# RISING STARS 2021: PATHOLOGY

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# RISING STARS 2021: PATHOLOGY

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# Histopathological Evidence of Occipital Involvement in Male Androgenetic Alopecia

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**Background:** The occipital region of the scalp is generally accepted as an unaffected area of androgenetic alopecia (AGA) for both genders. However, evidence of AGA involving the occipital scalp has been demonstrated in women; meanwhile, it is unclear whether occipital involvement also occurs in men.

**Objective:** We aimed to determine if there is occipital involvement in men with AGA.

**Methods:** This case-control study compared hair counts of scalp biopsy specimens from the occipital region of 82 men with Hamilton-Norwood III-VII and 82 unaffected men.

**Results:** The mean ages of men with AGA and controls were $40.1 \pm 8.9$ and $38.6 \pm 10.5$ years, respectively ($P = 0.291$). A significant decrease in total hair follicles, terminal hair follicles, follicular units and terminal to vellus (T:V) ratio, along with a significant increase in follicular stelae was indicated in the AGA group compared to controls (all $P < 0.05$). Subgroup analyses revealed that average counts of total hair follicles, terminal hair follicles and T:V ratios were also significantly lower in males with Hamilton-Norwood VI and VII than in controls (all $P < 0.05$). There were no correlations between increasing age and hair count parameters, but a significant negative association was found between total follicle numbers and disease duration ($r = -0.23$, $P = 0.02$).

**Conclusions:** AGA can involve the occipital area of male patients with advanced disease. Therefore, the occiput of particular cases should not be used to determine reference data for normal scalp hair, and preoperative measurements of miniaturized hairs in the donor site are strongly recommended in all persons undergoing hair transplantation.

Keywords: pattern hair loss, transverse section, hair count, donor site, hair transplantation, miniaturization

## INTRODUCTION

The most common cause of hair loss in both men and women is androgenetic alopecia (AGA), also known as pattern hair loss, and is characterized by gradual hair thinning within a specific distribution on the scalp in genetically susceptible individuals (1). Male patients typically present with hairline recession in the frontotemporal areas as well as balding of the vertex and mid-scalp whereas female patients usually manifest as diffuse hair thinning over the central scalp with a preserved frontal hairline (2). Androgens are apparently implicated in the pathogenesis of male

AGA; by contrast, the role of androgens in women is much less established (3, 4). Although the etiologies between men and women are not identical, they share similar features which include a miniaturization of hair follicles and a progressive shortening of anagen duration (1, 5).

The diagnosis of AGA is usually straightforward and based upon characteristic clinical findings; however, scalp biopsies, in particular transverse sections, may be required to achieve a definite diagnosis in uncertain cases. The main histopathological features of AGA are as follows: (i) decreased terminal hair follicles and increased vellus hair follicles resulting in a reduced ratio of terminal to vellus (T:V) follicles of <4:1; (ii) a slightly increased telogen count, standardly accounting for 15–20%; (iii) the presence of follicular stelae below miniaturized hair follicles; and (iv) normal follicular density, apart from long-standing and advanced stages, which reveal an actual decrease in total hair follicles (6, 7).

The occipital region of the scalp has been widely accepted as an uninvolved area of AGA for both sexes, under the concept that affected persons have androgen-sensitive and androgen-insensitive scalp regions. This principle has led to the subsequent introduction of donor dominance theory and hair transplantation as a treatment option. Nonetheless, in our practice, we have observed hair thinning extending toward the occipital scalp in some advanced AGA, with more frequent observations among female patients. This may be partly due to the fact that androgen-independent mechanisms also play a role in many women. Moreover, it was highlighted in the field of hair restoration surgery that hair miniaturization can occur in the back and sides of the scalp (the donor sites), which will negatively influence transplant outcomes (8).

Occipital involvement has been demonstrated in female patients with AGA. Previous phototrichogram studies comparing hair characteristics in the occipital area between female AGA and normal controls showed highly consistent results (9–12). Statistically significant decreases in hair density and thickness relative to controls were confirmed in subjects with Ludwig II and III. Additionally, Ekmekci et al. conducted hair counts in biopsy specimens from the mid-scalp and occipital areas of forty female subjects with Ludwig I and II. A quarter of the subjects possessed occipital findings compatible with AGA (T:V ratios of <4:1), and almost 40% of the subjects had findings of suspected AGA in the occipital scalp (T:V ratios ranged from 4:1 to 7:1) (13).

On the contrary, occipital involvement in male AGA has not been proved. The results of phototrichogram studies, performed on the occipital region of men with AGA and normal men, have also been inconsistent. One study showed no significant differences in all hair parameters (14). Another study found statistically significant decreases in hair density and hair diameter in male subjects with the U type of the Basic and Specific classification, corresponding to Hamilton-Norwood VI and VII (15). The aim of our study was to determine whether occipital involvement existed in male patients with AGA.

## MATERIALS AND METHODS

### Study Design and Participants

This study was approved by the Mahidol University Institutional Review Board for Ethics in Human Research (MURA2020/165) and was conducted in accordance with the principles of the Declaration of Helsinki and in compliance with the International Conference on Harmonization-Good Clinical Practice and local regulatory requirements. Informed consent was obtained from the study participants or their families, as appropriate. All male patients with biopsy-proven AGA, who underwent paired 4 mm punch biopsies from both affected frontal/vertex and clinically normal occipital regions of the scalp for transverse sectioning from 2014 to 2019, were enrolled into this case-control study. Patient demographics and clinical data were collected. Principal exclusion criteria included incomplete medical records and the presence of other hair and scalp disorders. Additional exclusion criteria were documented systemic diseases related to hair loss, the use of medications or energy-based devices which can affect hair growth within 6 months and a history of hair transplantation.

As being considered the most reliable method, histological examination of transverse sections from scalp biopsy specimens was chosen to confirm androgenetic changes. Regarding the biopsy protocol in patients with AGA at our institution, the biopsy landmark on the occipital scalp was the external occipital protuberance of the skull. Paired biopsy specimens from the balding and non-balding occipital scalp were horizontally sectioned using the techniques described by Whiting DA (6). Tissue slides of the biopsy specimen from the occipital scalp in each study subject were reviewed. Two cases were excluded as we were unable to obtain the slides and three cases were excluded on account of tangential cuts, which complicated histologic interpretation. A total of 82 cases remained for analysis.

### Control Subjects

The control group consisted of 82 male adult decedents who required an autopsy for legal documentation at our institution. These decedents had no type of hair loss and received a 4 mm punch biopsy on the normal occipital scalp, following our AGA biopsy protocol, for transverse sectioning within 8 h of death to avoid autolysis, after receiving written informed consent from the bereaved relatives. Controls were randomly selected from a pool of male deceased subjects in our previous studies on hair counts from scalp biopsy specimens in the Thai population (16, 17), with an equal number of cases and controls. The biopsy specimens of controls were processed using a protocol identical to those of the study cases. The exclusion criteria used were also indistinguishable.

### Assessments

Histology slides of specimens from the occipital scalp of both case and control subjects were re-examined by a blinded dermatopathologist. All follicular structures were identified at various anatomical levels from the epidermis to subcutis. The numbers of total, terminal, and vellus hair follicles, along with the number of follicular units, were recorded. Anagen and telogen

hair follicles, as well as follicular stelae were also counted. Catagen hair follicles were included in the group of telogen hair follicles. Half of the intermediate hair follicles were assigned to terminal hair follicles with the other half to vellus hair follicles. Differences in hair parameters between groups were subsequently analyzed.

## Statistical Analyses

The minimum sample size for estimation of population mean with a 10% margin of error was 26, calculated using data from the previous study assessing values of hair counts per 4 mm diameter punch biopsy from the occipital scalp of Koreans, demonstrating that the mean number of total hair follicles in male subjects was $15.3 \pm 3.9$ (18).

Comparisons between study and control groups were performed using the Student's $t$-test, the Mann-Whitney $U$ test, or the Chi-squared test as appropriate. For further analyses among study subgroups based on disease severity was conducted using the analysis of variance, the Kruskal-Wallis test, or the Chi-squared test. When the overall comparison $P < 0.05$, pairwise

comparisons of subgroups were performed using the Turkey's honest significance difference test, the Mann-Whitney $U$ test, or the Chi-squared test as appropriate. The impact of advancing age on hair changes was determined using the Pearson correlation coefficient. A $P < 0.05$ was considered significant, and all statistical analyses were performed with SPSS software (PASW version 18.0; SPSS Inc., Chicago, IL).

## RESULTS

### Comparisons of Hair Count Parameters Between AGA and Control Groups

Hair counts from the clinically normal occipital scalp of patients with AGA, compared to controls, are shown in **Table 1**. The mean age of 82 patients with AGA was $40.1 \pm 8.9$ years whereas the mean age of 82 control subjects was $38.6 \pm 10.5$ years. The age difference between groups was not statistically significant ($P = 0.291$), representing only 18 months; therefore, an age adjustment was not applied. The mean number of total hair follicles in AGA subjects was $17.6 \pm 4.2$ (95% confidence interval [CI]: 16.68–18.52), showing a significantly lower number compared with controls (mean: 19.1 $\pm$ 6.1, 95% CI: 18.56–21.24, $P = 0.005$). Average counts of terminal hair follicles and follicular units were also significantly lower in patients with AGA than in controls ($P = 0.001$ and $0.002$, respectively). In addition, there was a significant decrease in T:V ratio and a significant increase in follicular stelae in the AGA group ($P = 0.001$ and $0.035$, respectively).

### Subgroup Analyses of Subjects With AGA

The summary of hair counts in the control and AGA subgroups based on disease severity are exhibited in **Table 2**. As for the severity of AGA according to Hamilton-Norwood classification, 14 (17%), 18 (22%), 15 (18%), 19 (23%), and 16 (20%) patients were grades III, IV, V, VI, and VII, respectively. The overall comparisons among subgroups revealed statistically significant differences in total hair follicles, terminal hair follicles,

**TABLE 1** | Hair counts per 4 mm diameter punch biopsy from the occipital scalp of male patients with androgenetic alopecia and controls.

|  | Controls | AGA | *P*-value |
|---|---|---|---|
| Number of cases | 82 | 82 |  |
| Age, y, mean (SD) | 38.6 (10.5) | 40.1 (8.9) | 0.291 |
| Total hair follicles, mean (SD) | 19.9 (6.1) | 17.6 (4.2) | 0.005* |
| Terminal hair follicles, mean (SD) | 17.9 (4.2) | 15.9 (3.8) | 0.001* |
| Vellus hair follicles, median (range) | 2 (0–7) | 3 (0–14) | 0.359 |
| Follicular units, mean (SD) | 9.3 (1.9) | 8.4 (1.8) | 0.002* |
| Follicular stelae, median (range) | 1 (0–4) | 3 (0–12) | 0.035* |
| Terminal:vellus ratio | 8.9:1 | 7.4:1 | 0.001* |
| Anagen:telogen ratio | 92.2:7.8 | 87.6:12.4 | 0.889 |

*AGA, androgenetic alopecia; SD, standard deviation; y, year.*
*\*Statistically significant.*

**TABLE 2** | Hair counts in the occipital scalp of controls and patient subgroups with different disease severity of androgenetic alopecia.

|  | Controls | Hamilton-Norwood stages of AGA subjects | | | | | *P*-value |
|---|---|---|---|---|---|---|---|
|  |  | III | IV | V | VI | VII |  |
| Number of cases | 82 | 14 | 18 | 15 | 19 | 16 |  |
| Age, y, mean (SD) | 38.6 (10.5) | 40.5 (11.4) | 38.2 (12.4) | 44.6 (10.2) | 38.8 (8.4) | 49.5 (7.4) | 0.079 |
| Disease duration, y, mean (SD) | – | 3.6 (0.8) | 5.2 (1.5) | 6.5 (2.2) | 6.9 (1.9) | 8.3 (2.3) | 0.001* |
| Total HFs, mean (SD) | 19.9 (6.1) | 18.6 (4.9) | 19.1 (5.2) | 17.8 (5.1) | 15.3 (5.4) | 14.2 (4.4) | 0.002* |
| Terminal HFs, mean (SD) | 17.9 (4.2) | 17.9 (4.8) | 18.3 (4.9) | 16.1 (5.2) | 13.1 (4.2) | 11.9 (3.9) | 0.002* |
| Vellus HFs, median (range) | 2 (0–7) | 2 (0–5) | 2 (0–8) | 3 (0–10) | 3 (0–11) | 4 (0–14) | 0.146 |
| Follicular units, mean (SD) | 9.3 (1.9) | 8.7 (1.9) | 8.8 (2.1) | 8.5 (1.6) | 7.9 (1.4) | 8.1 (2.5) | 0.035* |
| Stelae, median (range) | 1 (0–4) | 1 (0–4) | 1 (0–6) | 2 (0–8) | 2 (0–7) | 3 (0–12) | 0.294 |
| Terminal:vellus ratio | 8.9:1 | 8.6:1 | 8.8:1 | 7.9:1 | 6.8:1 | 5.3:1 | 0.001* |
| Anagen:telogen ratio | 92.2:7.8 | 90.7:9.3 | 91.2:8.8 | 88.6:11.4 | 85.4:14.6 | 83.2:16.8 | 0.294 |

*AGA, androgenetic alopecia; HF, hair follicle; SD, standard deviation; y, year.*
*\*Statistically significant.*

**FIGURE 1** | Total hair follicles between controls and patient subgroups with different disease severity of androgenetic alopecia **(AGA)**. *$P < 0.05$ when compared to the control group.
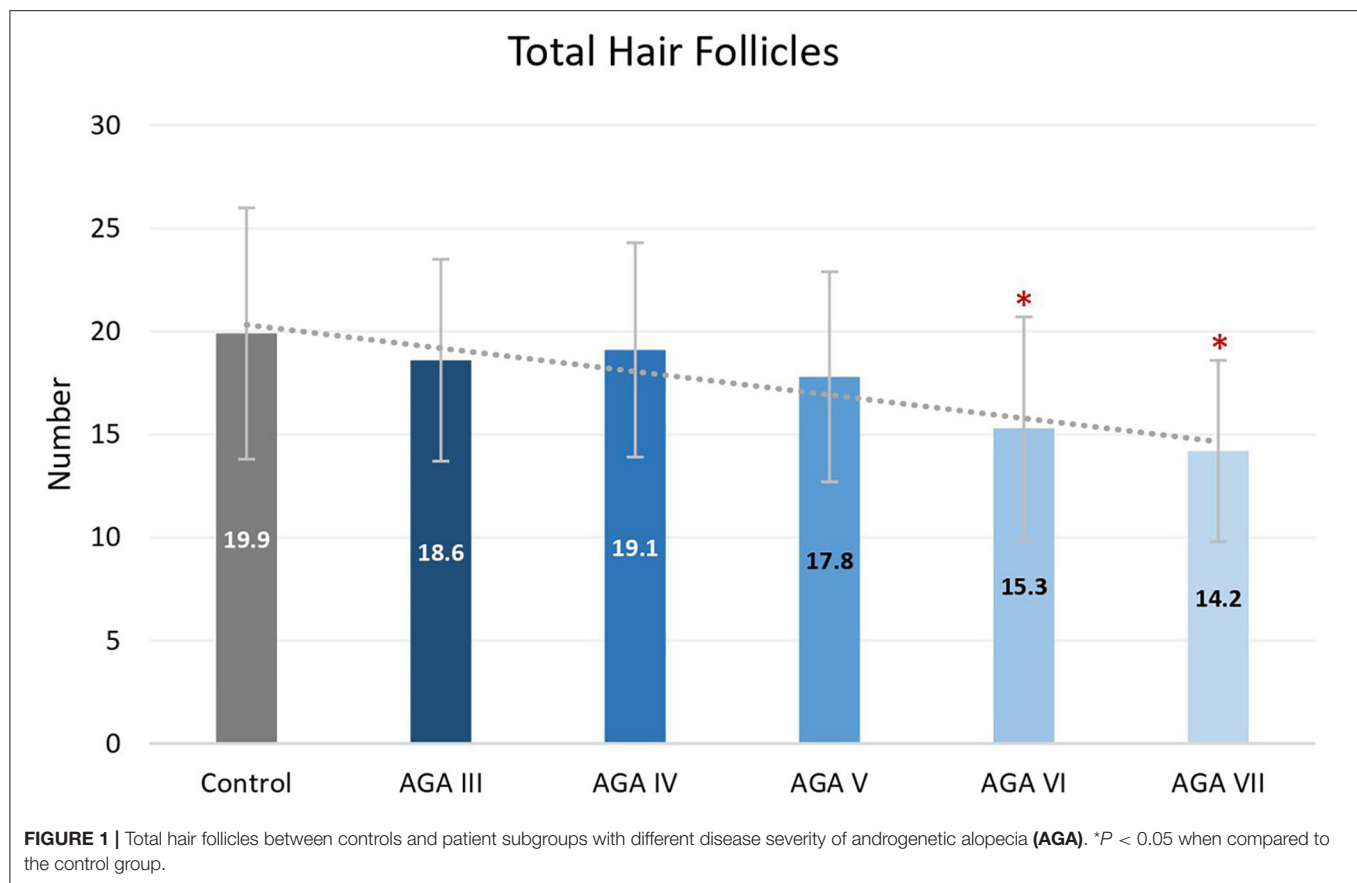
follicular units and T:V ratios ($P = 0.002$, 0.002, 0.035 and 0.001, respectively).

Subgroup analyses showed no significant differences in age between groups; nevertheless, pairwise comparisons displayed significant decreases in the average numbers of total and terminal hair follicles, as well as T:V ratios, in patients with AGA grades VI and VII, when compared to the control group (all $P < 0.05$) (**Figures 1–3**). There were no significant associations between increasing age and changes in any of the hair parameters; however, a significant negative association between total follicle numbers and disease duration was found (r = $-0.23$, $P = 0.02$).

## DISCUSSION

We assessed the histopathological changes in the occipital scalp of men with AGA compared with the normal occipital scalp of unaffected men and found a significant reduction in total hair follicles, terminal hair follicles and T:V ratio, along with a significant increase of follicular stelae in the occipital scalp of male subjects with AGA. Furthermore, AGA patients had a somewhat wider range of vellus follicle numbers than unaffected men, even though a significant difference in statistics was not achieved among median values. These results parallel the findings from previous histopathological studies analyzing biopsy specimens from the frontal or vertex area of men with

AGA compared to the same scalp sites of normal controls (6, 19, 20).

Based on subgroup comparisons, the more advanced the AGA, the more pronounced the changes in hair parameters, but statistically significant differences from controls were established only in subgroups with advanced disease (Hamilton-Norwood VI-VII). These findings are consonant with two previous comparative studies using phototrichogram analysis on the occipital scalp between male AGA and normal controls. The study showing no significant differences solely recruited subjects with mild to moderate severity (Hamilton-Norwood I–V) (14), whereas the other study revealed a significant change of hair density and diameter in patients with the U type, the most severe type of the Basic and Specific classification, compatible with Hamilton-Norwood VI–VII (15).

Androgenetic changes in the occipital region appear dissimilar to changes in the frontal and vertex regions. An approximate 25% reduction of total follicle numbers in patients with Hamilton-Norwood VI–VII was noticed while the average T:V ratios in these patients were 6.8:1 and 5.3:1, reduced from a normal ratio of 8.9:1 in controls. A noticeable depletion of total hair follicles can be observed in conjunction with an unsubstantial degree of hair miniaturization. In contrast to changes in the frontal and vertex regions, an actual reduction of total follicle numbers is particularly recognized

**FIGURE 2 |** Terminal hair follicles between controls and patient subgroups with different disease severity of androgenetic alopecia **(AGA)**. *$P < 0.05$ when compared to the control group.
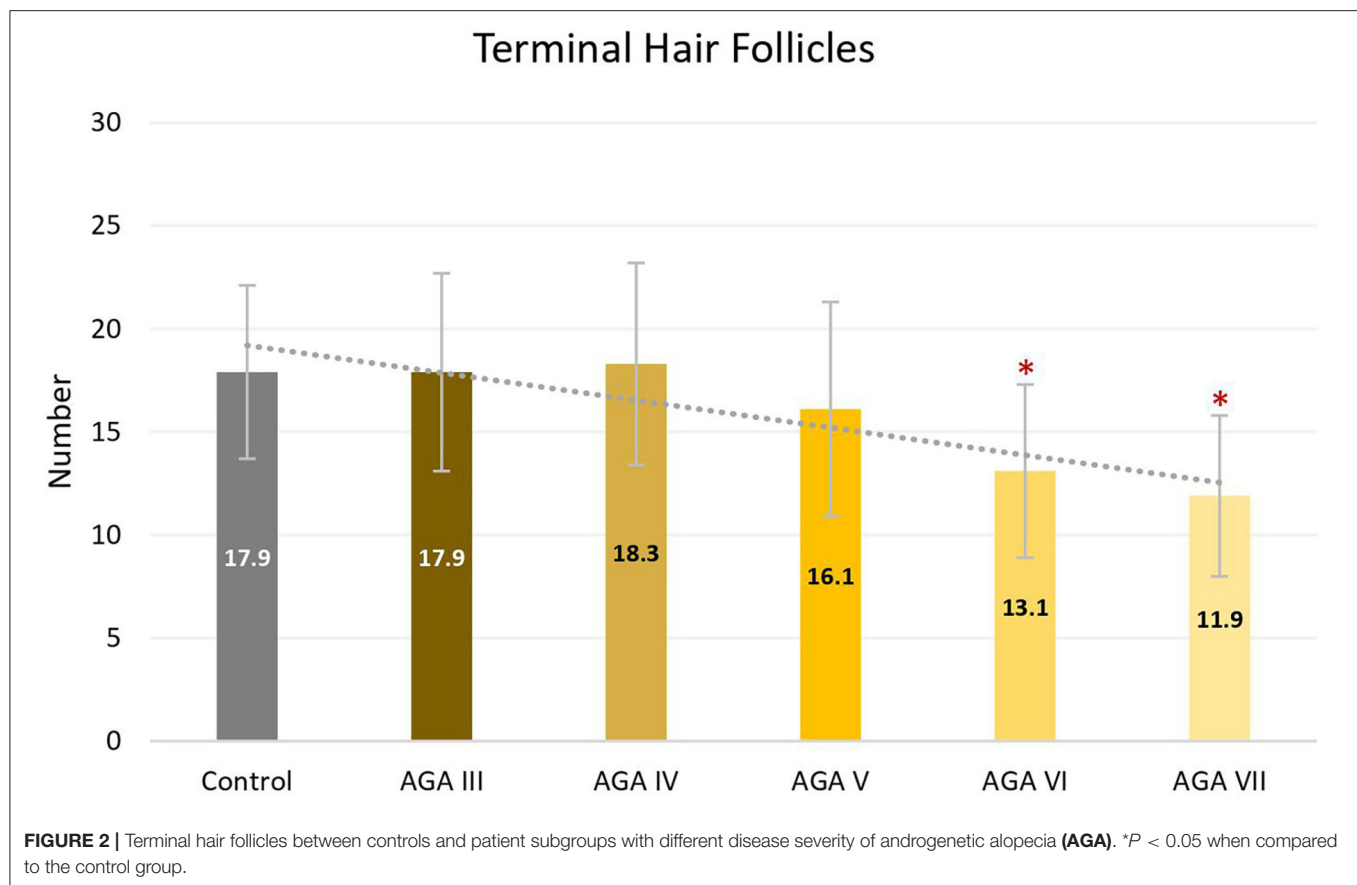
at advanced stages of disease, when a reversal of terminal to vellus hair follicles seems to be obvious (6, 19). Hence, these feature deviations may imply unique characteristics of the occipital scalp in the process of AGA. We hypothesize that occipital hair follicles comprise two populations: one small component influenced by AGA and the unaffected majority. Therefore, clinical changes of AGA in the occipital scalp can be detected only in very advanced disease when the small population experiences pronounced androgenetic changes, including follicular dropout. Meanwhile, the impact on the T:V ratio remains minimal as a large number of normal hair follicles from the unaffected population are included in the ratio calculation.

Distinct anatomical structures and properties related to androgens are suspected to be mainly responsible for site-specific alterations in the occipital scalp of individuals with AGA (21, 22). Accumulating evidence suggests that hair follicles in the occipital area might elicit less androgen-regulated cellular responses than hair follicles in the other scalp areas (22, 23). Frontal hair follicles contained a significantly higher level of androgen receptors and 5α-reductase enzymes, known for converting testosterone into a more potent androgen called dihydrotestosterone, compared to occipital hair follicles for both genders (24–27). Scalp biopsies also demonstrated a greater amount of aromatase, the enzyme accountable for lowering androgen levels by turning into estrogens, within the occipital

rather than the frontal hair follicles in both men and women (21, 24, 28). Moreover, distinctive characteristics of the occipital scalp are perhaps due to its unique embryological origin. In regard to structural development of the head in vertebrates, a large portion is of neural crest origin while some posterior parts of the head, including the occipital region, are derived from the mesoderm (29).

As no correlation was established in this study between increasing age and total follicle numbers, the follicular dropout that occurred among the men with AGA seems not to result from senescent alopecia. These male patients also displayed an increase of miniaturized hairs and telogen counts, which are not features of age-related alopecia (30–34). On the other hand, a negative correlation between total follicle numbers and disease duration was indicated, showing a coherence with histopathological features of AGA in the original sites where follicular dropout can be observed in long-standing disease (7, 35).

The limitation of this study includes the limited ethnic population. Physical properties of hair, such as hair shapes and density, differ between ethnicities. Our findings might not be generalized to other racial and ethnic groups. Further well-designed prospective studies with diverse racial subjects are required to confirm the present findings.

**FIGURE 3 |** Terminal to vellus hair ratio between controls and patient subgroups with different disease severity of androgenetic alopecia **(AGA)**. *$P < 0.05$ when compared to the control group.
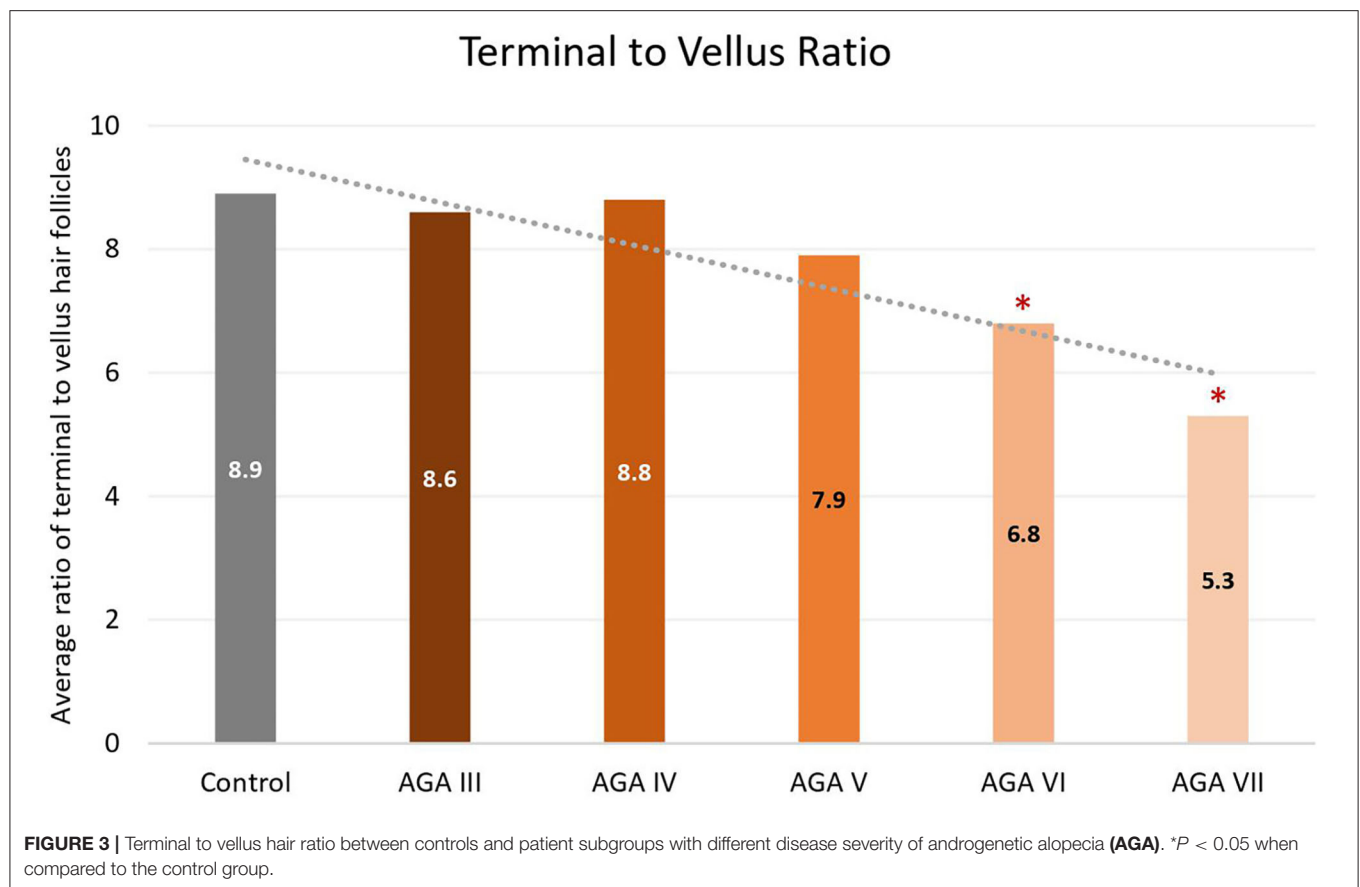
## CONCLUSION

Three main issues and recommendations are derived from this study. First, AGA extendedly involves the occipital area in male patients with advanced disease. Second, androgenetic changes can occur even in the clinically normal occipital scalp; therefore, the occiput of individuals with AGA, especially in advanced stages, should not be used to determine reference data for normal scalp hair. Third, preoperative measurements of miniaturized hairs present in the donor site are strongly recommended in all persons undergoing hair transplantation due to their impact on the success of surgery.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Mahidol University Institutional Review Board for Ethics in Human Research. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

PS and KC: conceptualization and writing—review and editing. PS and SR: methodology and validation. PS, KC, and SK: formal analysis. PS and SK: investigation and data curation. SK and KC: writing—original draft preparation. All authors have read and agreed to the published version of the manuscript.

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# Gastrointestinal Manifestations of COVID-19 Infection: Clinicopathologic Findings in Intestinal Resections Performed at Single Institution

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It is now known that COVID-19 not only involves the lungs, but other organs as well including the gastrointestinal tract. Although clinic-pathological features are well-described in lungs, the histopathologic features of gastrointestinal involvement in resection specimens are not well characterized. Herein, we describe in detail the clinicopathologic features of intestinal resection specimens in four patients with COVID-19 infection. COVID-19 viral particles by *in situ* hybridization and immunofluorescence studies are also demonstrated. All four patients were males, aged 28–46 years, with comorbidities. They initially presented with a severe form of pulmonary COVID-19 and showed gastrointestinal symptoms, requiring surgical intervention. Histopathologic examination of resected GI specimens, mostly right colectomies, revealed a spectrum of disease, from superficial mucosal ischemic colitis to frank transmural ischemic colitis and associated changes consistent with pneumatosis cystoides intestinalis. Three patients were African American (75%), and one was Caucasian (25%); three patients died due to complications of their COVID-19 infection (75%), while one ultimately recovered from their GI complications (25%), but experienced prolonged sequela of COVID-19 infection including erectile dysfunction. In conclusion, COVID-19 infection, directly or indirectly, can cause ischemic gastrointestinal complications, with predilection for the right colon.

Keywords: COVID-19, gastrointestinal manifestations, pneumatosis cystoides intestinalis, ischemic colitis, ISH

# INTRODUCTION

In December 2019, a new human coronavirus (SARS-CoV-2) type emerged in Wuhan, China. COVID-19, the infectious disease caused by SARS-CoV-2, is now a pandemic affecting countries throughout the world. Since spring 2020, the United States has seen a dramatic rise in number of cases, and currently the number of deaths attributable to COVID-19 infection is more than 500,000 (1).

Although the most common presentation of the infection is the development of respiratory symptoms 2–14 days following exposure, gastrointestinal (GI) presentation is becoming increasingly recognized. The receptor of SARS-CoV-2, angiotensin converting enzyme 2 (ACE2) is highly expressed both in GI epithelial cells and in liver (2). Almost the entire GI tract, including the stomach, small intestine, and rectal epithelial cells express the SARS-CoV-2 nucleocapsid protein and the ACE2 protein (3). The SARS-CoV-2 nucleocapsid protein, which encapsulates the viral genome, is essential for SARS-CoV-2 replication (4). High levels of these two proteins in cells of the GI tracts of SARS-CoV-2 infected patients can explain the concomitant digestive symptoms, including diarrhea, nausea, vomiting, and abdominal pain (3).

**TABLE 1 |** Patients' demographic, laboratory values and pathologic findings.

| Case # | Demographics | | | | Laboratory values | | | | | | Pathologic findings | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age | Sex | Race | Comorbidities | CRP (mg/L) | D-dimer (ng/ml) | LDH (Units/L) | Lactic acid (mMol/L) | Troponin I (ng/L) | Hgb/Hct | Gross findings | Histology |
| Case 1 | 47 | M | AA | HTN, Obesity (BMI 33.08 kg/m²) | 176.3 | 4,064 | NA | 4.0 | 39 | 9.9/26 | Large patches of erythematous colonic mucosa with focal loss of mucosal folds. | Ischemic colitis showing transmural acute and chronic inflammation and serositis. Features suggestive of pneumatosis. |
| Case 2 | 37 | M | AA | HTN, Obesity, Insulin Dependent DM | 282.6 | 978 | 556 | NA | 44 | 11/33 | Cecum showed several areas of ulceration ranging in size from 0.6 to 1.0 cm | Focal mucosal ulceration, with associated acute and chronic inflammation. Features suggestive of pneumatosis. Focal serositis. |
| Case 3 | 40 | M | C | Obesity (BMI 33.2 kg/m²) | NA | >20,000 | NA | 7.1 | 1,838 | 15.6/46 | Cecum: Necrotic with dark-brown discoloration. Ascending colon is hemorrhagic and dusky with obvious air bubbles | Mucosal hemorrhagic ischemia, fibrin microthrombi, and pneumatosis cystoides intestinalis |
| Case 4 | 28 | M | AA | DM | 24.87 | 1,380 | 681 | 10.6 | 98 | 7.3/22 | Small intestine: Granular/gritty brown mucosa with diffuse gray-brown exudate | Transmural ischemia with pseudomembrane formation and acute serositis. Features suggestive of pneumatosis. |

Here, we present a series of four SARS-CoV-2 patients who were admitted to the University of Alabama at Birmingham (UAB) Medical Center during June to August 2020 and who underwent GI resections. The course of their SARS-CoV-2 infections was remarkable for the development of GI complications, which necessitated surgical management by right hemicolectomy or segmental small bowel resection; three of the four patients ultimately died as a result of the complications of their COVID-19 infection. The clinical, macroscopic and histopathologic findings are described, which adds to the pathophysiology of SARS-CoV-2 infection and contributes to the ongoing management of the COVID-19 disease.

## MATERIALS AND METHODS

The Institutional Review Board of UAB approved the study. A retrospective review of surgical pathology archives for COVID-19 related intestinal resections was performed during the period of June to August 2020. There were four patients with bowel resections, three of whom underwent right hemicolectomies and one a segmental small bowel resection. The hematoxylin and eosin (H&E)-stained slides were reviewed by GI pathologists (SA, IE, DD, and CRP).

In this study, for detection of SARS-CoV2 virus, both immunofluorescence and *in situ* hybridization (ISH) assays were performed as follows: 5-$\mu$m formalin-fixed paraffin-embedded (FFPE) tissues sections on Plus Slides (VWR, Radnor, PA) were cut at 5 $\mu$m and baked for 2 h at 60°C. Tissues were then deparaffinized and rehydrated with sequential 5-min incubations in xylene (three times), twice in 100% ethanol, and twice in 95% ethanol. Slides were washed three times, for 5 min each, with distilled water. Antigen retrieval was then performed in Tris-EDTA pH-9 buffer at 70°C for 20 min in a steamer, followed by three 5-min washes in distilled water. Tissues were balanced with PBS for 10 min and blocked with 3% w/v bovine serum albumen (BSA) in PBS for 40 min at room temperature. Slides were then blotted dry, and antibody against SARS-CoV-2 nucleocapsid protein (clone GTX135361, GeneTex, Irvine, CA) was applied at a 1:500 dilution in PBS+3% w/v BSA for 1 h at room temperature. Tissues were then rinsed with PBS three times for 5 min under gentle agitation. 4′,6-Diamidino-2-phenylindole (100 ng/mL in PBS) staining was performed for 5 min in the dark at room temperature, followed by three 5-min washes in PBS under gentle agitation. Slides were then mounted with ProLong Gold antifade mounting media (Thermofisher) and stored in the dark until image acquisition. Confocal immunofluorescence images were acquired with a Nikon A1R confocal microscope.

ISH assay was performed using RNAscope® kit and ISH probes according to the manufacturer's instructions (Advanced Cell Diagnostics, ACD, Newark, CA). RNA probe, in C2 channel
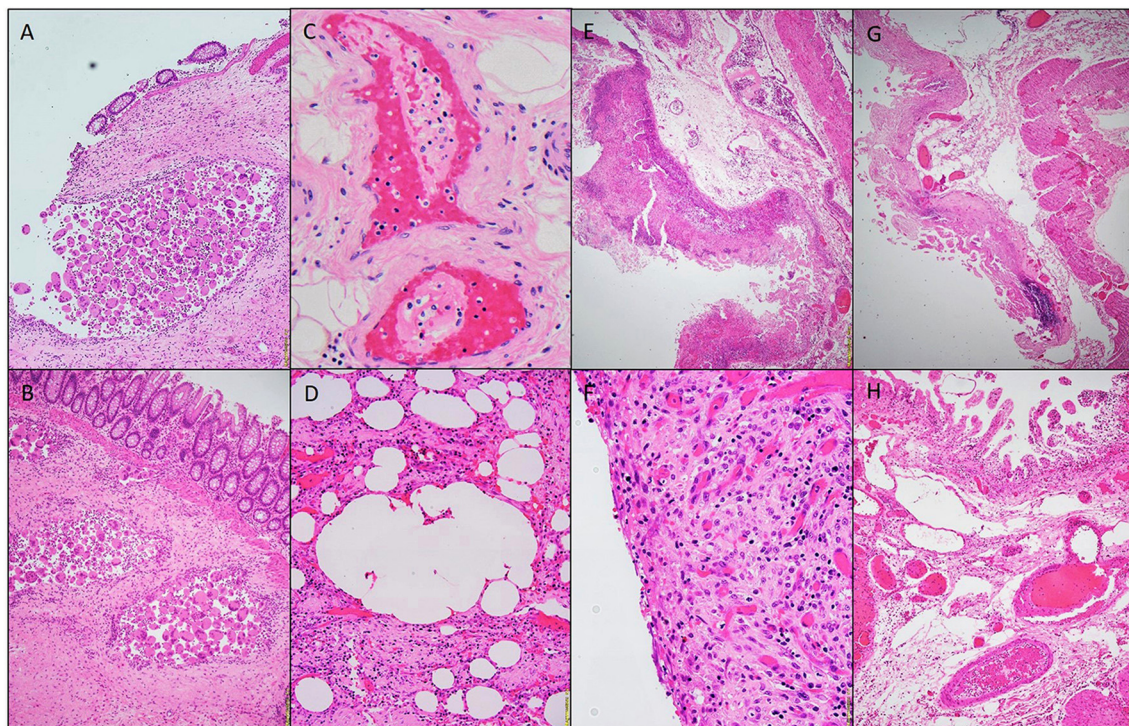


FIGURE 1 | Photomicrographs of a hemicolectomy specimen. Aggregates of multinucleated giant cells lining the cysts. H&E stain 20x **(A,B)**. Fibrin thrombi, H&E staining 40x **(C)**. Micro-cyst lined with multinucleated giant cells, H&E staining 10x **(D)**. Pseudomembrane, H&E 10x **(E)**. Acute serositis, H&E staining 40x **(F)**. Ischemic enteritis, H&E 10x **(G)**. Submucosal thrombi, H&E 10x **(H)**.

targeting SARS-CoV-2 replicative RNA intermediate, was used to detect the replicating virus as red signal (nCoV2019-orf1ab-sense-C2, cat. no. 859151-C2). RNAscope® 2.5 Duplex Reagent Kit (cat. no 322430) along with Human (Hs) Positive Control Probes for housekeeping genes PPIB-C1/ POLR2A-C2, (cat. no 321641) were used to assess the integrity of the RNA. Simultaneously, consecutive sections were probed with probes targeting dihydrodipicolinate reductase B mRNA of a *Bacillus subtilis* strain (DapB) as a negative control, (cat. no. 320751) to assess the specificity of the assay.

## RESULTS

### Clinical Data

Our cohort included four patients aged 28–46 years with a mean age of 38 years. Three of four patients were African-American (AA); one was non-Hispanic Caucasian. All patients had comorbidities that included essential hypertension (HTN), diabetes mellitus (DM, insulin-dependent or insulin-resistant), and obesity (BMI 33.08–34.8 kg/m$^2$). All patients initially presented with respiratory symptoms, including fever, dyspnea, and/or flu-like symptoms which developed into acute respiratory distress syndrome (ARDS) requiring intubation, ventilation,

and critical care management. The length of time between a positive COVID-19 test and significant GI symptomatology and resection varied widely from 1 to 53 days, with an average of 21.5 days. GI symptoms occurred on a spectrum, beginning as constipation, abdominal pain, or a lower GI bleed before progressing to ischemia and necrosis requiring surgical intervention. COVID-19 infections were confirmed by the presence of SARS-CoV-2 RNA, determined by PCR. The patients were enrolled in a clinical trial of remdesivir. Despite the treatment, they subsequently developed complications, such as lower extremity deep vein thrombosis, pulmonary thromboembolism, septicemia with septic shock (due to *Cutibacterium/Propionibacterium avidum*), multi-organ failure, hypotension, pulseless electrical activity and encephalopathy. For all four patients, acute renal failure was a complication, and all underwent continuous renal replacement therapy. Furthermore, the course of their disease was complicated by the development of GI symptoms, such as GI bleeding in the form of hematochezia, as well as an ileus. All patients underwent CT scans of the abdomen and pelvis, which showed changes compatible with GI bleeding with various degrees of intestinal pneumatosis and bowel ischemia. These findings were corroborated by the frank ischemia, ulceration, cecal dilation, and necrosis



**FIGURE 2 |** H&E and ISH images of patient #1 in focal mucosal ulcer with granulation tissue formation along with adjacent uninvolved mucosa. **(A)** H&E images of ulcerated colon tissue at 4X magnification **(B)** Images at 20X magnification after *in situ* hybridization using V-nCOV2019—orf1ab–sense probe (cat # 859151-C2), ACD, Bio-techne, detecting replicating SARS-COV-2 RNA (red chromogen) in ulcerated colon tissue sections. **(C)** H&E images at 10x magnification of adjacent uninvolved mucosa **(D).** Higher magnification images at 40X after *in situ* hybridization using V-nCOV2019—orf1ab-detected as red signal in adjacent uninvolved mucosa. **(E)** Negative control images at 10X after *in situ* hybridization using probe (cat # 320751), ACD, Bio-techne, targeting DapB (Bacillus subtilis stain) which did not detect any staining **(F)** Positive control images at 10X after *in situ* hybridization using probe (cat # 321641), ACD, Bio-techne, demonstrated strong positive staining (red chromogen) in sections probed for the housekeeping gene POLR2A.

