes or dyskeratotic cells	A total of 38 lesions in 24 patients with 7 AKs, 25 SCCs *in situ*, 3 invasive SCCs, and 3 keratoacanthomas
Bowen disease (BD) (33)	2012	Reflectance confocal microscopy (*in vivo*)	Two types of targetoid cells were seen: those presenting as large, homogeneous, bright cells with a dark halo; and round ones with a dark center, surrounding bright rim, and dark halo	Targetoid cells correlated dyskeratotic cells with condensed, eosinophilic cytoplasm and a retraction halo. Dyskeratotic cells were correlated with a dark central nucleus and a surrounding clear retraction halo	10 cases of BD
BCC (34)	2013	Comparison of reflectance confocal microscopy and multiphoton tomography findings (*in vivo*)	Elongated cells and palisading structures are easily recognized using both methods	Due to the higher resolution, changes in nucleus diameter or cytoplasm could be visualized using multiphoton tomography (MPT) Therefore, nucleus diameter, nucleus/cytoplasm ratio, and cell density are estimated for normal and BCC cells using MPT	9 patients with BCC

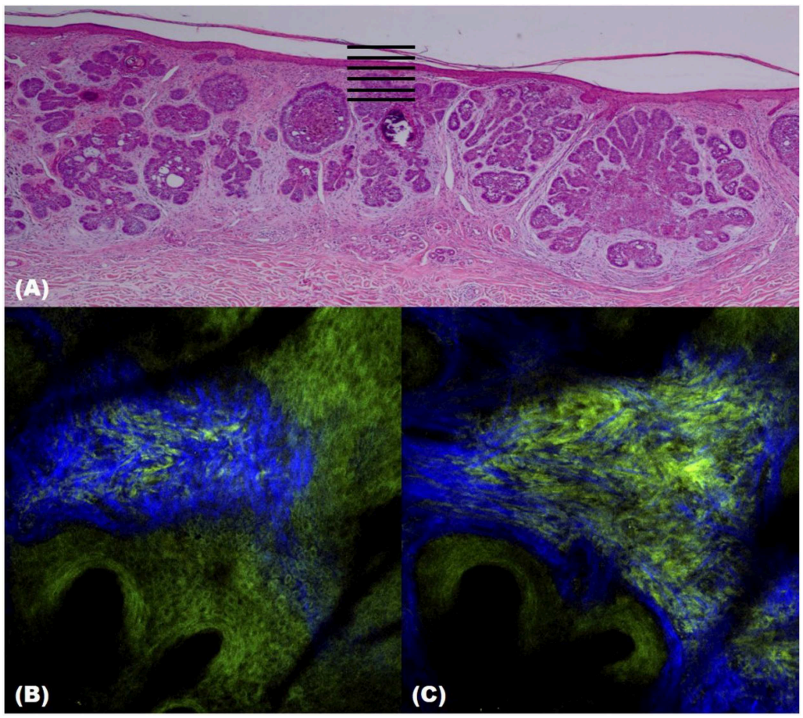

FIGURE 10 | Two-photon microscopy (TPM) images of basal cell carcinoma (BCC). **(A)** Histopathological finding. **(B,C)** TPM images showing parallel collagen fibers (blue) surrounding a BCC tumor nest.

TABLE 6 | Key articles comparing multiphoton microscopy and histopathology.

Tumor type	Year	Type	Main findings	Correlation with histopathological findings	Sample size
Basal cell carcinoma (BCC) (39)	2015	*In vivo* multiphoton microscopy (MPM)	1. Nests of basaloid cells palisading in the peripheral cell layer at the dermoepidermal junction and/or in the dermis 2. Parallel collagen and elastin bundles surrounding the tumors 3. Mucinous stroma adjacent to tumor was visualized using MPM	These features generally correlated well with histopathologic examination. However, histologic examination revealed palisading of peripheral layers in some of the tumor nests of the lesion, although this feature was not obvious in the nests imaged with MPM.	9 patients with a total of 10 BCC
Squamous cell carcinoma *in situ* (SCCIS), superfical BCC (SBCC) (40)	2008	*Ex vivo* MPM	The following findings were seen: SCCIS: bowenoid dysplasia, multinucleated cells, or hyperkeratosis SBCC: peripheral palisading of tumor cells	The morphologic features differed significantly between these lesions and perilesional skin.	5 specimens of SCCIS 6 specimens of SBCC
Actinic keratosis (AK), squamous cell carcinoma (SCC) (41)	2016	*In vivo* MPM	Changes in the morphology of the keratinocytes, such as broadened epidermis, large intercellular spaces, enlarged nucleus and a large variance in cell shape could easily be recognized.	AK: hyperparakeratosis and cell pleomorphism SCC: invasion of the dermis, keratin pearls and hyperchromatic nuclei	6 patients with AK 6 patients with SCC
Benign and malignant melanocytic nevi (BMMN) (42)	2014	*In vivo* MPM	They evaluate BMMN using 9-point scale showing different values according to two-photon excited fluorescence and second harmonic generation of nevi. Indices corresponding to common nevi (0–1), dysplastic nevi (1-4), and melanoma (5-8) were significantly different ($P < 0.05$).	Prominent qualitative correlations included the morphology of epidermal keratinocytes, the appearance of nests of nevus cells surrounded by collagen fibers, and the structure of the epidermal–dermal junction.	5 common nevi 5 dysplastic nevi 5 melanoma
BCC, SCC, dermatofibrosarcoma protuberans (DFSP) (43)	2019	*Ex vivo* moxifloxacin labeling-based MPM	Moxifloxacin MPM imaged both cells and collagen in the skin, similarly to label-free MPM, but with enhanced fluorescence intensities in cells and enhanced imaging speeds.	Moxifloxacin MPM could detect specific cellular features of various skin cancers in good correlation with histopathological images at the higher imaging speed than label-free MPM.	10 patients with BCC 1 patient with SCC 1 patient with DFSP