**FIGURE 3 |** H&E and ISH images of patient #2 in focal mucosal ulcer with granulation tissue formation along with adjacent uninvolved mucosa. **(A)** H&E images of ulcerated colon tissue at 4X magnification **(B,C)** Images after *in situ* hybridization using V-nCOV2019—orf1ab–sense probe (red chromogen) at lower 4X and higher magnification 40x, respectively. **(D)** H&E images at 10X magnification of adjacent uninvolved mucosa **(E,F)** *in situ* hybridization using V-nCOV2019—orf1ab- sense probe detected as red signal in adjacent intact epithelium of uninvolved mucosa at higher magnification images at 40x. Images show pronounced viral load in the surface epithelial lining and the crypts (Basal cytoplasmic localization).

encountered intraoperatively. Additionally, laboratory findings were remarkable for elevated C-reactive protein (ranging from 24.87 to 282.6 mg/L), d-dimer (ranging from 978 to > 20,000 ng/mL), lactate dehydrogenase (556–681 U/L), lactic acid (4.0–10.6 mMol/L), and troponin I (39–1838 ng/L). Most patients were also anemic (Hgb between 7.3 and 11 g/dL; Hct 22–33%). The three patients who expired due to the infection demonstrated the most dramatic clinical courses, with pronounced laboratory, gross, and microscopic abnormalities (**Table 1**). For all four patients with SARS-CoV-2 infection confirmed by nasal swab PCR testing, there was colonic pneumatosis with or without ischemic changes for whom CT scans of the abdomen showed evidence of gas in the mesentery, or features suggestive of lower GI bleeding.

## Gross and Microscopic Characteristics
Three patients had right hemicolectomies and one had a segmental small bowel resection. All four specimens showed variable degrees of ischemic changes. Gross examination of one of sample showed transmural ischemic necrosis with hemorrhage, resulting in perforation. The mucosa showed, microscopically, deep ulceration and focal pseudomembrane formation. In an additional patient, there was diffuse transmural acute and chronic inflammation with necrosis and hemorrhage, and associated acute serositis. The lamina propria demonstrated congestion and edema (in all patients); rare vascular fibrin thrombi were identified (in 2 patients). Pneumatosis cystoides intestinalis (cyst formation with associated multinucleated giant cells lining) were also recognized in two patients (**Figures 1**–**5** and **Table 1**).

ISH with a V-nCOV2019-sense probe detected replicating SARS-COV-2 RNA. Furthermore, all four samples were positive on immunofluorescence for antibody against SARS-CoV-2 nucleocapsid protein with a distinctive pattern and a variable viral load (**Figures 2**–**4**). All patients underwent exploratory laparotomies for GI complications of COVID-19 disease, and three of the four patients underwent a right hemicolectomy procedure. The morphologic findings in all specimens included ischemic changes, acute and chronic inflammation, and fibrin microthrombi in small blood vessels in underlying areas of mucosal ulceration. Moreover, all cases, regardless of extent of the disease, showed changes consistent with pneumatosis cystoides intestinalis, including prominent submucosal edema with occasional cyst formation and aggregates of multinucleated giant cells.
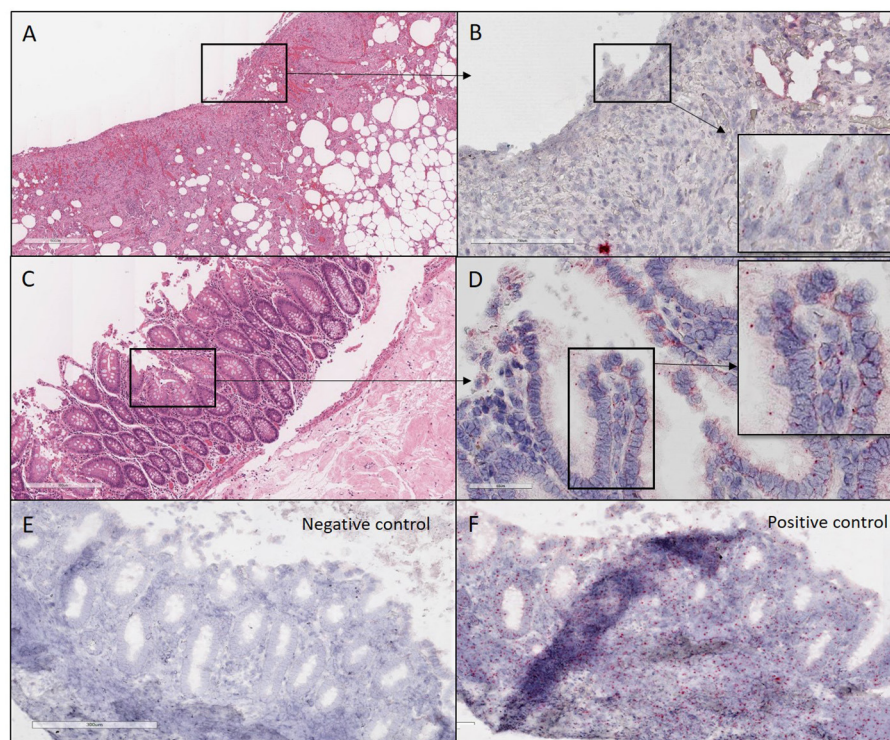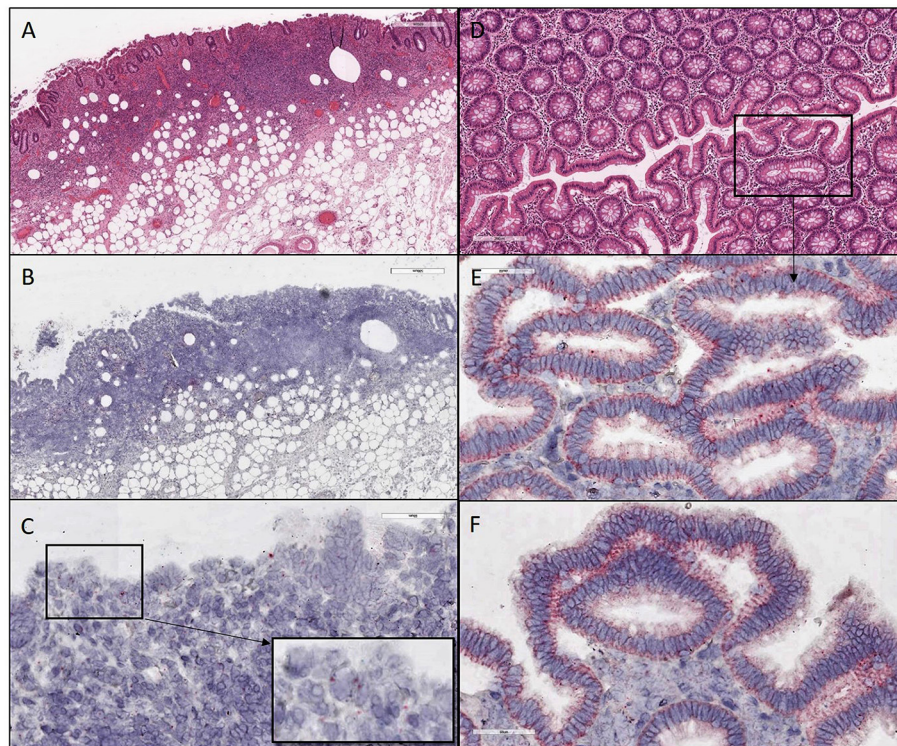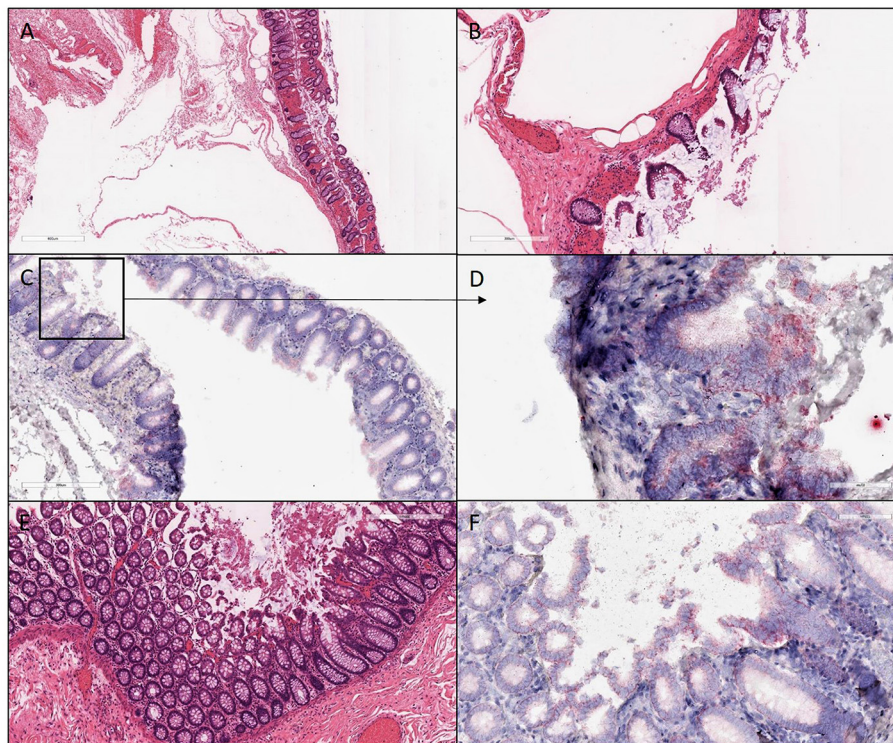
**FIGURE 4 |** H&E and ISH images of patient #3 in focal mucosal ulcer with granulation tissue formation along with adjacent uninvolved mucosa. **(A,B)** H&E images of ischemic colon tissue sections at 4X and 10X magnification, respectively. **(C,D)** Images at lower (10X) and higher magnification (40X), respectively, after *in situ* hybridization using V-nCOV2019—orf1ab–sense probe (red chromogen) More viral load of replicating SARS-COV-2 RNA was detected in this ischemic section as compared to its uninvolved mucosa represented in **(F)**. **(E)** H&E images at 10X of adjacent uninvolved mucosa **(F)** *in situ* hybridization using V-nCOV2019—orf1ab-sense probe detected as red signal in adjacent intact epithelium of uninvolved mucosa at 20X magnification.

## DISCUSSION

GI symptoms associated with COVID-19 are present in up to 30% of patients, with diarrhea, abdominal pain, and hematochezia occasionally evident as the initial presentation (5). Although patients with significant pulmonary disease have detectable SARS-CoV-2 RNA in fecal samples, a substantial number of patients with GI manifestations also have SARS-CoV-2 RNA in fecal samples (6). Others have now individualistically established that the virus can be cultured from the feces during an active infection (7). In the present study, we demonstrated by immunofluorescence (**Figure 6**) and ISH studies (**Figures 2–5**) evidence of the virus in affected area of the intestine.

Notably, neither of the patients had a medical history of established GI disease, nor etiology for colitis. Extensive infectious work up including *Clostridium difficile* serology was conducted on two of the four patients, which was negative. Reactivity for CMV IgG was noted in the serum of one patient indicating a previous infection; however, no evidence of viral cytopathic effects were noted on histology. One patient had clinical and microbiological evidence of septicemia due to *Cutibacterium (Propionibacterium) avidum*, the commensal skin microorganism, which is unlikely to be implicated as a primary agent in the etiology of hemorrhagic colitis. One

patient developed HIT following anticoagulation, resulting in septic shock. While this further exacerbated the bowel ischemia and thrombosis, it was a contributing factor rather than a primary cause. Moreover, extensive chart review failed to reveal definitive evidence of mesenteric thrombosis, radiologically or intraoperatively, to explain the segmental ischemic findings.

The target viral receptor for SARS-CoV-2 is ACE2, which is highly expressed on type II alveolar epithelial cells, as well as in glandular cells of gastric, duodenal, and rectal epithelia (8). This suggests that the SARS-CoV-2 gains entry into, and potentially damages, host GI tissue, explaining the digestive symptoms. Microthrombi also contribute to GI insults (9).

Although several studies have attempted to identify histologic findings indicative of SARS-CoV-2 infection, the presentation and expression of SARS-CoV-2 RNA remains variable (10). In our cohort, not all GI specimens demonstrated findings of coagulopathy and ischemia, but all samples revealed changes consistent with pneumatosis cystoides intestinalis. While pneumatosis cystoides is commonly associated with bacterial infection, its pathogenesis is poorly understood and multifocal, and can also be attributed to mechanical and pulmonary causes (11). In our cohort, those patients that underwent infectious work-up for gas producing bacteria were negative. Moreover, these patients were acutely ill, requiring repeated surgical
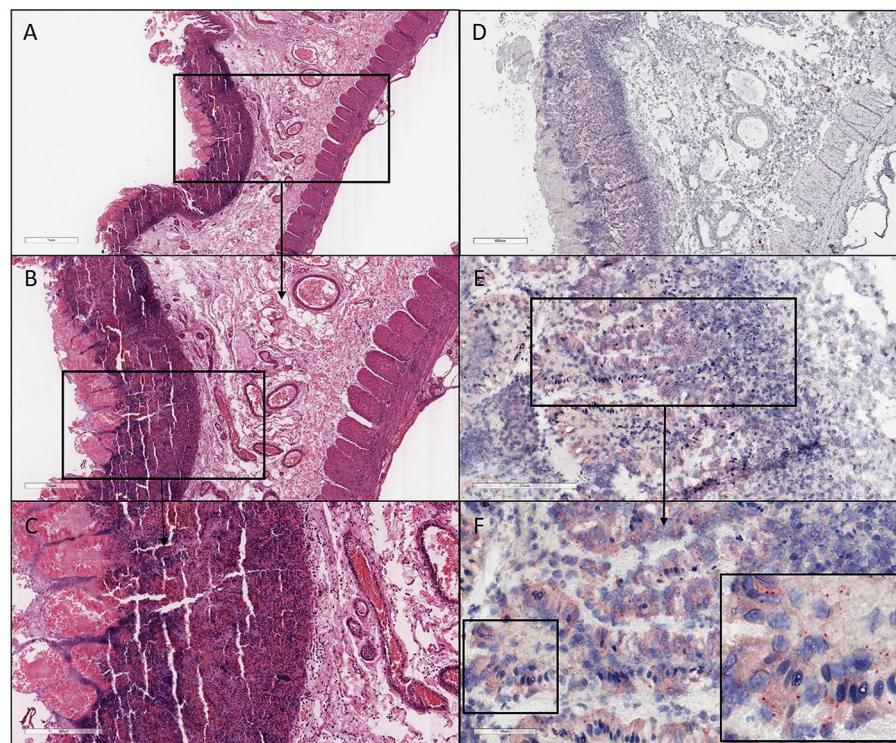
FIGURE 5 | H&E and ISH images of patient #4 in focal mucosal ulcer with granulation tissue formation. **(A–C)** H&E images of ischemic colon tissue sections at 2X, 4X, and 10X magnification, respectively. **(D–F)** Images at lower (4X) and higher magnification (20X and 40X), respectively, after *in situ* hybridization using V-nCOV2019—orf1ab–sense probe (red chromogen) illustrating replicating SARS-COV-2 RNA was detected.

intervention, intubation and ventilation. This suggested that the etiology of pneumatosis could be the result of iatrogenic trauma or manipulation increasing intraluminal pressure (11), or the result of free air entering the perivascular spaces of the intestinal wall following alveolar rupture in the setting of pulmonary obstruction (12); the clinical significance failed to be elucidated. The pathogenic pathway of COVID-19 remains largely undetermined, and its relationship with the gastrointestinal findings remains a challenge. It is widely accepted that the viral infection results in microthrombi which can lead to ischemic injury; however, in the setting of critical illness, HIT, and intragenic interventions (ECMO) it is difficult to quantify the exact role SARS-CoV-2 virus plays. The clinicopathologic findings in our cohort caused us to consider the relationship between the ischemic enterocolitis and hypercoaguable state of our patients with COVID-19 infection; however, comparison with non-COVID associated acute ischemic bowels should be performed to further characterize and ultimately define the histopathological features of the virus.

Moreover, each GI specimen demonstrated SARS-CoV-2 positivity via ISH, regardless of the extent of disease. Coronaviruses are also found by electron microscopy in intestinal cells of animals (13). Comparable to our study, SARS-CoV-2 RNA ISH is reported to be positive in a few mucosal epithelial cells and lymphocytes of the GI tract in humans (7). Also what supports our findings is that SARS-Cov-2

RNA in stool, determined by amplification is now widely accepted (7).

In a recent study that included a large number of SARS-CoV-2 patients (n=95), 58 patients experienced GI symptoms including diarrhea, anorexia and nausea (14). Fecal samples of 65 hospitalized patients were tested for SARS-CoV-2, including 42 patients with and 23 without GI symptoms, of which 22 (52.4%) and 9(39.1%) were positive, respectively. Six of the patients who had GI symptoms underwent endoscopic examination; one showed erosion/ulcer. In two, SARS-CoV-2 RNA was detected in the esophagus, stomach, duodenum, and rectum (14).

In the present study, there were pathologic findings for the four patients with confirmed SARS-CoV-2, for whom CT scans of the abdomen showed either evidence of colonic pneumatosis with or without ischemic changes and evidence of gas in the mesentery, or features suggestive of lower GI bleeding. All four patients underwent exploratory laparotomies and three of the four were subjected to a right hemicolectomy procedure for COVID-19 GI related complications. The presence of microthrombi in the watershed area suggested that SARS-CoV-2 demonstrates partiality toward the right colon. Moreover, all cases, regardless of extent of the disease, showed pathologic changes consistent with pneumatosis cystoides intestinalis.

For some patients, GI symptoms may be associated with pulmonary SARS-CoV-2 infection, and surgical management of
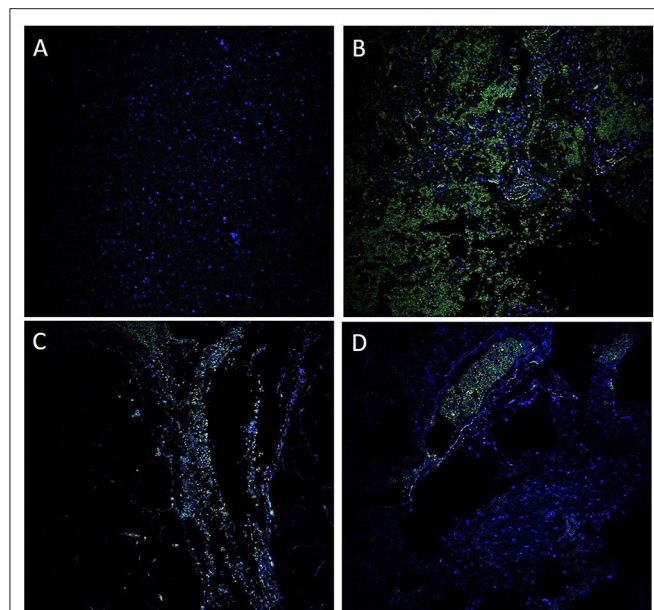
**FIGURE 6 |** Immunofluorescence for antibody against SARS-CoV-2 nucleocapsid protein. A negative control using a probe targeting DapB (Bacillus subtilis strain) did not detect any staining **(A)**; a positive control demonstrated strong positive staining in sections probed with the housekeeping gene **(B)**. These findings suggest that the GI tract is infected with SARS-CoV-2 **(C)**. A unique observation in our case study is the presence of the virus within the blood vessels **(D)**.

GI complications, including bleeding, is sometimes necessary. The morphologic findings observed in the resection specimens varied from superficial acute colitis with or without hemorrhage and ischemic changes, to a frank transmural necrotic colitis with fibrin microthrombi. Changes consistent with pneumatosis cystoides intestinalis were also evident. In conclusion, COVID-19 disease, directly or indirectly, can cause ischemic GI complications, with a predilection for the right colon. Awareness of these morphologic changes may prompt pathologists to include potential SARS-CoV-2 infection to the differential diagnosis when the etiology for ischemic colitis is unclear.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

SA principal investigator and corresponding author, conceptualized the study with SS and AB, finalized the manuscript with the assistance of AB. CM, PB, and UM performed the *in-situ* Hybridization and reviewed the results and critically reviewed the manuscript. SP reviewed and revised the manuscript. CP, II, DD, GL, and PVB, contributed in data collection. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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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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# From Macroscopy to Ultrastructure: An Integrative Approach to Pulmonary Pathology

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Pathology and radiology are complimentary tools, and their joint application is often crucial in obtaining an accurate diagnosis in non-neoplastic pulmonary diseases. However, both come with significant limitations of their own: Computed Tomography (CT) can only visualize larger structures due to its inherent–relatively–poor resolution, while (histo) pathology is often limited due to small sample size and sampling error and only allows for a 2D investigation. An innovative approach of inflating whole lung specimens and subjecting these subsequently to CT and whole lung microCT allows for an accurate matching of CT-imaging and histopathology data of exactly the same areas. Systematic application of this approach allows for a more targeted assessment of localized disease extent and more specifically can be used to investigate early mechanisms of lung diseases on a morphological and molecular level. Therefore, this technique is suitable to selectively investigate changes in the large and small airways, as well as the pulmonary arteries, veins and capillaries in relation to the disease extent in the same lung specimen. In this perspective we provide an overview of the different strategies that are currently being used, as well as how this growing field could further evolve.

**Keywords:** microCT, lung, histology, lung disease, imaging

# INTRODUCTION

Computed tomography (CT), positron emission tomography (PET) and histopathology are major components in the differential diagnosis and follow-up of patients with acute and chronic non-neoplastic respiratory diseases. Given the diversity in CT and histologic presentation and the inherent morphologic differences in strategies of diagnosis between neoplastic and non-neoplastic disease, we will specifically focus on non-neoplastic diseases. Especially as these tend to result in significant day-to-day challenges in the differential diagnosis.

Particularly in the field of interstitial lung diseases (ILD), there is an important diagnostic and prognostic role for CT and histopathology, with a growing emphasis on the relative weight of chest imaging as a diagnostic tool (1). Thus, the need for an invasive video-assisted or robot- assisted thoracic surgery biopsy to confirm differential diagnosis has drastically reduced. Consequently, lung biopsies are currently only recommended for those challenging cases where major discrepancies are observed between CT and clinical findings and a clear diagnosis cannot be rendered. Given this important role of imaging in the management of ILD, it is primordial to integrate the existing clinical, imaging and histopathologic data as much as possible.

Initially, assessment of remodeling and fibrosis patterns by experienced radiologists was the only way to leverage the available imaging data, but gradually and partly due to the large inter-observer variability, automated image analysis tools for characterization and quantification of CT signs and patterns have been developed of which some are on the verge of making their appearance in routine clinical care. CT can provide 3D insight into the (gross) morphological changes in the lung. However, CT patterns are often nonspecific and may change during the evolution of a disease. Importantly, CT changes may also have more than one (histo-) pathological correlate. For example, a study demonstrated that more than 90% of patients presenting with CT findings that were inconsistent with usual interstitial pneumonia demonstrated histological evidence of usual interstitial pneumonia (2). Additionally, *in vivo* resolution is rather limited with a slice thickness of 1 mm most commonly being used for routine patient CT scans due to radiation concerns. This is sufficient to investigate airways and vessels >1 mm in diameter and gross changes in the secondary pulmonary lobule and the lung parenchyma. However, the small airways, the cellular composition and specific smaller sized structures are impossible to address, although morphologic secondary effects of primary changes such as hyperinflation and air trapping may be observed.

Histopathology on the other hand provides the advantage of a detailed analysis of the morphologic and cellular changes within the lung, whereby structures in the micrometer range can be readily resolved. However, (histo-) pathology is limited due to relatively small tissue samples and only allows for a 2D investigation. This can lead to discordance between initial diagnosis made on transbronchial cryobiopsy or surgical lung biopsy and the final diagnosis made in explant lung specimens at the time of transplantation (15–22 and 12.4% respectively) (3, 4). In addition, the lung is considered quite

fragile, making it vulnerable for deformation and distortion during the preparation resulting in histologic artifacts potentially hampering microscopic examination.

As, especially non-neoplastic pulmonary diseases, show a great spatial heterogeneity in disease activity and progression, targeted sampling is essential. It is reasonable to assume that often important patterns/changes in the lung can be overlooked due to sampling bias or sectioning artifacts upon histologic examination.

Proof of concept data of the huge potential of microCT, a tool designed to combine the 3D aspect of CT with almost full histological resolution, to investigate human lung (disease) was provided in 2005. However, at the time, the authors were still struggling to obtain sufficient contrast between air and tissue (5). A subsequent porcine study by Litzlbauer et al. (6) extensively validated the use of microCT with histology, showing great promise in measuring morphological changes such as alveolar surface density and mean linear intercept. Therefore, we and others have further optimized these protocols for *ex-vivo* scanning of human lung tissue to bridge the gap between radiology and histopathology, and thus between macroscopy and near-cellular resolution. In this article, we describe a unique approach of air-inflating whole human lung/lobe explants and subjecting these to a wide spectrum of investigations. We propose to use this macro to micro approach leveraging CT and microCT to bridge the gap between radiologic data and histopathology with illustrative examples. Further, we propose to use this approach to selectively investigate changes in the large and small airways, the interstitial compartment as well as the pulmonary arteries, veins and capillaries in relation to the disease extent in the same lung specimen. In addition, other promising tools are discussed in more detail including their potential applications. We acknowledge that a plethora of techniques has also been investigated in animal studies, which can readily be leveraged to combine imaging data with mechanistical insights (7); however, we deliberately focus on studies in humans that can be implemented more easily into routine clinical practice.

# METHODOLOGY OF LUNG PROCESSING

Obtaining sufficient contrast between tissue and air is of major importance in the imaging of lung explants. Therefore, immediately following surgical resection, the lung is collected and cannulated via the main stem bronchus. Applying water-controlled air pressure, inflating the lung with 30 cm of water pressure and subsequently lowering the pressure to 10 cm of water pressure allows for an even and uniform recruitment of the lung specimen. The lungs are then mounted in a styrofoam box and frozen in liquid nitrogen vapors. At this moment, the lungs can be safely stored at −80°C until further use. *Ex vivo* CT scanning can be used to correlate with the last available *in vivo* CT, which already allows a higher spatial resolution due to higher radiation doses that can be used. MicroCT scanning of entire lung specimens is more complicated and a dedicated device is needed, where large samples can be mounted while keeping the lung frozen solid inside a styrofoam box. Depending on the size of

the specimen, a resolution of 90–150 micron is feasible, thereby increasing the resolution 6–8 fold compared to conventional *in vivo* CT imaging.

After CT and microCT imaging, which can be used to identify structures of interest, the lung is cut in even slices using a band saw and smaller sized samples with a diameter typically between 12 and 22 mm can be extracted with a core bore or power drill depending on the degree of fibrosis within the samples. These samples can subsequently be re-scanned with high-resolution microCT preferably while keeping the sample frozen (8), where a further increase in resolution can be achieved given the smaller size of the respective specimen, typically up to 5–15 microns.

Subsequently, the lung can be further processed for histopathologic and molecular assessment, e.g., by formalin fixation and dehydration of samples. Our workflow is further illustrated in **Figure 1**. The quality of standard histology is not severely impaired by the prior process of freezing and scanning the lung. Although minor freezing artifacts can be observed, the quality of the histology is much better compared to frozen sections, explained by the fact that the tissue is never in direct contact with ice-cold solutions such as glutaraldehyde. It is very important to emphasize that this process of freezing the lung *in toto* and using microCT also has limited negative impact on further downstream molecular analysis illustrated by e.g., similar gene expression signatures in scanned vs. non-scanned specimens (8). Also bulk RNA-sequencing (9, 10), immunohistochemistry (11), and microbiome sequencing (Einarsson et al. accepted for publication) have already been performed, making ours a valid tool for more molecular-based research.

## CT AND MICROCT OF EXPLANT LUNGS

Leveraging CT and whole lung microCT, an easier separation of the lung into morphologically inconspicuous, moderate and severely diseased areas can be made, that can be specifically sampled using the targeted sampling protocol described above. The healthy appearing areas as evident from the imaging can be used as a proxy of early disease. This is valuable given the temporal heterogeneity in non-neoplastic lung diseases, since genuine specimens with early disease features for research purposes are difficult to obtain. There is certainly some ground truth in such a hypothesis as distinct morphological and molecular processes have been demonstrated in minimally affected vs. severely affected regions (9, 10).

Next to pattern and structure analysis, it is also possible to visually identify and segment the entire bronchial tree from the main stem bronchus until the last branch of conducting airways (i.e., terminal bronchioles). This approach can therefore be used to investigate airway abnormalities like airway collapse, airway obstruction or bronchiectasis on a whole lung scale. In addition quantitative data such as the numbers of airways per generation, airway diameters and segment lengths can be generated as has been described already in COPD (12), graft-vs.-host-disease post-allogenic stem cell transplantation (13), lymphangioleiomyomatosis (11) and physiologic aging

(14). The use of a contrast agent, for example osmium staining, also allows for the investigation of (micro-)vascular structures, making the estimation of vascular volumes relative to the total tissue volume possible (15). Specifically in rare pulmonary diseases, whole lung microCT can bridge the gap between research and clinical routine as exemplified in **Figure 2**, where a representative *ex-vivo* CT scan (**Figure 2A**), whole lung microCT scan (**Figure 2B**) and the segmented airway tree based on whole lung microCT (**Figure 2C**) are shown from a patient who underwent pneumonectomy for unilateral congenital emphysema (**Figures 2D,E**). The airway segmentation shows an aberrant airway bifurcation pattern with a marked decrease in the number of visible airways. Remarkably, there are large airway segments without notable airway branching; especially airway segments in severely emphysematous areas show a lack of airway bifurcations (**Figure 2C**).

## CORRELATION WITH HISTOPATHOLOGY

Numerous insights can be generated by high-resolution imaging, however the correlation between imaging and histopathology is often difficult, given the inherent differences in spatial resolution. While high-resolution imaging data allows for the investigation of morphological changes, it only provides black and white images and changes at the cellular level or minimal density differences cannot be resolved, making routine pathology still an indispensable tool for both research and routine clinical purposes. Some attempts have, however, been made to correlate imaging with histopathology data, especially with the recent introduction of synchrotron imaging, an extremely powerful source of X-rays, of paraffin embedded samples where sub-stacks of images with (sub)micrometer resolution can be created. Hierarchical phase contrast imaging is another application of synchrotron imaging, where specialized sample preparation and equipment enable the scanning of entire lungs, but also other organs, with a resolution of 25 micron. Furthermore, this technology allows the acquisition of sub-scans with a resolution of 2.5 micron due to the high X-ray photon flux and coherence achieved at modern fourth-generation synchrotron sources (16). This technique has been employed by Ackermann and colleagues to reveal microvascular changes (i.e., broncho-pulmonary shunting) in the bronchial circulation of COVID-19 patients (17). Synchrotron imaging of paraffin embedded samples on the other hand has been used by another group to study morphological changes in the vascularity in pulmonary vascular diseases. Westroo and colleagues leveraged synchrotron imaging to demonstrate a previously unappreciated plexiform lesion heterogeneity, the pathological hallmark of (idiopathic) pulmonary hypertension (18). The same group also used synchrotron-based phase-contrast microCT in combination with vascular dye injection to investigate the vascular morphology in alveolar capillary dysplasia with misaligned pulmonary veins, with vascular dye injection being of significant help in the analysis and further segmentation of the vascular structures (19). A 3D approach for virtual histology and histopathology based on multi-scale phase contrast x-ray tomography has also been

**FIGURE 1 |** Overview of the proposed protocol that can be used to investigate the lung from macroscopy to microscopical ultrastructure. The lung is firstly cannulated via the main stem bronchus and inflated using a compressed air source and subsequently fixed in liquid nitrogen fumes. Following an *ex-vivo* CT and microCT, the lung is sliced in 2 cm slices and samples are systematically extracted using a core bore or power drill. Pre-selected or randomly selected samples can be scanned with microCT to further improve the spatial resolution. Further validation can be performed with histopathology. More downstream possible molecular analysis includes compartment-specific gene expression analysis or single nuclear analysis which can be used to elucidate specific pathways in pathological areas. *blood vessel.

applied to punches of tissue samples with a maximum cross section of 8 mm derived from COVID-19 victims, which allows the segmentation of individual cells with a minimal resolution of 167 nm (20). Lastly, a 36 M-pixel synchrotron radiation microCT has been successfully used to study the secondary pulmonary lobule from a large human lung specimen (21).

However, this approach is not readily available due to the specific nature of the required equipment for both acquisition and analysis, the need for extensive experience with the interpretation of the images and is therefore not (yet) ready for high-throughput screening.

## MICROCT IMAGING AS A SCOUTING TOOL

Histopathologic analysis remains the only reliable option for identifying cellular and morphologic patterns keeping the limitation of small tissue samples and the lack of 3D insight in mind. Serial sectioning of paraffin blocks has been used to extensively demonstrate the correlation between microCT and histology (14), however the process of aligning, matching and cutting the samples is tedious and time-consuming. As an alternative, microCT scanning of paraffin blocks or microCT as a scouting tool have been proposed (22). This can especially

be interesting for retrospective studies, in situations where no whole organ is available or when detailed and fast histopathologic assessment is essential for routine patient care such as surgical lung biopsies in the context of a possible interstitial lung disease or suspicion of neoplasms in a resected lung specimen. Interesting and relevant regions can be selected for further sectioning based on the microCT selection which enables time-efficient preparation of conventional histological sections (22). In that aspect, it is also of interest that a novel X-ray microtomosynthesis of unstained pathology tissue samples has also been proposed. Its unique design maximizes the photon flux density through the sample as no rotation is required and therefore samples can be scanned closer to the source resulting in higher resolution (23).

In addition to its potential as a scouting tool, X-ray imaging can also assist in generating a 3D overview of morphological changes in the lung structure. Jones et al. leveraged microCT of paraffin embedded samples to investigate the 3D morphology of fibroblast foci, a key histologic feature of active fibroproliferation in the context of interstitial lung disease. In contrast to the general belief at that time, the fibroblast foci in the lungs of IPF patients were not interconnected and displayed a wide plasticity (24). Wells et al. also used microCT, histopathology, and immunohistochemistry to investigate necrotic granuloma, a characteristic feature of tuberculosis and showed that necrotic
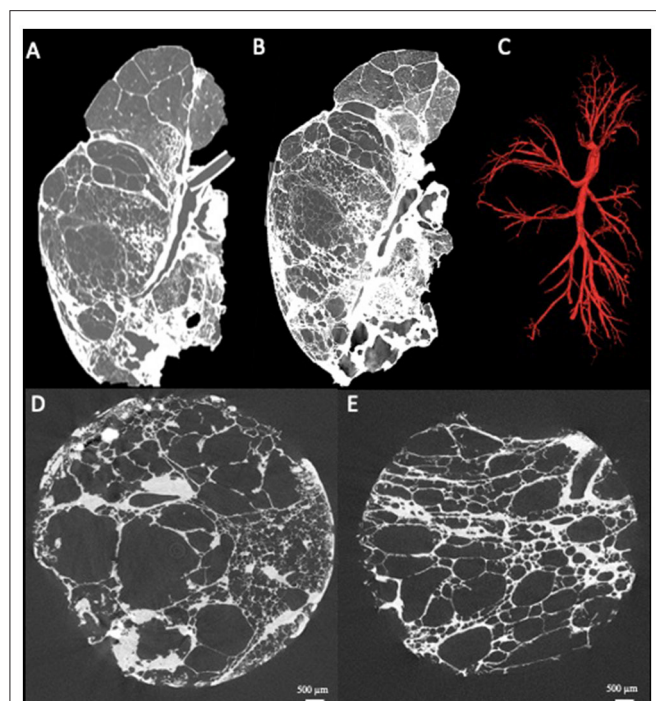
**FIGURE 2 |** Presentation of a case where clinical routine meets research. Patient with unilateral congenital emphysema that underwent pneumonectomy. The *ex-vivo* clinical CT scan shows severe emphysematous destruction **(A)** with some fibrosis in the lower lung lobe. The whole lung microCT shows greatly improved details providing near alveolar resolution **(B)**. Airway segmentation of the whole lung by microCT demonstrating the simplification of the airway tree characterized by a low number of visible airways and remarkable long airway segments without airway branching **(C)**. MicroCT imaging of extracted lung specimens showing the severe emphysematous destruction in this lung **(D,E)**.

granulomas exhibit more complex shapes than anticipated, including cylindrical, branched morphologies that are connected to the airways and shaped by the bronchi (25).

The 3D overview provided by microCT is not only helpful to investigate morphologic changes but can also be used to investigate structural changes in the airways. Mcdonough et al. were the first to employ microCT to quantify the size and number of terminal bronchioles, the last generation of conducting airways before the respiratory bronchioles and alveoli, in tissue samples removed from explanted lungs from COPD patients. Terminal bronchioles can be visually identified on microCT imaging by the loss of the airway wall and appearance of alveolar buds and are of considerable importance in chronic non-neoplastic lung diseases. A significant decrease in the number and size of terminal bronchioles in end-stage COPD lungs was found compared to controls. Given that repeated sampling of the same lung was possible and given that the tissue volume of the sample could be determined, it not only allowed the assessment of the number of terminal bronchioles in the sample, but by carefully measuring the lung volume, these numbers could be extrapolated to the entire lung. This approach provided quantitative data of the actual small airway involvement in COPD

for the first time, something that was always assumed but never conclusively demonstrated in a quantitative way (26). Later on, this approach has also been successfully leveraged to demonstrate small airway involvement in post lung transplantation rejection, cystic fibrosis and idiopathic pulmonary fibrosis (27–30). Over the last years, this approach has been further refined to allow scanning of the samples in frozen condition, making destructive dehydration which was applied at first, unnecessary. In addition, an improvement in the analysis tools has allowed the researchers to extract additional quantitative parameters such as the number of alveolar attachments, the thickness of the airway wall, the circularity of the lumen, but also size and diameter of the preterminal bronchioles (31).

## DISCUSSION

Given the above evidence, it is clear that microCT can serve as a complementary, and in the future perhaps even an indispensable tool for routine pathology (32). Although significant technical advances have been made, there is still some work to be done. Indeed, specific microCT devices have already been optimized for 3D imaging of non-stained soft tissue at a resolution of 5–10 micron. These scans could be implemented in routine care and could assist in histology-guided identification of a range of tissue structures and diagnostically relevant histologic criteria and moreover may allow easy quantification of relevant measures such as for example tissue thickness (33). It is our belief that similar advances could also assist in improving the differential diagnosis, particularly in the field of non-neoplastic diseases. However, these approaches could also be leveraged to better understand neoplastic lung diseases such as micro-metastasis, the role of the tumor micro-environment and the role of the vasculature in neoplastic lung disease. Our approach also allows for structurally targeted molecular investigation with both fresh frozen–or formalin fixed tissue being available after non-destructive imaging, allowing panel-based gene expression analysis or broad next-generation sequencing approaches (34). Additionally, microorganisms such as bacteria from biofilms can be recultivated from native flash frozen tissue and used in *in-vitro* assays.

Applying microCT or synchrotron imaging directly to living patients remains an elusive dream, yet a recent study applied propagation-based phase-contrast CT on a human-scale chest phantom prepped with an inflated fresh porcine lung. The authors demonstrated that a resolution of 100 micron could be obtained in a limited local area of interest with significant less radiation than used in conventional CT scanning (35), indicating the possibilities of *in vivo* high resolution scanning. This indicates that we could dream big and hope to apply similar high-resolution imaging *in vivo*, making it perhaps even possible to avoid invasive biopsies. Caution is however needed as rigorous interpretation of the, often immense, imaging data is needed, which could cause a new wave for artificial intelligence based analysis of microCT scans, similar to the current wave of research in the use of artificial intelligence analysis of conventional CT images (36).

Therefore, the imaging-based approaches to close the gap from macroscopy to ultrastructure seems a safe and trustworthy option, which can be relatively fast implemented in the routine care of patients suffering from acute or chronic lung disease and which can facilitate the differential diagnosis.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Hospital of Antwerp Ethical committee. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SV, PB, CW, MA, JH, TL, JK-V-E, and DJ: responsible for conception and design of the study. EP, MK, AS, HE, TW, AH, FL, SD, PP, VV, and PV: wrote sections of the manuscript. All authors contribute to sample preparation, processing, contributed to manuscript revision, read, and approved the submitted version.

## FUNDING

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# Up-to-Date Pathologic Classification and Molecular Characteristics of Intrahepatic Cholangiocarcinoma

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Intrahepatic cholangiocarcinoma (iCCA) is an aggressive primary liver malignancy with an increasing incidence worldwide. Recently, histopathologic classification of small duct type and large duct type iCCA has been introduced. Both these types of tumors exhibit differences in clinicopathological features, mutational profiles, and prognosis. Small duct type iCCA is composed of non-mucin-producing cuboidal cells, whereas large duct type iCCA is composed of mucin-producing columnar cells, reflecting different cells of origin. Large duct type iCCA shows more invasive growth and poorer prognosis than small duct type iCCA. The background liver of small duct type iCCA often shows chronic liver disease related to hepatitis B or C viral infection, or alcoholic or non-alcoholic fatty liver disease/steatohepatitis, in contrast to large duct type iCCA that is often related to hepatolithiasis and liver fluke infection. Cholangiolocarcinoma is a variant of small duct type iCCA composed of naïve-looking cuboidal cells forming cords or ductule-like structures, and shows better prognosis than the conventional small duct type. Fibrous tumor stroma, one of the characteristic features of iCCA, contains activated fibroblasts intermixed with innate and adaptive immune cells. The types of stroma (mature versus immature) are related to tumor behavior and prognosis. Low tumor-infiltrating lymphocyte density, *KRAS* alteration, and chromosomal instability are related to immune-suppressive tumor microenvironments with resistance to programmed death 1/ programmed death ligand 1 blockade. Data from recent large-scale exome analyses have revealed the heterogeneity in the molecular profiles of iCCA, showing that small duct type iCCA exhibit frequent *BAP1*, *IDH1/2* hotspot mutations and *FGFR2* fusion, in contrast to frequent mutations in *KRAS*, *TP53*, and *SMAD4* observed in large duct type iCCA. Multi-omics analyses have proposed several molecular classifications of iCCA, including inflammation class and proliferation class. The inflammation class is enriched in inflammatory signaling pathways and expression of cytokines, while the proliferation class has activated oncogenic growth signaling pathways. Diverse pathologic features of iCCA and its associated multi-omics characteristics are currently under active

investigation, thereby providing insights into precision therapeutics for patients with iCCA. This review provides the latest knowledge on the histopathologic classification of iCCA and its associated molecular features, ranging from tumor microenvironment to genomic and transcriptomic research.

Keywords: intrahepatic cholangiocarcinoma, pathology, small duct, large duct, tumor microenvironment, genomics, transcriptomics

# INTRODUCTION

Cholangiocarcinomas (CCAs) include intrahepatic CCA (iCCA), perihilar CCA, and distal CCA (1). Anatomically, iCCA, often called as "peripheral CCA," is defined as a tumor located in the periphery of the second-order bile ducts, ranging from segmental bile ducts to smaller branches of the intrahepatic biliary tree. Perihilar CCA, also known as Klatskin tumor, is defined as a tumor that arises at the junction where the right and left hepatic ducts meet, with the insertion site of the cystic duct as its distal limit. CCAs involving more of the distal area, such as the common bile duct, is defined as distal CCA. This review mainly focuses on iCCA.

Recent evaluation indicates that iCCA comprises approximately 10–15% of primary liver malignancies (2, 3), and its incidence worldwide has increased over the past decades (4). However, changes in the nomenclature, classification, and the disease coding system of CCA have hampered the accurate estimation of the incidence of iCCA (5). Countries with the highest incidence include South Korea (2.8 per 100,000 people/year), where *Clonorchis sinensis* infection was prevalent in the past, and Thailand (2.2 per 100,000 people/year), which still is an endemic area for infections due to *Opisthorchis viverrini* (4, 6, 7). In other countries where parasites are not endemic, the incidence of iCCA is low, usually below or around 1 per 100,000 people/year. Moreover, there are proposed risk factors such as choledochal cyst, primary sclerosing cholangitis, chronic B or C viral hepatitis, and non-alcoholic fatty liver disease caused by obesity or metabolic syndromes. However, a significant proportion of patients with iCCA have no known risk factors (4, 8). Since these patients rarely present symptoms in the early stage, they are often diagnosed with advanced disease with a dismal prognosis and 5-year overall survival rate of approximately 10% even in developed countries (9–11).

Intrahepatic cholangiocarcinoma is an epithelial neoplasm with biliary differentiation, and usually presents with abundant fibrous tumor stroma containing cancer-associated fibroblasts (CAFs), innate and adaptive immune cells, etc. Recently, iCCAs have been classified into two subtypes namely, small duct type and large duct type (3, 12). Furthermore, the molecular characteristics of iCCA are under active investigation owing

**Abbreviations:** CCA, cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; CAFs, cancer-associated fibroblasts; TCGA, The Cancer Genome Atlas; WHO, World Health Organization; MUC, mucin; CRP, c-reactive protein; EBER, Epstein–Barr virus-encoded small RNA; CKs, cytokeratins; FGB, fibrinopeptide B; CLC, cholangiolocarcinoma; HCC, hepatocellular carcinoma; DPM, ductal plate malformation; IPNB, intraductal papillary neoplasm of the bile duct; BilIN, biliary intraepithelial neoplasia; IPMN, intraductal papillary mucinous neoplasm; TAMs, tumor-associated macrophages.

to technological advances in nucleotide sequencing and the availability of massive data sources on cancer, such as The Cancer Genome Atlas (TCGA) and cBio Cancer Genomics Portal (13, 14). The combination of histopathological and multi-omics data have provided novel insights into understanding the molecular pathology and thereby, developing therapeutic options for iCCA.

This review aims to provide the latest knowledge on the histopathologic classification of iCCA based on the fifth edition of the World Health Organization (WHO) classification of digestive system tumors. In addition, we discuss the associated molecular features based on tumor microenvironment, genomic and transcriptomic research results presented so far.

# PATHOLOGICAL FEATURES OF INTRAHEPATIC CHOLANGIOCARCINOMA

Intrahepatic cholangiocarcinoma is an adenocarcinoma arising in the intrahepatic biliary tree. Fibrous tumor stroma is one of the characteristic features of iCCA, and the fibrous stroma is various in amount and distribution. The tumor center is usually more fibrotic than the tumor periphery, showing proliferating tumor cells invading into the surrounding liver. Lymphovascular and perineural invasion are often detected even at an early stage.

## Gross Features of Intrahepatic Cholangiocarcinoma

Macroscopically, iCCAs can be classified into three types: mass-forming, periductal infiltrating, and intraductal growing. Based on gross appearance, the mass-forming type is most common, and often exhibits mixed features (**Figure 1**) (15). The mass-forming type shows a definite round tumor mass with invasive border. The cut surface is usually white, pale tan or yellowish in color with firm consistency due to fibrous tumor stroma. The periductal infiltrating type shows a growth pattern that extends along the bile duct, exhibiting a whitish fibrotic and thickened bile duct wall. The intraductal growing type grows into the lumen of the bile duct, forming single or multiple soft papillary masses attached to the bile duct wall. The tumor mass and resultant obstruction often dilate the bile duct and make the tumor symptomatic. Since most of the intraductal growing type iCCA cases are now being considered as malignant transformations of intraductal papillary neoplasm of the bile duct (IPNB), in the latest fifth edition of WHO classification, gross morphologic types of iCCA include mass forming, periductal infiltrating and mixed type of these two (3, 16).

**FIGURE 1 |** Macroscopic types of intrahepatic cholangiocarcinoma. **(A)** Mass-forming (MF) type showing a whitish tan tumor mass invading adjacent liver parenchyma. **(B)** Periductal infiltrating (PI) type showing a whitish periductal tumor growth along the bile duct branches. **(C)** Mixed type of MF and PI showing a solid tumor of MF type (white arrow) and a periductal growth of PI type (black arrow). **(D)** Intraductal growing type showing a friable tumor mass in the dilated bile duct (white arrow). This type of tumor is re-classified as intraductal papillary neoplasm of bile duct.

## Classification of Small Duct Type and Large Duct Type

Conventional iCCA can be further classified into two histopathological types according to the level or size of the affected duct. Recently, small duct type and large duct type iCCA have been introduced in the WHO classification (3).

Small duct type iCCA, which has been reported as a peripheral, ductular, and cholangiolar type, accounts for 36–84% of iCCA (12, 17–19). Small duct type iCCA shows small-sized tubular growth of cuboidal or low-columnar tumor epithelial cells. There is little or no mucin production, and occasional areas of growth exhibit a pattern resembling ductular reaction with slit-like glandular lumen (**Figures 2A,B**). Background liver of small duct type iCCA often shows chronic liver disease related to B viral hepatitis, C viral hepatitis, alcoholic hepatitis, and non-alcoholic steatohepatitis (12, 17, 20).

Large duct type iCCA, which has been reported as bile duct type or perihilar type, arises in large intrahepatic bile ducts and comprises 8–60% of iCCA cases (12, 17–19). Large duct type iCCA is composed of mucin-producing columnar cells forming irregular shaped-and-sized tubules or gland-like structures. This type usually shows a highly invasive growth pattern accompanied by a desmoplastic reaction (**Figures 2D,E**) (3). Pathological examination of the background liver of large duct type iCCA often reveals chronic bile duct injury due to hepatolithiasis, parasitic infection in bile ducts, or primary sclerosing cholangitis (8, 20).

Furthermore, histopathological features of small duct type and large duct type iCCA are related to their gross appearance. The periductal infiltrative type of iCCA is exclusively large duct type, whereas the mass-forming type is more heterogeneous, including small duct and large duct types (17, 21). Mass-forming type iCCA with small duct type histology showed better prognosis than other types (17).

## Putative Cells of Origin

Small duct type iCCA occurs in smaller intrahepatic bile ducts compared to large duct type. Canals of Hering, which are histological structures that link hepatic canaliculi and the biliary tree, cuboidal cholangiocytes of bile ductules, and interlobular bile ducts are considered as the putative cells of origin (22, 23). In contrast, large duct type iCCA might be derived from columnar biliary epithelium producing mucin or peribiliary glands around them (8, 20). However, the cellular origin of iCCA is still controversial, since various lineage tracing animal studies showed mixed results indicating hepatic stem or progenitor cells, cholangiocytes or hepatocytes as the cells of origin of iCCA (24, 25). Further research is required to conclusively define the origin of small duct and large duct type iCCAs.

## Immunohistochemical Markers for Small Duct Type and Large Duct Type Intrahepatic Cholangiocarcinoma

Examination of a panel of immunohistochemical (IHC) markers is useful to differentiate small duct type and large duct type. In large duct type iCCAs, high expression of mucin (MUC) core protein 5AC, MUC6, and S100 calcium-binding protein P (S100P) has been reported (26, 27), whereas neural cell adhesion molecule (NCAM, also known as CD56) and N-cadherin have been found to be highly expressed in the small duct type. NCAM and N-cadherin are normally expressed in cholangioles (28, 29). Intra- and/or extracellular mucin, detected by mucicarmine or Alcian blue staining, is abundant in the large duct type

in contrast to its scarcity or absence in the small duct type. Examining a panel of these markers, including S100P, N-cadherin, NCAM, and Alcian blue, has been reported to be more effective in differentiating small duct and large duct types (12). Additionally, c-reactive protein (CRP) was recently found to be an effective marker for the diagnosis of small duct type iCCA (30) (**Figures 2C,F**). Biliary cytokeratins (CKs) such as CK7 and CK19 are useful for confirming biliary differentiation or biliary origin. However, their ability to differentiate between small duct type and large duct type iCCA is limited (31, 32).

Histopathological features of hematoxylin and eosin-stained slides usually provide insight to distinguish small duct and large duct type iCCA. In addition, application of immunohistochemical (IHC) markers (S100P, NCAM, and N-cadherin) and special stain for mucin is useful to support the diagnosis of iCCA subtypes, especially when the tissue is limited in biopsies.

## Comparison of Prognosis and Treatment Response Between Small Duct Type and Large Duct Type Intrahepatic Cholangiocarcinoma

The prognosis of small duct type iCCA is generally favorable compared to that of large duct type iCCA (12, 17, 33, 34). Accordingly, inflammation-related markers [CRP and fibrinopeptide B (FGB)] and proliferation-related markers [extracellular signal-regulated kinases (ERK) 1/2 and Ki-67] are highly expressed in small duct type iCCA and large duct type iCCA, respectively (17). It has also been reported that the response to conventional chemotherapy is better with small duct type than with large duct type iCCA (35).

## Differential Diagnosis of Intrahepatic Cholangiocarcinoma in a Biopsied Tissue

For liver primary tumors, diagnosis of iCCA requires differentiation from combined hepatocellular-cholangiocarcinoma (cHCC-CC), since both components of cHCC-CC may not clearly present due to the limitations of the biopsied tissue. Application of IHC markers for hepatocellular carcinoma (HCC) is helpful to identify portions of hepatocytic differentiation. Hepatocyte paraffin-1 (Hep Par 1) and arginase-1 (ARG1) are highly sensitive and specific (both exceeding 80%) markers, and addition of glypican-3 (GPC) is shown to be useful for the diagnosis of poorly differentiated areas of hepatocytic differentiation with sensitivity over 80% (36).

Since an iCCA is histopathologically an adenocarcinoma, it is necessary to differentiate it from metastatic adenocarcinoma from other organs. Application of the following IHC markers is helpful. Caudal-type homeobox 2 (CDX2) is a widely used marker for the diagnosis of metastatic colorectal adenocarcinoma with sensitivity over 90%. Since its specificity is relatively low (70%) (37), combination with CK7 and CK20, which are usually negative and positive in colorectal adenocarcinoma, respectively, is recommended (32). Adenocarcinoma of the lung and ductal carcinoma of the breast, which can be differentiated by IHC staining with antibodies of thyroid transcription factor-1 (TTF-1; 75% sensitivity and specificity) (38, 39) and GATA binding protein 3 (GATA-3; over 90% sensitivity and specificity),

respectively (38, 40, 41). Paired box 8 (PAX8) is a sensitive marker for ovarian and endometrial carcinomas, as well as for renal cell carcinomas with sensitivity approaching 90% (40). Metastatic prostate adenocarcinoma is usually positive for the antibodies against prostate specific antigen (PSA) and prostate specific acid phosphatase (PSAP), with sensitivity and specificity exceeding 95% (40, 42). However, in cases of metastatic adenocarcinomas originating from organs adjacent to the liver including gallbladder, pancreas, and stomach, etc., it is difficult to differentiate iCCA from these tumors, due to the lack of specific IHC marker. Some potentially promising markers have been introduced, and filamin A was reported to show high positivity (63%) by immunohistochemistry on iCCA (43). Recently, an *in situ* hybridization assay for albumin RNA was reported to show 90% sensitivity and 100% specificity for iCCA, particularly for the differentiation of small duct type iCCA and metastatic tumors (44–46).

# Variants of Intrahepatic Cholangiocarcinoma

## Cholangiolocarcinoma

Cholangiolocarcinoma (CLC) is a variant of iCCA that belongs to the small duct type. It is defined as an iCCA with more than 80% of the tumor area showing cholangiolocellular differentiation without hepatocellular differentiation. The prefix "cholangiolo" implies histopathological similarity to the cholangiole or canals of Hering (47). CLCs show small cuboidal cells forming cords or tubular structures with antler-like growth resembling the ductular reaction of non-tumorous liver (48). Often, the lumina of tumor cords are inconspicuous, the atypia or pleomorphism of tumor epithelial cells is minimal, and regularly spaced intervening stroma is also a characteristic feature (**Figure 3A**).

CLC is thought to arise at the bile ductule, containing hepatic stem or progenitor cells, and canals of Hering. It was previously classified as a subtype of combined hepatocellular-cholangiocarcinoma (49), however, molecular profiling studies favor the classification of CLC as part of iCCA (50). According to the current WHO classification, CLC without components of HCC or intermediate carcinoma is an iCCA and is not considered as combined hepatocellular-cholangiocarcinoma.

CLC is distinguished from conventional small duct type iCCAs based on its excellent outcome, which shows significantly higher overall and disease-free survival (49, 51). Even iCCAs with cholangiolocellular differentiation (>10% of the tumor area) were found to have a better prognosis than those without (17, 52). A transcriptomic profiling study reported that iCCA with cholangiolocellular differentiation correlated with inflammation class, while iCCA without cholangiolocellular differentiation correlated with proliferation class (the molecular classification is discussed in more detail later in the genomic-transcriptomic profiles section) (52, 53).

## Intrahepatic Cholangiocarcinoma With Ductal Plate Malformation Pattern

Ductal plate malformation (DPM) refers to a developmental anomaly characterized by pathologically existing embryonic bile duct structures ("ductal plates"). The percentage of iCCAs
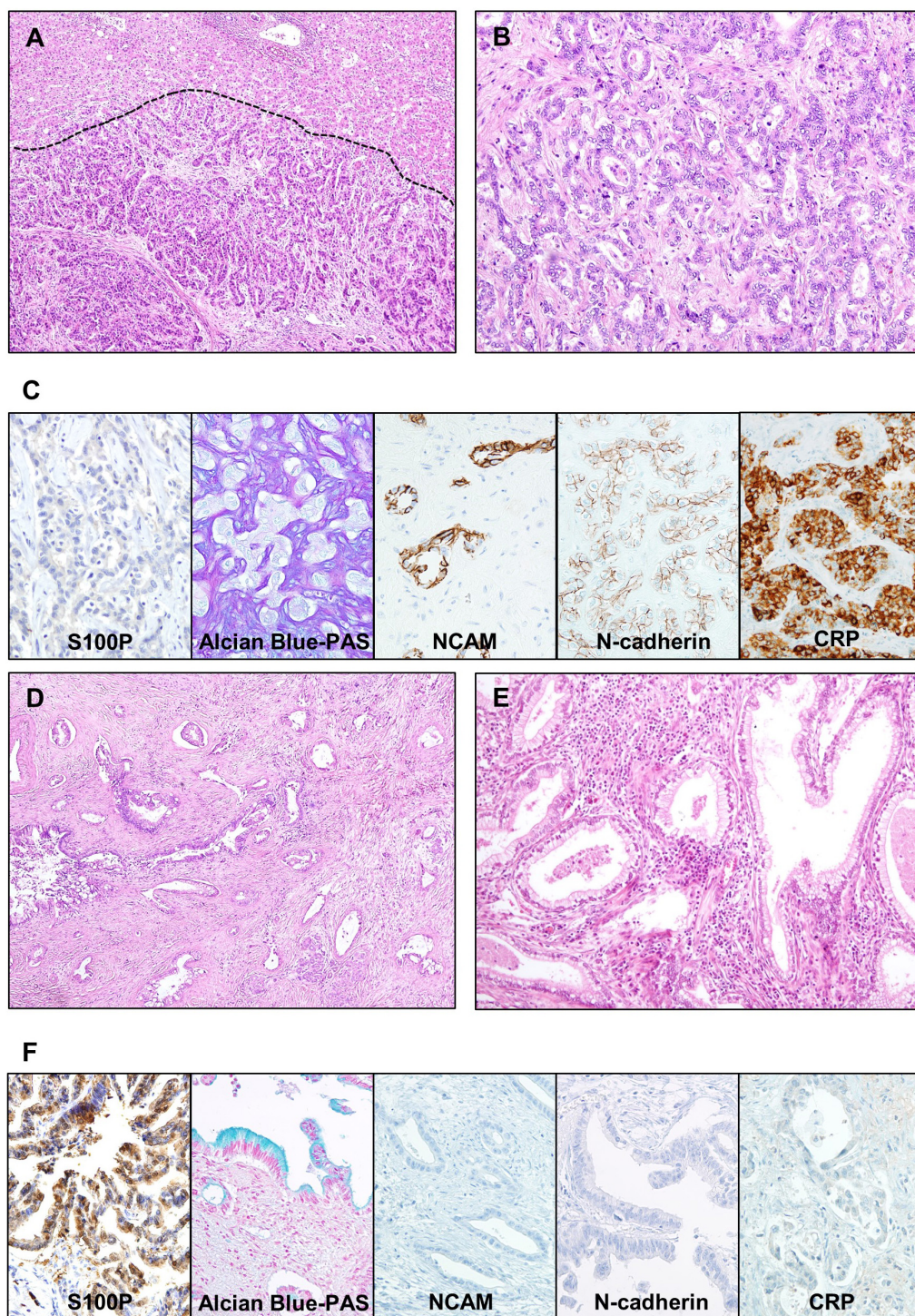
FIGURE 2 | Representative microscopic images of small duct type and large duct type intrahepatic cholangiocarcinoma (iCCA). (A–C) Small duct type iCCA. (A) A low-power view showing uniform-shaped tumor glands replacing hepatocellular trabeculae at the border (indicated by dashed line). (B) A higher magnification image showing the growth of cuboidal cells forming cords and small glandular structures, without intra- or extracellular mucin. (C) Microscopic images of special and immunohistochemical panel staining for small duct type iCCA; positive expression of NCAM, N-cadherin and CRP, negative expression of S100P, and absence of mucin in the Alcian blue staining is characteristic. (D–F) Large duct type iCCA. (D) A low magnification image shows infiltrative growth of adenocarcinoma with rich fibrous stroma. (E) A higher magnification image showing columnar cells with intracellular mucin forming irregular glandular spaces. (F) Microscopic images of special and immunohistochemical panel staining for large duct type iCCA; positive expression of S100P, presence of mucin in the Alcian blue staining, and negative expression of NCAM, N-cadherin, and CRP is characteristic. Original magnification: 40× for (A,D), 100× for (B,E), 200× for (C,F). S100P, S100 calcium-binding protein P; NCAM, neural cell adhesion molecule; CRP, c-reactive protein; PAS, periodic acid–Schiff.
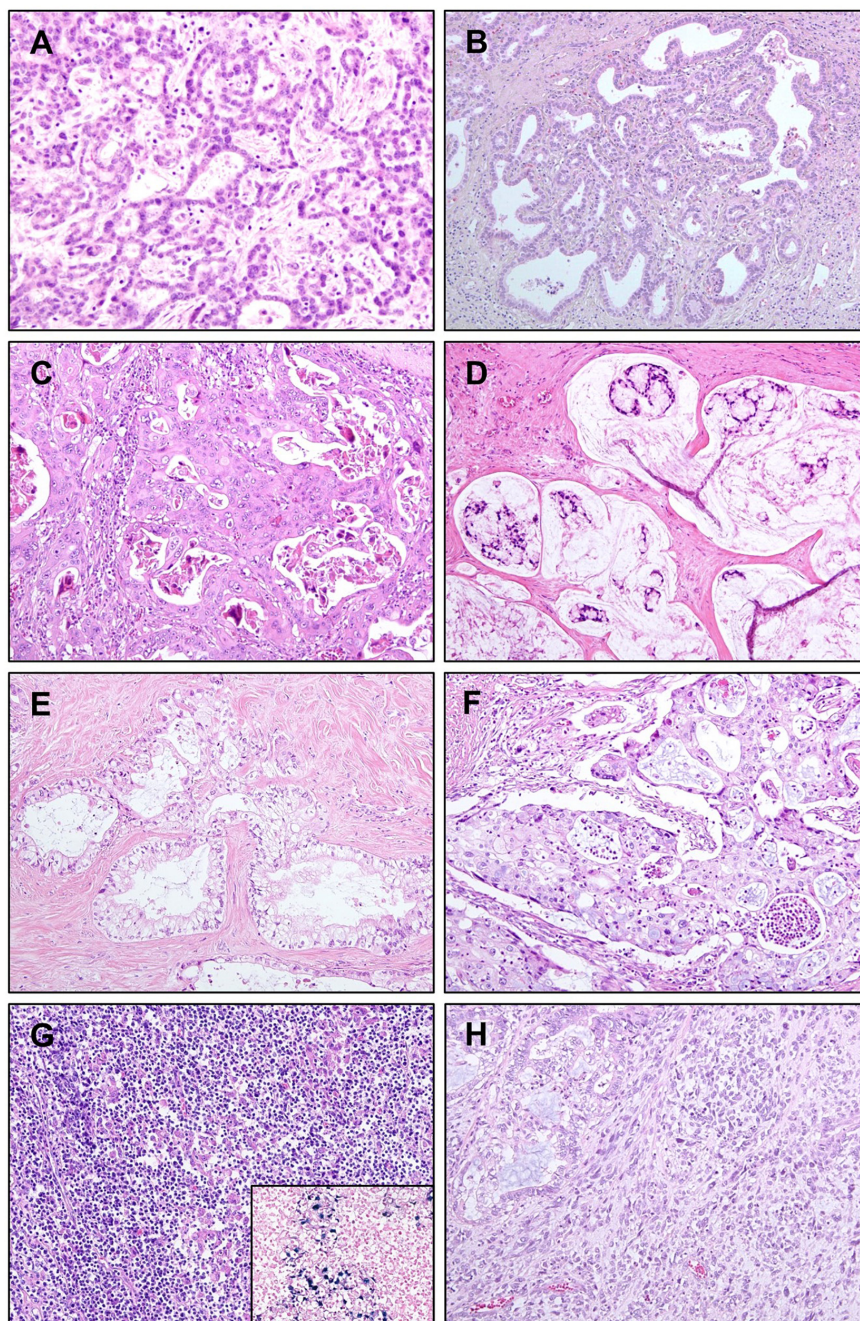
**FIGURE 3 |** Variants of intrahepatic cholangiocarcinoma (iCCA). **(A)** Cholangiolocarcinoma. Bland-looking small cuboidal tumor epithelial cells are forming cords or ductules, with antler-like branching pattern. **(B)** iCCA with ductal plate malformation pattern. Cuboidal tumor cells are forming irregularly dilated and coalesced spaces, resembling developmental anomaly of ductal plate. **(C)** Adenosquamous carcinoma showing both of gland-forming portion and portions with squamous differentiation. **(D)** Mucinous carcinoma. Mucin-producing tumor cell clusters are floating in mucin pools. **(E)** Clear cell carcinoma. Tumor cells have large clear cytoplasm with eccentric nuclei. **(F)** Mucoepidermoid carcinoma showing squamoid tumor cells intermixed with mucin-producing cells. **(G)** Lymphoepithelioma-like carcinoma showing marked lymphocytic infiltration into tumor epithelial component. Tumor epithelial cells are positive for Epstein–Barr virus (EBV), detected by *in situ* hybridization of EBV-encoded small RNA (inset). **(H)** Sarcomatous iCCA showing mainly pleomorphic spindle cells, with adenocarcinoma components in the upper left corner. Original magnification: 100×.

that are diagnosed as iCCAs with DPM pattern is very small, approximately 2.9% of cases in a cohort of 175 resected iCCAs (54). Histopathologically, the tumor epithelial cell lining is usually benign-looking cuboidal cells without mucin production, and they form glandular structures that are elongated, tortuous, and coalesced, mimicking ductal plates (**Figure 3B**) (55). Genetic

alterations in iCCA with DPM pattern include point mutations in *FGFR2*, *PTPRT*, *ARID1A*, and *CDKN2A*, and fusion of *FGFR2* (54, 56). Patient survival seems better than that of conventional small duct type iCCAs (54).

## Adenosquamous Carcinoma/Squamous Carcinoma

Adenosquamous carcinoma of the liver has both squamous epithelial and glandular components (**Figure 3C**), and its incidence is rare (57). Squamous carcinoma, showing squamous differentiation in the entire tumor is extremely rare. This type of variant iCCA is reported to be correlated with chronic cholangitis caused by liver flukes or hepatolithiasis (58). The prognosis of adenosquamous carcinoma of the liver is usually poor, with a median survival of approximately 6 months (57).

## Mucinous Carcinoma/Signet Ring Cell Carcinoma

Mucinous carcinoma is a variant that belongs to large duct type iCCA. It contains an overwhelming amount of extracellular mucin in the luminal space of tumor glands, usually over 50% of the total tumor volume by convention (59), often causing tumor epithelial cells to float in the mucin pool (**Figure 3D**). This type of tumor usually occurs due to the malignant transformation of the IPNB. Signet ring cell carcinoma occasionally presents as a mucinous carcinoma with varying distribution; however, pure signet ring cell carcinoma of the liver is extremely rare. The absence of ovarian-like stroma differentiates this variant of iCCA from mucinous cystic neoplasm (60).

## Clear Cell Carcinoma

Clear cell carcinoma is characterized by bulky cytoplasmic clearing and eccentrically located nuclei in most tumor epithelial cells with glandular and trabecular growth patterns (**Figure 3E**) (58, 61). Primary clear cell carcinoma of the liver can be differentiated from HCC with clear cell change, metastatic clear cell carcinoma of the kidney, and metastasis from other gastrointestinal tract tumors by IHC staining for hepatocyte paraffin 1 (HepPar-1), CD10, and CK20, respectively (62).

## Mucoepidermoid Carcinoma

Primary mucoepidermoid carcinoma of the liver shows features similar to those in other organs, including the salivary glands. It reveals a more intimate mixture of epidermoid or squamous and mucin-secreting elements, compared to adenosquamous carcinoma where mucin-secreting cells and foci of squamous differentiation exist separately (**Figure 3F**) (63). There have been only a few reports, and most of them have shown a poor prognosis (64).

## Lymphoepithelioma-Like Carcinoma

Lymphoepithelioma-like carcinoma is characterized by dense lymphoid stroma around the tumor epithelial cells, often forming lymphoid follicles. Tumor epithelial cells show an undifferentiated or gland-forming pattern, rarely with well-differentiated or bland-looking glands (**Figure 3G**). Almost all cases are Epstein–Barr virus-encoded small RNA (EBER) positive and usually have favorable outcomes (65, 66).

## Sarcomatous Intrahepatic Cholangiocarcinoma

Sarcomatous iCCA usually shows mixed features of conventional iCCA and undifferentiated components of cells with spindle or rhabdoid features (**Figure 3H**). When a conventional iCCA component is not present, a definite diagnosis is difficult, since the sarcomatoid component is often negative for epithelial markers by IHC staining (67). Sarcomatous iCCA usually has a worse prognosis than conventional iCCAs (68).

# Precursor Lesions
## Biliary Intraepithelial Neoplasia

Biliary intraepithelial neoplasia (BilIN), a precursor lesion of CCA, occurs at the epithelium of intra- and extrahepatic bile ducts and in the peribiliary glands. Large duct type iCCA, but not small duct type, is often accompanied by BilIN (17, 21). BilIN is virtually invisible upon gross examination, although it may be associated with subtle changes such as mucosal thickening. Microscopically, BilIN consists of flat or micropapillary (less than 3 mm in height) epithelial lesions that are graded as low-grade or high-grade (carcinoma *in situ*) based on the highest degree of cytoarchitectural atypia (69, 70). This two-tiered classification replaces the former three-tiered classification, wherein the former BilIN-1 and BilIN-2 are now classified as low-grade, and the former BilIN-3 is now classified as high-grade.

Low-grade BilIN shows mild cytoarchitectural atypia, including flat pseudopapillary and/or micropapillary growth pattern, nuclear stratification, hyperchromatic nuclei, and increased nuclear-cytoplasmic ratio; however, nuclear polarity is preserved. High-grade BilIN is characterized by moderate to severe cytoarchitectural atypia, including more complex patterns, complete loss of polarity, marked nuclear atypia, and frequent mitosis. While IHC staining for p53 is usually negative in low-grade BilIN, it is often overexpressed in high-grade BilIN (71). The expression of p16 is relatively preserved in low-grade BilIN and decreased in high-grade BilIN (72). A notable mutation in BilIN lesions is alterations in *KRAS*, which is reported to be approximately 30% (73) (**Figures 4A,B**).

Differentiating BilIN from reactive epithelial atypia may be difficult, especially in biopsy samples. Reactive atypia shows overlapping attenuated basophilic cells with nuclei having fine and diffuse chromatin. The nucleoli are small or conspicuous. Mitotic activity may be prominent. Reactive epithelial atypia usually shows a gradual transition from uninvolved epithelium, in contrast to the abrupt change usually seen in BilIN. The IHC detection of S100P was shown to be useful, being mostly negative in reactive epithelial atypia. However, its expression increased sequentially from low-grade BilIN to high-grade BilIN and subsequently in iCCA (74).

## Intraductal Papillary Neoplasm of the Bile Duct

Intraductal papillary neoplasm of the bile duct (IPNB) is defined as a grossly visible premalignant neoplasm showing intraductal papillary or villous growth of biliary-type epithelium (70). It is considered to be a counterpart of a similar tumor arising in the pancreas, the so-called intraductal papillary mucinous neoplasm (IPMN). IPNB is divided into low-grade and high-grade based on the highest degree of cytoarchitectural atypia.
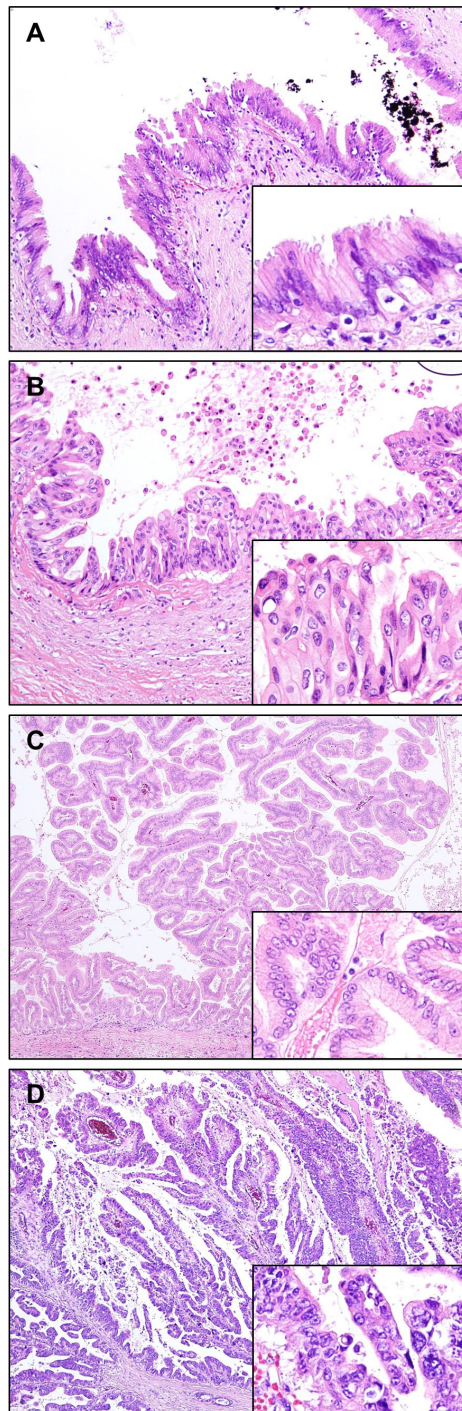
FIGURE 4 | Precursor lesions of intrahepatic cholangiocarcinoma. (A,B) Biliary intraepithelial neoplasia (BilIN). (A) Low-grade BilIN composed of columnar cells with intact nuclear polarity and minimal atypia. (B) High-grade BilIN showing stratification of cells with marked nuclear atypia and loss of polarity. (C,D) Intraductal papillary neoplasm of the bile ducts (IPNB). (C) Low-grade IPNB showing a papillary growth of columnar biliary type epithelial cells with mild pleomorphism and preserved nuclear polarity. (D) High-grade IPNB showing irregular papillary projections, composed of highly pleomorphic and stratified cells with increased nuclear-cytoplasmic ratio. Original magnification: 100× for (A,B), 40× for (C,D), 200× for inset images.

When invasive carcinoma develops in this lesion, it is diagnosed as IPNB with associated invasive carcinoma. High-grade IPNBs are often associated with stromal invasive carcinoma, usually consisting of tubular adenocarcinoma and occasionally mucinous carcinoma (75).

Grossly, IPNBs appear as polypoid masses with dilatation of the bile ducts. These are usually isolated papillary lesions, whereas some IPNBs appear as multiple contiguous papillary or polypoid lesions. Some IPNBs are characterized by mucus hypersecretion, forming mucin-containing fusiform dilatation or cysts, similar to those observed in IPMNs (76). Microscopically, IPNBs form papillary structures with fine fibrovascular cores (**Figures 4C,D**). Four histological subtypes are generally accepted based on cytological appearance and immunophenotype, namely, – pancreatobiliary, intestinal, gastric, and oncocytic. Immunohistochemically, MUC1 is mostly expressed in the pancreatobiliary type. Gastric type usually express MUC5AC and MUC6, and the intestinal type frequently express MUC2. CK20 is positive in the intestinal type, but not in the gastric and oncocytic types (70, 77). The presence of two or more histopathological types is common in IPNB, therefore these tumors are diagnosed based on the most prevalent histopathological type. The pancreatobiliary type is most common, with higher prevalence in western countries than in Asia. In contrast, intestinal type is more common in Asian populations than in western populations, while oncocytic and gastric types are least frequent (78). Although the clinical implications of histopathologic subtypes are still controversial, the pancreatobiliary type is reported to be linked with a higher frequency of associated invasive carcinoma, frequent lymph node metastasis, and recurrence (77).

A recent consensus has proposed a different classification for IPNBs of type 1 and type 2 (79, 80). Type 1 IPNB shows more homogeneous appearance than type 2 IPNB, and is composed of regular villous, papillary, or tubular structures usually with low-grade dysplasia, but may present with high-grade dysplasia with foci of low-grade dysplasia. Mucin overproduction is frequently observed, whereas stromal invasion is uncommon. This is most commonly found in intrahepatic bile ducts. Histological similarity with IPMN of the pancreas is also characteristic. Type 2 IPNB exhibits heterogenous appearance composed of irregular and complicated villous, papillary or tubular structures. This is usually composed of high-grade dysplasia, and foci of low-grade dysplasia are absent or minimal. Invasive carcinoma is more frequently associated with type 2 IPNB than type 1 IPNB. Mucin overproduction is not common. Type 2 IPNB arises throughout the biliary tree, including intrahepatic and extrahepatic bile ducts.

The mutational profile studies on IPNB have reported that diverse cancer driver mutations are frequently observed, including *KRAS*, *TP53*, *STK11*, *CTNNB1*, *APC*, *SMAD4*, and *GNAS*. Type 1 IPNBs show higher mutation rates of *KRAS*, *GNAS*, and *RNF43*, whereas type 2 IPNBs have higher *TP53* and *SMAD4* mutation rates (81, 82).

Although IPNBs present papillary morphology, sometimes tubular growth pattern of epithelial components with less mucin production is predominantly observed, similar to intraductal tubulopapillary neoplasm (ITPN) of the pancreas (83). Such cases have been described as intraductal tubular neoplasms
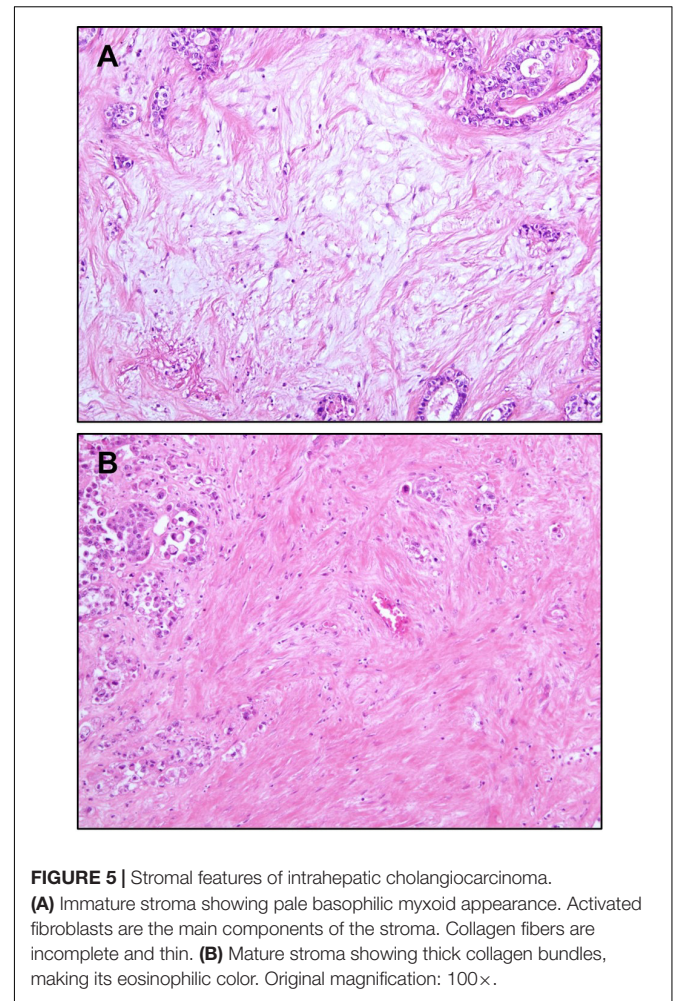
or ITPNs. Recently, ITPN has been reported to show more frequent intrahepatic occurrence in contrast to IPNB, which favors both intra- and extrahepatic locations. Furthermore, IPNB and ITPN differ in their genomic and epigenomic profiles. Recently, IPNB has been reported to share mutational profiles with extrahepatic CCA, including mutations in *TP53*, *SMAD4*, and *KRAS* and deletions on chromosomes 9q, 17p, and 18q. However, ITPN shows low overall mutational burden, and distinct DNA methylation pattern that clustered together with iCCA rather than extrahepatic CCA, suggesting that IPNB and ITPN are distinct entities (84).

# TUMOR MICROENVIRONMENT OF INTRAHEPATIC CHOLANGIOCARCINOMA

One of the characteristics of iCCA is the abundance of fibrous stroma (85). The amount of fibrous stroma has been reported to be associated with poor prognosis (86). Furthermore, so-called "scirrhous type" iCCA, which is defined as iCCA having scirrhous area (where the amount of fibrous stromal component is at least equal to the area of epithelial component) more than 70% of the largest cut surface, has been reported to show worse prognosis than conventional iCCAs (87). More recently, the characteristics of immature and mature fibrous tumor stroma have been reported to be related to tumor behavior (88, 89). Immature stroma is composed of myxoid stroma with randomly oriented short keloid-like collagen bundles. In contrast, mature stroma shows multilayered mature collagen fibers (**Figure 5**). Accordingly, iCCAs with immature stroma have been reported to show poorer prognosis compared to iCCAs with mature stroma (88, 89).

Activated CAFs, one of the major components of the tumor microenvironment, have been demonstrated to facilitate tumor growth and progression, and promote immunosuppression in tumors (90). CAFs are thought to be recruited from hepatic stellate cells, portal fibroblasts, or circulating mesenchymal cells, but the exact source is currently unknown (91). Transforming growth factor β (TGF-β) and platelet-derived growth factor D (PDGF-D) secreted by tumor epithelial cells recruit CAFs. Recruited CAFs not only promote desmoplastic reaction by collagen and matrix metalloproteinases, but also cause tumor epithelial cells to proliferate, invade, and resist antitumor mechanisms by secreting growth factors such as PDGF-B and epidermal growth factors (92, 93). Patients with iCCA with a high proportion of activated CAFs were reported to have a shorter survival rate than patients with low CAF proportion (94).

Immune cells, including tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes, are also main components of the tumor microenvironment. The hepatic macrophage population consists of activated macrophages derived from Kupffer cells or bone marrow-derived macrophages (95), and activated macrophages can be classified as M1- (classical) and M2 (alternative)-polarized types (96). The M2 phenotype forms the majority of TAM population in iCCA, having anti-inflammatory and pro-tumor functions mediated by



**FIGURE 5 |** Stromal features of intrahepatic cholangiocarcinoma. **(A)** Immature stroma showing pale basophilic myxoid appearance. Activated fibroblasts are the main components of the stroma. Collagen fibers are incomplete and thin. **(B)** Mature stroma showing thick collagen bundles, making its eosinophilic color. Original magnification: 100×.

the secretion of anti-inflammatory cytokines, including IL-4, IL-10, IL-13, and TGF-β (97). These cells also promote intratumoral angiogenesis, which is vital for tumor survival and metastasis (85, 98). A high proportion of M2 TAMs in iCCA is correlated with increased invasiveness of tumor cells and poor disease-free survival (97, 99). In contrast, M1 TAMs have been reported to exert pro-inflammatory functions, including secretion of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β (99).

Tumor-infiltrating lymphocytes include B cells (CD20$^+$), helper T cells (CD4$^+$), cytotoxic T cells (CD8$^+$), and regulatory T cells (Tregs, FOXP3$^+$). The major proportion of tumor-infiltrating lymphocytes comprises T cells rather than B cells (100). The distribution and proportion of CD4$^+$ and CD8$^+$ T cells varies among iCCAs, and increased population of these cells is correlated with better prognosis (8, 85). In addition, iCCAs with B cell infiltration have been reported to be associated with better survival than iCCAs without B cells (101). Treg cells are a subset of CD4$^+$ T cells that suppress innate and adaptive immune responses mainly by secreting IL-10 and TGF-β, which are known to promote tumor progression by inhibiting antitumor immune response. Regarding iCCA, while there are a few studies on

the clinical aspects of the presence of Treg cells, there is no sufficient evidence to draw a conclusion. Therefore, additional investigation is required (101–103).

With the advent of immune checkpoint blockade therapeutics, the expression status of cell surface proteins with immune escape mechanisms is currently under active investigation. Cytotoxic T-lymphocyte antigen-4 (CTLA-4), expressed on the surface of Treg cells, suppresses cytotoxic T cell activity by binding to CD80 of antigen-presenting cells (85). High CTLA-4 expression has been reported to be related to worse relapse-free survival of patients with CCA, raising the possibility of effective immunotherapy targeting CTLA-4 (104). Programmed death 1 (PD-1), expressed on T cells, and its ligand programmed death ligand 1 (PD-L1), on tumor epithelial cells, are other major immune checkpoints of interest. Binding of PD-L1 to PD-1 diminishes the immunological function of cytotoxic T cells. Approximately 9–30% of iCCA has been reported to be PD-L1 positive as observed by IHC staining (105–107). Recently, our group reported that *KRAS* alteration and chromosomal instability were associated with resistance to PD-1/PD-L1 blockade immunotherapy, whereas high intratumoral tumor-infiltrating lymphocyte density was associated with a favorable immunotherapy response in patients with CCA (35). Many clinical trials for iCCA using immune checkpoint inhibitors are ongoing based on the expression status of markers, including PD-1, PD-L1, and CTLA-4, with expectations of promising results in the near future (108).

# MULTI-OMICS FEATURES OF INTRAHEPATIC CHOLANGIOCARCINOMA

## Germline and Somatic Mutational Profile of Intrahepatic Cholangiocarcinoma

### Germline Predisposition

There are a few germline predispositions for cancers, including proto-oncogenes and tumor suppressor genes, either by inheritance from parents or *de novo* mutation at the zygote level. Approximately 8–12% of iCCAs have been reported to have known pathogenic or possibly deleterious germline mutations, and the most commonly found germline-mutated genes are *BRCA1* and *BRCA2*, which are associated with DNA repair mechanism and hereditary cancer syndromes (109–112) (**Table 1**). Other germline variants linked with iCCA include *APC*, an antagonist of the Wnt signaling pathway, *BAP1*, which mediates deubiquitination, and mismatch repair mechanism-related genes, namely *MLH1* and *MSH2* (109, 111, 113). However, evidence regarding the association between known hereditary cancer syndromes and iCCA is currently not fully established and requires further investigation.

## Somatic Short Mutations, Structural Variations, and Copy Number Aberrations

Among somatic mutations identified in iCCA, the most well-known and frequent variants are at exons 5–8 of *TP53* and

**TABLE 1** | Summary of germline mutations reported in intrahepatic cholangiocarcinoma.

| Gene | Frequency of occurrence (%) | References |
|------|------|------|
| *BRCA1* | 1–3 | (109–111) |
| *BRCA2* | 1–3 | (109–111) |
| *MLH1* | 2 | (109) |
| *MSH2* | 2 | (109) |
| *MUTYH* | 2 | (111) |
| *BAP1* | 1 | (111) |
| *PMS2* | 1 | (111) |
| *APC* | 1 | (111) |

hotspots at codons 12/13 of *KRAS*, which are involved in cell cycle arrest/DNA repair and mitogen-activated protein kinase (MAPK) signaling pathway, respectively (114–116). Other MAPK pathway genes such as *NRAS* and *BRAF* are also frequently mutated in iCCA (117). Owing to the advancement and wide use of massive parallel sequencing techniques, many other driver gene mutations have been discovered in the last decade. Single-nucleotide variants of chromatin remodeling-related genes such as *ARID1A*, *BAP1*, and *PBRM1* have been reported with frequencies ranging from 6 to over 30% (115, 118–120). Moreover, mutations in *IDH1/2,* which acts as an epigenetic regulator, are most frequently observed, with an average incidence of approximately 15% (115, 121–124). Other somatic short mutations include Akt signaling pathway-associated genes such as *PTEN*, *PIK3CA*, and *PIK3C2A*, and *SMAD4*, a TGF-β signaling pathway gene (115, 118, 125, 126).