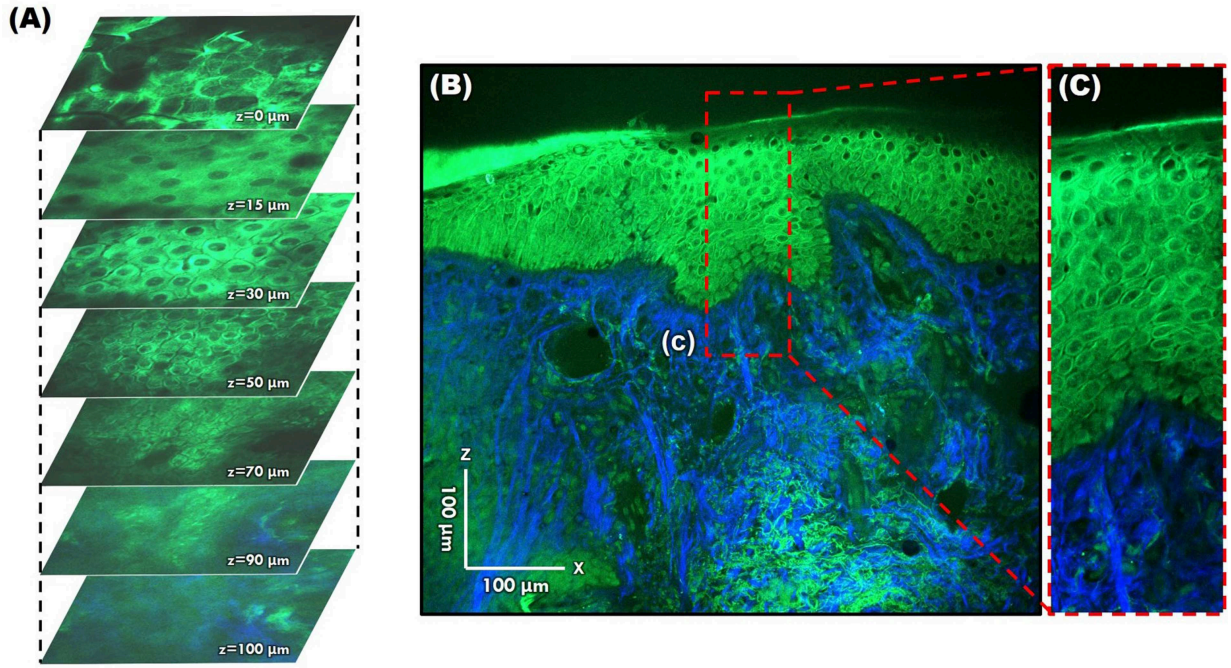

FIGURE 11 | Moxifloxacin-based multi-photon microscopy images of normal skin. **(A)** En face images at different depths. **(B,C)** Cross-sectional view of the epidermis and dermis.

multi-photon microscopy (MPM) imaging also reportedly provides label-free contrast and reveals several characteristic features of basal cell carcinoma lesions (39). This feature correlates well with histopathological examination, findings, and SHG in particular shows collagen and elastin bundles around the tumor (**Figure 10**) (**Table 6**).

However, since TPM and MPM utilize weak endogenous fluorescence in tissue, there is a need for high excitation laser power and extension of pixel duration (44, 45). To overcome this limitation and reduce photodamage, moxifloxacin, an FDA-approved antibiotic, has been reported as a cell-labeling agent for MPM (46). Moxifloxacin has bright intrinsic multi-photon fluorescence, good tissue penetration, and high intracellular concentration. In addition, moxifloxacin-based MPM imaging is 10 times faster than imaging based on endogenous fluorescence (**Figure 11**) (46).

Although imaging depth remains a limitation, various methods to achieve a clear and high-resolution image are being developed. It is also expected that the diagnosis rate can be increased by tumor marker labeling. A recent report stated that in patients with EMPD, a subclinical extension can be assessed by MPM using whole-mount immunostaining with anti-cytokeratin 7 antibody to label Paget cells (35). These trials will be used in the *ex vivo* skin tissue to find the tumor's margins, and it is anticipated that it may replace frozen sections in the future. For more generalized clinical applications, the cost of the equipment is the greatest hinderance. MPM equipment is expensive because it uses a femtosecond laser (36).

CONCLUSION

In addition to ultrasonic devices that can closely observe the skin and deep structures, the development of dermatological equipment that unites laser and optical technology has shown visible progress. The principle of these devices is to analyze signals reflected or scattered from the skin, and there is a fundamental limitation that it is evaluated by looking into the mirror. These limitations are expected to improve in the near future by the development of fluorescent probes targeting tumors or diseases and will be used more actively for the diagnosis and treatment of skin lesions.

For dermatologists, this is a good opportunity to strengthen the specialty of dermatology. We are already familiar with laser equipment and have demonstrated a correlation between clinical and histopathological findings. When we use imaging equipment to further investigate a patient's skin and present objectively explainable data by linking "clinical imaging–histopathological findings," a more robust doctor–patient relationship can be established.

AUTHOR CONTRIBUTIONS

BO conceived the concept and wrote the manuscript. KK co-conceived the concept and drafted the figures and tables. KC co-conceived the concept and edited and improved 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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