The most frequently found structural variation in iCCA is *FGFR* gene fusion, notably *FGFR2*, which has been reported in 6–14% of iCCAs (127, 128). The most common fusion partner is *BICC1*; however, several other genes were also found, including *AHCYL1* and *PPHLN1* (128–130). Driver gene amplification was found in *ERBB2* (2–12%), *MDM2* (0–13%), *EGFR* (1–16%), and *CCND1* (10–13%). Deletion of 9p21.3, or the locus including genes *CDKN2A* and *CDKN2B* is found in 10–20% of iCCA (13, 126, 127, 131).

Microsatellite instability-high cases are usually determined by three methods namely, observing the size change in more than three out of five marker loci by polymerase chain reaction, IHC for mismatch repair proteins including, MLH1, MSH2, MSH6, and PMS2, or estimation of tumor mutation burden by NGS. Such cases are known to be rare (around 1%) in iCCA (126, 132–134). The mutational characteristics of iCCA are summarized in **Table 2**.

Genetic alterations are also correlated with pathological features. Hotspot mutations in *KRAS* have been reported in periductal infiltrating type, but not in mass-forming type (135). Histopathologically, small duct type has been reported to have more frequent *BAP1* and *IDH1/2* hotspot mutations and *FGFR2* fusion, and lower incidence of *KRAS* mutation than large duct type (12, 26, 51, 122, 136, 137). On the contrary, large duct type is known to have frequent mutations in *TP53*, *KRAS* and some

**TABLE 2 |** Major somatic variants and reported incidence in intrahepatic cholangiocarcinoma.

| | Groups | Gene or locus | Frequency of occurrence (range, %) | References |
|---|---|---|---|---|
| Small nucleotide variants | DNA repair | TP53 | 2.5–39.3 | (13, 35, 115, 120, 123, 125, 126, 131) |
| | Chromatin remodeling | ARID1A | 7–36 | (13, 35, 115, 120, 123, 126, 131) |
| | | BAP1 | 6–16 | (13, 35, 116, 121, 123, 126, 131) |
| | | PBRM1 | 9–14.3 | (13, 35, 116, 119, 121, 123, 126, 131) |
| | MAPK signaling pathway | KRAS | 2–30.3 | (13, 35, 115, 121, 123, 125, 126, 131) |
| | | NRAS | 3–9.3 | (13, 115, 117, 119, 125, 126, 131) |
| | | BRAF | 3–5 | (13, 35, 117, 125, 126, 131) |
| | Epigenetic regulator | IDH1 | 5–36 | (13, 35, 115, 126, 131) |
| | | IDH2 | 3.7–36 | (13, 115, 117, 119, 121, 126, 131) |
| | TGF-β signaling pathway | SMAD4 | 0–9 | (13, 35, 115, 126, 131) |
| | Akt signaling Pathway | PTEN | 0.6–11 | (13, 115, 117, 125, 126, 131) |
| | | PIK3CA | 3–7 | (13, 35, 115, 117, 119, 120, 125, 126, 131) |
| | | PIK3C2A | 0–7.1 | (117) |
| Structural variation | Translocation | FGFR2 | 6–14 | (13, 127, 128, 131) |
| | Amplification | CCND1 | 10–13 | (13, 35, 131) |
| | | EGFR | 1–16 | (126, 131) |
| | | ERBB2 | 2–12 | (35, 126, 131) |
| | | MDM2 | 0–13 | (131) |
| | Deletion | 9p21.3 (CDKN2A/B) | 10–20 | (126, 131) |
| Microsatellite instability | | | ~1 | (126, 131, 133) |

TGF-β pathway genes, including *SMAD4*, *TGFBR2*, *FBXW7*, and *MYC* (35).

From an etiological point of view, liver fluke *O. viverrini* infection-related iCCA had a higher *TP53* mutation rate, while *BAP1* and *IDH1/2* mutations were more frequently found in non-fluke-related cases (131, 138). *TP53* mutation was also found to be significantly correlated with hepatitis B virus (HBV) infection (127, 139). Regarding patient outcome, worse overall survival of patients with mutated *TP53*, *KRAS*, and *TERT* or deleted *CDKN2A* has been reported (126).

# Genomic-Transcriptomic Profiles: Molecular Classification of Intrahepatic Cholangiocarcinoma

Several multi-omics approaches have been reported in the past decade, and several molecular classifications of iCCA have been presented (**Table 3**).

## Inflammation/Proliferation Class

Integrated gene expression and mutational analyses performed by Sia et al. revealed two classes (inflammation and proliferation) of iCCA (53). The inflammation class accounted for approximately 40% of iCCA, and it was characterized by activation of immune response-related pathways, including dendritic cell signature and cytokines such as IL-4 and IL-10. The proliferation class showed activation of several oncogenic pathways including receptor tyrosine kinase pathway genes, such as *EGF*, *RAS*, *AKT*, *MET*, and other growth factor genes. Patient outcomes were worse in the proliferation class than in the inflammation class.

## Prognosis-Based Classes

Transcriptomic profiling of iCCA and perihilar CCA by Andersen et al. revealed two prognostic groups (C1 and C2) with 5-year survival rate. The group with poor prognosis (C2) indicated increased activation of *VEGF/ERBB*, *CTNNB1/MYC*, and *TNF* signaling network and *KRAS* mutation, whereas these characteristics were not seen in the group with good prognosis (C1) (140). Recently, the gene expression pattern of iCCAs with cholangiolocellular differentiation trait, having favorable prognosis, was reported to be similar to that of C1, and has a signature including upregulated expression of inflammation-related genes and downregulated expression of proliferation-related genes based on Gene Ontology terms (52, 141).

## Tumor Microenvironment-Based Classes

A recent study on the classification of iCCA according to its tumor microenvironment presented four subtypes based on gene signature analysis: "immunogenic," which shows high innate and adaptive immune cell infiltration, "myeloid-rich," which has strong macrophage and myeloid signatures, "mesenchymal," with strong activated fibroblast signature, and "immune-desert," which is characterized by lowest expression of all signatures (142). The immunogenic subtype had the best outcome, whereas the mesenchymal subtype had the worst outcome, in agreement with the prognostic features of the tumor microenvironment of iCCA discussed previously.

## Other Classifications

Multi-omics data from TCGA project revealed that *IDH1/2-* and *PBRM1*-mutant subgroups showed upregulation of

**TABLE 3 |** Notable classification of intrahepatic cholangiocarcinoma from multi-omics studies.

| Base of classification | Number of cases | Molecular classification and characteristics | References |
|---|---|---|---|
| Inflammation versus proliferation signature | 149 | ● Inflammation class<br> - Enriched in immune response-related pathways<br> - Overexpression of *IL-4* and *IL-10* (Th2 marker)<br> - Favorable prognosis<br>● Proliferation class<br> - Enriched in oncogenic pathways including RTK and angiogenic pathways, increased expression of *EGF*, *RAS*, *AKT*, *MET*, and growth factors<br> - Worse outcome compared to inflammation class | Sia et al. (53) |
| Prognosis | 104* | ● Cluster 1 (group with good prognosis)<br> - No *KRAS* mutation<br> - Absence or weak expression of *HER2* and *MET*<br>● Cluster 2 (group with poor prognosis)<br> - Enriched *VEGF/ERBB*, *CTNNB1/MYC*, and TNF pathway and *KRAS* mutation | Andersen et al. (140) |
| Tumor microenvironment | 78 | ● Immune desert subtype<br> - Minimal expression of all TME signatures<br>● Immunogenic subtype<br> - High innate and adaptive immune cell presence<br> - Strong activation of fibroblasts and inflammatory and immune checkpoint pathways<br> - Best outcome<br>● Myeloid-rich subtype<br> - Strong monocyte-derived myeloid cell signatures<br> - Low lymphoid signatures<br>● Mesenchymal subtype<br> - Strong active fibroblast signatures<br> - Worst outcome | Job et al. (142) |
| TCGA project | 32 | ● *IDH*-mutant cluster<br> - *IDH1/2* mutation<br> - Enriched mitochondrial gene expression<br> - Loss of function of *ARID1A* and *PBRM1*<br>● *CCND1* amplification cluster<br> - Highly methylated<br> - *BAP1/FGFR* cluster<br>● *BAP1* mutation or *FGFR2* fusion<br>● Survival difference is not significant between clusters | Farshidfar et al. (13) |
| Etiologic factor-associated | 69 | ● Cluster 1<br> - Liver fluke-related<br> - *ARID1A*, *BRCA1/2*, and *TP53* mutations<br> - *ERBB2* amplification<br> - CpG island hypermethylation<br>● Cluster 2<br> - Partly liver-fluke-related<br> - *TP53* mutation<br> - High expression of *CTNNB1*, *WNT5B* and *AKT1*<br>● Cluster 3<br> - High CNA burden<br> - Enriched immune-related pathways<br>● Cluster 4<br> - Associated with viral hepatitis<br> - *BAP1* or *IDH1/2* mutation<br> - High expression of *FGFR* family proteins<br> - CpG shore hypermethylation<br> - Favorable prognosis | Jusakul et al. (131) |

*Whether only intrahepatic cholangiocarcinoma was included is not certain.*
*CNA, copy number aberration; HCC, hepatocellular carcinoma; IL, interleukin; RTK, receptor tyrosine kinase; TCGA-CHOL, The Cancer Genome Atlas-Cholangiocarcinoma Consortium; TME, tumor microenvironment.*

mitochondrial genes and downregulation of chromatin-modifying genes such as *ARID1A* and *ARID1B* due to hypermethylation of the promoter CpG region, while cases with

*FGFR2* fusion showed downregulation of mitochondrial genes (13). Furthermore, another study has proposed a classification based on the correlation of multi-omics features with etiologic

**FIGURE 6 |** Clinico-pathologic and molecular summary of intrahepatic cholangiocarcinoma (iCCA). Macro and microscopic, immunohistochemical, mutational, and clinical overview of iCCA. HBV, hepatitis B virus; HCV, hepatitis C virus; PSC, primary sclerosing cholangitis; S100P, S100 calcium-binding protein P; NCAM, neural cell adhesion molecule; CRP, c-reactive protein. *Based on the classification by Sia et al. (53).

background, showing that liver fluke-associated clusters 1 and 2, which harbor *TP53* mutation and *ERBB2* amplification in common, can be differentiated based on hypermethylated CpG island (for cluster 1). Furthermore, liver fluke-negative clusters 3 and 4 can be subdivided based on immune-related pathway enrichment (for cluster 3), and *IDH1/2* mutations, *FGFR2* fusion, and hypermethylated CpG promoter shores associated with viral hepatitis (for cluster 4) (131).

## Moleculo-Pathological Correlation

The two major pathological types, small duct type and large duct type iCCA differ in their molecular characteristics. Small duct type iCCAs frequently have *BAP1*, *IDH1/2*, and *FGFR* mutations, while large duct type iCCAs more commonly show *KRAS*, *TP53*, and *SMAD4* mutations. Interestingly, iCCA with cholangiolocellular differentiation trait, which belongs to the small duct type, has been reported to be correlated with inflammation class and group with good prognosis (C1) (52, 53, 140). The pathological, clinical, and molecular characteristics of iCCA based on currently available evidence are summarized in **Figure 6**.

## Perspectives on Targeted Therapies

Large-scale genomic analyses have identified target molecules for chemotherapy of patients with iCCA. Thus far, use of fibroblast growth factor receptor (FGFR) inhibitors and isocitrate dehydrogenase (IDH) 1 and 2 inhibitors are promising strategies against iCCA (130, 143).

Fibroblast growth factor receptor family proteins are localized on the cell membrane and transfer extracellular growth signals through intracellular tyrosine kinase domains. Pemigatinib, an oral inhibitor of FGFR1-3, has been approved by the United States Food and Drug Administration (FDA) for the treatment of patients with refractory advanced CCA with *FGFR2* fusion (130).

Isocitrate dehydrogenase 1 and 2 proteins are essential components of the tricarboxylic acid cycle, that normally generate NADPH *via* conversion of isocitrate into α-ketoglutarate. Mutant IDH1/2 proteins accelerate this process, resulting in excess production of the byproduct, 2-hydroxyglutarate, which acts as an oncometabolite by interfering with histone and DNA methylation regulation (143). A recent phase 3 clinical trial of the IDH1 inhibitor ivosidenib in patients

with advanced iCCA has shown promising results in increasing overall survival, and the FDA has approved its use in previously treated *IDH1*-mutated iCCA patients (143, 144).

Even though there is hope for these approved target agents, application is limited to those who harbor specific mutations, and antitumor efficacy is limited by intratumoral heterogeneity and drug resistance. Other targeted protein inhibitor molecules are in active clinical trials, including other inhibitors of FGFR family; multi-kinase inhibitors that act on epidermal growth factor receptor, vascular endothelial growth factor receptor and platelet-derived growth factor receptor; specific tyrosine kinase inhibitors targeting HER2, BRAF inhibitors, and immune checkpoint inhibitors (145, 146). To detect a variety of potential actionable mutations, the European Society for Medical Oncology has recommended routine use of NGS in patients with advanced cholangiocarcinoma (147).

## CONCLUSION

Intrahepatic cholangiocarcinoma is a very heterogenous malignancy with respect to histomorphology and molecular perspectives. The tumor microenvironment of iCCA also varies significantly depending on the type of immune cell infiltration and tumor stromal characteristics. Histopathological classification of small duct and large duct types shows differences

in etiology, molecular features, and clinical outcomes. Analyses of NGS and multi-omics studies have suggested molecular classifications of iCCA and identified *FGFR2* fusion and *IDH1/2* mutations as indications for targeted drugs. Further studies are needed for better pathological-molecular correlation and marker development for targeted therapy as well as immunotherapy to improve the treatment efficacy of patients with iCCA.

## AUTHOR CONTRIBUTIONS

TC and YP contributed to the conception and design of this manuscript. TC performed the literature search, data analysis, and wrote the first draft of the manuscript. YP critically revised the manuscript. Both authors approved the submitted version.

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# Senescence-Associated Molecules and Tumor-Immune-Interactions as Prognostic Biomarkers in Colorectal Cancer

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**Background and Aims:** The initiation of cellular senescence in response to protumorigenic stimuli counteracts malignant progression in (pre)malignant cells. Besides arresting proliferation, cells entering this terminal differentiation state adopt a characteristic senescence-associated secretory phenotype (SASP) which initiates alterations to their microenvironment and effects immunosurveillance of tumorous lesions. However, some effects mediated by senescent cells contribute to disease progression. Currently, the exploration of senescent cells' impact on the tumor microenvironment and the evaluation of senescence as possible target in colorectal cancer (CRC) therapy demand reliable detection of cellular senescence *in vivo*. Therefore, specific immunohistochemical biomarkers are required. Our aim is to analyze the clinical implications of senescence detection in colorectal carcinoma and to investigate the interactions of senescent tumor cells and their immune microenvironment *in vitro* and *in vivo*.

**Methods:** Senescence was induced in CRC cell lines by low-dose-etoposide treatment and confirmed by Senescence-associated β-galactosidase (SA-β-GAL) staining and fluorescence activated cell sorting (FACS) analysis. Co-cultures of senescent cells and immune cells were established. Multiple cell viability assays, electron microscopy and live cell imaging were conducted. Immunohistochemical (IHC) markers of senescence and immune cell subtypes were studied in a cohort of CRC patients by analyzing a tissue micro array (TMA) and performing digital image analysis. Results were compared to disease-specific survival (DSS) and progression-free survival (PFS).

**Results:** Varying expression of senescence markers in tumor cells was associated with in- or decreased survival of CRC patients. Proximity analysis of p21-positive senescent tumor cells and cytotoxic T cells revealed a significantly better prognosis for patients

in which these cell types have the possibility to directly interact. *In vitro*, NK-92 cells (mimicking natural killer T cells) or TALL-104 cells (mimicking both cytotoxic T cells and natural killer T cells) led to dose-dependent specific cytotoxicity in >75 % of the senescent CRC cells but <20 % of the proliferating control CRC cells. This immune cell-mediated senolysis seems to be facilitated via direct cell-cell contact inducing apoptosis and granule exocytosis.

**Conclusion:**   Counteracting tumorigenesis, cellular senescence is of significant relevance in CRC. We show the dual role of senescence bearing both beneficial and malignancy-promoting potential *in vivo*. Absence as well as exceeding expression of senescence markers are associated with bad prognosis in CRC. The antitumorigenic potential of senescence induction is determined by tumor micromilieu and immune cell-mediated elimination of senescent cells.

Keywords: cellular senescence, colorectal cancer, senescence-associated secretory phenotype (SASP), prognostic biomarker, senolysis

## INTRODUCTION

Malignant neoplasia of colon and rectum are associated with high morbidity and mortality and account for 10 % of cancer cases and 9.4 % of cancer deaths (1). Molecular mechanisms of colorectal carcinogenesis are increasingly understood, yet the role of cellular senescence and its contribution to survival and treatment outcome of cancer patients remain unclear.

One mechanism in tumor biology that only recently started to gain more attention due to its role in carcinogenesis is cellular senescence. Cellular senescence describes a permanent cell cycle arrest following potentially protumorigenic DNA-damaging incidents in premalignant cells, thereby counteracting malignant progression (2). There is a multitude of trigger mechanisms leading to the initiation of cellular senescence. Eroded telomeres which occur after repetitive cell divisions (3) or cumulative DNA erosions due to sublethal stressful conditions such as oxidative stress (4), proliferative stress due to oncogene-induced mitogenic hyperstimulation (5–7), loss of tumor suppressors (8, 9) or the presence of DNA damaging agents can induce a DNA damage response, arresting the cell cycle of impaired cells (10). Anticancer treatment such as chemotherapeutic agents, ionizing radiation (11–15) as well as targeted therapies are capable of evoking cellular senescence (16–21). Therapy-induced senescence (TIS) has been observed in tumor cells both *in vitro* and *in vivo* (15). Apart from ceasing proliferation, senescent transformation involves characteristic morphological and metabolic changes (22). *In vitro*, senescent cells adopt a characteristic flat, enlarged "fried egg" morphology as well as nuclear alterations (23–26). Increased lysosomal activity, detected by visualization of the lysosomal enzyme Senescence-associated β-galactosidase (SA-β-GAL) at pH 6, is a widely established biomarker of senescent cells (27). While detection of SA-β-GAL may be used for identification of cellular senescence in fresh or frozen cells (28, 29), the enzyme activity-dependent assay cannot be carried out on formalin-fixed, paraffin-embedded (FFPE) tissues (29) and therefore this distinctive feature may not be used to study cellular senescence

*in vivo* to a large extent. Due to the irreversible proliferation arrest, the senescent state is strongly associated with an absence of proliferation markers such as Ki-67 and the expression of anti-proliferative proteins (30). The onset of the senescence program involves cell cycle suppressors such as p53, p21, and p16 (22). The extent to which these features are displayed may vary (23) and none of these characteristics are exclusively linked to cellular senescence. Consequently only a combination of markers allows for distinctive identification of senescent cells (31). Recently, there have been approaches to identify novel markers of senescence (32, 33).

Although no longer proliferating, senescent cells remain highly metabolically active and display an altered secretory and signaling activity. Apart from autocrine enforcement of the senescent state, senescent cells induce non-cell-autonomous effects via direct cell-cell contact with nearby cells, paracrine signaling, and secretion of a multitude of factors affecting angiogenesis and immune surveillance of the tissue environment. The SASP, adopted by arrested cells in the presence of DNA impairment, consists of a distinct composition of secreted molecules involving signaling factors like inflammatory cytokines, enzymes and extracellular matrix components (34). The SASP highly depends on the cell type (34) and enables senescent cells to attract immune cells such as macrophages, NK cells and T cells to the site of a tumorous lesion, activating them to specifically eliminate senescent cells and thus promoting the immunosurveillance of the tumor (35, 36). While some senescent cells remain in the tissue for years (29, 37, 38) and eventually contribute to age-related diseases (39, 40), there are settings where the SASP signaling activates an immediate immune response, resulting in the installation of a proinflammatory micromilieu and eventually the removal of the senescent cells (41, 42), termed "senolysis." This immune cell driven clearance of senescent cells involves the innate (41, 42) as well as the adaptive immune cells (43, 44). There is evidence that senescent cells under senescence surveillance are eliminated by macrophages (45) or NK cell-mediated induction of granule exocytosis (46, 47).

Cellular senescence has been linked to colorectal carcinogenesis (40). The silencing of the senescence-regulating cell cycle suppressors p16 and p53 typically involved in cellular senescence induction (22) is a crucial step to overcome cellular senescence in colorectal carcinogenesis (48, 49). There is first evidence that measurement of cellular senescence might be a predictive parameter in CRC patients (50) but the clinical implications of the contribution of cellular senescence to colorectal carcinogenesis have not yet been studied in a large patient cohort. Since TIS occurs during various CRC therapies, the influence of this biomechanism on disease progression in CRC needs to be investigated in a clinical setting. Furthermore, it might be a promising approach in colorectal cancer therapy to use the potential of the senescence-induced immunosurveillance to counteract malignant progression (51). Evaluating the impact of cellular senescence and the potential of therapy-induced senescence in CRC demands reliable detection methods and biomarkers applicable to FFPE tissue to explore this key mechanism in colorectal carcinogenesis *in vivo*. We further explored the potential of senescence-associated molecules as prognostic and predictive biomarkers in CRC and conducted both *in vitro* and *in vivo* studies to gain a better understanding of the functional role of the interaction between senescent colorectal tumor cells and the immune system.

## MATERIALS AND METHODS

### Material

A list of antibodies and inhibitors used in this study can be found in **Supplementary Table 1**.

### CRC Cell Culture

After preliminary experiments with various CRC cell lines, Caco-2 cells were cultivated in MEM (+15 % fetal bovine serum + 1 % pyruvate, 1 % NEAA, 1 % glutamine, 1 % penicillin, 1% streptomycin) at 37°C and 5% $CO_2$ on 12-well-plates. Senescence was induced by low dose (5 μM) Etoposide treatment. 24 h after seeding, the growth medium was replaced by medium containing 5 μM Etoposide. Cells treated with equal volumes of Dimethyl Sulfoxide (DMSO) were used as negative control. After 48 h the medium was replaced by growth medium, and cells were allowed to recover. Analyses were performed after 72 h.

Cytoblocks of Etoposide-treated and control cells were generated after harvesting using Accutase (Sigma Aldrich) treatment, formalin (Sigma Aldrich) fixation and embedding in 1 % Agarose. For following analyses, samples were transferred to paraffin and standard sectioning (2 μm) and subsequent staining was performed according to standard protocols used for routine pathology or as published previously (52). Furthermore, transmission electron microscopy (TEM) was performed according to protocols established for routine diagnostics at our institute (53).

### Immune and CRC Cell Culture

NK-92 cells were cultivated in α-MEM + 12.5% fetal bovine serum + 12.5% horse serum + 1% penicillin + 1% streptomycin + 100–200 U/ml IL-2 (48 h)/ 5 ng/ml IL-2 (every 48 h) at 37°C

and 5% $CO_2$. TALL-104 cells were cultivated in Iscove's Modified Dulbecco's Medium (ATCC) + 20% fetal bovine serum, 2.5 μg /ml human albumin, 0.5 μg/ml D-manitol + 50–100 U/ml recombinant human IL-2 (48 h) at 37°C and 5% $CO_2$. For co-culture experiments, Caco-2 cells were cultivated on 6-well-plates and senescence was induced as according to 3.1. After 72 h of recovery, the growth medium was replaced by immune cell growth medium containing immune cells in different target-to-effector ratios. Cells were co-cultivated for up to 180 min. Following 120 min of co-incubation, cells were washed, and non-adherent cells (immune cells and non-vital Caco-2 cells) were removed. The quantity of remaining adherent Caco-2 cells after Co-culture was measured using cell viability assays such as crystal violet (CRV) staining of the remaining adherent cells.

### Senescence and Cell Viability Assays

Cellular senescence was detected by SA-β-Gal staining using the Senescence β-Galactosidase Staining kit according to the manufacturer's instructions (Cell Signaling). In addition, cells were subjected to FACS analysis using the cellular senescence live cell analysis assay (Enzo) and a Becton Dickinson FACScalibur cytometer and Cell Quest Software (BD Bioscience). For viability analysis, Caco-2 cells were treated as described. At the indicated times, cells were washed with PBS, fixed with methanol:ethanol (2:1) and stained with 0.1 % crystal violet for 30 min. The plates were washed in running tap water and air dried for 24 h. Crystal violet was solubilized using 33 % acetic acid for 30 min. The absorbance was measured at 600 nm using a microplate reader (Tecan).

### Live Cell Imaging

Immune cells were added to Etoposide-treated Caco-2 cells as described above. Cells were incubated at 32°C for 180 min. Cell-cell interactions were observed using a Jenoptik GRYPHAX SUBRA camera system in 100 x magnification. Pictures of representative areas were taken with a 30 s interval.

### Patient Cohort

The patient cohort consisted of up to 598 patients diagnosed with primary colorectal carcinoma at the Institute of Pathology of the University Medical Center, Mainz. These patients had not received neoadjuvant treatment prior to their surgery and were treated according to national and WHO guidelines in place at the time. Patients with a hereditary cancer syndrome or history of inflammatory bowel disease were not included in this study. Retrospective use of these and other patients' data as well as material for research purposes was approved by the ethical committee of the medical association of the State of Rhineland-Palatinate [ref. no. 837.075.16 (10394)]. All experiments were in accordance with the Declaration of Helsinki. Characteristics of the patients can be found in **Supplementary Table 2**.

### Human Tissue Analyses

From each patient, FFPE tissue samples containing tissue of the primary tumor and non-cancerous tissue were obtained from routine procession of the surgery specimens. Clinical data such as age, DSS, PFS, localization and stage of the tumor

were obtained. Representative areas of tumor center, invasive margin and non-cancerous epithelium were identified by review of hematoxylin and eosin (H&E)-stained sections from each sample and cores of 1 mm in diameter were obtained using the TMArrayer (Pathology Devices, San Diego, USA) and included in the TMA. From each patient, 3 samples containing representative areas of the primary tumor were included. TMA sections were stained for various senescence-associated molecules and other cell types. Staining of the slides was carried out using an automated staining system (Agilent Technologies) and its respective reagents. IHC-stained TMA sections were digitalised using a Hamamatsu Nanozoomer Series scanner (Hamamatsu Photonics, Hamamatsu, Japan) at 20 x magnification. Slides were thoroughly annotated by a pathology expert, thereby cancerous epithelium and stroma were marked. Digital image analysis was performed using HALO (Indica Labs, Albuquerque, USA). A random forest classifier was trained to discern (cancerous) epithelium and stroma. The percentage of positive cells within the classified tumor cells was obtained. Consecutive sequential TMA sections were co-registered for additional comprehensive morphometric analyses such as distance-measurements.

## Statistical Analyses

Statistical analyses were carried out using GraphPad Prism version 9. Cell viability data was compared with the control group using $t$-test or ordinary one-way ANOVA. Dunnett T3 test (statistical hypothesis testing) was used to correct for multiple testing. For each marker, the values' distribution was analyzed, and cutoff values were chosen to represent meaningful biological groups, while at the same time finding optimal cutoff values. This was done similar to the method proposed by Budczies et al. where "[t]he optimal cutoff is defined as the point with the most significant (log-rank test) split." These authors have implemented their approach as open source software named the Charité Cutoff Finder (54). Additionally, we also applied the surv_cutpoint capability of the R survminer package which functions in a similar fashion (54). Cutoff values can be found in **Supplementary Table 3**. Survival analyses were performed using Kaplan-Meier-plots, differences in survival were calculated by performing log-rank Mantel-Cox test.

## RESULTS

Low dose Etoposide treatment induces senescence-related morphological changes and SA-ß-Gal activity. Morphological changes commonly found in senescent cells such as enlarged size and vacuolation could be detected in cells treated with Etoposide using white light microscopy and electron microscopy (**Figure 1A**). Increased SA-β-GAL activity was observed in 71.9 % of Etoposide-treated Caco-2 cells but only 1.1 % of control cells ($p < 0.0001$) (**Figure 1B**). Senescence induced by Etoposide treatment was also confirmed by FACS analysis (**Figure 1C**).

To assess the prognostic potential of senescence-associated molecules suggested by previous studies (32, 33) in a clinical setting, we evaluated various markers in our cohort of CRC patients immunohistochemically (**Figure 2A**) and observed mixed effects. For NTAL, ARMCX3, p21, and EBP50 the percentage of positive tumor cells showed a statistically significant prognostic effect. High expression of NTAL was linked to a better DSS and PFS (**Supplementary Figure 1**). A high expression of p21 was linked to a higher PFS (**Supplementary Figure 1**), underlining the important role of p21-mediated senescence in tumor defense. Evaluating expression of ARMCX3 and EBP50 (**Supplementary Figure 1**), we found that a high expression was associated with a decreased DSS compared to the group of patients with lower expression levels. This surprising finding led us to try a three-tier cutoff system into excessive, moderate, and low expression (**Figures 2B–F**, cutoffs on the right and in **Supplementary Table 3**). Interestingly, for all markers (including gH2AX), using this approach showed that moderate expression was associated with the best prognosis, while both low and excessive expression showed a worse prognosis. This was statistically significant for NTAL, ARMCX3, and EBP50 (**Figures 2B–E**, **Supplementary Figure 1**). Taken together, this highlights the dual role of cellular senescence, with both low and excessive expression of senescence-associated markers showing worse DSS and PFS.

In search of an explanation for this plurivalent effect we hypothesized that the negative prognosis in patients with large numbers of senescent cells might result from a defective interaction between senescent cells and the tumor micromilieu. The excessive numbers of senescent cells in patients with a negative prognosis might reflect accumulation of these cells within the tumor tissue due to an ineffective tumor immunosurveillance and a failure of the immune system to clear of senescent cells. To investigate the immunosurveillance of senescent cells in our clinical cohort and analyse immune cells targeting senescent cells, we visualized the spatial relationship of senescent cells and cytotoxic T cell as stained by CD8 (**Figure 3A**). Using digital image analysis, consecutive sections with cores of one patient stained for different molecules were co-registered and corresponding tissue areas on the different sections were identified. The average distance between these two cell populations as well as the percentage of CD8-positive cells within 100 μm of p21-positive cells were determined (**Figure 3C**). To identify a possible impact on survival, proximity data was correlated with DSS and PFS. Interestingly, both a lower average distance between these two cell populations as well as a higher percentage of CD8-postive cells within 100 μm of p21-positive cells were linked to a significantly increased DSS and PFS (**Figures 3B,D**). This suggests that a closer immunosurveillance of the lesion improves the prognosis of CRC patients.

To explore the senescence-induced immunosurveillance of colonic cancer cells in depth, we conducted a series of co-culture experiments. After 2 h of co-incubation, the number of adherent senescent Caco-2 cells decreased depending on the ratio of immune cells that was added. Addition of NK-92 (displaying properties of natural killer cells) or TALL-104 cells (displaying properties of both cytotoxic T cells and NKT cells) to Caco-2 cells lead to dose-dependent detaching of adherent Caco-2 cells and cell death in >75 % of senescent cells but <20 % of proliferating control cells which was confirmed by CRV staining. This dose-dependent cytotoxicity was not
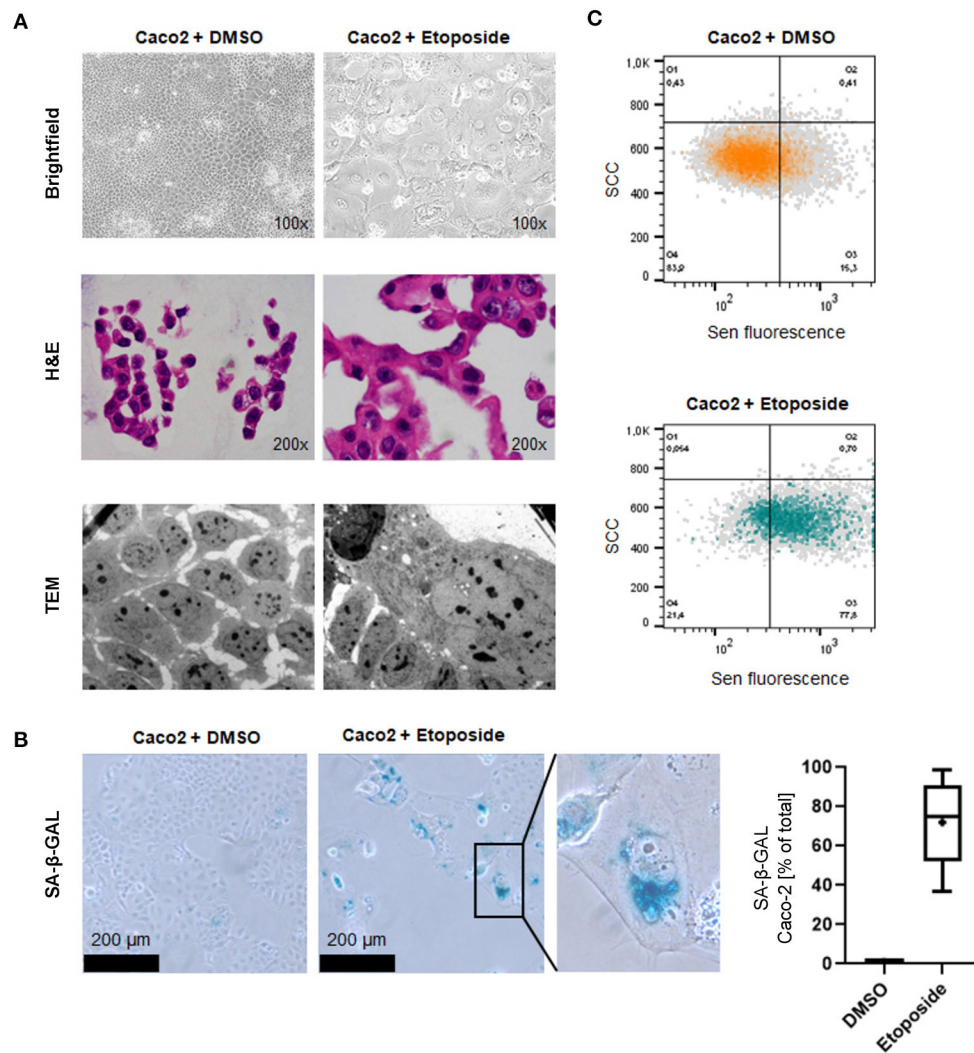
**FIGURE 1 |** Induction of cellular senescence *in vitro* by Etoposide treatment. **(A)** Morphological appearance of Etoposide (Eto)-treated and control (DMSO) Caco2 cells using brightfield microscopy (upper panel), normal H&E-stained sections of cytoblocks (middle panel) and TEM (lower panel). Senescent cells adopt a characteristic "fried egg" morphology, including an enlarged shape and nucleus. Cytoplasmatic vacuolation is apparent. **(B)** Increased senescence SA-β-GAL staining indicates senescence induction. SA-β-GAL-positive cells were counted after DMSO and Etoposide-treatment. **(C)** FACS analysis confirms senescence induction in Etoposide-treated cells.

observed in the control group of proliferating cells that had not been exposed to Etoposide (**Figures 4A,B**). To discern whether this specific elimination of senescent cells was facilitated via factors secreted into the growth medium by the immune cells, we incubated senescent Caco-2 cells with immune cell supernatant. Importantly, addition of conditioned supernatant of TALL-104 or NK-92 cells to Etoposide-pre-treated Caco-2 cells did not decrease cell viability measured by CRV absorption (**Figures 4C,D**). To confirm the hypothesis that direct cell-cell contact with immune cells accounts for the cell death of senescent Caco-2 cells and to visualize this interaction, we conducted electron microscopy and live cell imaging during co-incubation. Live cell imaging proves directed movement of immune cells toward senescent cells followed by detaching of senescent cells.

Non-senescent cells in the environment of senescent cells were not eliminated by the immune cells to the same extent. Electron microscopy of the co-culture experiments shows direct cell-cell contact between TALL-104 cells and senescent Caco-2 cells (**Figures 4E,F**, **Supplementary Video 1**).

To determine how immune cells execute the elimination of senescent cells, a set of co-culture experiments was conducted under inhibition of different pathways of cell death. By adding inhibitors of apoptosis, granule exocytosis and necroptosis, the relevance of those pathways for immune cell-mediated elimination was determined. ZVAD has been demonstrated to decrease death receptor mediated cell death in senescent cells (46). Previous studies had not found an impact of caspase-dependent apoptosis on NK cell-mediated senolysis (46).
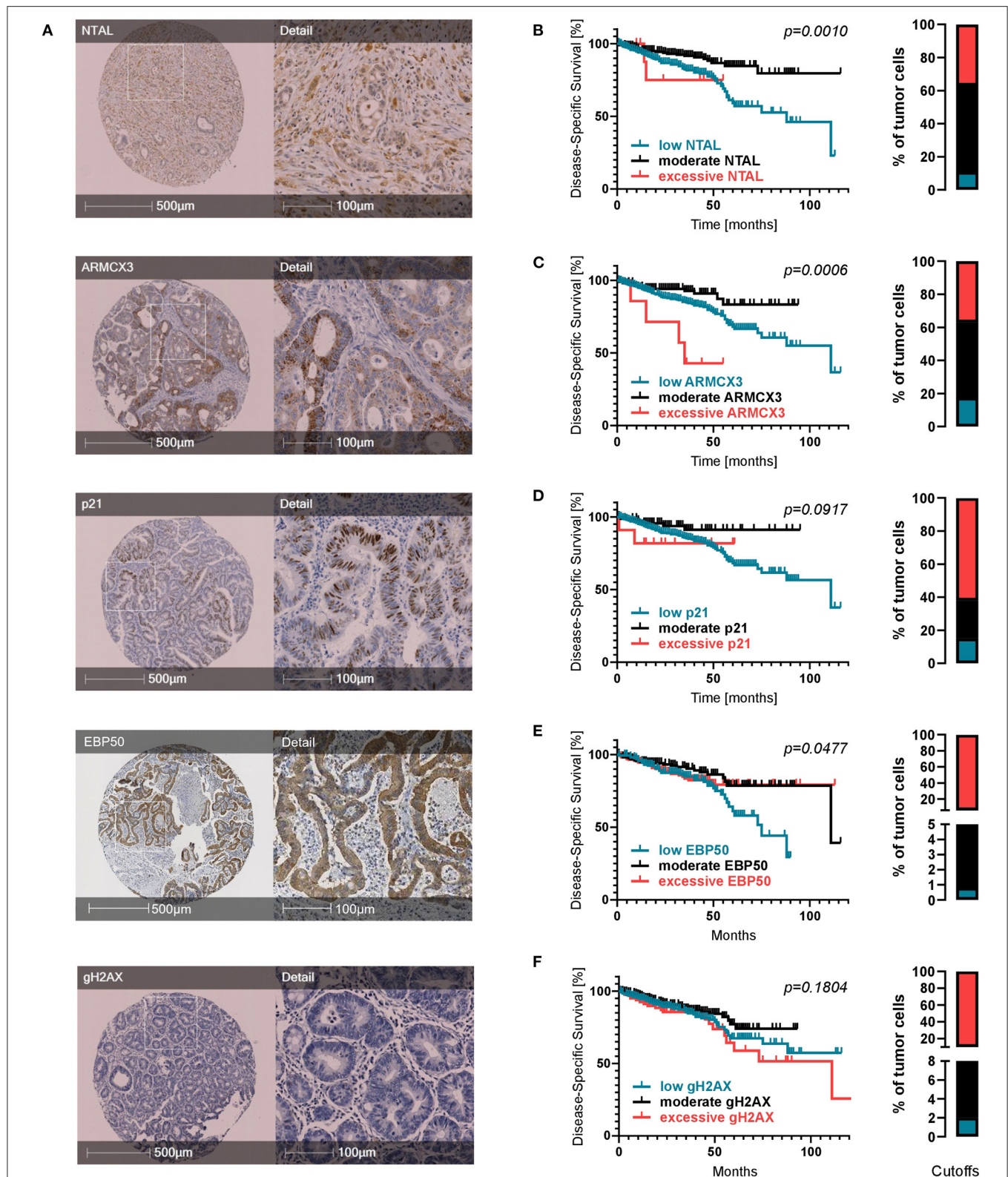
**FIGURE 2 |** Expression of senescence-associated molecules in CRC patients. **(A)** Representative TMA cores for each IHC marker. **(B–F)** Kaplan-Meier survival analyses regarding expression of senescence markers when divided into three subcohorts: low expression (petrol), moderate expression (black) and excessive expression (red). Cutoffs are displayed as bar graphs on the right of each curve and were calculated using a modification of the Charité Cutoff Finder from (54). Disease-specific survival is shown. Two tier subdivision, progression-free survival and detailed individual cutoff values can be found in the **Supplementary Material**.

FIGURE 3 | Proximity analysis of senescent cells and immune cells in CRC patients. (A) Schematic drawing of how the distance between immune cells and senescent tumor cells was measured. (B) Kaplan-Meier survival analyses regarding the average distance between CD8- and p21-positive cells. (C) Schematic drawing of how the proportion of immune cells within close proximity (within 100 μm) of senescent tumor cells was measured. (D) Kaplan-Meier survival analyses regarding the percentage of CD8-positive cells within 100 μm of p21-positive cells. Again, cutoff values are displayed as bar graphs.

However, we found that using pan-caspase inhibitor ZVAD to block death-receptor-mediated apoptosis resulted in significantly higher quantity of remaining adherent senescent cells after co-incubation resulting from abrogated senolysis of both NK-92 and TALL-104 cells ($p < 0.0001$). Addition of the SMAC-mimetic and apoptosis-sensitizer BV6 however did not have a measurable effect. Inhibiting the necroptosis pathway using Necrostatin-1, an allosteric inhibitor of RIP1, did not reverse the cytotoxicity of TALL-104 or NK-92 cells, suggesting a necroptosis-independent mechanism responsible for the targeted elimination of senescent

cells. To assess the role of granule exocytosis for the immune-mediated depletion of senescent cells, we conducted a set of experiments in the presence of Concanamycin A (Conc A) which inhibits perforin-based cytolytic activity by inhibition of vacuolar type $H^+$-ATPase. Conc A decreased the cytotoxic effect of TALL-104 cells ($p < 0.0001$) but did not significantly prevent killing of senescent cells by NK-92 cells. HMGB1-Inhibititor glycyrrhizinic acid (Gly. Acid) was used to address HMGB1-dependent metabolic cell death. Spautin-1 ($p < 0.0230$) was used to address autophagy-associated cell death mechanisms.

**FIGURE 4 |** Co-incubation of senescent cells and immune cells. **(A,B)** CRV absorbance of remaining adherent cells after co-incubation of senescent Caco-2 cells and immune cells. Co-incubation with TALL-104 **(A)** or NK-92 **(B)** immune cells lead to dose-dependent elimination of senescent cells. **(C,D)** Elimination of senescent cells under the influence of immune cell conditioned supernatant and various inhibitors of cellular clearance mechanisms as measured by CRV absorbance. TALL-104 **(C)** and NK-92 **(D)** experiments are shown. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, ns = not significant. **(E)** Life cell imaging of co-incubation of senescent cells and immune cells. Specific elimination of senescent cells (blue) by NK-92 cells while proliferating cells (orange) are spared. **(F)** Electron microscopy. Cell-cell contact of TALL-104 cells (red) and a senescent cell (blue).

ABT263 partly abrogated the cytotoxic effect of NK-92 cells ($p < 0.0001$) but did not significantly prevent killing of senescent cells by TALL-104 cells. Altogether, our co-incubation, electron microscopy and live cell imaging results indicate that direct cell-cell contact between immune cells and targeted senescent cells is a key mechanism for immune-cell-mediated senolysis. In the presence of inhibitors of apoptosis or (to some extent) granule exocytosis, immune cell-mediated elimination of senescent cells is decreased, suggesting that killing of senescent cells is mainly

facilitated via apoptosis induction and via induction of granule exocytosis (**Figures 4C,D**).

## DISCUSSION

Arresting the cell cycle of premalignant cells as a response to oncogenic signaling and DNA impairment strongly supports the idea of senescence as a beneficial anti-cancer-mechanism (55–57). A premalignant cell's ability to senesce involves major

tumor-suppressor pathways and has been proven crucial to fight neoplastic transformation *in vivo* (6, 9, 58–60) and affects treatment outcome of cancer patients (61, 62). Recent studies point to a crucial role of cellular senescence in gastrointestinal diseases including colorectal carcinogenesis (40). Studies report that oncogene-induced senescence (OIS) prevents progression of benign KRAS-mutated sessile serrated adenomas to invasive carcinomas and provides an important barrier opposing malignancy in these early lesions. Malignant transformation to serrated adenocarcinoma requires overcoming this OIS-facilitated cell cycle arrest by downregulation of p16Ink4a (49). There is evidence to suggest that senescence detection might be of predictive value for CRC patients (50), however an extensive clinical study to evaluate the prognostic potential of senescence markers had been missing.

Our study reflects the important and complex role of cellular senescence in colorectal carcinogenesis. In many previous studies, cellular senescence has been described as a "double-edged sword" (34), referring to both the pro- and antitumorigenic effects senescent cells do have on disease progression. We demonstrate that absence of intratumoral senescence and therefore the lack of a basic antitumor defense mechanism is linked with a negative prognosis. Regarding the expression of p21, NTAL, EBP50 and ARMCX3, our results show the important role of senescence induction in tumor defense and underline the relevance of cell cycle regulator p21 and p21-mediated senescence. Moreover, we show that the occurrence of extremely high percentages of senescent cells in CRC is linked to a negative prognosis when compared to patients with moderate expression of senescence markers. These findings point to a complex role of cellular senescence in CRC, suggesting that both non-existent and extensive detection of senescent cells correlate with a negative prognosis. However, further analyses in the context of various molecular and disease subtypes are also necessary to validate our findings.

Senescent cells within the tumor do not automatically imply an effective tumor defense. An effect contributing to the negative outcome of those patients with large numbers of senescent cells might be the inflammatory micromilieu developing in close distance to senescent cells as a driver of further cell damage and therefore accelerating disease progression. While some of the factors secreted after SASP initiation contribute to maintaining the cell cycle arrest and reinforce the senescence program in premalignant cells (63–66) the SASP-driven proinflammatory alterations to the micromilieu–despite enhancing immune surveillance –do as well have malignancy-promoting effects (67).

Secreted factors may provide protumorigenic conditions and stimulate growth, dedifferentiation, and invasiveness of premalignant epithelial cells (68–70). Senescent fibroblasts signaling might contribute to growth-enabling changes to the microenvironment of dormant metastases (71). Increased VEGF expression by senescent cells increases angiogenesis in lesions at risk of malignant transformation and facilitates tumor vascularization, hereby contributing to malignant transformation (72). In CRC, VEGFR2 signaling silences the tumor-antagonizing effect of cellular senescence by actively bypassing p21 (73). There is evidence that the SASP-mediated inflammatory response

enhances immune control of senescent tumorous lesions in colorectal carcinoma and prevents malignant transformation in the presence of functional p53 but is protumorigenic in p21/p53-deficient lesions (74).

The perception of senescence as a beneficial anticancer mechanism (55–57) depends on the ability of the immune system to clear senescent cells and prevent negative effects mediated by senescent cells that remain in the tissue (39). Disruption of the tumor immunosurveillance results in accumulation of senescent cells (39), which might be the cause for the negative prognosis we observed in patients with extensive expression of senescence markers. Our study demonstrates that a dichotomous classification does not apply when describing the impact of cellular senescence detection on CRC prognosis. Senescence-associated molecules do have significant prognostic value concerning the outcome of patients with CRC. Moreover, we demonstrate that immune cells *in vitro* specifically eliminate senescent colon cancer cells while somewhat sparing proliferating cells. As Sagiv et al. showed for liver fibrosis in mice (46), we could demonstrate that NK cell-mediated clearance of senescent colorectal carcinoma cells is dependent on granule exocytosis. However, in contrast to Sagiv et al. (46) we found that suppression of the death receptor pathway by ZVAD abrogated the immune cell-mediated elimination of senescent cells in both the NK cell and the cytotoxic T cell model. Thus, our findings lead to the conclusion that induction of both apoptosis and granule exocytosis contribute to the targeted elimination of senescent cells by the immune system.

To reflect this interaction in the clinical setting, proximity analyses of the spatial relation of senescent tumor cells and immune cells are of prognostic relevance and could constitute a prognostic tool in colorectal cancer. Interestingly, we found that the spatial relation of p21-positive tumor cells and cytotoxic T cells is indicative of prognosis regarding DSS and PFS of CRC patients. There is evidence for an immune-infiltration-preventing effect of SASP signaling under certain circumstances (75). High percentage of senescent cells within the tumor–indicative of a negative prognosis as demonstrated in our study–might be the result of impeded tumor immune infiltration due to SASP signaling by senescent cells inhibiting immune cells (75). Furthermore, we showed that patients with close distance of senescent tumor cells and cytotoxic T cells do have a significant better survival which might indicate an antitumorigenic, preferable SASP signaling in these patients. How to impact SASP and induce the preferable, immunosurveillance-promoting secretory activity in senescent cells needs further evaluation and bares great potential in future therapy development. There is *in vitro* (18, 76, 77) and *in vivo* (78–80) evidence for a therapeutic approach inducing cellular senescence in cancerous lesions, evoking immune-cell mediated elimination of cancer cells and enhancing tumor surveillance (41, 44, 81). There are first therapeutic approaches of altering the senescence-induced immune response to induce an antitumorigenic microenvironment (51). Furthermore, therapeutic agents specifically eliminating senescent cells, called senolytics, have demonstrated great potential in various age-associated diseases, including cancer (82).

Taken together, cellular senescence is a key mechanism in opposing malignant transformation of impaired cells. The antitumorigenic effect of cellular senescence is dependent on an intact immune surveillance of the lesion. Therefore, the interaction of immune cells and senescent cells within the tumor microenvironment is of crucial prognostic relevance and provides targets for CRC therapy.

## DATA AVAILABILITY STATEMENT

Datasets presented in this article are not readily available because of the General Data Protection Regulation regarding patient data. Requests to access the datasets should be directed to sebastian.foersch@unimedizin-mainz.de.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the Medical Association of the State of Rhineland-Palatinate [ref. no. 837.075.16 (10394)]. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

FK and SF: conception and design. FK, SF, AF, and AH: acquisition and data. FK, SF, BK, KT, and MJ: analysis, interpretation of data, and critical revision of the manuscript. FK, SF, and KT: drafting of the manuscript. FK, SF, and MS: statistical analysis. SF: obtaining funding, administrative, and technical, or material support. SF and WR: supervision.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.865230/full#supplementary-material

**Supplementary Figure 1 |** Additional Kaplan-Meier curves showing **(A)** disease-specific survival with a two-tier cutoff and **(B)** complementary progression-free survival. Cutoffs are displayed as bar graphs and were calculated using a modification of the Charité Cutoff Finder from (54).

**Supplementary Table 1 |** Antibodies and inhibitors used in this study.

**Supplementary Table 2 |** Clinicopathological characteristics of the patient cohort.

**Supplementary Table 3 |** Cutoff values for survival analysis as calculated using a modification of the Cutoff Finder from (54).

**Supplementary Video 1 |** Elimination of senescent Caco-2 cells by TALL-104 immune cells.

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# Myxoinflammatory Fibroblastic Sarcoma of the Parotid Gland: First Case Report and Literature Review

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Myxoinflammatory fibroblastic sarcoma (MIFS) is a rare, low-grade malignant soft tissue tumor. Most of the previously reported cases about this tumor were diagnosed within the soft tissues. Here, we report a unique case of MIFS of the right parotid gland in a 39-year-old Chinese male. The tumor primarily consisted of an inflammatory area and a mucus-like area in a migratory distribution. A number of lymphocytes, neutrophils, viral-like cells with large nucleoli, and eosinophilic cytoplasm or Reed-Sternberg-like cells, as well as spindle cells and epithelial-like aberrant cells, were observed within the tumor. They were found to express Vimentin and CD10 protein and no other specific immunohistochemical markers. The various cytomorphology and immunohistochemical features of this tumor were highly consistent with MIFS found in other sites. Therefore, several leading pathologists ultimately confirmed the final diagnosis of MIFS in the right parotid gland after repeated deliberation. To our knowledge, this is the first case of MIFS occurring in the parotid gland. Thus, our study provides a novel basis for identifying the biological behavior of the tumor in MIFS and also allows us to better understand the pathology of this rare tumor.

Keywords: myxoinflammatory fibroblastic sarcoma, MIFS, soft tissue tumor, Vimentin, parotid gland

## INTRODUCTION

Myxoinflammatory fibroblastic sarcoma (MIFS) is a rare, slow-growing, low-grade malignant soft tissue tumor (1). It was first described by Montgomery et al. (2), and several cases have since been reported around the world (3, 4). Clinically, MIFS is considered to be a superficial tumor found within the soft tissues, and generally manifests itself as a painless subcutaneous mass in the distal limb in adults. However, in recent years, a proportion of cases have been reported in several other non-extremity sites, such as the breast, buttocks, chest wall, and thighs (3, 5, 6). However, MIFS of the parotid gland has not been reported and described previously. In this study,

we present a unique case of MIFS of the parotid gland in a Chinese patient with a characteristic histomorphology, consisting of inflammatory and mucin-like areas. It was characterized by the prominent morphological presentation of aberrant large cells, containing huge nucleoli with a typical immunophenotype (Vimentin protein positive), and other features that were consistent with the previously reported MIFS in other sites. The study was approved by the Institutional Review Board of the Cancer Hospital of Guangxi Medical University and was performed according to the principles of the "Declaration of Helsinki." Written informed consent for this study was obtained from the patient prior to the study.

## CASE PRESENTATION

### Clinical Findings

A 39-year-old male patient was diagnosed with a right submandibular mass in the outpatient clinic of our hospital 2 months ago. The physical examination showed that the neck curvature and mobility were normal, no lateral curvature was noticed, and the right lower jaw could reach the size of a 2 × 1-cm mass. Additionally, the mass was hard, had unclear boundary and blunt edge with poor activity, but no other abnormality was observed in other parts. After the patient was admitted to the hospital, the clinician used a fine needle for aspiration of cells from the tumor. The pathological examination further revealed that the nuclear atypical cells were visible, and the malignant possibility was not excluded. The ultrasound of the neck showed a substantial lesion of the right parotid gland, and the CT examination displayed a small

nodule below the parotid gland (**Figures 1A,B**), which was initially considered as a benign tumor or tumor-like lesion of the parotid gland. His laboratory tests for the blood routine analysis, liver function, renal function, blood sugar, and thyroid function were found to be within the normal range. His HIV, HBsAg, and HCV serology were also negative. Under the general anesthesia, the right parotid mass resection and right facial nerve anatomy were performed and, thereafter, submitted for the histopathological examination.

## Pathologic Findings

Moreover, on the gross specimen, there was a gray-red, gray-yellow nodular mass within the parotid gland tissue, the size was 1.8 × 1.5 × 1 cm, and there was a complete capsule. The cut surface was grayish-yellow, gray, and white, soft, mucoid, and exhibited no hemorrhagic necrosis.

Histopathological microscopy showed that the tumor consisted of the different inflammatory regions as well as mucin-like regions, and the inflammatory cells were mostly lymphocytes or neutrophils. The inflammatory regions showed large nucleoli, and the cytoplasm was eosinophilic with viral-like cells or Reed-Sternberg-like cells, and it exhibited different degrees of fibrosis. The mucus region was primarily composed of spindle cells and tissue-like or epithelial-like malformed cells. The interstitial was hyaline degeneration, and small amounts of scattered multinucleated giant cells were visible in the lesion. Transitional distribution of inflammatory and mucous areas, visible large cells of nucleoli, and mitotic figures were not commonly observed, but mildly shaped spindle cells were seen in the inflammatory area. A part of the area was characterized by mucus and fibrotic regional composition, accompanied by
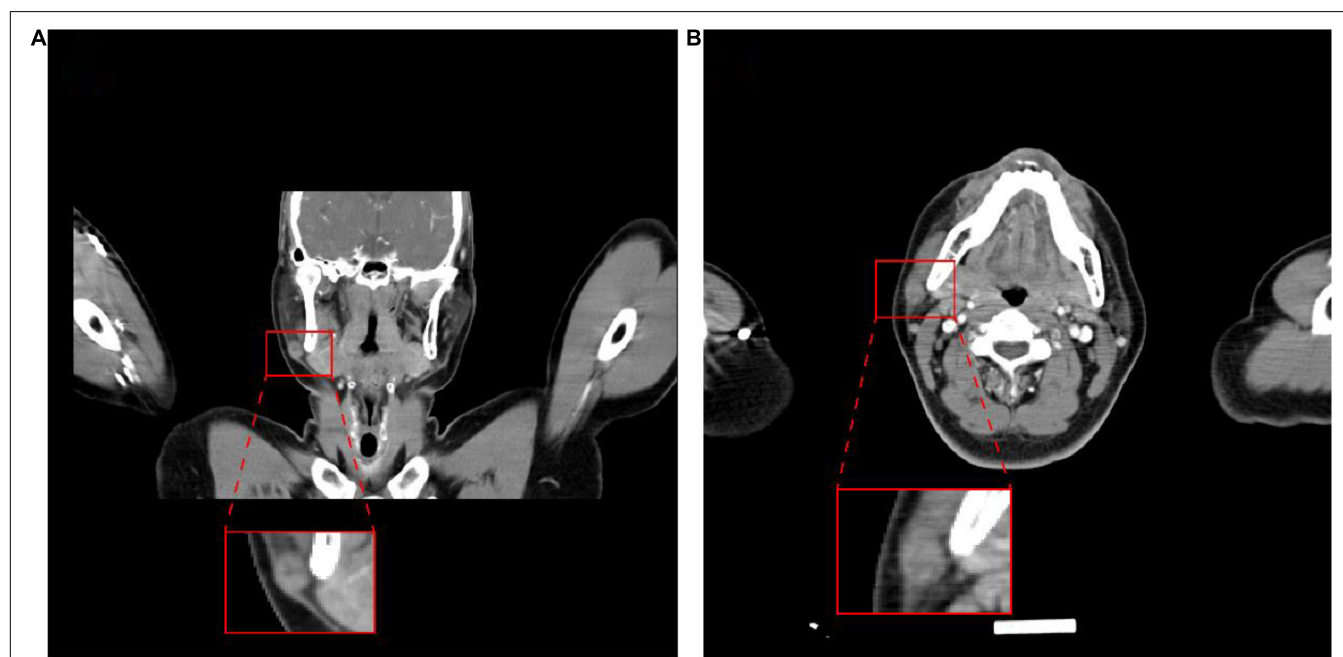


**FIGURE 1** | Images of the tumor. Computed tomography showed a nodule on the lower right side of the parotid gland. **(A)** Coronal plane; **(B)** transverse plane.
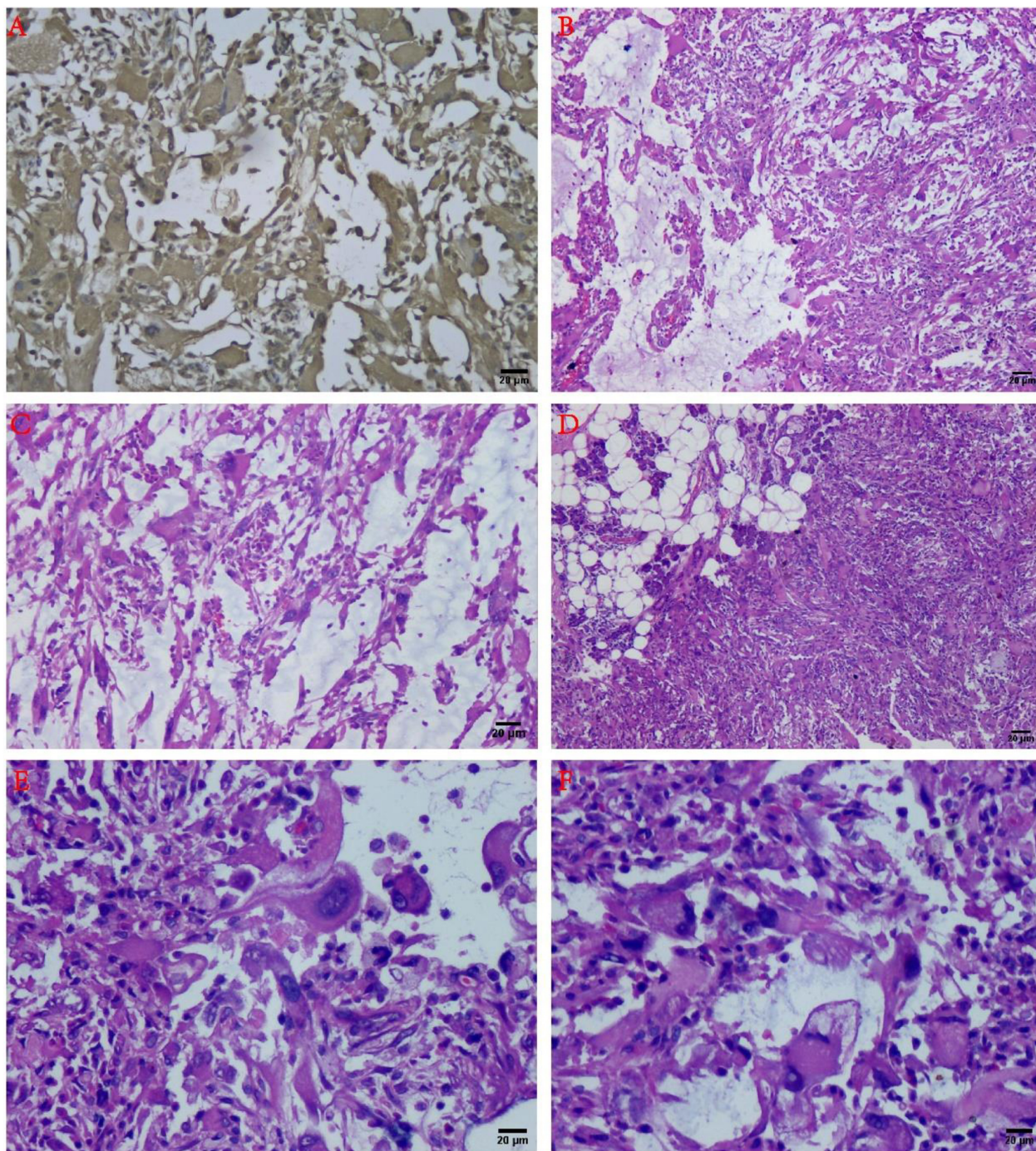
**FIGURE 2** | Histology and immunohistochemistry of the tumor. **(A)** Immunohistochemical results showed Vimentin diffuse positive (+) (IHC staining, ×100). **(B)** The tumor consisted of the mucous area and the fibrous area with inflammatory cell infiltration in the mucus-like area, and the various fusiform cells can be connected to each other to form a network structure (H&E staining, ×100). **(C)** At the junction of the mucous area and the fiber area, inflammatory cell infiltration could be observed (H&E staining, ×100). **(D)** The tumor can be infiltrated into the parotid tissue (H&E staining, ×100). **(E)** Large malformed cells with large nucleoli can be noted (H&E staining, ×200). **(F)** The "inclusion body" nucleolus resembling Reed-Sternberg cells has been recognized as one of the characteristics of mucinous inflammatory fibroblastic sarcoma (H&E staining, ×200).

lymphocyte or neutrophil infiltration. The most characteristic morphological manifestations were the presence of the large cells with huge nucleoli, with interstitial inflammatory cell reactions. Moreover, visible in the mucus area were the pseudo-fat cells, spindle cells involving adipose tissue, whereas the phagocytic cells can also be seen in some areas (**Figures 2B–F**, **3**).
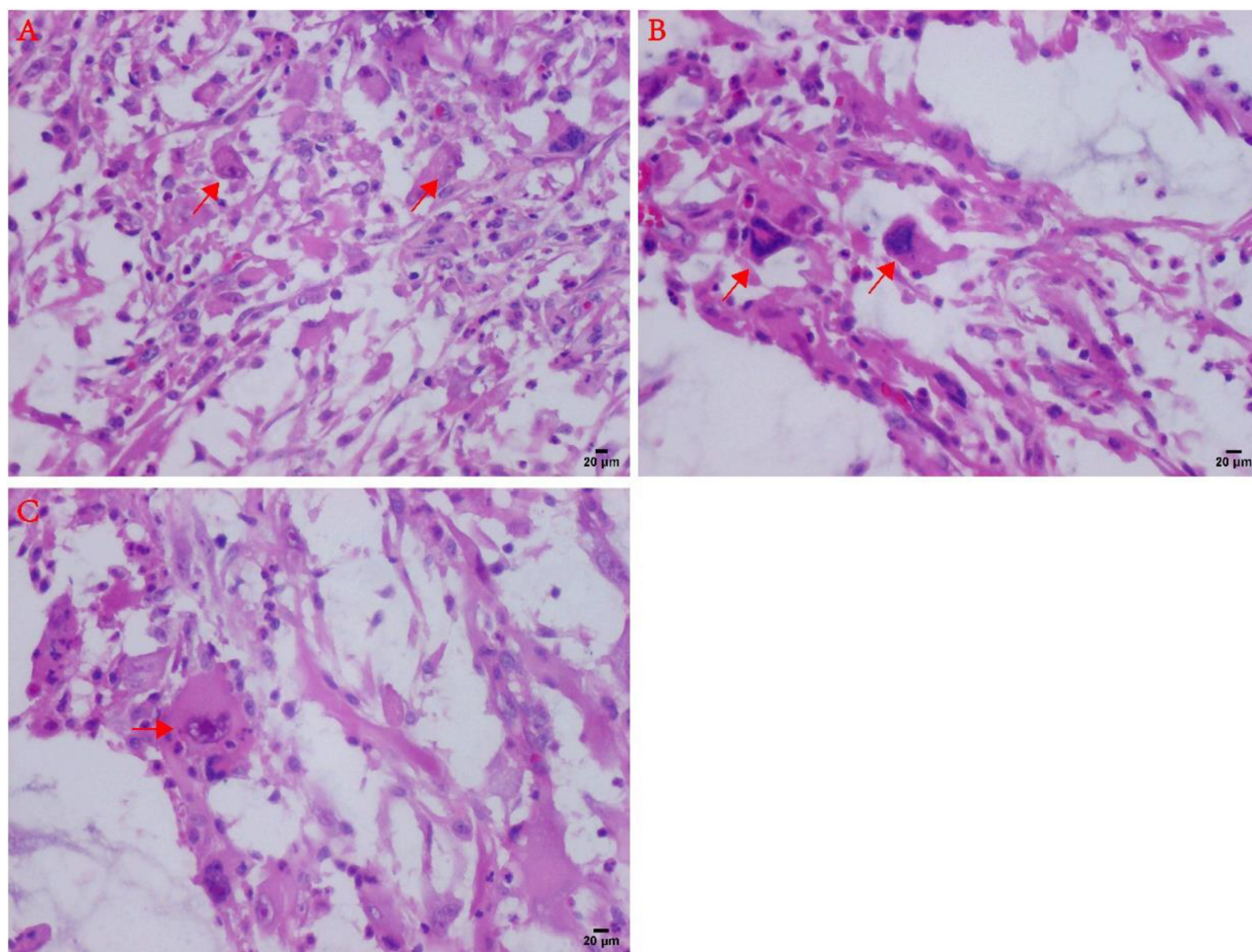
FIGURE 3 | Atypical microscopic features in MIFS. (A) Virus-like cells with abundant cytoplasm and nucleoli (arrows). (B) Large malformed cells with two enlarged nuclei shown by arrows; (C) R-S cell-like tumor cells with markedly large nucleoli (arrows).

## Immunohistochemical Findings

Immunohistochemistry revealed that the tumor cells expressed Vimentin and CD10 (diffuse positive) (**Figures 2A**, **4A**), lymphocytes expressed LCA, macrophages expressed CD163 and CD68, Ki-67 index was 10%, CK, SMA, P63, CD34, CD1a, D2-40, and S-100 were negative (**Table 1** and **Figure 4**).

According to tumor imaging, microscopic histomorphology, and comprehensive immunohistochemical analysis, the authors finally diagnosed the tumor as parotid myxoinflammatory fibroblastic sarcoma. The patient is currently in good condition and is undergoing regular follow-up in our hospital, and no recurrence or metastasis has been found.

## DISCUSSION

Based on the previously published review of domestic and foreign literature, this is the first report describing the occurrence of MIFS in the parotid gland. It has been reported that MIFS usually presents as a slow-growing painless mass, mostly in adults, and

the peak age of the onset is 30–50 years old, the average age is about 40 years old. A small number of cases also occur in children or adolescents, and tumors primarily arise in the soft tissue location of the distal part of the limb (1, 7). In this study, we have summarized the clinicopathological features of MIFS reported in the literature in the rare sites (non-bone and soft tissue, since 1998) (**Table 2**) (6, 8, 9). Clinically, MIFS may be similar to benign lesions and can be often misdiagnosed as the slippery membrane inflammation, ganglion cysts, or giant cell tumor of the tendon sheath. CT or MRI usually presents as diffuse enhancement, lobulated, subcutaneous mass with non-specific magnetic resonance imaging features, thereby suggesting the presence of benign or malignant lesions (1).

The rich and diverse histological performance of MIFS is a major challenge in the diagnosis of this tumor (10). For example, the ratio of mucin-like areas, fibroinflammatory areas, and the cell area can effectively vary from case to case, and the number of cells with inclusion body-like nuclei is generally considered to be the major marker of MIFS. Another major challenge in diagnosis is the presence of severe inflammatory
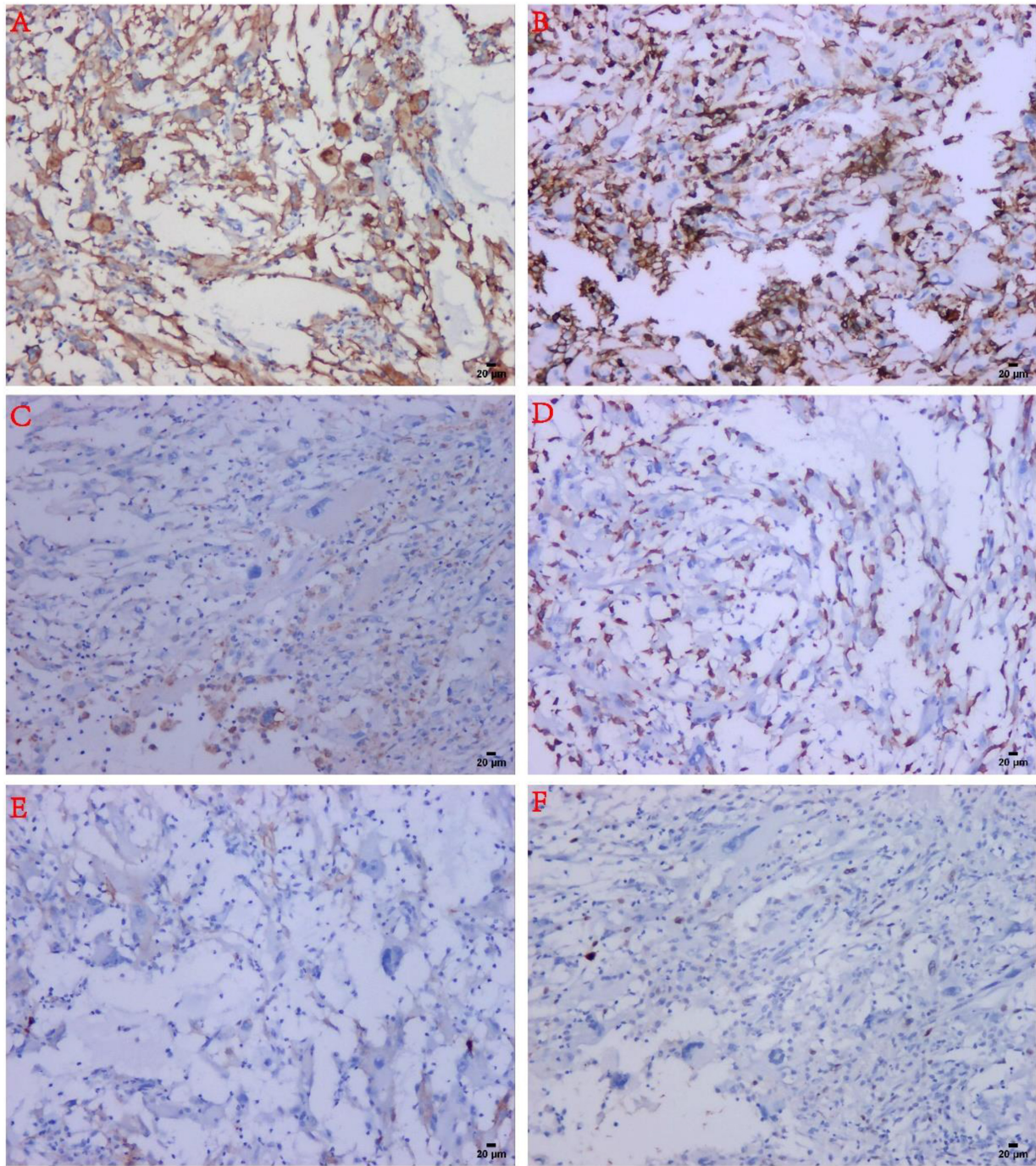
**FIGURE 4 |** Immunohistochemical findings in MIFS. **(A)** The tumor spindle cells and malformed cells were strongly positive for CD10. **(B)** Infiltrated inflammatory cells were strongly positive for LCA. **(C)** The reactive histiocytes were positive for CD68. **(D)** Reactive histiocytes were positive for CD163. **(E)** The tumor cells were negative for D2-40. **(F)** The tumor cells expressed low-proliferative Ki-67 (about 10%).

reactions, which are mediated primarily by the lymphocytes and lymphoid aggregates-infiltrated tumor cells. These may be mistaken for inflammatory processes or other malignant tumors, such as lymphoma mucinous fibrosarcoma (11). In the mucus background, the tumor spindle cells and epithelioid cells are usually connected to each other to form a complex network structure, and a large number of the neutrophils, macrophages, and plasma cells can be seen, but the mitotic figures and necrosis are not commonly observed in this tumor. Immunohistochemistry has been found to play a very limited

**TABLE 1** | A summary of the primary antibodies used and the results of immunohistochemistry.

| Antibody | Source | Dilution | Result |
|----------|--------|----------|--------|
| CKpan | Maixin, Fuzhou, China | 1:100 | − |
| Vimentin | Maixin, Fuzhou, China | 1:150 | +++ |
| LCA | Maixin, Fuzhou, China | 1:100 | + |
| CD68 | Maixin, Fuzhou, China | 1:100 | + |
| Ki-67 | Maixin, Fuzhou, China | 1:200 | + (10%) |
| SMA | Maixin, Fuzhou, China | 1:100 | − |
| P63 | Maixin, Fuzhou, China | 1:100 | − |
| CD34 | Maixin, Fuzhou, China | 1:100 | − |
| Calponin | Maixin, Fuzhou, China | 1:100 | − |
| SMMHC | Maixin, Fuzhou, China | 1:100 | − |
| CD1a | Maixin, Fuzhou, China | 1:100 | − |
| S-100 | Maixin, Fuzhou, China | 1:100 | − |
| CD163 | Maixin, Fuzhou, China | 1:100 | + |
| CD10 | Maixin, Fuzhou, China | 1:100 | + |
| D2-40 | Maixin, Fuzhou, China | 1:100 | − |

role in the diagnosis of MIFS because it has no specific tumor markers (12).

In the present study, the main features for the diagnosis of MIFS included areas of solid spindle cells, alternating with myxoid foci with the diffuse infiltration of inflammatory cells. Unlike other previously reported cases, a random distribution of the larger atypical cells was seen in our case, including Reed-Sternberg cells, variants of virus-like cells with inclusion-like nucleoli, and large malformed cells. Immunohistochemistry has been widely used in MIFS, but no specific immunophenotype has been found. However, differential responsiveness to the various markers has been observed in the previous studies, including Vimentin, D2-40, CD68, bcl-1, CD10, and CD163 (3, 12). In the present study, we found consistent and strong immunoreactivity for Vimentin and CD10 in the tumor cells in this case. Moreover, another novel finding of our study was the low expression of the proliferative marker Ki-67, and the most characteristic

phenomenon was that, in the large malformed cells and R-S like-cells, Ki-67 was almost absent. These pieces of evidence suggested that these atypical cells were the degenerative tumor cells that were biologically inactive.

The cytogenetic analysis of MIFS further revealed complex genetic abnormalities (13), including a circular chromosome formed on the Chromosome 3, a translocation of chromosome t (1:10) (p22: q24), rearrangement of *TGFBR3* and *MGEA5* genes (14). Some other cases showed *TGFBR3* and *MGEA5* gene rearrangement, which led to an upregulation of NPM3 and FGF8, accompanied by amplification of the *VGLL3* locus (10, 15).

Most cases of MIFS have demonstrated low malignant potential. However, 22–67% of cases can potentially recur, and only a few cases can metastasize (3), but high-grade MIFS may lead to more adverse progression (16). The best treatment for MIFS is complete surgical resection to ensure that the incisal margin is negative; however, the beneficial impact of radiotherapy and chemotherapy remains unclear (17). Our patient remained in good condition after 7 months of follow-up, and no recurrence or metastasis was observed.

## CONCLUSION

In summary, we report that the first case of MIFS was detected in the parotid gland. However, due to its complex histological morphology and inflammatory background, we need to distinguish it from other similar lesions, such as inflammatory myofibroblastic sarcoma and myxofibrosarcomas, to facilitate accurate diagnosis. Although no specific immunohistochemical or molecular markers are available for the diagnosis of this tumor. In addition to describing the classical histological features in this study. Our results supported the use of a distinct panel of immunohistochemical markers, including CD10 and Vimentin, might be helpful support for MIFS diagnostics in many equivocal cases. At present, the diagnosis of MIFS is still mainly based on clinicopathological diagnosis, which needs to be combined with the appropriate clinical environment

**TABLE 2** | A summary of clinicopathological features of MIFS reported in the literature from the rare sites (non-bone and soft tissue, reviewed since 1998).

| Author/year | Age/gender | Location | Pathological features | Treatment | Follow-up (mo) | Outcome |
|-------------|------------|----------|----------------------|-----------|----------------|---------|
| Current study | 39/M | Parotid gland | The tumor consisted of inflammatory regions and mucin-like regions, the inflammatory cells were mostly lymphocytes or neutrophils. The most characteristic morphological manifestations are large cells with large nucleoli, with interstitial inflammatory cell reaction. | Surgery | 28 | No recurrence or metastasis |
| Jain et al. (8) | 7-month-old infant | Eyeball | A mixed cellular infiltrate composed of numerous neutrophils, larger cells appeared to be binucleate (Reed-Sternberg-like or virocyte-like) | Resection | NA | NA |
| Auw-Haedrich et al. (6) | 27/F | Iris | Tumor contained mucinous and cellular areas with high grade of polymorphy and a large variety of cell sizes and forms | Resection | 15 | No recurrence |
| Numminen et al. (9) | 51/M | Nose | The tumor was heterogeneous in composition, with myxoid, relatively acellular areas alternating with cellular areas | Surgery | 48 | No recurrence or metastasis |

*NA, not available; mo, month; F, female; M, male.*

and characteristic histomorphological findings to significantly improve the treatment outcome.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the Affiliated Cancer Hospital of Guangxi Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

JC and SZ read and approved the final manuscript. CW performed the writing of the manuscript. XY and PG organized the material and helped with the analysis. XC carried out experiments. CL and JC performed the pathological analysis and diagnosed the patient. All authors wrote and revised the manuscript.

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# CK5/6 and GATA3 Defined Phenotypes of Muscle-Invasive Bladder Cancer: Impact in Adjuvant Chemotherapy and Molecular Subtyping of Negative Cases

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**Introduction and Objective:** Identifying patients that benefit from cisplatin-based adjuvant chemotherapy is a major issue in the management of muscle-invasive bladder cancer (MIBC). The purpose of this study is to correlate "luminal" and "basal" type protein expression with histological subtypes, to investigate the prognostic impact on survival after adjuvant chemotherapy and to define molecular consensus subtypes of "double negative" patients (i.e., without expression of CK5/6 or GATA3).

**Materials and Methods:** We performed immunohistochemical (IHC) analysis of CK5/6 and GATA3 for surrogate molecular subtyping in 181 MIBC samples. The mRNA expression profiles for molecular consensus classification were determined in CK5/6 and GATA3 (double) negative cases using a transcriptome panel with 19.398 mRNA targets (HTG Molecular Diagnostics). Data of 110 patients undergoing radical cystectomy were available for survival analysis.

**Results:** The expression of CK5/6 correlated with squamous histological subtype (96%) and expression of GATA3 was associated with micropapillary histology (100%). In the multivariate Cox-regression model, patients receiving adjuvant chemotherapy had a significant survival benefit (hazard ratio [$HR$]: 0.19 95% confidence interval [$CI$]: 0.1–0.4, $p < 0.001$) and double-negative cases had decreased OS ($HR$: 4.07; 95% $CI$: 1.5–10.9, $p = 0.005$). Double negative cases were classified as NE-like (30%), stroma-rich (30%), and Ba/Sq (40%) consensus molecular subtypes and displaying different histological subtypes.

**Conclusion:** Immunohistochemical-based classification was associated with histological subtypes of urothelial MIBC. IHC markers like CK5/6 and GATA3 that are used in pathological routine could help to identify patients with basal and luminal

tumor characteristics. However, a two-sided classification system might not sufficiently reflect the heterogeneity of bladder cancer to make treatment decisions. Especially the group of IHC-double negative cases, as further analyzed by mRNA expression profiling, are a heterogeneous group with different implications for therapy.

## INTRODUCTION

Bladder cancer (BCa) is the second most common genitourinary malignancy with about 570,000 new cases worldwide every year (1). About 25% of patients present with the muscle-invasive disease at the time of diagnosis and >90% of cases are urothelial carcinomas. The current standard of care for muscle invasive BCa (MIBC) is radical cystectomy with pelvic lymphadenectomy. However, relapse rates after surgery are high and 5-year overall survival (OS) rates are about 43% for pT3-tumors and 25% if the tumor has spread to local lymph nodes (2, 3). Cisplatin-based adjuvant chemotherapy may prolong survival rates and should be offered to patients with pT3/4 and/or pN + tumors (4). Patient selection for adjuvant chemotherapy is based on pathological tumor stage and chemo-eligibility, but no biomarker-based selection criteria for cisplatin-based chemotherapies exist or are included in current guidelines (4). The predictive biomarker-based decisions are needed to identify potential responders to chemotherapy and those that would be unnecessarily exposed to adjuvant therapy toxicities.

In recent years, genomic sequencing techniques have advanced, leading to comprehensive genomic characterization of BCa cohorts. This led to transcriptomic-based molecular subtyping of cancers and drove our understanding of BCa biology (5–9). It has been proposed that patients with basal tumors benefit most of neoadjuvant chemotherapy (NAC), whereas luminal tumors have a better prognosis regardless of the application of NAC (10, 11). However, contradictory results have been published and different nomenclatures, definitions, numbers of molecular subtypes, and inter-/intra-tumoral heterogeneity of BCa have hindered prospective validation and clinical translation.

To facilitate the clinical implementation of subtyping into clinical routine, immunohistochemical (IHC) markers that refer to "basal" and "luminal" molecular subtypes have been proposed (12–14). Guo et al. reported that IHC-staining with GATA3 and CK5/6 can classify the BCa correctly in over 80% of the cases into luminal and basal molecular subtypes. However, it remains unclear to which molecular subtype tumors without GATA3, nor CK5/6 expression (double negative) can be assigned.

In the present study, we explore IHC markers as surrogate markers for molecular subtyping, correlations with histologic subtypes, and impact on survival with adjuvant chemotherapy in a mono-institutional cystectomy cohort of urothelial MIBC. In addition, we performed RNA-sequencing of the group of "double negative" cases (CK5/6 and GATA3 negative), which are a heterogenous group on the molecular level.

## MATERIALS AND METHODS

### Cohort

Tissue/tumor samples and patient data used in this study were provided by the University Cancer Center Frankfurt (UCT). The written informed consent was obtained from all patients and the study was approved by the institutional review boards of the UCT and the ethical committee at the University Hospital Frankfurt (project-number: SUG-6-2018 and UCT-53-2021) which was conducted according to local and national regulations and according to the Declaration of Helsinki.

A total of 186 FFPE tissue samples from 181 patients with MIBC treated at the Department of Urology, University Hospital Frankfurt from 2010 to 2020 were retrieved from the archive of the Senckenberg Biobank of the Senckenberg Institute of Pathology.

Clinico-pathological and follow-up data were gathered from medical charts and records of the University Cancer Center and independently reviewed by two authors.

Histopathology of all cases was systematically re-reviewed by two experienced genitourinary pathologists according to current WHO-criteria (15). Histological subtypes were reported if at least 10% of tumor showed subtype histology including pure and mixed tumors.

### Immunohistochemical Analysis

For construction of the tissue microarray (TMA), one tissue core (diameter 1 mm) of a representative tumor area was taken from a "donor" block and was arranged in a new "recipient" block using the TMA Grandmaster (3DHISTECH, Budapest, Hungary).

Hematoxylin and eosin stain slides were automatically developed on a Tissue-Tek Prisma Plus staining device (Sakura Finetek, Torrance, CA, United States). All IHC-analyses were conducted using the DAKO Omnis staining system (Agilent, Santa Clara, CA, United States) with the DAKO FLEX-Envision Kit (Agilent) according to manufacturer's instruction. We performed staining of CK5/6 (Clone: D5/16 B4; ready-to-use kit; Dako/Agilent, Santa Clara, CA, United States) and GATA3 (Clone: L50-823; ready-to-use kit; Cell Marque, Rocklin, CA, United States). IHC "double negative" was defined as negative for expression of CK5/6 and GATA3 and "double positive" was defined as positive for expression of CK5/6 as well as GATA3.

Stained slides were scanned with the Pannoramic slide scanner (3DHISTECH, Budapest, Hungary). The quantitative analysis of IHC was annotated by two genitourinary pathologists. TMA cores with either absence of representative tumor tissue or presence of staining artifacts were excluded from the analysis.

## RNA Isolation and Molecular Subtype Calling

A 1 mm punch was taken from the FFPE blocks of a representative tumor area with at least 50% tumor content. RNA was isolated using the "truXTRAC FFPE total NA Kit–Column" (Covaris, Woburn, MA, United States) and RNA-concentration was measured by using the QuantiFluor RNA System (Promega, Madison, WI, United States) according to the manufacturer's protocol. The mRNA expression of 19,398 mRNA targets was determined using the HTG Transcriptome Panel (HTG Molecular Diagnostics, Tucson, AZ, United States) on Illumina NextSeq 550 system (Illumina, San Diego, CA, United States). Gene counts were normalized using median normalization and log2-transformed for further analysis. Sequencing data have been uploaded to the Gene Expression Omnibus (GSE198607). IHC double negative samples were defined as negative for CK5/6 and GATA3 in IHC analyses and classified according to the six molecular consensus classes of MIBC using the R-based consensus MIBC classification tool and the Bioconductor-package for R (6).

## Statistical Analysis

We performed descriptive statistics of all data.

For the survival analysis, only patients with radical cystectomy in "curative intent" ($n = 110$) were included to create a homogenous cohort. In total, 71 patients that did not fulfill this requirement were excluded, i.e., no radical cystectomy, with primary metastatic disease, NAC or missing follow up data. We defined the OS as main endpoint of interest, which was defined as time interval between surgery and death. Secondary endpoint was disease-free survival (DFS), defined as time interval between surgery and death due to BCa or recurrence.

Kaplan–Meier method was used to estimate and illustrate survival probabilities. We used uni- and multivariable Cox's proportional hazards models to estimate the hazard ratio (HR) and corresponding 95% confidence interval (CI) for covariates for OS and DFS. All tests were two-tailed, and a significance level of $\alpha = 5\%$ was used. The statistical analyses were performed using the R Statistical Software (Version 4.1) and R Studio (Version 2021.09.1 + 372).

## RESULTS

### Patient Characteristics

Overall, we included specimen of 181 patients with MIBC on the TMA. Samples were obtained from transurethral resection of the bladder (TURB) in 86 (47.5%) cases and from cystectomy in 95 (52.5%) cases. The median age was 71 years (IQR: 62–78). A total of 140 (77%) patients were male and 41 (23%) were female. The clinico-pathological details of the cohort are summarized in **Table 1**.

### Immunohistochemical-Classification

176 TMA-Spots were evaluable for the IHC-status of CK5/6 and GATA 3. Cases were classified into CK5/6 positive, GATA3 positive, "double negative" or "double positive" (representative images in **Figure 1**). CK5/6 positive cases were significantly associated with female gender (63% of tumors of female patients had CK5/6 expression vs. 37% tumors of male patients had CK5/6 expression, $p = 0.004$). CK5/6 positivity correlated with squamous histological subtype (24 of 25 cases [96%] had positive CK5/6 expression), whereas all 9 micropapillary cases were negative for the basal marker CK5/6 and positive for GATA 3 ($p < 0.0001$). All cases with neuroendocrine subtype were double negative. Twelve cases with other histological subtypes were negative for CK5/6, positive for GATA3 in 6 cases (one sarcomatoid, three plasmacytoid, one undifferentiated [giant cell], one glandular), double negative in 4 cases (one clear cell, one sarcomatoid, one undifferentiated [giant cell], one lymphoepithelial), and double positive in 2 cases (one sarcomatoid, one undifferentiated [giant cell]). Correlation of gender, tumor stage, age, and histology subtype with the two IHC markers are shown in **Table 2**.

### Survival Analysis

We assessed survival rates of 110 patients with adequate follow up that received radical cystectomy in curative intend. Median follow-up was 66 months (IQR: 34–98 months). In this group, 35 patients received at least two cycles of adjuvant chemotherapy. **Table 3** shows patients characteristics, tumor stage, and IHC markers for patients with and without adjuvant chemotherapy.

Tumor and lymph node stage as well as the application of adjuvant chemotherapy were significantly associated with OS (**Figure 2** and **Supplementary Figures 1, 2**). The 12-month OS and DFS rates were 49% and 41 without and 77 and 62% with adjuvant chemotherapy, respectively.

In the total cohort, neither IHC markers (CK5/6, GATA3) nor the histological subtype were significantly associated with OS or DFS (**Table 4** and **Supplementary Figure 3**). After stratification for patients receiving only the cystectomy vs. patients receiving an adjuvant chemotherapy, expression of CK5/6 had a HR of

**TABLE 1 |** Clinico-pathological details of 181 patients on the TMA analyzed for histological subtype of urothelial carcinoma and immunohistochemistry.

| Median Age (IQR) | | 71 (62–78) |
|---|---|---|
| **Gender** | Male | 140 (77%) |
| | Female | 41 (23%) |
| **Max. tumor-Stage** | pT2 | 87 (48%) |
| | pT3 | 67 (37%) |
| | pT4 | 27 (15%) |
| **Histological subtype on TMA spot** | NOS | 131 (72%) |
| | Squamous | 25 (14%) |
| | Micropapillary | 9 (5%) |
| | Neuroendocrine | 4 (2%) |
| | Sarcomatoid | 3 (2%) |
| | Plasmacytoid | 3 (2%) |
| | Other (1 lymphoepithelial, 1 clear cell, 1 glandular, 3 giant cell) | 6 (3%) |

*IQR, interquartile range; NOS, not otherwise specified; TMA, tissue micro array.*
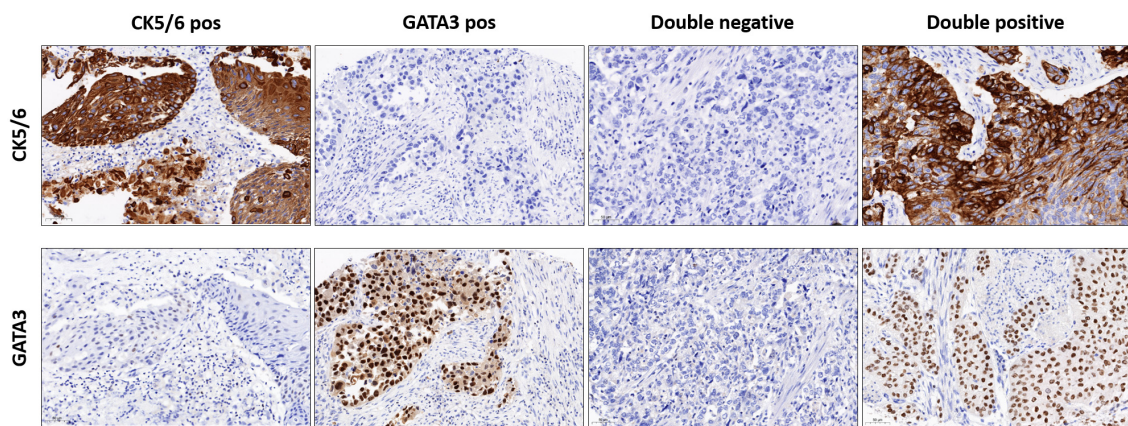
**FIGURE 1 |** Representative images of IHC staining of CK5/6 positive, GATA 3 positive, double negative, and double positive cases (magnification 200 ×).

**TABLE 2 |** Association of clinic-pathological findings with the IHC-markers CK5/6 and GATA3.

| | | *n* (%) | CK5/6 positive (*n* = 32) | GATA3 positive (*n* = 81) | Double negative (*n* = 17) | Double positive (*n* = 46) | *p* |
|---|---|---|---|---|---|---|---|
| **Age** | **<70** | 73 (41%) | 15 (47%) | 29 (36%) | 9 (53%) | 20 (44%) | *0.5* |
| | **≥70** | 103 (59%) | 17 (53%) | 52 (64%) | 8 (47%) | 26 (15%) | |
| **Gender** | **Male** | 135 (77%) | 23 (72%) | 67 (83%) | 16 (94%) | 29 (63%) | **0.021** |
| | **Female** | 41 (23%) | 9 (28%) | 14 (17%) | 1 (6%) | 17 (37%) | |
| **Max. Tumor-Stage** | **pT2** | 83 (47%) | 11 (34%) | 45 (56%) | 8 (47%) | 19 (41%) | *0.138* |
| | **pT3** | 66 (38%) | 18 (56%) | 23 (28%) | 5 (29%) | 20 (44%) | |
| | **pT4** | 27 (15%) | 3 (10%) | 13 (16%) | 4 (24%) | 7 (15%) | |
| **Histological subtype** | **NOS** | 127 (72%) | 18 (59%) | 65 (80%) | 10 (59%) | 34 (74%) | **<0.0001** |
| | **Squamous** | 25 (14%) | 14 (44%) | 1 (1%) | 0 | 10 (22%) | |
| | **Micropapillary** | 9 (5%) | 0 | 9 (11%) | 0 | 0 | |
| | **Neuroendocrine** | 3 (2%) | 0 | 0 | 3 (18%) | 0 | |
| | **Sarcomatoid** | 3 (2%) | 0 | 1 (1%) | 1 (6%) | 1 (2%) | |
| | **Plasmacytoid** | 3 (2%) | 0 | 3 (4%) | 0 | 0 | |
| | **Undifferentiated (giant cell)** | 3 (2%) | 0 | 1 (1%) | 1 (6%) | 1 (2%) | |
| | **Glandular** | 1 (0.6%) | 0 | 1 (1%) | 0 | 0 | |
| | **Clear cell** | 1 (0.6%) | 0 | 0 | 1 (6%) | 0 | |
| | **Lymphoepithelial** | 1 (0.6%) | 0 | 0 | 1 (6%) | 0 | |

*A total of 176 spots were evaluated. The p-values were calculated using Pearson–Chi square test. NOS, not otherwise specified. Bold values indicate significant differences with p-values < 0.05.*

0.4 (95% *CI*: 0.2–1.2, $p = 0.09$) in the adjuvant chemotherapy group. A multivariate cox-regression model adjusting for tumor and lymph node (LN) stage, adjuvant chemotherapy, and IHC-staining is shown in **Figure 3**. In addition to tumor- and LN stage, the double negative cases were associated with an increased risk of death (*HR*: 4.07; 95% *CI*: 1.5–10.9, $p = 0.005$). Adjuvant chemotherapy was associated with the survival benefit (*HR*: 0.19; 95% *CI*: 0.10–0.36, $p < 0.001$). The multivariate cox-regression model for DFS is shown in the **Supplementary Material**.

## Molecular Analysis of CK5/6 and GATA3 Negative Cases

Ten double negative cases were analyzed using the HTG Transcriptome Panel and molecular subtypes were determined according to the consensus classification of Kamoun et al. (6).

Three cases were classified as stroma-rich, three as NE-like and four as Ba/Sq. **Table 5** shows molecular subtypes with histological subtype and pathological stage. The Ba/Sq cases showed heterogeneous histology with lymphoepithelial, undifferentiated (giant cell) and sarcomatoid subtypes. According to the UNC classification system, all cases were classified as basal (8). And according to the TCGA classification system, cases were classified as basal-squamous and neuronal (5). Representative HE-stained pictures of histological subtype and their molecular subtype are shown in **Figure 4**.

## DISCUSSION

This study shows a high concordance of an IHC-subclassification based on the two markers CK5/6 and GATA3 with histological

**TABLE 3** | Characteristics of patients with and without adjuvant chemotherapy.

| | | CE only $n = 75$ | Adjuvant Chemotherapy $n = 35$ | p |
|---|---|---|---|---|
| Age (IQR) | | 72 (64.25–76.75) | 60 (52.75–71) | |
| Age | <71 | 32 (43%) | 26 (74%) | **0.002** |
| | ≥71 | 43 (57%) | 9 (26%) | |
| Gender | Male | 59 (79%) | 28 (80%) | *1.0* |
| | Female | 16 (21%) | 7 (20%) | |
| Stage Max. | pT2 | 23 (31%) | 5 (14%) | *0.12* |
| | pT3 | 37 (49%) | 24 (69%) | |
| | pT4 | 15 (20%) | 6 (17%) | |
| Lymph node status | pN0 | 42 (56%) | 13 (37%) | *0.084* |
| | pN1 + pNx | 33 (44%) | 22 (63%) | |
| R-Status | R0 | 60 (80%) | 28 (80%) | *1.0* |
| | R1/R2/Rx | 15 (20%) | 7 (20%) | |
| Histological subtype | NOS | 57 (77%) | 23 (66%) | *0.39* |
| | Squamous | 8 (11%) | 4 (11%) | |
| | Micropapillary | 5 (7%) | 2 (6%) | |
| | Neuroendocrine | 1 (1%) | 2 (6%) | |
| | Other | 3 (4%) | 4 (11%) | |
| Type of chemotherapy | Gem/Cis | | 28 (80%) | |
| | Gem/Carbo | | 4 (11%) | |
| | Platin/Etoposid | | 2 (6%) | |
| | other | | 1 (3%) | |
| Recurrence | No | 54 (72%) | 18 (51%) | *0.058* |
| | Yes | 21 (28%) | 17 (49%) | |
| CK5/6 + GATA3 | CK5/6 pos | 14 (19%) | 5 (15%) | *0.45* |
| | GATA 3 pos | 36 (48%) | 13 (38%) | |
| | Double neg | 7 (9%) | 6 (18%) | |
| | Double pos | 18 (24%) | 10 (29%) | |

*CE, cystectomy; NOS, not otherwise specified; Gem/Cis, gemcitabine/cisplatin; Gem/Carbo, gemcitabine/carboplatin; R-Status, resection status. Bold values indicate significant differences with p-values < 0.05.*

subtypes in MIBC. Double negative patients without expression of CK5/6 nor GATA3 had decreased *OS* rates. Subtyping of double negative cases revealed a histological and molecular heterogeneous subgroup. Strengths of our study are the



**FIGURE 2** | Kaplan–Meier curve for overall survival (OS) for patients with and without adjuvant chemotherapy.

pathological re-review of all cases, evaluation of standardized IHC-staining, the use of a broad transcriptome panel for molecular phenotyping, and the analysis of adjuvant chemotherapy in a clinically well-annotated cohort of patients with MIBC.

The use of molecular subtyping to guide the selection of systemic therapies has been proposed for NAC and immune checkpoint inhibitors (7, 10, 16). However, contradictory results published in the past years and the diversity of molecular subtype taxonomy until the publication of the consensus classification have hindered the use and clinical translation (6, 17–19). Data to provide guidance to select patients for adjuvant chemotherapy based on molecular subtypes are sparse.

It has been suggested that CK5/6 and GATA3 expression can identify basal and luminal molecular subtypes in 80–90% of cases which could be a useful tool in routine pathological assessments to identify the basic molecular subtypes of BCa (12). Other studies using more markers confirmed a good correlation of IHC-based subtyping with gene expression-based subtypes (determined by targeted NanoString panels), but still with a risk of differing classification in about 15–20% of cases (13, 20).

Our results show that CK5/6 expression was associated with squamous histological subtype and female gender, which
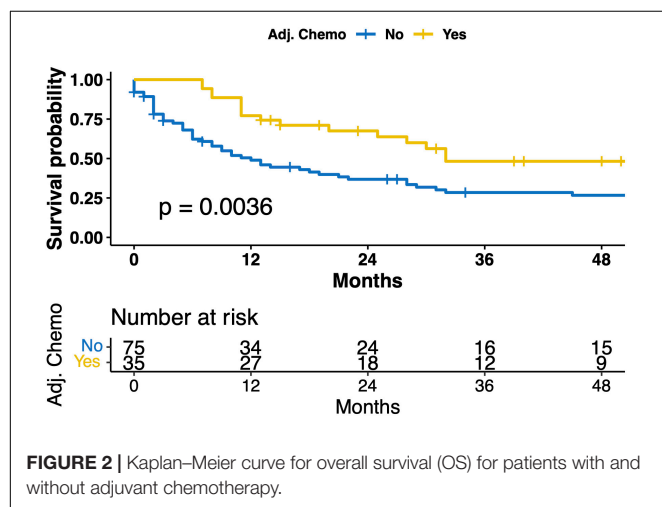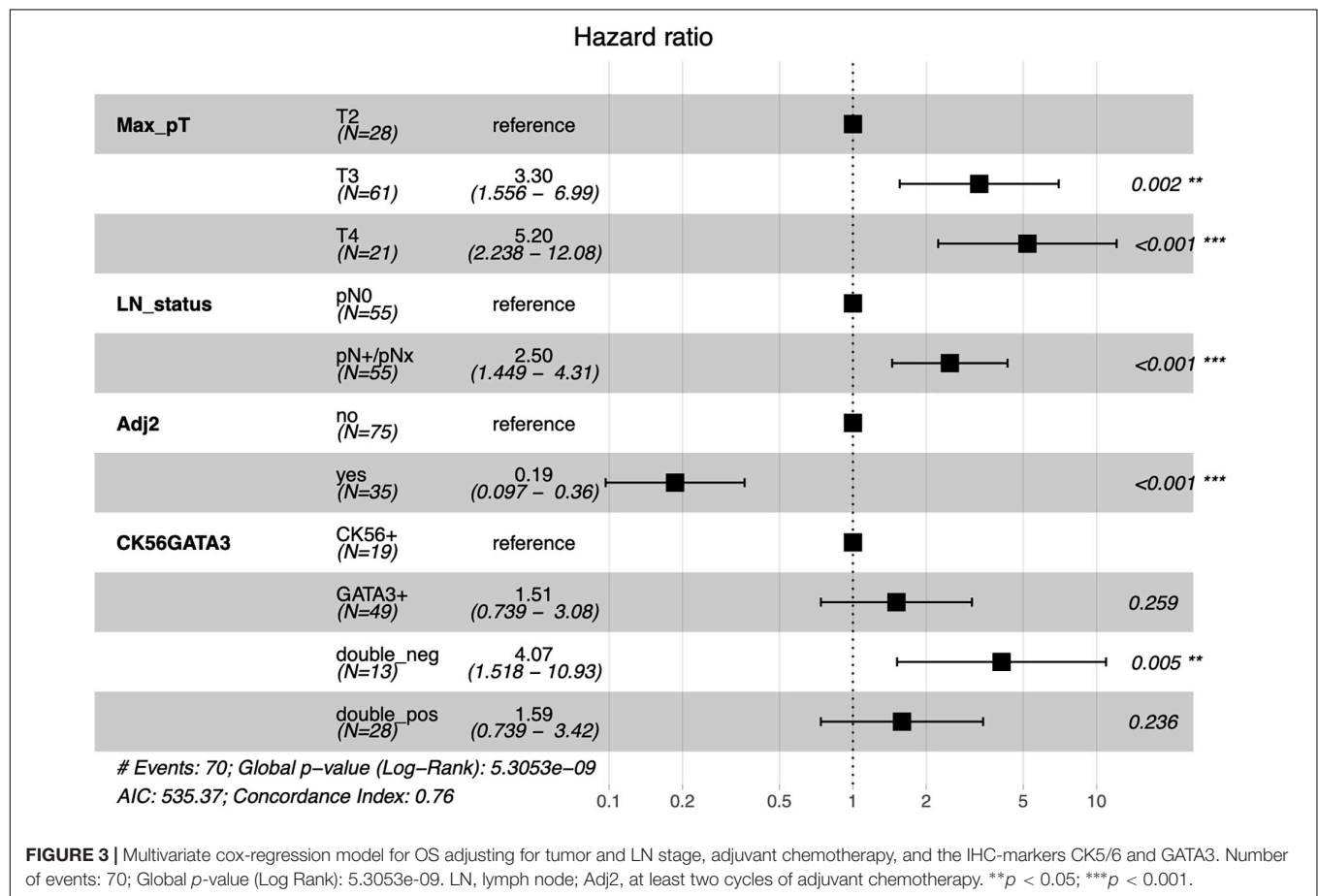
**TABLE 4 |** Univariate cox-regression model for histological subtype and IHC-markers CK5/6 and GATA3 stratified for patients with and without adjuvant chemotherapy.

| | | Total cohort (n = 110) | p | Adjuvant chemotherapy (n = 35) | p | CE only (n = 75) | p |
|---|---|---|---|---|---|---|---|
| **Histological subtype on TMA** | NOS | Reference | | Reference | | Reference | |
| | Squamous | 0.8 (0.3–1.7) | 0.5 | 0.4 (0.06–3.4) | 0.6 | 0.9 (0.4–2.2) | 0.9 |
| | Micropapillary | 1.2 (0.5–3.1) | 0.7 | 2.9 (0.6–13.1) | 0.2 | 0.89 (0.3–2.9) | 0.9 |
| | Neuroendocine | 0.9 (0.2–4.1) | 0.9 | 1.8 (0.2–14.1) | 0.6 | NA | |
| | Other | 0.7 (0.3–1.9) | 0.5 | 0.9 (0.2–4.2) | 0.9 | 0.8 (0.2–3.5) | 0.8 |
| **CK56** | Pos | 0.9 (0.5–1.4) | 0.5 | 0.4 (0.2–1.2) | 0.09 | 1.1 (0.7–1.9) | 0.7 |
| **GATA3** | Pos | 0.8 (0.5–1.3) | 0.3 | 0.7 (0.3–1.8) | 0.4 | 0.8 (0.4–1.4) | 0.4 |
| **CK56 + GATA3** | CK56 pos | Reference | | Reference | | | |
| | GATA3 pos | 1.0 (0.5–2.0) | 0.9 | 1.9 (0.4–8.8) | 0.4 | 0.9 (0.4–1.8) | 0.71 |
| | Double neg | 1.6 (0.7–3.7) | 0.3 | 0.3 (0.5–16.7) | 0.2 | 1.7 (0.6–4.7) | 0.31 |
| | Double pos | 1.0 (0.5–2.1) | 1.0 | 1.1 (0.1–5.0) | 0.8 | 1.3 (0.6–2.8) | 0.59 |

*CE, cystectomy; NOS, not otherwise specified.*



**FIGURE 3 |** Multivariate cox-regression model for OS adjusting for tumor and LN stage, adjuvant chemotherapy, and the IHC-markers CK5/6 and GATA3. Number of events: 70; Global *p*-value (Log Rank): 5.3053e-09. LN, lymph node; Adj2, at least two cycles of adjuvant chemotherapy. **$p < 0.05$; ***$p < 0.001$.

is in concordance with published data (6, 13). The same applies to the positive correlation between GATA3-expression and micropapillary histological subtype. Four cases with neuroendocrine histological subtype were double negative in IHC-analyses.

To test the two-marker-based IHC-classification for its predictive value of chemotherapy efficacy, we performed analyses in the cohort of adjuvant-treated patients. We are aware, that our survival analyses, which demonstrated a significant

benefit for patients receiving adjuvant chemotherapy, hold the risk of selection bias since patients receiving chemotherapy were younger and kidney function or comorbidities were not considered. However, when analyzing only patients receiving chemotherapy, none of the markers predicted survival, but cases expressing the basal marker CK5/6 tend to have improved survival with adjuvant chemotherapy, with the level of significance missed (*HR* 0.42 95% *KI* 0.15–1.2, *p* = 0.09). These results are in line with the available data for NAC

**TABLE 5** | Description of double negative cases analyzed for mRNA expression profiles, including histological subtypes on TMA and whole slide, and molecular subtypes according to the consensus, UNC, and TCGA (5) classifier.

| Patient | Age at surgery | Gender | Max. pT stage | LN metastases | Predominant histological subtype on TMA | Histological subtype on whole slide | Adjuvant chemotherapy | Consensus class | UNC subtype | TCGA subtype |
|---|---|---|---|---|---|---|---|---|---|---|
| 001_069 | 68 | m | 3b | Yes | Lymphoepithelial | Lymphoepithelial | Yes | Ba/Sq | Basal | Basal_squamous |
| 001_004 | 71 | m | 4a | No | Undifferentiated/Giant cell | Undifferentiated/Giant cell | No | Ba/Sq | Basal | Basal_squamous |
| 001_033 | 71 | m | 3b | Yes | NOS | NOS + Squamous | No | Ba/Sq | Basal | Basal_squamous |
| 001_039 | 60 | m | 2b | Yes | Sarcomatoid | Sarcomatoid | Yes | Ba/Sq | Basal | Basal_squamous |
| 001_012 | 57 | m | 4b | Yes | NOS | NOS | Yes | Stroma-rich | Basal | Basal_squamous |
| 001_040 | 78 | m | 4a | Yes | NOS | NOS | Yes | Stroma-rich | Basal | Basal_squamous |
| 001_065 | 70 | m | 4a | No | NOS | NOS + Pseudoglandular | No | Stroma-rich | Basal | Basal_squamous |
| 001_021 | 62 | m | 3b | No | Neuroendocrine | Neuroendocrine | Yes | NE-like | Basal | Neuronal |
| 001_050 | 62 | m | 3b | Yes | Neuroendocrine | Neuroendocrine | Yes | NE-like | Basal | Neuronal |
| 001_071 | 67 | m | 2b | No | Neuroendocrine | Neuroendocrine | No | NE-like | Basal | Neuronal |

*Ba/Sq, basal/squamous; m, male; NE, neuroendocrine; NOS, not otherwise specified; UNC, University of North Carolina; TCGA, the cancer genome atlas.*

proposing that patients with basal-like tumors respond better to chemotherapy, whereas luminal-like tumors have an inferior response to chemotherapy.

For the neoadjuvant setting, Seiler and colleagues developed a single-sample genomic subtyping classifier to subdivide patients into four groups revealing that patients with basal tumors had the most improvement in OS with NAC compared with surgery alone (10). More recent studied showed similar results for non-luminal tumors (11, 20). Contrary results from Sjödahl and colleagues as well as Taber and colleagues, however, indicated that basal tumors less frequently respond to NAC and according to Kamoun and colleagues, the consensus molecular subtypes do not correlate with response to NAC (6, 17, 18). Therefore, further research will have to clarify the role of molecular subtypes as predictive markers of chemotherapy efficacy.

In addition to the findings in cases with IHC expression of at least one marker, we detected a subgroup of double negative cases without expression of CK5/6 or GATA3. The cohort seems to be of biological significance, as patients in this group had an increased risk of death in multivariate analysis. This finding has recently been described also by other groups (13, 14). However, no detailed molecular analyses were reported, as no full transcriptomic analyses were performed (12, 13). We, therefore, conducted further molecular analyses of the double negative group using an mRNA transcriptome panel covering approximately 19,300 targets, thus enabling us to call consensus class molecular subtypes.

Double negative cases tended to be molecularly basal-like according to the UNC classifier but are more heterogenous than a two tailed-classification can represent. Three molecular subtypes (Ba/Sq, stroma-rich, neuronal) and five histological subtypes (NOS, neuroendocrine, sarcomatoid, undifferentiated/giant cell, lymphoepithelioma-like) were present in this group. This highlights the heterogeneity of double negative cases and implicates that a more complex system than a two-tailed classification might be needed for more individualized treatment decisions. For example, the (molecular and histological) neuroendocrine subtype is known to have a poor prognosis and thus should be treated aggressively with upfront chemotherapy and might be responsive to immune checkpoint inhibition (4, 16). On the other hand, the lymphoepithelioma-like subtype of urothelial carcinoma is associated with a more favorable prognosis and might be more responsive to immune checkpoint inhibition as proposed by the results of the PURE-1 trial (21, 22). The well-known fact of intra-tumoral heterogeneity in urothelial BCa adds to these facts.

The limitations of our study are the retrospective design and the low number of molecular analyses. Due to low numbers, we did not include patients receiving NAC in our survival analysis. Selecting patients for NAC, which is the recommended treatment for MIBC, might be of higher clinical relevance than for adjuvant chemotherapy. In addition, we performed two IHC-marker analyses only to surrogate basal and luminal molecular subtypes. However, this was intentionally done to create comparability with the literature. Other studies using more markers for protein-based subtyping reported similar
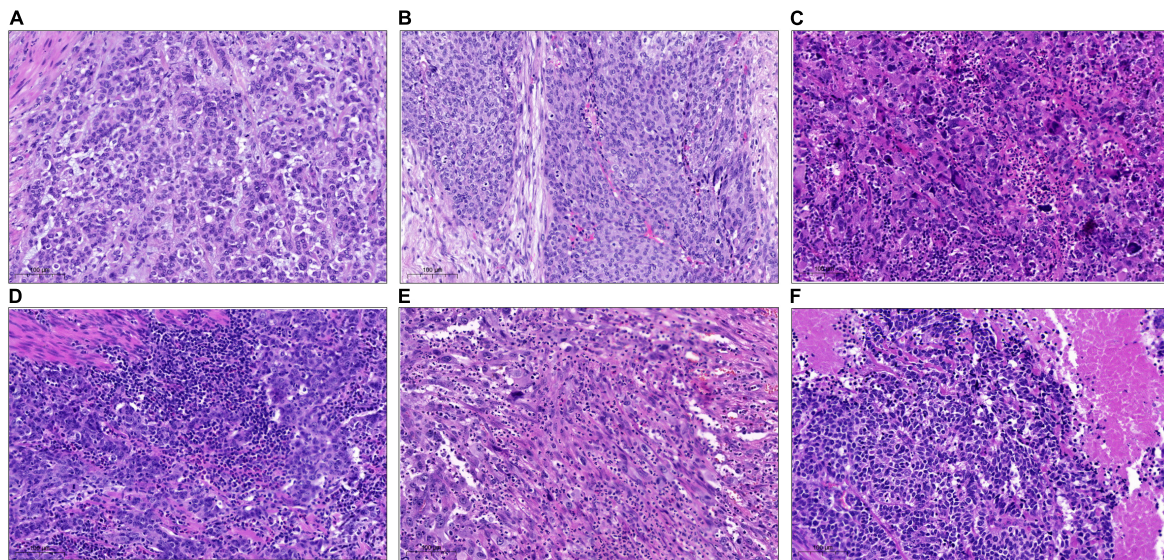
**FIGURE 4 |** Representative pictures of different histological subtypes on the whole slides classified as double negative in tissue micro array (TMA)-analysis and their molecular consensus subtypes. **(A)** Histological subtype: NOS; molecular Subtype: Stroma-rich. **(B)** Histological subtype: NOS (+ squamous on whole slide); molecular subtype: Ba/Sq. **(C)** Histological subtype: poorly differentiated/giant cell; molecular subtype: Ba/Sq. **(D)** Histological subtype: lymphoepithelial; molecular subtype: Ba/Sq. **(E)** Histological subtype: sarcomatoid; molecular subtype: Ba/Sq. **(F)** Histological subtype: neuroendocrine; molecular subtype: NE-like. Ba/Sq, basal/squamous; NE, neuroendocrine; NOS, not otherwise specified.

results with high concordance of histological subtypes and IHC, but also demonstrated that protein-based subtyping did not predict survival in population-based cystectomy cohorts (13, 19). Thus, the question remains if a limited protein-based assessment of surrogate markers for molecular subtypes can serve as a predictor for chemotherapy response or if mRNA-expression profiles are necessary for adequate determination of molecular subtypes or other markers like immune cell infiltration are necessary for patient selection (18). So far, the histopathological staging remains the most important prognostic factor (23). However, efforts to bring the results of the large transcriptomic studies into a clinical routine are underway (24).

In conclusion, we demonstrated that an IHC-based classification was associated with histological subtypes of urothelial MIBC. Although IHC markers used in pathological routine might help to identify patients with basal and luminal tumor characteristics, a two-sided classification system might not sufficiently reflect the heterogeneity of BCa to guide treatment decisions. Especially the group of IHC-double negative cases is a heterogeneous group with different implications for therapy.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The gene expression omnibus (GEO) with accession GSE198607 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198607).

## ETHICS STATEMENT

Written informed consent was obtained from all patients, and the study was approved by the Institutional Review Boards of the UCT and the Ethical Committee at the University Hospital Frankfurt (project numbers: SUG-6-2018 and UCT-53-2021).

## AUTHOR CONTRIBUTIONS

FK, HR, and PW contributed to conception and design of the study. FK, LK, SB, CW, AS, and KB organized the database and samples. AS, NB, KB, JK, HR, and PW performed data curation and formal analysis. FK and CD performed the statistical analysis and analysis of sequencing data. AS, NB, and KB performed IHC. FK wrote the first draft of the manuscript. HR, PW, and FC performed review and editing of the manuscript and supervision. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.875142/full#supplementary-material

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# The Role of Chronic Liver Diseases in the Emergence and Recurrence of Hepatocellular Carcinoma: An Omics Perspective

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Hepatocellular carcinoma (HCC) typically develops from a background of cirrhosis resulting from chronic inflammation. This inflammation is frequently associated with chronic liver diseases (CLD). The advent of next generation sequencing has enabled extensive analyses of molecular aberrations in HCC. However, less attention has been directed to the chronically inflamed background of the liver, prior to HCC emergence and during recurrence following surgery. Hepatocytes within chronically inflamed liver tissues present highly activated inflammatory signaling pathways and accumulation of a complex mutational landscape. In this altered environment, cells may transform in a stepwise manner toward tumorigenesis. Similarly, the chronically inflamed environment which persists after resection may impact the timing of HCC recurrence. Advances in research are allowing an extensive epigenomic, transcriptomic and proteomic characterization of CLD which define the emergence of HCC or its recurrence. The amount of data generated will enable the understanding of oncogenic mechanisms in HCC from the CLD perspective and provide the possibility to identify robust biomarkers or novel therapeutic targets for the treatment of primary and recurrent HCC. Importantly, biomarkers defined by the analysis of CLD tissue may permit the early detection or prevention of HCC emergence and recurrence. In this review, we compile the current omics based evidence of the contribution of CLD tissues to the emergence and recurrence of HCC.

Keywords: hepatocellular carcinoma, chronic liver disease, genomics, epigenetics, transcriptomics, proteomics, metabolomics

## INTRODUCTION

Despite the extensive characterization of the molecular landscape of hepatocellular carcinoma (HCC), the mechanisms through which the liver tissue drives the emergence and recurrence of HCC remain poorly understood. The most common etiologies of chronic liver disease (CLD) are chronic hepatitis B or C viral infection (HBV and HCV, respectively), alcohol-related liver disease (ALD), and non-alcoholic fatty liver disease (NAFLD) (1, 2). The unresolved

inflammation associated with CLD often leads to fibrosis and subsequent cirrhosis, characterized by a buildup of scar tissue in the liver. Moreover, the resulting oxidative stress, through generation of reactive oxygen and nitrogen species in proliferating hepatocytes, as well as inflammatory cells responding to molecules released by damaged hepatocytes, promotes an environment that accelerates malignant transformation and survival of hepatocytes (3).

Despite surgical treatment in HCC yielding high survival outcomes at 5 years, the incidence of intrahepatic recurrence after curative resection remains unsatisfactory at 70% (1). Whereas, early recurrences (<2 years) typically arise from micrometastases following resection, late recurrences (>2 years) are consistent with a phenomenon known as the "field effect," where de novo tumors arise due to an inflammatory carcinogenic microenvironment (4). In recent years, the focus of next generation sequencing profiling has shifted from delineating genomic biomarkers in tumoral tissue to acquiring a multi-omic overview of the tumor microenvironment (TME) and the characterization of the contribution of CLD tissue to HCC development. This review summarizes the current omics knowledge about HCC emergence and recurrence by investigating the putative involvement of CLD tissues.

## THE ROLE OF CHRONIC LIVER DISEASE IN HCC EMERGENCE

### The Role of the Genetic Landscape in CLD in HCC Emergence

Several monogenic diseases trigger the development of cirrhosis and increase the risk for CLD-HCC initiation. These diseases are characterized by germline mutations of genes including HFE1 (encoding MHC-I like molecules (5), ATP7B liver copper homeostasis (6), FAH [required during the tyrosine catabolic pathway (7), UROD essential for heme biosynthesis (8)], glucose-6-phosphatase (G6Pase) (9, 10) and SERPINA1 [cofactor for TTR expression (11, 12)]. In addition, numerous single-nucleotide polymorphisms (SNPs) have been reported to be associated with HCC development (13). HCC-associated SNPs are not by themselves pathogenic per se, but require an additional cause of CLD. For example, the interplay between aflatoxin B1, HBV and SNPs of GSTM1 and GSTT1 (null alleles) increases the risk of HCC emergence (14). While SNP of PNPLA3 (rs738409) is strongly associated with CLD (ALD and NAFLD) related HCC (15–17), its contribution is minor in HCC promoted by HCV infection (18, 19). Interestingly, in the context of hepatic fibrosis, cells overexpressing PNPLA3 promoted immune cells chemotaxis (monocytes and macrophages) as compared to their wild type PNPLA3 carriers (20). In addition to SNP of PNPLA3 (rs738409), SNPs in TM6SF2 (rs58542926), and HSD17B13 (rs72613567) increase and decrease the risk of alcohol-related cirrhosis HCC, respectively (21). Whereas, SNPs in WNT3A-WNT9A (rs708113) were found to exert a protective role in hepatocarcinogenesis (22).

The higher mutational burden found in CLDs compared to the normal liver suggests positive selection shapes the genomic landscape during HCC emergence (23–27). Analysis of somatic mutations across healthy, ALD- and NAFLD-livers revealed that somatic mutations in FOXO1, CIDEB, and GPAM detected in CLD tissue showed convergent evolution. Of note, mutations in metabolism genes were observed in both ALD and NAFLD but less frequently in HCC (28). Ng et al. identified 7 significantly mutated genes across 122 HCC biopsies and showed 7% of samples harbored GPAM mutations (7 of 9 were frameshift mutations), which were more frequent than mutations in cancer-related genes such as ARID2 and RB1 (29). Mutations were identified in CLD tissues with allelic frequencies suggesting clonal expansion in CLD (26). Some non-driver mutations may become an advantageous asset for the liver's ability to not only withstand injuries but also to regenerate. In this study, genes involved in the control of liver regeneration were the most recurrently mutated within cirrhotic nodules, including driver mutations in cancer-related gene ARID1A, which controls gene expression associated with emergence of injury-induced liver progenitor-like cells (30). Recently, the relationship between ARID1A expression and immune cells that infiltrate tumors was clarified, whereby ARID1A expression in hepatocytes was positively associated with activated eosinophils, T helper cells, central memory T and Th2 cells (31). Frequent and cancer-promoting mutations in ARID1A and TP53 were also acquired in subpopulations of hepatocytes in cirrhotic regenerating nodules, where an increase in clonal size was primarily observed in CLD but rarely in HCC cases (24). It is now well-established that alterations in TP53 signaling pathways affect the TME at all stages of HCC development from initiation to metastasis, i.e., via hepatocytes, hepatic stellate cells (HSCs), immune cells, as well as cancer stem cells (32, 33). In one of the studies exploring the role of p53 in the immune response arising from embryonic liver progenitor cells, mutual H-Ras expression and TP53 downregulation resulted in HCC development. The data indicated that p53 exerted a cytostatic effect by blocking proliferation and triggering cellular senescence, rendering cancer cells susceptible to recognition by the immune system (34). Cancer removal was affected by immune cells, including macrophages, neutrophils and NK cells (34–36). Non-hepatocyte tumor suppressive effects of TP53 were mediated by its activity in HSCs. Lujambio et al. tested the conditional inactivation of p53 in HSCs during liver fibrosis. Depletion of p53 resulted in increased fibrosis and excessive extracellular matrix production, which eventually led to tumor occurrence and liver failure. It was shown that the formation of HCC did not directly evolve from p53-depleted HSCs, rather its deletion in these cells created a niche for hepatocytes malignant transformation (36). In addition, p53 expression levels (high or depleted) of HSCs were shown to modulate the different tumor-inhibiting phenotype of macrophages (M1 or M2, respectively) (36). It is important to stress that the previously mentioned experiments were performed in immunologically-compromised mice, thereby without an appropriate immune response. Nevertheless, these results support a model whereby physiological p53 activity in hepatocytes and HSCs facilitate tumor clearance. Liver injury also affects p53 in HSCs. For instance, following liver injury, the increase of p53 levels trigger

senescence of HSCs, thus promoting clearance of the scar tissue by immune cells. This response can be viewed as a balancing factor between regeneration and malignant transformation: p53 signaling protects from transformation while activating progenitor cells for compensatory liver repopulation (36, 37).

Mutational signatures in cirrhotic liver are conserved in HCC (27), including aging-associated single base substitution and indel mutational signatures in CLD, relating to increased proliferation after tissue regeneration (38). Together with senescence, a state of permanent cell cycle arrest, short telomeres in hepatocytes are hallmarks of cirrhosis (39), where shortening occurs more quickly than through aging alone. *TERT* promoter mutations occur predominantly at two hotspots of chromosome 5 (40) and one of the key mechanisms involved in malignant transformation of cirrhotic hepatocytes is the reactivation of telomerase by mutations in its promoter such as the well-characterized −124 and −146 C>T transitions. In line with these observations, the *TERT* promoter is frequently mutated in premalignant cirrhotic nodules early during hepatocarcinogenesis (23). Recently, hotspot mutations in the *TERT* promoter were found to be altered in a focal nodular hyperplasia lesion and clonally related HCC lesions (41). In contrast to other studies, Kim et al. found that *TERT* promoter mutations were only detectable in HCC, yet not in the cirrhotic nodules (24). These findings suggest that *TERT* promoter mutations can frequently act as a gatekeeper event during the transformation sequence, but are not always necessary for HCC emergence. Importantly, mutations arising in regenerating nodules of CLD tissues suggest an early carcinogenic event depicting clonal expansion in the stepwise malignant transformation from CLD to HCC (**Figure 1**).
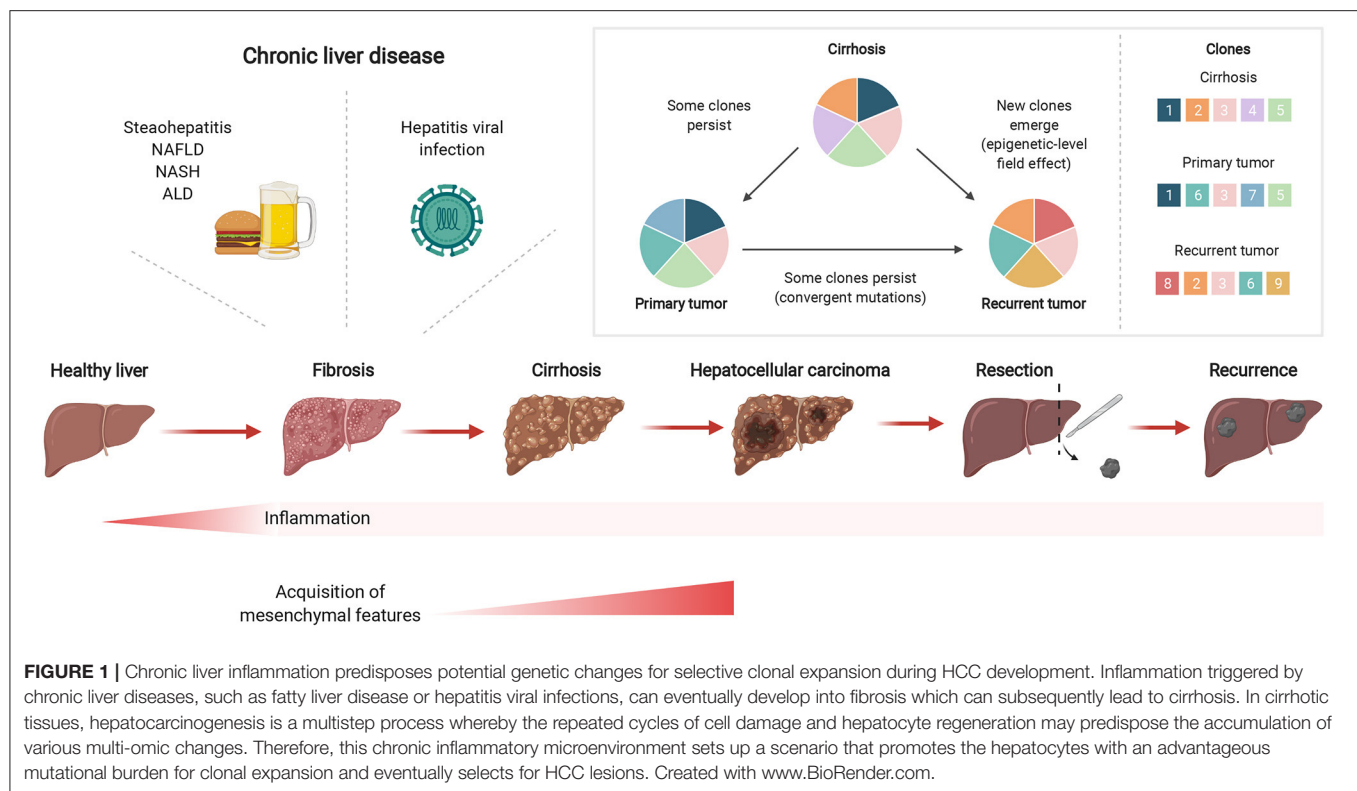
## Epigenetic Alterations Predispose Cells to Tumorigenesis

CLDs trigger mechanisms that lead not only to the accumulation of genetic mutations but also of various cooperative epigenetic changes. Epigenetic silencing plays a key role in HCC emergence, where promoter methylation of tumor suppressor genes such as *RASSF1A* are noted at high frequency during the progression from cirrhosis to HCC (42). Okamoto et al. reported that hepatitis infections trigger an innate immune response dependent on natural killer (NK) cells to elicit DNA methylation, including methylation of *RASSF1A* (43). Comparison of promoter methylation profiles in the CLD tissue from HCC patients (cirrhotic vs. non-cirrhotic) identified aberrant promoter methylation of *UGT1A7* and *PLG,* genes which function in fibrogenesis and detoxification of carcinogens, respectively (44). These results highlight the importance of elucidating the underlying mechanisms of CLD beyond the genomic level to better understand the evolution of HCC. In line with this, our group recently demonstrated that CLDs trigger the formation of an epigenetic niche that predisposes cells to oncogenic alteration (45). We developed a CLD-associated DNA methylation score with prognostic value in HCC. High CLD methylation score was associated with higher levels of *TP53* somatic alterations in HCC, a poor prognostic

marker (46). Additionally, we showed that despite not harboring genetic alterations observed in HCC, CLD samples showed hypomethylation of genes such as *HDAC11,* which is highly expressed in immune cells (47), *UBD (FAT10),* involved in the inflammatory response and immune cell infiltration (47, 48), and *TAGLN2* that controls liver cancer cell motility (47–49). These genes are reported to be upregulated in HCC and in certain cases also to promote tumor progression (50–52). In this way, epigenetic changes during CLD influence the emergence of HCC by epigenetic regulation of gene expression, and could also create a permissive environment for the acquisition of genetic alterations that influence HCC prognosis.

## Transcriptomic Dysregulation in CLD Contributes to Emergence of HCC

Gene expression in CLD patients is associated with HCC outcomes, suggesting that the environment in which HCC develops exerts an influence on tumor development (12, 45, 53, 54). One study investigated gene expression in non-tumoral liver tissues in patients with early-stage HCC and in liver tissue from patients with cirrhosis without HCC (55). They identified an immune-regulatory gene expression signature which is associated with risk of HCC development in cirrhotic patients (median follow up, 10 years). Our group observed increased expression of immune gene sets in CLD but not HCC samples, in line with the shift toward a suppressive immune environment in HCC to allow tumor growth (45, 56). The overexpression of *CTNNB1*, associated with mutations in this gene, occurred in both cirrhotic tissues and tumors and correlated with higher expression of Wnt pathway target genes involved in apoptosis and proliferation, such as *c-Myc* (57). Interestingly, missense mutations in exon-3 of *CTNNB1* do not promote *CTNNB1* overexpression but cause an aberrant activation of the Wnt signaling pathway by preventing phosphorylation and the degradation of ß-catenin. The subset of cases with exon-3 mutations shows a more aggressive phenotype of HCC (58). Of note, *Wnt/CTNNB1* mutations correlate with a significant reduction of activated immune cells and immune activation pathways with an increase of M2-type macrophages in HCC patients (58–61). Although often driven by mutation affecting its promoter, TERT overexpression was shown to be associated with macrophage activation in patients with ALD. The study unveiled the cross-talk of TERT with the NF-κB pathway during macrophage polarization (62). Transcriptomic analysis of premalignant cirrhotic nodules and early HCCs also suggests that the MYC transcription signature may play a role in malignant transformation (63). Indeed, E2F and MYC-targets gene sets were also found to be upregulated compared to normal liver in a progressive manner from CLD to HCC (45). In addition, current data indicates a significant link between hepatic inflammation mediators and c-Myc in CLDs. The relationship between c-Myc and aberrant expression of inflammatory mediators play a central role in fibrosis, cirrhosis, and liver cancer. For example, c-Myc upregulates IL-8, IL-10, TNF-α, and TGF-β, while its expression is promoted by IL-1, IL-2, IL-4, TNF-α, and TGF-β (64). Cancer-related pathways,

**FIGURE 1** | Chronic liver inflammation predisposes potential genetic changes for selective clonal expansion during HCC development. Inflammation triggered by chronic liver diseases, such as fatty liver disease or hepatitis viral infections, can eventually develop into fibrosis which can subsequently lead to cirrhosis. In cirrhotic tissues, hepatocarcinogenesis is a multistep process whereby the repeated cycles of cell damage and hepatocyte regeneration may predispose the accumulation of various multi-omic changes. Therefore, this chronic inflammatory microenvironment sets up a scenario that promotes the hepatocytes with an advantageous mutational burden for clonal expansion and eventually selects for HCC lesions. Created with www.BioRender.com.

including epithelial-to-mesenchymal-transition (EMT), were identified to be transcriptionally dysregulated in precancerous lesions, independent of genetic alterations (45). The process of EMT in fibrogenic disorders includes phenotypic plasticity, where epithelial cells may dedifferentiate to a mesenchymal-like state in the hostile inflammatory environment, and acquire features such as increased motility (65, 66). Overall, these data highlight how dysregulation of transcriptional programmes may already be activated in CLD and contribute to tumorigenesis.

Tumors occur in a liver microenvironment comprising endothelial, immune and hepatic stellate cells, which contributes to the development of HCC through a complex network of intercellular interactions. Single cell RNA-sequencing (scRNA-seq) enables the study of this heterogeneous environment at the level of individual cells and captures the mutual regulatory mechanisms within a tissue. The transcriptomic profiles of individual cell populations in the liver microenvironment from 4 patients with healthy liver tissues, 3 patients with cirrhosis and 16 cases of HBV-related HCC tissues have been investigated using scRNA-seq and demonstrated the increasing immune cell involvement in each tissue from normal to HCC, relating to inflammatory damage from cirrhosis and the immune response (54). From eight identified genes whose expression in endothelial and stellate cells (liver-resident fibroblasts activated in CLD to support the tumor-promoting microenvironment) correlated with survival in HCC patients (54), four were potential oncogenes with high expression in HCC (*CKS2, HSP90AB1, RPL12, S100A6*) and two were potential tumor-suppressor genes (*CCL14* and *CD5L*) with low expression. The importance of

these genes has been corroborated in other studies, for example the silencing of *CKS2* has indeed been shown to inhibit cancer cell proliferation in HCC cells (67). Xing et al. reported that *CD5L* was decreased in liver tissues (54), however CD5L has also been found to be upregulated in HCC and liver cirrhosis (68–72) with a functional involvement in cellular proliferation promotion and antiapoptotic responses by binding to HSPA5 (73). CD5L further emerges as a key player in fibrosis in the setting of CDL as it exerts a protective effect on fibrosis by a combination of mechanisms, including protection from damage, prevention from fibrosis and immune cell infiltration (74). Indeed, CD5L prevents neutrophil and monocyte-derived macrophage infiltration in the inflamed liver (74). Zhang et al. performed scRNAseq on healthy liver, cirrhosis and HCC patients to reveal the landscape of cirrhosis-associated immune cell infiltration in HCC (75). By looking both at differential genes in the B cell developmental trajectory from healthy liver to CLD to HCC and the tumor-associated genes between cirrhotic and malignant hepatocytes, the authors identified enrichment for the humoral immune response pathway and response to oxidative stress (75). In the hepatocytes, they identified key prognostic genes (*FTCD, MARCKSL1, CXCL3, RGS5, KNG1,* and *S100A16*) and JUN transcription factor was suggested to play a role in malignant transformation. In a study investigating tumor-infiltration in HCC at the multi-omics level, *SPP1* was indicated as an immune-related predictor of poor survival and mediator of macrophage-HCC cell interactions (76). Dong et al. used scRNA-seq to compare gene expression between tumor and inflammatory para-tumor tissue, inferring *MLXIPL* as an

important transcription factor in the route from normal liver cells to HCC subclones (77). Profiling gene expression in single cell populations in CLD is an expanding area and has the potential to reveal the role of individual cell types in HCC emergence, thus far revealing expression of genes in hepatocytes, and also endothelial cells, stellate cells and B cells, for regulating tumor proliferation and the immune response in the damaged CLD microenvironment.

## Elucidating the Role of CLD in HCC Emergence Through Proteomics and Metabolomics Profiling

Proteomic- and metabolomic-based profiling adds valuable layers of information to the molecular landscape, since proteins and interactions between them are fundamental to cellular behavior. Functional proteomic analysis with regard to three major pathways in the liver (metabolism, complement and coagulation cascades) revealed a division of labor and complementarity among the different liver cell types. Upon mapping liver-disease-related genes to the healthy liver cell types, a high degree of enrichment was noted in the non-parenchymal cells (HSCs, Kupffer cells, liver sinusoidal endothelial cells and immune cells around the hepatocytes (78), highlighting the important regulative role of these cells in immunologic responses and disease development (79). During the progression of chronic liver injury toward fibrosis, the local interaction between Kupffer cells and resident liver macrophages is initiated, triggering the release of cytokines and chemokines (80, 81). The activation and proliferation of HSCs produce Extracellular matrix (ECM) proteins that subsequently form a fibrous scar (82–84). A further comprehensive serum proteomic analysis evaluated changes in protein levels between cirrhosis patients with and without HCC and discovered 21 novel diagnostic biomarkers for HCC enriched for the complement and coagulation cascade pathway (85). Proteins found differentially expressed in cirrhotic samples, FGFR4, TPM4, TPM2, LAALS3BP, and APOA1, were identified as the center of respective protein-protein interaction networks in CLD (86). Pathway enrichment of these networks suggest key roles for these proteins not only in the pathology of cirrhosis but potentially in HCC progression. Aberrant expression of FGFR4 has been reported to contribute to HCC progression (87), and proteins in the corresponding network were enriched for the MAPK cascade, which plays a pivotal role in HCC development due to activation by upstream growth factors (88), whereas APOA1 is prognostic for survival in HCC patients and predictive of early recurrence. Further research is required in following CLD cases to identify predictive biomarkers and to assert the role of CLD in shaping the proteome in HCC development, as large proteogenomics studies have thus far focused on characterizing the tumoral tissue in HCC (89, 90).

Metabolomics provides holistic information on dynamic metabolic responses resulting from perturbations caused by CLD. Metabolites related to ammonia recycling, the urea cycle, and amino acid metabolism were found to discriminate between HCC and cirrhosis. A panel of metabolites including methionine, proline, ornithine, pimelylcarnitine, and octanoylcarnitine
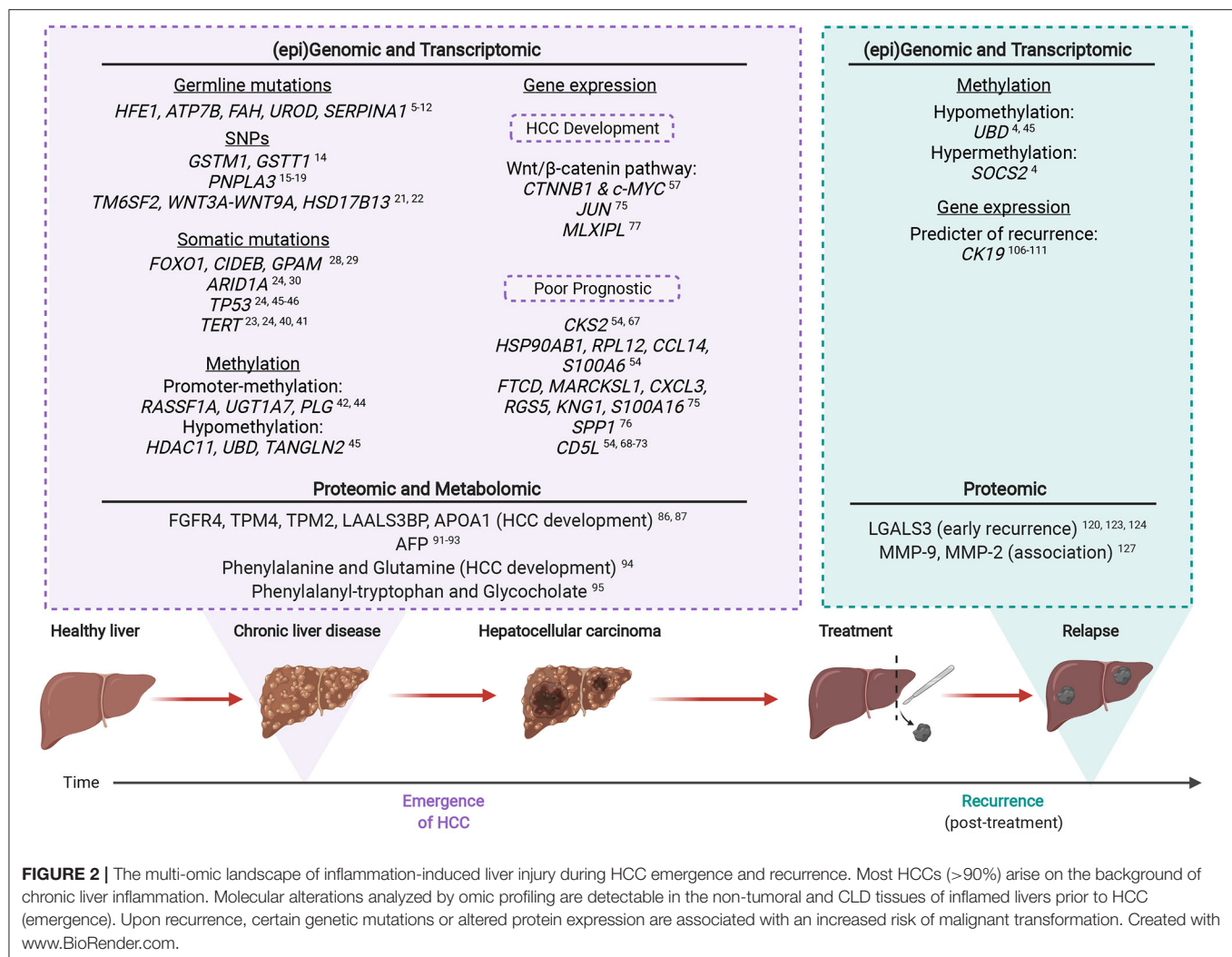
demonstrated higher diagnostic accuracy compared to alpha-fetoprotein (AFP) alone and was validated in another cohort (91). Nevertheless, AFP plays an important role in the development and progression of HCC as it activates the PI3K/AKT/mTOR pathway to promote expression of oncogenes such as Ras, Src, and CXCR4. It also leads to immune evasion by inducing apoptosis in antigen-presenting cells and inhibiting proliferation of infiltrating immune cells (92, 93). In an exploratory research, phenylalanine and glutamine were associated with development of HCC in patients with cirrhosis (94) while a larger multicentre study, analyzing a panel of metabolites, identified and phenylalanyl-tryptophan and glycocholate and validated these in an independent cohort. The panel showed a reliable HCC diagnosis in high-risk cirrhosis which, in combination with AFP, improved the panel's sensitivity (95). Metabolomics results indicate the utility of identifying perturbed metabolic pathways for determining biomarkers for cirrhotic patients that are at high risk of developing HCC, but metabolomic profiles of cirrhotic patients differ depending on the underlying etiology (96). There have been a number of studies on the metabolome of cirrhotic patients, as listed by Khan et al. (96), or in studies that discriminate HCC from cirrhotic background (97) which may explain altered pathways during hepato-carcinogenesis. However, there is not a uniform consensus regarding shift of metabolite expression in the emergence of HCC from CLD. This is in part due to the technical limitations including large numbers of metabolites exceeding analysis capabilities, too few metabolites in the database to garner true positive results, or challenges in experimental reproducibility, as well as differences in clinical characteristics in the HCC cohorts between studies.

# MOLECULAR ALTERATIONS IN NON-RESOLVED LIVER INFLAMMATION PREDICT HCC RECURRENCE

Recurrence of HCC after therapy or resection represents an important clinical issue as it affects up to 70% of patients within 5 years (1, 2), with risk of late recurrence doubling upon the presence of liver cirrhosis (98). Current evidence suggests that inflammatory signals might increase tumor fitness and reduce expression of "stress ligands" on cancer cells (99–101) thereby allowing the outgrowth of malignant clones (102). This is especially evident in HCC where inflammation-induced tissue injury in CLD, cell death and liver regeneration following resection potentiate tumor outgrowth.

## The Epigenetic Predisposition of CLD Tissues for HCC Recurrence

Recurrent tumors have been shown to harbor distinct genomic profiles and more aggressive phenotypes compared to primary tumors in HCC, suggesting an intracellular reprogramming during tumor recurrence (103). The carcinogenic environment of CLD may foster tumor recurrence through selective pressures for clonal outgrowth as micrometastases, or by formation of multiple *de novo* tumors, with the latter clinically defined as recurrence despite being genetically independent from the

**FIGURE 2 |** The multi-omic landscape of inflammation-induced liver injury during HCC emergence and recurrence. Most HCCs (>90%) arise on the background of chronic liver inflammation. Molecular alterations analyzed by omic profiling are detectable in the non-tumoral and CLD tissues of inflamed livers prior to HCC (emergence). Upon recurrence, certain genetic mutations or altered protein expression are associated with an increased risk of malignant transformation. Created with www.BioRender.com.

primary. Recently, Ding et al. profiled matched initial/recurring tumors and neighboring cirrhotic/fibrotic liver in 133 HCC patients at the genetic and epigenetic (DNA methylation) levels (4). In this study, whole genome sequencing identified a patient with independent multifocal tumors originating from different clones. By contrast, aberrant methylation patterns were maintained from cirrhosis to both tumors, suggesting epigenetic alterations already existed in the CLD tissue. Many early methylation changes linking CLD to HCC were shown to target genes involved in inflammation-associated tumorigenesis, including *SOCS2* hypermethylation and *UBD* hypomethylation, the latter of which was also found hypomethylated in CLD samples by Gallon et al. (45). Interestingly, SOCS2 has a critical role during tumor-immune surveillance (104) and in restraining deleterious immune responses upon severe liver injury (105).

Overall, aberrant methylation patterns and epigenetic alterations in HCC reflect their corresponding cirrhotic/fibrotic livers in the majority of cases, including both initial and recurring tumors. Therefore, these underlying mechanisms that exist in the liver before carcinogenesis highly influence the risk of carcinogenesis from liver diseases to HCC and evidence the relevance of the epigenetic-level field effect.

## Expression of Inflammatory Pathway Genes in CLD Tissues Associate With HCC Recurrence

Persistent liver damage and concomitant non-resolved inflammation is known to alter the genomic landscape of the liver. As discussed above, this specific genetic background subsequently promotes the transformation of hepatocytes into progenitor cells. However, the switch from a normal to a malignant setting has been shown to be marked by the expression of cytokeratin *CK19* in hepatocytes in the presence of inflammation or other stimuli (106). A large number of studies showed that *CK19* also is aberrantly expressed in HCC and gene signatures of this progenitor marker were independent predictors of HCC recurrence after liver transplant (107–111). Despite a lack of association between gene expression profiles in tumor tissues and HCC recurrence in a cohort of 106 patients,

a gene-expression signature in the non-tumoral adjacent liver tissues was found to be predictive of late recurrence and overall survival (53). In this study, the genes found to be indicative of poor prognosis were not only enriched for the NF-kB inflammatory response but also for oxidative stress and IL-6 growth signaling pathways. The gene set was further validated in non-tumor tissues with varying etiology, indicating the robustness of the late recurrence signature. Interestingly, death of hepatocytes is known to trigger expression of IL-6 and other growth factors to promote survival and growth of neighboring mutated hepatocytes (112). IL-6 signaling mediated by tumor-associated macrophages was found to promote a cancer stem cell phenotype in HCC, which due to the self-renewal and tumor-initiating capacity of stem cells, is involved in recurrence and metastasis. NF-kB (activated by IL-6) and STAT3 pathways are key orchestrators connecting non-resolving inflammation with HCC, which control the expression of genes involved in cell proliferation and immune functions (3). Transcriptional studies also demonstrate enhanced expression of distinct inflammatory cytokines and chemokines in primary tumors and metastatic lesions (113, 114). In line with this, intrahepatic metastasis in HBV-related HCC was found to be associated with a Th2-dominant cytokine gene expression profile in non-cancerous hepatic tissues from metastatic HCC patients (115). Chronic hepatitis infections result in the production of a cirrhotic liver which can promote the formation of *de novo* tumors, leading to late recurrence. In an effort to identify gene signatures related to HCC recurrence, Okamoto et al. profiled the gene expression of liver tissue of HCV patients and created a prediction score which could stratify the risk of patients of multicentric HCC recurrence (116). To understand gene expression signatures in HCV patients, Tsuchiya and colleagues profiled the tumor and non-tumor tissues of HCV-positive HCC patients. The resulting gene set successfully separated patients with and without HCC recurrence and further analysis revealed that hepatic nuclear factor 4-alpha and interferon gamma were the two main networks involved during HCC recurrence (117). Overall, these results demonstrate that non-tumoral transcriptional profiles can predict HCC recurrence independent of expression in the tumors, pointing toward the tumor microenvironment as being implicated in recurrence after resection. Furthermore, non-resolved inflammation and activation of inflammatory pathways are key in HCC late recurrence and disease progression.

## Proteomics-Level Evidence of Immune Cell Involvement in HCC Recurrence

Tumor-promoting inflammation closely resembles inflammatory mechanisms typically found during immune responses. Hence, the study of the proteome within the inflamed non-tumoral background of HCC provides a framework for analyzing cellular networks and a reference for altered communication associated with pathology. The control of liver inflammation and regeneration is mainly orchestrated by infiltrating inflammatory cells. Upon hepatic injury, resident Kupffer cells, circulating monocytes, macrophages, dendritic cells and neutrophils interact to maintain the liver tissue architecture. Interestingly, studies

have shown that upon inflammation triggered by CLDs or surgery, myeloid cells can act in a dual mode, that is by promoting regeneration or, on the contrary, driving inflammatory processes that could aid tumor recurrence (118). In healthy tissues, myeloid cells play a vital role in tissue repair, as well as protective immunity through cytokine secretion and phagocytosis. However, in the underlying environment of non-resolving inflammation in HCC, myeloid cells are shifted toward immunosuppressive and tumor-promoting states such as tumor-associated macrophages and myeloid-derived suppressor cells (119). Between patients with recurrent and non-recurrent HCC, most differentially expressed proteins also belonged to immune system pathways (120). This included LGALS3, which was associated with early recurrence and ability of tumors to suppress the immune system. LGALS3 secreted by the cells of the TME alters immune cell function by inhibiting the expression of T cell receptors and preventing NK cell receptor binding to antigen (121, 122). Interestingly, this lectin was previously found to be expressed in HCC and focal regenerating nodules of cirrhotic tissue, despite being absent in normal hepatocytes (123, 124), possibly representing an early neoplastic event. Autophagy is a fundamental pathway that has multiple effects on immunity and inflammation, by influencing the development, homeostasis and survival of both immune and inflammatory cells. Of note, low concentration of the autophagy-related marker LC3 on HCC and adjacent liver microenvironment can be a significant predictor of both early and late HCC recurrence (125). Interestingly, autophagy proteins in the myeloid cells of the TME contribute to immune suppression of T lymphocytes by effecting LC3-associated phagocytosis. Altered LC3-associated phagocytosis in the myeloid compartment, subsequently induces control of tumor growth by tumor-associated macrophages upon phagocytosis of dying tumor cells (126). An additional class of enzymes involved in HCC recurrence which are typically expressed by the "pro-restorative" macrophage infiltrate (118) are matrix metalloproteinases (MMPs). MMPs are a family of proteolytic enzymes that perform multiple roles in the normal immune response to infection. MMP-9 overexpression by tumor-associated macrophages is associated with higher invasive potential of HCC (118). Interestingly, MMP-9 and MMP-2 positive staining in stromal tissue adjacent to tumors is associated with HCC recurrence in patients with underlying cirrhosis (127). Taken together, these results highlight the key roles of cells in the inflamed tumor microenvironment in cancer development and recurrence.

The key omic factors involved in CLD-HCC are summarized in **Figure 2**, from studies which indicate that HCC emergence and recurrence highly depend on the genomic, transcriptomic, proteomic and metabolomic background of CLD tissues.

## CONCLUSION

The molecular mechanisms underlying HCC tumorigenesis from a background of CLD are gradually being unveiled through recent sequencing efforts. Omics based studies facilitate the study of global changes in biomolecules in a high

throughput manner, and hence are well-poised to reveal the complex changes which lead to HCC emergence and recurrence. Convergent mutations underlie clonal expansion from CLD, indicating selective pressure from the damaged environment, with aberrant methylation patterns from CLD persisting in HCC. In the emergence of HCC from CLD, *TERT* promoter mutations aid hepatocyte fitness and there is evidence of hypomethylation associated with upregulation of tumor-promoting genes, as well as epigenetic silencing of tumor suppressor genes. Current studies support the "epigenetic priming" model, whereby epigenetic changes induced by a prolonged chronic state sensitize cells to genetic mutations and altered transcriptional landscape, triggering tumorigenic progression and recurrence. Further implicated in the emergence of HCC are transcriptional dysregulation of genes involved in the WNT/b-catenin pathway and enrichment of the humoral response pathway, whereas pathways implicated in recurrent HCC include IL-6 growth signaling, NF-kB, IFN-gamma and HNF4a pathways. During both emergence and recurrence, evidence from multi-omic studies predominantly highlights members of the inflammatory response, underlying the key role of a chronically inflamed microenvironment as the molecular link between CLD and HCC. We are only now starting to appreciate the dynamicity, heterogeneity and complexity of the initial steps required to predispose CLD tissues to full blown HCC. Overall, the expansion of ongoing analysis of non-tumoral tissue and CLD will have a major impact in the management of HCC, thus supporting the necessary development and refinement of preventive therapeutic strategies that reflect the molecular dysregulations associated with the CLD background.

## AUTHOR CONTRIBUTIONS

SP, MC-L, and JG: conceptualization. SZ and GB: literature research and preparation of the first draft of the manuscript. SZ: figure design. SP, CN, MC-L, GH, SS, OK, JG, and GB: critical revision and editing. All authors have read and agreed to the published version of the paper.

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# Investigation of Functional Synergism of CENPF and FOXM1 Identifies POLD1 as Downstream Target in Hepatocellular Carcinoma

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**Background:** Lines of evidence implicate CENPF and FOXM1 may have novel co-operative roles in driving hepatocellular carcinoma (HCC).

**Objective:** We investigated the clinicopathological correlation, functional characterization, molecular mechanism and translational significance of CENPF and FOXM1.

**Methods:** We carried out integrative studies investigating functional synergism of CENPF and FOXM1 in HCC and its metastasis. Human HCC samples, HCC cell lines and mouse model were used in the studies. Stable knockdown, q-PCR, Western blotting, whole-transcriptomic sequencing (RNA-seq), as well as cell and mouse assays were performed.

**Results:** Upon clinicopathological correlation, we found that co-overexpression of CENPF and FOXM1 in human HCCs was associated with more aggressive tumor behavior including presence of venous invasion, tumor microsatellite formation, and absence of tumor encapsulation. Moreover, co-silencing FOXM1 and CENPF using shRNA approach in HCC cell lines resulted in significantly reduced cell proliferation. Furthermore, our RNA-seq and differential gene expression analysis delineated that CENPF and FOXM1 co-regulated a specific set of target genes in various metabolic processes and oncogenic signaling pathways. Among them, POLD1, which encodes the catalytic subunit of DNA polymerase δ, was ranked as the top downstream target co-regulated by CENPF and FOXM1. POLD1 expression was positively correlated with that of FOXM1 and CENPF in HCCs. In addition, POLD1 expression was significantly upregulated in HCC tumors. Functionally, *in vivo* orthotopic injection model showed that stable knockdown of POLD1 in HCC cells suppressed tumor incidence and tumorigenicity and had a trend of diminished lung metastasis.

**Conclusion:** Taken together, our data suggest that CENPF and FOXM1 could synergistically support hepatocarcinogenesis via the regulation of POLD1. CENPF and FOXM1 may represent new vulnerabilities to novel drug-based therapy in HCC.

**Keywords: CENPF, FOXM1, POLD1, liver cancer, HCC, functional synergism**

# INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide (1, 2) and the second and third commonest cancer, respectively, in China and Hong Kong. Indeed, 55% of all new liver cancers worldwide each year occur in China including Hong Kong, due to a high prevalence of hepatitis B viral (HBV) infection (3, 4). It has a poor prognosis and only few effective treatment options are available. Despite years of efforts in studying the molecular mechanism of HCC carcinogenesis, current understanding on this lethal disease is still limited. In a recent study of our group (5), we have utilized whole-transcriptome sequencing technology to perform a differential gene expression (DGE) analysis using the dataset of 50 pairs (tumor and the corresponding non-tumorous liver tissue) of HCC cases from The Cancer Genome Atlas (TCGA). By comparing the gene expression in HCC tumors and their corresponding non-tumorous liver tissues, differentially expressed genes in HCC were identified. Among the 734 differentially expressed genes, CENPF and FOXM1 were listed as the first and third most upregulated genes respectively in HCC. Both FOXM1 and CENPF are crucial for cell-cycle progression, especially in the G2/M phase.

In a study on mitosis regulation and aging, CENPF was demonstrated to be a direct target of the cell cycle master regulator FOXM1 (6). Hence, it is traditionally believed that CENPF is a downstream target of FOXM1 and under the regulation by FOXM1. Interestingly, CENPF and FOXM1 were predicted to be master regulators of prostate cancer malignancy in a cross-species computational analysis by comparing interactomes of human and mice (7), and experimental validation demonstrated that they function synergistically to promote tumor growth by regulating prostate cancer-associated target gene expression profiles. Knockdown of CENPF and FOXM1 synergistically reduced the proliferation of cancer cells and tumor growth in cell-line-derived xenografts. It was further demonstrated that knockdown of CENPF expression reduced the binding of FOXM1 to its targets, suggesting CENPF is required for appropriate genomic binding by FOXM1. Additional data showed that they were co-localized in nucleus and their subcellular co-localization was mutually dependent.

Taken together, multiple lines of evidence imply CENPF and FOXM1 may have novel cooperative roles in regulating the expression of shared target genes. Hence, we postulate that they may have functional synergism in driving hepatocarcinogenesis.

# MATERIALS AND METHODS

## Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted by Trizol (Thermo Fisher Scientific, Waltham, MA, United States) and cDNA was synthesized by a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, United States). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with target-specific TaqMan probes (Thermo Fisher Scientific, Waltham, MA, United States)

listed in **Supplementary Table 1**. The mRNA expression was normalized by the expression of the housekeeping gene *HPRT*.

## Clinical Specimens and Clinicopathological Correlation Analysis

The primary HCC specimens and their corresponding non-tumorous liver tissues of randomly selected 118 human HCC cases were surgically resected from HCC patients in Queen Mary Hospital of Hong Kong between year 1991 and 2017. None of the patients had received therapies before hepatic tumor resection. Total RNA of these 118 pairs clinical specimens were isolated for subsequent qRT-PCR and clinicopathological correlation analysis using SPSS24.0 software, as previously described (8). We did not differentiate into micro- or macrovascular invasion. However, although the venous invasion included both microvascular and macrovascular invasion, mostly it was microvascular invasion. The tumor microsatellites were defined as microscopic or small tumor nodules less than 1 cm in diameter and in close proximity to the main tumor. Direct liver invasion was defined as invasion of the tumor cells into the non-tumorous liver parenchyma without separation by tumor capsule or fibrous layer (9). The use of clinical specimens was approved by the Institutional Review Board of the University of Hong Kong and the Hospital Authority.

## Cell Lines and Culture Conditions

Hepatocellular carcinoma cell line Hep3B (HB-8064) was obtained from the American Type Culture Collection (ATCC) and HCC cell line Huh7 (JCRB0403) was obtained from JCRB Cell Bank. MHCC97L was a gift from Liver Cancer Institute, Fudan University. Hep3B cells were cultured in Minimum Essential Medium (MEM) supplemented with 1mM sodium pyruvate (NaPy). MHCC97L cells were cultured in Dulbecco's modified Eagle minimal high glucose essential medium (DMEM-HG) supplemented with 1mM NaPy. Huh7 cells were cultured in DMEM-HG media. All cell culture media mentioned above were further supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin unless otherwise specified. Cell line cultures were maintained in 37°C and 5% $CO_2$ incubator.

Authentication of HCC cell lines used in this study was performed by short tandem repeat (STR) DNA Profiling in March 2018 and no cellular cross-contamination was detected. STR result for MHCC97L is provided in **Supplementary Figure 1**. Cell cultures were tested negative for Mycoplasma contamination. "Xenome," utilizing RNA-seq data, estimated a negligible 0.04–0.42% (*n* = 3) for MHCC97L, while 0.15–0.40% for clinical human NTL and HCC samples (*n* = 6) with mouse contamination, thus indicating our MHCC97L cells do not contain cells of murine origin (10). Furthermore, MHCC97L used in this study contains HBV integration in the TERT locus of the genome (8).

## Stable Lentiviral-Based Short-Hairpin RNA Knockdown Cell Models

FOXM1, CENPF, and POLD1 were stably knocked down in MHCC97L HCC cells by the lentiviral-based short-hairpin

RNA (shRNA) approach as previously described (11, 12). The oligonucleotide sequences encoding non-targeted control (shNTCC) shRNA and shRNAs that specifically target FOXM1 (shFOXM1), CENPF (shCENPF), and POLD1 (shPOLD1) (Integrated DNA Technologies, Coralville, IA, United States) were summarized in **Supplementary Table 2**. The forward and reverse oligonucleotides were reannealed to generate shRNA cassettes and each of them was individually cloned into pLKO.1-Puro plasmid with puromycin resistance gene as selection marker. For lentiviral packaging, individual shRNA plasmid was co-transfected with the packaging mix into 293FT cells by lipofectamine 2000 transfection reagent (Invitrogen) at a plasmid to lipofectamine ratio (μg:μL) of 1:2. The viral supernatants were harvested 48 h post-transfection and used to transduce MHCC97L cells. About 1 μg/mL puromycin was applied to the viral-transduced cells for at least 4 days to select for stable knockdown clones. The knockdown efficiencies of FOXM1 and CENPF were examined by RT-qPCR and western blot.

## Western Blot Analysis

Cells were lysed by 6X protein sample buffer (0.35 M Tris-HCl, pH 6.8, 30% glycerol, 20% SDS, 9.3% DTT and 0.05% bromophenol blue) and resolved by SDS-polyacrylamide gel electrophoresis. Immunoblots were incubated with primary antibody rabbit anti-human FOXM1 (D12D5) XP (Cell Signaling Technology, Danvers, MA, United States), rabbit anti-human CENPF (D6X4L) (Cell Signaling Technology, Danvers, MA, United States) and mouse anti-human β-actin (Sigma Aldrich, St. Louis, MO, United States) at 4 C overnight, followed by horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (Sigma Aldrich, St. Louis, MO, United States) at room temperature for 2 h. The chemiluminescence signal was detected with the ECL detection system (GE Healthcare, Lafayette, CO, United States).

## Cell Proliferation Assay by Direct Cell Counting

About $2 \times 10^4$ cells of each cell line were seeded into each well of multiple 24-well culture plates in triplicates and incubated in a 37°C humidified incubator with 5% $CO_2$. Cell growth was assessed by determining the number of cells in each well every 24 h for 4 consecutive days. Direct cell counting was performed using COULTER COUNTER Z1 Cell and Particle Counter (Beckman Coulter).

## Cell Growth Inhibition Assay by XTT Assay

Cell Proliferation II Kit (XTT) (Roche, Basel, Switzerland) was used to determine the percentage of cell growth inhibition according to the manufacturer's instructions.

## Animal Studies

All animal studies were approved by the Animals (Control of Experiments) Ordinance of Hong Kong and the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (CULATR number: 3848-15), and strictly following the institutional regulations and guidelines. All animal studies were performed on 4 to 6 weeks old BALB/c-nu/nu (nude) athymic male mice, which were provided by the Centre for Comparative Medicine Research of the University of Hong Kong.

## *In vivo* Orthotopic Liver Injection Model

*POLD1* was knocked down in luciferase-labeled MHCC97L (MHCC97L-Luc) HCC cells by shRNA approach. The orthotopic liver injection was performed on 4 to 6 weeks old BALB/c-nu/nu (nude) athymic male mice to assess the metastatic potential of the injected HCC cells as previously described (13). About $1 \times 10^6$ of MHCC97L-luc stable cells were resuspended in 15 μL of Matrigel Basement Membrane Matrix (Corning) diluted with serum-free cell culture medium in a 1:1 ratio and then injected into the left lobe of the livers of mice by a 29-gauge needle (Hamilton, Reno, NV, United States). The abdominal wound was sutured after the injection. At 6-week post-injection, bioluminescence imaging of the xenografts was performed. Mice were anesthetized with Pentobarbital at 80 mg/kg, followed by injection of D-luciferin at 100 mg/kg (Perkin-Elmer, Waltham, MA, United States) into the tumor-bearing mice intraperitoneally. Bioluminescence images were acquired by IVIS Spectrum *in vivo* imaging system (Perkin-Elmer, Waltham, MA, United States) to measure the total flux of the bioluminescent signals emitted from the dissected liver tumor xenografts and the distant lung metastases.

## Identification of FOXM1 and CENPF Co-regulated Genes by RNA-Sequencing

RNA-sequencing (RNA-seq) was performed to identify downstream target genes that were co-regulated by FOXM1 and CENPF. FOXM1 and CENPF were either individually silenced or co-silenced in Huh7, Hep3B and MHCC97L using small-interfering RNA (siRNA) approach and subjected to RNA-seq subsequently. Before transfection, $1.5 \times 10^5$ per well of cells were seeded onto a 6-well plate (for RNA extraction) and $3.5 \times 10^5$ per well of cells were seeded onto a 60-mm plate (for protein extraction) and were incubated in a 37°C humidified incubator with 5% $CO_2$ overnight. The siRNAs of the non-target control (NTC), FOXM1 and/or CENPF (Dharmacon, GE Healthcare, Lafayette, CO, United States) listed in **Supplementary Table 3** were transfected into the HCC cells with DharmaFECT 1 transfection reagent (Dharmacon, GE Healthcare, Lafayette, CO, United States). The cells were incubated for further 72 h and then subjected to RNA and protein extraction. Real-time PCR and western blot analysis were performed to confirm the knockdown efficiencies of FOXM1 and CENPF. Successful individual and co-knockdown cells were subjected to bioanalyzer analysis (Agilent) for RNA sample quality control followed by RNA-seq analysis (polyA and mRNA) at the Center for PanorOmic Sciences of the University of Hong Kong. The RNA-seq data has been deposited to NCBI SRA (PRJNA800214).

The DGE profiles of FOXM1 and/or CENPF knockdown HCC cell lines were analyzed by edgeR. Furthermore, additional filters were applied to confine the candidate gene list co-regulated
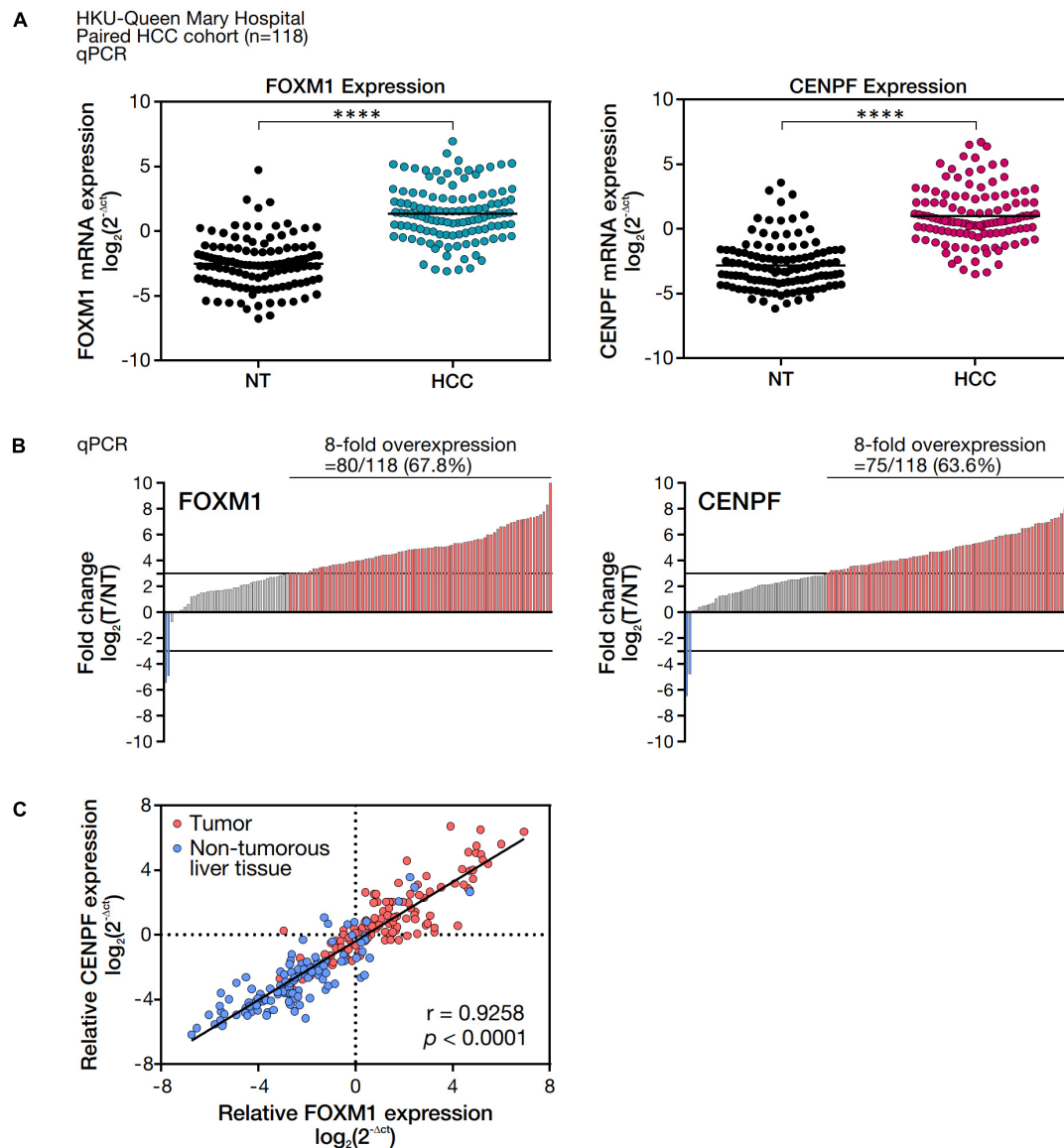
**FIGURE 1 |** FOXM1 and CENPF mRNA expression level and correlation analysis in HCC tumor and the corresponding non-tumorous liver tissues. **(A)** mRNA expression levels of FOXM1 and CENPF in 118 pairs of HCC and NT samples. mRNA expressions were normalized by housekeeping gene, *HPRT*. **(B)** Waterfall plot showing overall distribution of the fold change of FOXM1 and CENPF mRNA expression in 118 HCC clinical paired samples. **(C)** Pearson correlation analysis between FOXM1 and CENPF relative mRNA expression. Statistical significance was calculated by one-sample *t*-test (****$p < 0.001$).

by FOXM1 and CENPF based on four criteria: (i) they were differentially expressed with a *p*-value less than 0.05; (ii) with log CPM (counts per million) greater than 1; (iii) with an absolute log fold change greater than 1; and (iv) they were commonly differentially expressed in all 3 HCC cell lines with co-knockdown of FOXM1 and CENPF. Criteria (i)–(iii) applied to DGE analysis using both TCGA and our in-house whole-transcriptome sequencing of HCC clinical cases. To validate with DGE analysis results from the knockdown cell lines, correlation analysis between the candidate genes and FOXM1 and/or CENPF was performed using the TCGA and our in-house whole-transcriptome sequencing cohort of HCC clinical cases (14). The

activated and repressed candidate genes that were potentially co-regulated by FOXM1 and CENPF were also subjected to gene set enrichment analysis (15).

## RESULTS

### Expression and Clinical Relevance of FOXM1 and CENPF in Human HCCs

To validate FOXM1 and CENPF expression in our cohort of HCC patients, we examined the abundance of mRNA expression of FOXM1 and CENPF in 118 randomly selected pairs of HCC

**TABLE 1 |** Clinicopathological correlation analysis of FOXM1 and CENPF.

| Clinical parameters | | FOXM1 expression(T/NT) | | | CENPF expression(T/NT) | | | FOXM1 and CENPF expression (T/NT) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ≥8-Fold | <8-Fold | *p*-Value | ≥8-Fold | <8-Fold | *p*-Value | ≥8-Fold | <8-Fold | *p*-Value |
| Gender | Male | 63 | 28 | 0.348 | 60 | 31 | 0.223 | 54 | 37 | 0.446 |
| | Female | 17 | 10 | | 15 | 12 | | 15 | 12 | |
| Venous invasion | Yes | 48 | 19 | 0.204 | 49 | 18 | **0.011*** | 45 | 22 | **0.022*** |
| | No | 32 | 19 | | 26 | 25 | | 24 | 27 | |
| Tumor encapsulation | Yes | 23 | 15 | 0.146 | 20 | 18 | 0.057 | 18 | 20 | 0.059 |
| | No | 57 | 22 | | 55 | 24 | | 51 | 28 | |
| Tumor microsatellite formation | Yes | 48 | 16 | **0.045*** | 47 | 17 | **0.010*** | 44 | 20 | **0.009*** |
| | No | 31 | 22 | | 27 | 26 | | 24 | 29 | |
| Direct liver invasion | Yes | 32 | 10 | 0.123 | 29 | 13 | 0.254 | 27 | 15 | 0.225 |
| | No | 44 | 25 | | 42 | 27 | | 38 | 31 | |
| Tumor size | >5 cm | 53 | 22 | 0.221 | 50 | 25 | 0.204 | 46 | 29 | 0.228 |
| | ≤5 cm | 26 | 16 | | 24 | 18 | | 22 | 20 | |
| Cirrhosis | Yes | 35 | 23 | 0.066 | 36 | 22 | 0.445 | 31 | 27 | 0.183 |
| | No | 45 | 15 | | 39 | 21 | | 38 | 22 | |
| Chronic liver disease | Yes | 73 | 37 | 0.205 | 68 | 42 | 0.140 | 62 | 48 | 0.084 |
| | No | 7 | 1 | | 7 | 1 | | 7 | 1 | |
| Cellular differentiation (Edmonson's grading) | I–II | 34 | 18 | 0.403 | 33 | 19 | 0.560 | 31 | 21 | 0.459 |
| | III–IV | 45 | 20 | | 41 | 24 | | 37 | 28 | |
| Tumor stage | I/II | 25 | 16 | 0.195 | 22 | 19 | 0.093 | 21 | 20 | 0.195 |
| | III/IV | 53 | 22 | | 51 | 24 | | 46 | 29 | |

*Clinicopathological correlation analysis between the indicated clinical parameters and the individual and co-expression of FOXM1 and CENPF in human HCC (n = 118) at an 8-fold difference cut-off. Statistical analyses were done by SPSS software 24.0. Asterisks (\*) indicate statistically significant (p < 0.05). T; HCC tumorous tissue; NT; corresponding non-tumorous liver tissue. P values are shown in bold to emphasize they are < 0.05.*
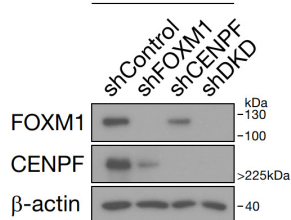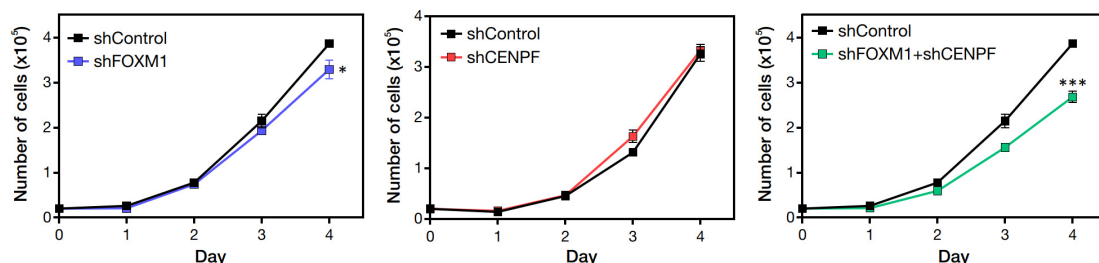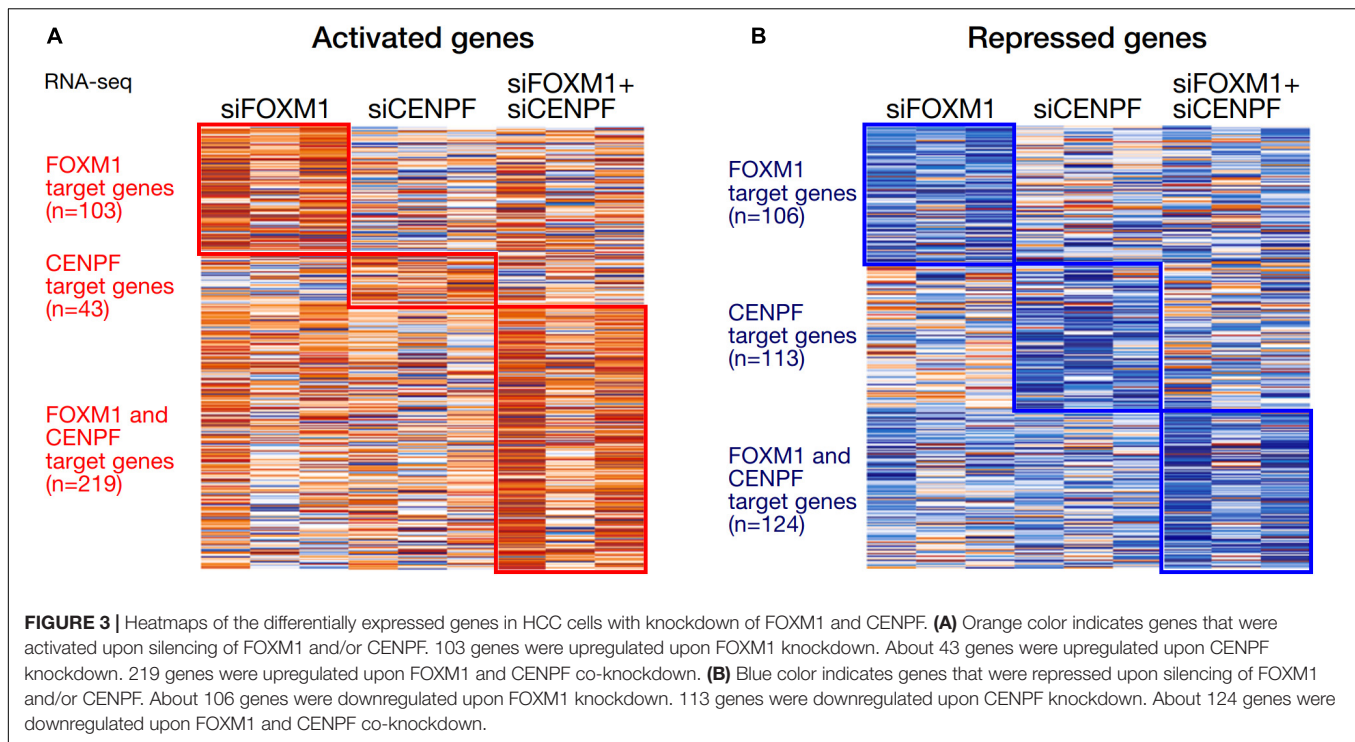


**FIGURE 2 |** Pro-proliferative role of FOXM1 and CENPF. **(A)** Western blot analysis showing single and double knockdown efficiencies of FOXM1 and CENPF in MHCC97L-Luc cells. **(B)** Representative cell proliferation assay showing the growth curves of HCC cells with single and co-knockdown of FOXM1 and CENPF for four consecutive days. Statistical significance was calculated by one-sample *t*-test (\**p* < 0.05; \*\*\**p* < 0.001). shDKD, shRNA double knockdown of FOXM1 and CENPF cells.

**FIGURE 3** | Heatmaps of the differentially expressed genes in HCC cells with knockdown of FOXM1 and CENPF. **(A)** Orange color indicates genes that were activated upon silencing of FOXM1 and/or CENPF. 103 genes were upregulated upon FOXM1 knockdown. About 43 genes were upregulated upon CENPF knockdown. 219 genes were upregulated upon FOXM1 and CENPF co-knockdown. **(B)** Blue color indicates genes that were repressed upon silencing of FOXM1 and/or CENPF. About 106 genes were downregulated upon FOXM1 knockdown. 113 genes were downregulated upon CENPF knockdown. About 124 genes were downregulated upon FOXM1 and CENPF co-knockdown.

clinical samples (tumor, HCC, and corresponding non-tumorous liver tissues, NT) by real-time RT-PCR analysis. By comparing the mRNA expression levels between the HCC and NT samples, both FOXM1 and CENPF were shown to be significantly upregulated (****$p < 0.0001$), respectively (**Figure 1A**). The overall distribution of FOXM1 and CENPF expression in our cohort was displayed in the waterfall plot (**Figure 1B**). Using a cut-off at 8-fold difference between HCC and NT, 80 of the 118 HCC cases (67.8%) showed an overexpression of FOXM1, and 75 of the 118 HCC cases (63.6%) showed an overexpression of CENPF, whereas only 2 HCC cases (1.69%) had under-expression of either FOXM1 or CENPF. Interestingly, when we compared the relative expression of FOXM1 and CENPF in HCC and NT, respectively, we observed that they were positively correlated with each other ($r = 0.9258$, $p < 0.0001$), and substantially higher expressions in the tumor samples (**Figure 1C**).

To examine the clinical relevance of FOXM1 and CENPF, either in terms of their expression alone or co-expression, clinicopathological correlation analysis was performed. Using a threshold of 8-fold difference between T and NT, we showed that the overexpression of FOXM1 was positively correlated with the presence of tumor microsatellite formation ($p = 0.045$), whereas the overexpression of CENPF was positively correlated with the presence of venous invasion ($p = 0.011$) and the presence of tumor microsatellite ($p = 0.010$). Remarkably, co-overexpression of FOXM1 and CENPF were positively correlated with the presence of venous invasion ($p = 0.022$) and the presence of tumor microsatellite formation ($p = 0.009$), while the absence of tumor encapsulation ($p = 0.059$) was also suggested but did not reach statistical significance (**Table 1**).

## FOXM1 and CENPF Were Critical for Cell Proliferation

FOXM1 and CENPF were individually silenced and co-silenced in MHCC97L-Luc HCC cell line by shRNA approach. We confirmed the successful knockdown by Western blot analysis (**Figure 2A**). To investigate the role of FOXM1 and CENPF in cell proliferation *in vitro*, the established stable knockdown cell lines were analyzed by anchorage-dependent cell proliferation assay. Co-knockdown of FOXM1 and CENPF cells showed the most significantly reduced proliferation rate ($p < 0.05$) (**Figure 2B**).

## Identification of Commonly Regulated Genes by FOXM1 and CENPF Using RNA-Seq

To investigate the potential synergistic underlying mechanism of FOXM1 and CENPF in HCC, we have established single and co-knockdown FOXM1 and CENPF cell lines using small-interfering RNA (siRNA) transient knockdown approach for subsequent RNA-seq. siRNA knockdown cell lines were established in Huh7, Hep3B and MHCC97L HCC cells. The knockdown efficiencies were confirmed by real-time RT-PCR and western blot analysis (**Supplementary Figure 2**). Among all, siFOXM1-6, siCENPF-10 and siFOXM1-6/siCENPF-10 exhibited strongest knockdown efficiencies consistently in all 3 HCC cell lines. Thus, they were used for RNA-seq, together with the siRNA control cells.

We analyzed the expression profiles from the HCC cell lines in which they were individually silenced or co-silenced by DGE analysis. From the DGE analysis across all 3 sets of knockdown cell lines, we have identified 209 genes that
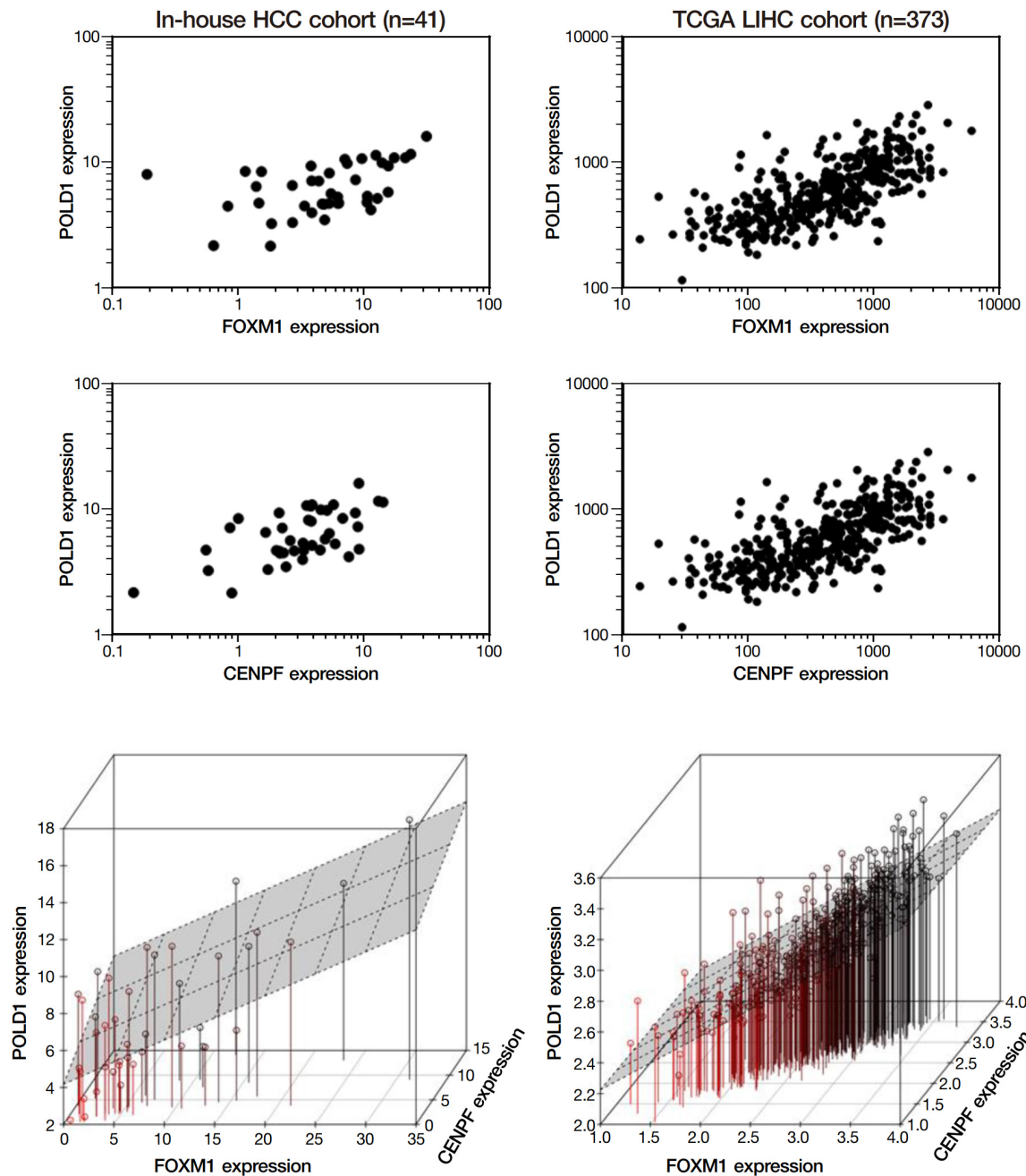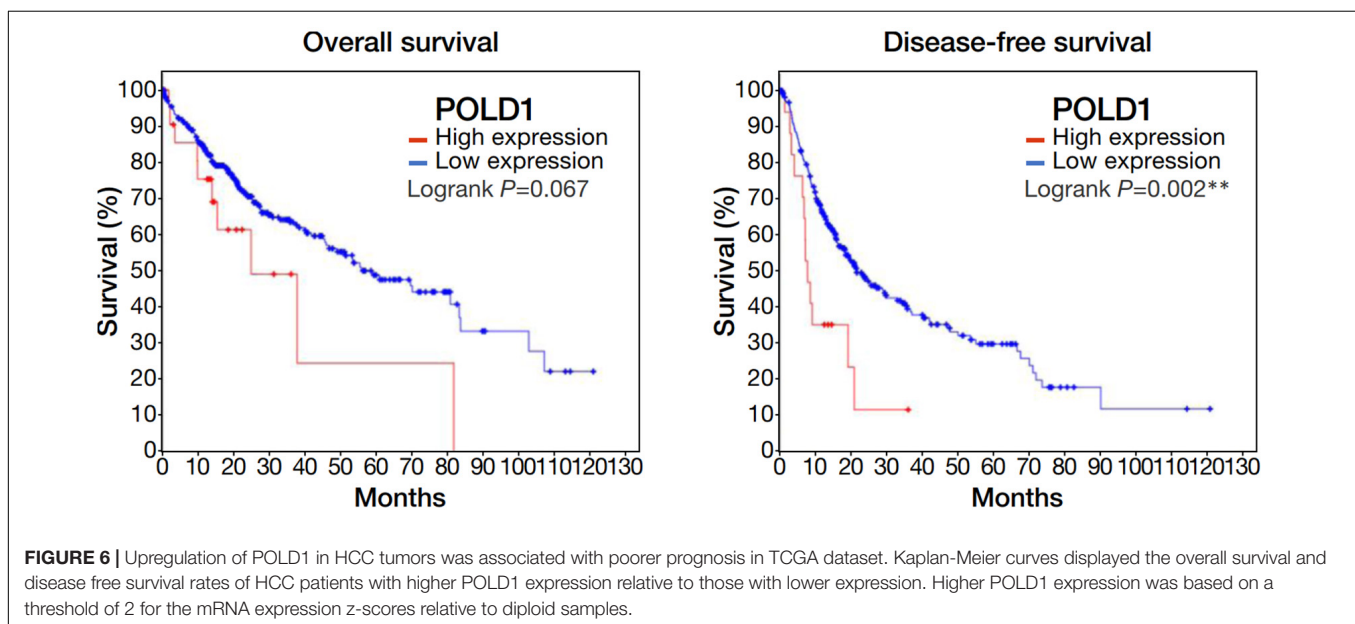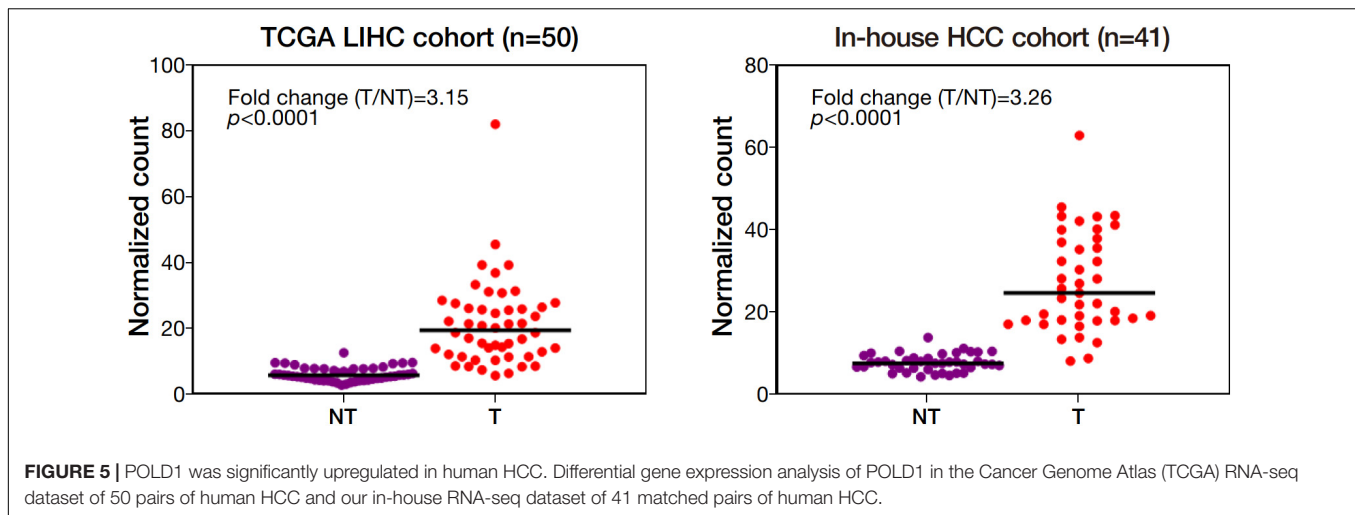
**FIGURE 4 |** POLD1 was positively correlated with FOXM1 and CENPF. Correlation analysis among the list of repressed candidate genes using our in-house RNA-seq dataset and the TCGA RNA-seq dataset. Both analyses indicated POLD1 was significantly correlated to both FOXM1 and CENPF ($p < 0.05$). Three-dimensional plot showed the correlation between the expressions of FOXM1, CENPF and POLD1 in both datasets.

were regulated by FOXM1, with 103 being activated and 106 being repressed upon FOXM1 knockdown; 156 genes that were regulated by CENPF, with 43 being activated and 113 being repressed upon CENPF knockdown; and 343 genes that were co-regulated by FOXM1 and CENPF, with 219 being activated and 124 being repressed upon co-knockdown of FOXM1 and CENPF (**Figure 3**). Among 343 candidate genes that were potentially being co-regulated by FOXM1 and CENPF, we further filtered

out 29 genes that were activated and 4 genes that were repressed upon co-knockdown of FOXM1 and CENPF in HCC cells (**Supplementary Figure 3**).

To elucidate the molecular pathways underlying the synergistic interaction of FOXM1 and CENPF, we subjected the list of activated and repressed candidate genes for gene set enrichment analysis using two databases, including Gene Ontology (biological process) and Reactome Pathway. We found

**FIGURE 5** | POLD1 was significantly upregulated in human HCC. Differential gene expression analysis of POLD1 in the Cancer Genome Atlas (TCGA) RNA-seq dataset of 50 pairs of human HCC and our in-house RNA-seq dataset of 41 matched pairs of human HCC.



**FIGURE 6** | Upregulation of POLD1 in HCC tumors was associated with poorer prognosis in TCGA dataset. Kaplan-Meier curves displayed the overall survival and disease free survival rates of HCC patients with higher POLD1 expression relative to those with lower expression. Higher POLD1 expression was based on a threshold of 2 for the mRNA expression z-scores relative to diploid samples.

that the differentially expressed genes were mainly enriched in biological pathways associated with various metabolic processes and biological signaling pathways. Notably, these genes were also enriched in several signaling pathways that are associated with tumorigenesis, including insulin growth factor (IGF) and platelet-derived growth factor (PDGF) signaling pathways (**Supplementary Figure 4**).

Among the 4 repressed gene candidates (**Supplementary Figure 3**), correlation analysis was performed using our in-house RNA-seq and the TCGA dataset. We found that *POLD1* (DNA Polymerase Delta 1) is positively correlated with both FOXM1 and CENPF, respectively and concurrently, in both RNA-seq datasets (**Figure 4**). In addition, *POLD1* expression was significantly upregulated in HCC tumor when compared with the corresponding liver tissue in TCGA RNA-seq dataset (fold change = 3.15, $p < 0.0001$) and in our in-house RNA-seq dataset (fold change = 3.26,

$p < 0.0001$) (**Figure 5**). Furthermore, we found that HCC patients with higher expression of *POLD1* had shorter overall survival and disease-free survival rates from the TCGA data analysis (**Figure 6**).

## Effect of Knockdown of POLD1 in Tumorigenicity and Lung Metastasis in HCC

We knockdown *POLD1* (shPOLD1) in MHCC97L HCC cells by shRNA approach and performed *in vivo* liver orthotopic injection assay to assess the functional role of *POLD1* in liver tumorigenicity and lung metastasis. We showed that shPOLD1 strongly reduced tumor incidence. The tumor sizes (measured by total flux signal) were significantly reduced in mice injected with shPOLD1 HCC cells. We also observed a trend of diminished lung metastasis upon knockdown
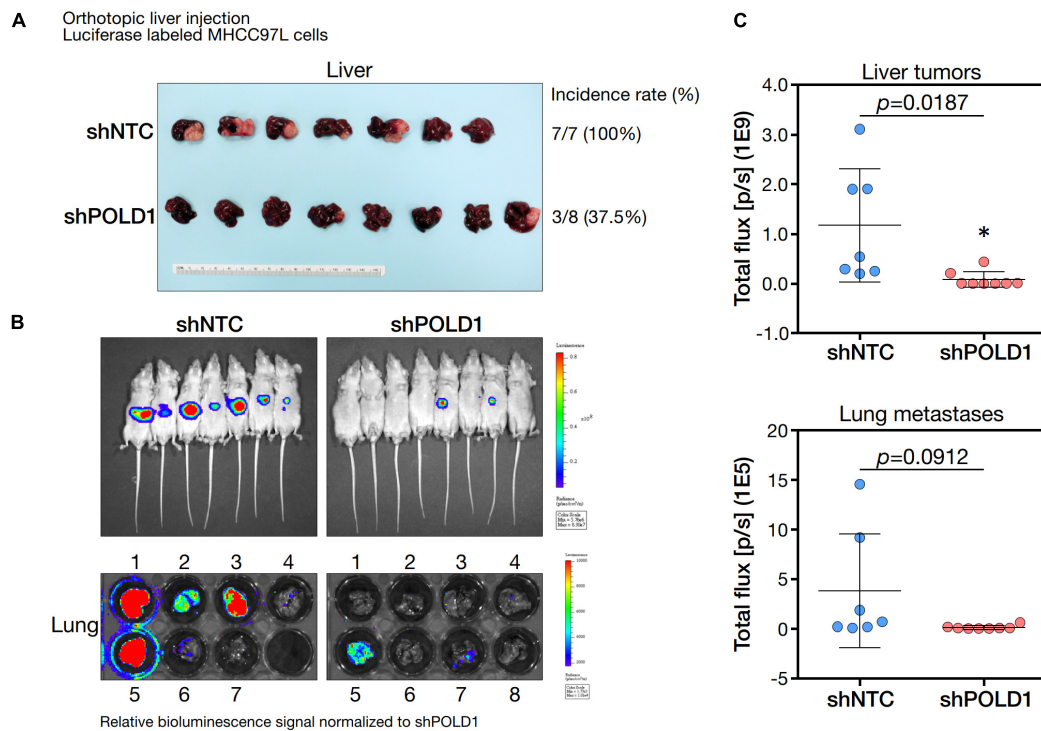
**FIGURE 7 |** Knockdown of POLD1 in MHCC97L-luc cells reduced tumor incidence, tumor sizes and lung metastases in orthotopic liver injection model. The *in vivo* functional role of POLD1 knockdown was evaluated as compared to the non-target control (NTC). Comparison was based on **(A)** tumorigenicity and **(B,C)** tumor size and lung metastasis.

of POLD1, but the difference could not reach statistical significance (**Figure 7**).

## DISCUSSION

Development of HCC is a multistep process results from accumulation of mutational events in cancer driver genes. The alterations in driver genes promote oncogenic functions, such as proliferation, survival, cell motility and immune evasion. Moreover, accumulation of genetic and epigenetic alterations could contribute to tumor initiation, progression, metastasis and resistance to therapy (16). The malignant phenotype of cancer entails the dysregulation of cell cycle machinery, which serves as a convergence point for cellular transformation (17).

In this study, we demonstrated the significant roles of FOXM1 and CENPF in hepatocarcinogenesis. First, we found the co-overexpression of FOXM1 and CENPF in HCC was associated with aggressive tumor behavior, including the presence of venous invasion, tumor microsatellite formation, and the absence of tumor encapsulation by clinicopathological correlation analysis. Second, we demonstrated that FOXM1 and CENPF are important for cell growth. Third, our RNA-seq study further delineated that FOXM1 and CENPF co-regulated a set of genes that play essential roles in various metabolic processes and oncogenic signaling pathways. Among all differentially expressed genes between the wild-type HCC cells and FOXM1 and/or

CENPF knockdown cell lines, *POLD1*, which encodes for the catalytic subunit of DNA polymerase δ, was ranked as the top downstream target co-regulated by FOXM1 and CENPF.

In addition to the delineation of underlying mechanism of FOXM1 and CENPF in HCC, we have also been able to demonstrate their therapeutic implications in HCC using *in vitro* models. Despite the efficacy of existing molecularly targeted drugs, e.g., sorafenib and regorafenib, there is still an unmet medical need for patients with advanced HCC. With the successful advent of immune checkpoint inhibitors (ICIs), the combo of atezolizumab and bevacizumab has become the standard of care. However, although early clinical outcomes are impressive, a significant proportion of patients do not respond to this regimen. Uncontrolled cell growth is one of the characteristics of cancer, which entails aberrant activities of cell cycle proteins. Upregulation of FOXM1 and CENPF were shown to be crucial for the deregulated cell proliferation in HCC in this study. HCC may be uniquely dependent on FOXM1 and CENPF for cell growth; thus, we speculated that targeting these two cell cycle regulators offer considerable potentials in treating HCC. Thiostrepton is a potential inhibitor of FOXM1 (18, 19) while zoledronic acid is a potential inhibitor of CENPF (20–22). Indeed, we observed thiostrepton and zoledronic acid inhibited HCC cells selectively, as compared to the normal liver cells (**Supplementary Figure 5**). Further studies are awaited to confirm their potential usage in HCC treatment. It would also be interesting to test these inhibitors in the presence of ICI

treatment, for instance in immunocompetent mouse models.

Taken together, our study provided new insight into the underlying synergistic mechanism of FOXM1 and CENPF via the regulation of POLD1, which plays a significant role in HCC progression. Moreover, FOXM1 and CENPF also represent new vulnerabilities to novel drug-based therapy in HCC.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA800214.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong.

## AUTHOR CONTRIBUTIONS

DH and IN: study concept and design. DH, W-LL, L-KC, and IN: acquisition of data, analysis and interpretation of data, and drafting of the manuscript. IN: acquisition of clinical samples. All authors reviewed and approved the final draft of the manuscript.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.860395/full#supplementary-material

**Supplementary Figure 1 |** Short tandem repeat (STR) DNA profiling of MHCC97L.

**Supplementary Figure 2 |** Transient knockdown efficiencies of FOXM1 and CENPF in Huh7, Hep3B and MHCC97L. **(A)** mRNA expression and **(B)** protein expression of FOXM1 and CENPF upon siRNA transfection.

**Supplementary Figure 3 |** Candidate genes that were co-regulated by FOXM1 and CENPF. Additional filters (differential expression with $p < 0.05$; logCPM $> 1$; and | logFC| $> 1$) were used to narrow down the list of candidate genes. Among all, there were 29 genes being activated and 4 being repressed upon co-silencing of FOXM1 and CENPF across Huh7, Hep3B and MHCC97L.

**Supplementary Figure 4 |** Gene set enrichment analysis of the candidate genes co-regulated by FOXM1 and CENPF. The analysis was performed using Gene Ontology (GO) biological processes and Reactome Pathway.

**Supplementary Figure 5 |** Half-maximal inhibitory concentration ($IC_{50}$) of thiostrepton and zoledronic acid in HCC cell and non-tumorigenic liver cells. The percent growth inhibition curve showed the response of the Huh7 HCC cell line and two non-tumorigenic liver cell lines, including MIHA and LO2, to thiostrepton or zoledronic acid treatments at varying dosage. Thio, thiostrepton; ZOL, zoledronic acid.

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# Ferroptosis in Intrahepatic Cholangiocarcinoma: $IDH1^{105GGT}$ Single Nucleotide Polymorphism Is Associated With Its Activation and Better Prognosis

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**Objectives:** Intrahepatic cholangiocarcinoma (ICC) has a dismal prognosis and often demonstrates an anti-apoptotic landscape, which is a key step to chemotherapy resistance. Isocitrate dehydrogenase 1 or 2 (*IDH1-2*)-mutated ICCs have been described and associated with better prognosis. Ferroptosis is a regulated iron-mediated cell death induced by glutathione peroxidase 4 (GPX4) inhibition, and may be triggered pharmacologically. GPX4 is overexpressed in aggressive cancers, while its expression is inhibited by $IDH1^{R132C}$ mutation in cell lines. We investigated tissue expression of ferroptosis activation markers in ICC and its correlation with clinical-pathological features and *IDH1-2* status.

**Materials and Methods:** We enrolled 112 patients who underwent hepatic resection or diagnostic liver biopsy for ICC. Immunostaining for transferrin-receptor 1 and GPX4, and Pearls' stain for iron deposits were performed to evaluate ferroptosis activation. Immunostaining for STAT3 was performed to study pro-inflammatory and anti-apoptotic landscape. Main *IDH1-2* mutations were investigated in 90 cases by real-time polymerase chain reaction.

**Results:** GPX4 overexpression was seen in 79.5% of cases and related to poor histological prognostic factors (grading and perineural and vascular invasion; $p < 0.005$ for all) and worse prognosis (OS $p = 0.03$; DFS $p = 0.01$). STAT3 was expressed in 95.5% of cases, confirming the inflammation-related anti-apoptotic milieu in ICC, and directly related to GPX4 expression ($p < 0.0001$). A high STAT3 expression correlated to a worse prognosis (OS $p = 0.02$; DFS $p = 0.001$). Nearly 12% of cases showed $IDH1^{105GGT}$ single nucleotide polymorphism, which was never described in ICC up to now, and was related to lower tumor grade ($p < 0.0001$), longer overall survival ($p = 0.04$), and lower GPX4 levels ($p = 0.001$).

**Conclusion:** Our study demonstrates for the first time that in most inflammatory ICCs ferroptosis is not active, and its triggering is related to *IDH1-2* status. This supports the possible therapeutic role of ferroptosis-inducer drugs in ICC patients, especially in drug-resistant cases.

**Keywords:** intrahepatic cholangiocarcinoma, ferroptosis, *IDH1*[105GGT] single nucleotide polymorphism, STAT3, GPX4

# INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver tumor, whose incidence and mortality have increased worldwide over the last decades (1–6). Because of a frequent diagnosis at an advanced stage, ICC prognosis remains dismal. Surgical resection is the only potentially curative treatment option, but recurrence rates remain high. Patients with metastatic or unresectable disease undergo palliative non-curative systemic therapies, with only modest increases in overall survival and frequent development of chemoresistance, often due to an escape from drug-induced apoptosis by cancer cells (1, 3–5, 7, 8).

Advances have been made in the last decade regarding ICC molecular background. In particular, Sia et al. identified two molecular subclasses, named the proliferative and the inflammation-related classes (9). The latter is defined by the triggering of pro-inflammatory signaling pathways *via* different interleukins and the signal transducer and activator of transcription 3 (STAT3) protein (4, 9). STAT3 is involved in several cellular processes, inhibits apoptosis, and it is known to play a role in many cancer types, being associated with a worse prognosis (4, 10–13).

A subgroup of ICCs showing missense mutations in the isocitrate dehydrogenase 1 and 2 (*IDH1-2*) genes has also been described (14, 15). *IDH*-mutated ICCs exhibit high expression of mitochondrial genes and low expression of chromatin modifier genes and were demonstrated to have a better prognosis than cases with wild type *IDH1-2* (3, 4, 14–17).

Ferroptosis is a newly described form of regulated iron-mediated cell death type, whose activation requires high intracytoplasmic iron concentrations and the inhibition of the reduced glutathione (GSH)-dependent enzyme glutathione peroxidase 4 (GPX4) (18, 19). GPX4 overexpression has been described in many aggressive cancers, making it a potential therapeutic target able to promote death in drug-tolerant tumor cells. Many molecules currently exist that are able to trigger the ferroptotic cascade, known as ferroptosis inducers, either by decreasing GSH levels, such as erastin, or by directly inhibiting GPX4 activity, such as RSL3. Some of them are currently under investigation in clinical trials for cancer treatment (19, 20). So far, no data are reported in the literature regarding the possible role of ferroptosis and ferroptosis inducers in ICC.

An *in vitro* study by Wang et al. (21) conducted on different cell lines firstly demonstrated that tumor-derived *IDH1*[R132C] mutation sensitizes cells to ferroptosis, by reducing GPX4 levels through the production of the oncometabolite 2-hydroxyglutarate (2-HG). In their work, mutated cells were able to undergo ferroptosis in response to erastin but not to RSL3, which acts in a concentration-dependent manner, implying that 2-HG acts directly on GPX4 expression (21).

Basing on this background, the aim of our study was to investigate tissue expression of ferroptosis activation markers in ICC cases, and to correlate it with clinical-pathological features, STAT3 expression, and *IDH1-2* status.

# MATERIALS AND METHODS

## Case Selection

We retrospectively collected a total of 112 consecutive patients with a diagnosis of ICC. Among them, 90 patients underwent laparoscopic hepatic resection with curative intent from January 2006 to May 2021. The remaining 22 patients were patients with liver mass who underwent diagnostic liver biopsy, eventually diagnosed as ICC, who subsequently underwent surgery in a different hospital. Thirty patients included in our cohort were already investigated before, as part of a previous study (22). Exclusion criteria were (i) the administration of any systemic or loco-regional therapy prior to surgery/biopsy, (ii) a survival of less than 3 months after surgery (for patients who underwent resection), to exclude deaths due to surgical complications, and (iii) the absence of available residual tumor tissue for immunohistochemical (IHC) stains and molecular tests. Only surgical specimens were considered for molecular analyzes. The study complies with the ethical guidelines of the 1975 Declaration of Helsinki and obtained the approval from the local Ethics Committee (Ethics Committee for Clinical Research—University Hospital of Padova, Italy; protocol #: 0038038/17). All of the patients gave their appropriate informed consent to any procedure.

## Clinical Data

Patients' relevant clinical and laboratory data were retrieved from medical records, including sex, age, serum ferritin levels, the presence of any underlying chronic hepatic or biliary disease, the presence of cirrhosis, and the administration of adjuvant chemotherapy. We also reported whether lymphadenectomy was performed during surgery or not.

All patients who underwent surgery were clinically followed-up, and regularly subjected to ultrasonography and computed tomography to detect any recurrence of the disease. Overall and disease-free survival time was obtained from medical charts.

## Histological Study

All of the cases were blindly and contemporarily reviewed by an experienced (MG) and two trainee (SS and DS) liver pathologists, and relevant histological features were recorded, including macroscopic tumor type, histotype, grade of differentiation, T stage (according to the revised 8th edition of the UICC staging system) (23), margin status (for surgical resections), and the presence of vascular and perineural invasion and lymph node metastasis.

## Immunohistochemical Study

Tissue microarrays (TMAs) made of formalin-fixed paraffin-embedded ICC tissue cores (with a diameter of 2 mm) were obtained by selecting two or three representative tumor areas from each liver resection case, depending on tumor dimension. All of the samples were processed by using the TMA Master platform (3DHistech, Budapest, Hungary), a semi-automatic and computer-assisted TMA platform.

Immunostains were performed on TMA and liver biopsy sections by using the following antibodies: anti-STAT3 (clone F-2; Santa Cruz Biotechnology, Dallas, TX, United States; dilution 1:200; mouse monoclonal), anti-GPX4 (clone E-12; Santa Cruz Biotechnology, Dallas, TX, United States; dilution 1:400; mouse monoclonal), and anti-transferrin receptor 1 (TFR1; also known as CD71) (clone 10F11; Leica Biosystems, Newcastle upon Tyne, United Kingdom; dilution 1:100; mouse monoclonal). All IHC stains were conducted according to standard protocols by using the Dako Omnis autostainer (Dako, Glostrup, Denmark), and all of the slides were counterstained with hematoxylin. Appropriate positive and negative controls were used for each run. In evaluating the expression of all markers, only cytoplasmic staining was considered. STAT3 and GPX4 expression was semi-quantitatively scored from 0 to 2 +, as follows: 0 = negative; 1 + = expression in $\leq$ 50% of tumor cells (TCs); 2 + = expression in > 50% of TCs (**Figure 1**). Finally, TFR1 positivity was evaluated as follows: 0 = negative or positive in $\leq$ 10% of TCs; 1 + = expression in 11–50% of TCs; 2 + = expression in > 50% of TCs (**Figure 2**).

In every case, we also performed histochemical Perls' stain (Artisan Iron Stain Kit, Dako, Glostrup, Denmark) to detect intratumoral iron deposits, which were recorded as absent/present (**Figure 3**).

## Molecular Study

As reported above, only surgical specimens (90 cases) were considered for molecular tests. For each case, DNA extraction was performed on selected representative areas of formalin-fixed paraffin-embedded ICC tissue, with Qiasymphony DSP DNA Mini Kit (Qiagen, Hilden, Germany). DNA quantity was measured spectrophotometrically by using NanoDrop ND 100 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Detection of the main *IDH1* (codons 105 and 132) and *IDH2* (codons 140 and 172) gene mutations was performed by real-time polymerase chain reaction (PCR) analysis (Easy PGX ready IDH1-2, Easy PGX platform, Diatech Pharmacogenetics, Jesi, Italy), according to the manufacturer's
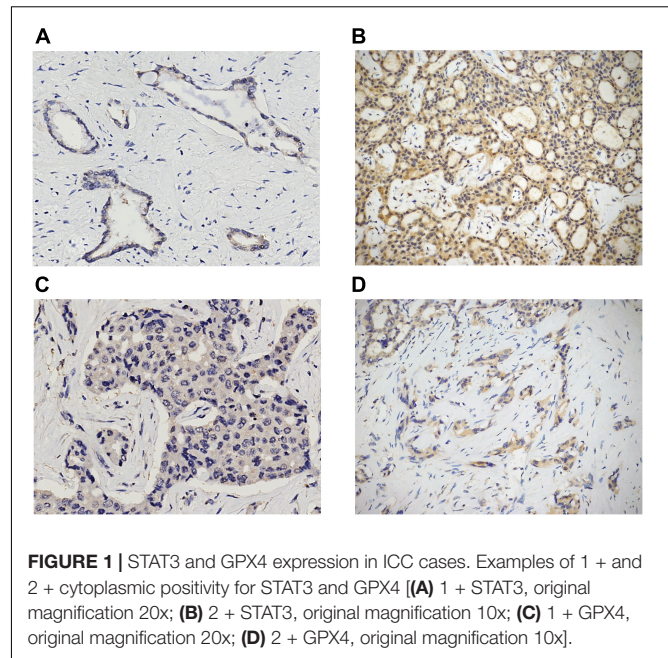


**FIGURE 1 |** STAT3 and GPX4 expression in ICC cases. Examples of 1 + and 2 + cytoplasmic positivity for STAT3 and GPX4 [**(A)** 1 + STAT3, original magnification 20x; **(B)** 2 + STAT3, original magnification 10x; **(C)** 1 + GPX4, original magnification 20x; **(D)** 2 + GPX4, original magnification 10x].

instructions. When necessary, the identified mutations were confirmed by PCR analysis and Sanger sequencing, by using the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, United States).

## Statistical Analysis

Continuous variables were expressed as median (range) while categorical variables as frequency and percentage. For clinical-pathological correlations, appropriated tests were used, including Student's *t*-test, one-way ANOVA test, Spearman rank correlation test and Fisher exact probability test. The Kaplan–Meier method was used to create survival curves, which were compared by using both the log-rank and the Breslow (generalized Wilcoxon) tests. Multivariate Cox backward stepwise regression analyzes were performed including all of the variables identified as significant on univariate Cox regression analyzes. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were calculated. $p$ values < 0.05 were considered statistically significant. Data analyzes were performed by applying Statistical Package for the Social Science (SPSS, version 25, IBM SPSS Statistics, Chicago, IL, United States) and GraphPad (version 6, GraphPad Software, San Diego, CA, United States) statistical software.

## RESULTS

### Clinical Features

Overall, in our cohort of 112 patients there were 58 males (51.8%) and 54 females (48.2%), with a median age of 68 years (range 34–92 years). Seventeen patients (15.2%) had a cirrhotic liver, and 36 patients (32.1%) received adjuvant chemotherapy after resection/biopsy. Patients had a median follow-up of 1.7 years (range 0.3–8.8 years). Lymphadenectomy was performed in 33/90 patients who underwent surgery. Clinical and laboratory
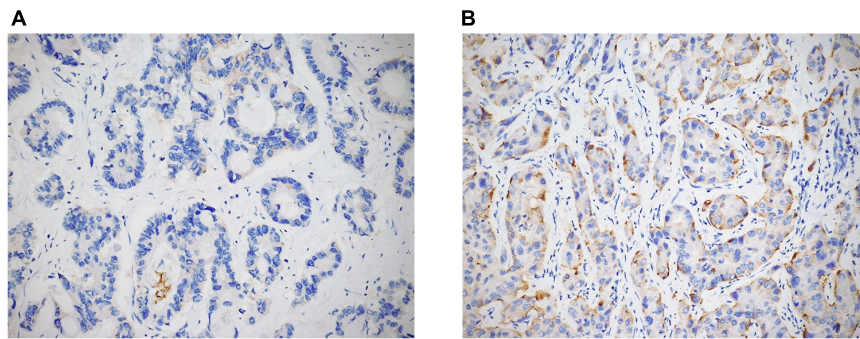
**FIGURE 2 |** TFR1 expression in ICC cases was semi-quantitatively evaluated as negative, 1 + [**(A)** original magnification 20x], and 2 + [**(B)** original magnification 20x].
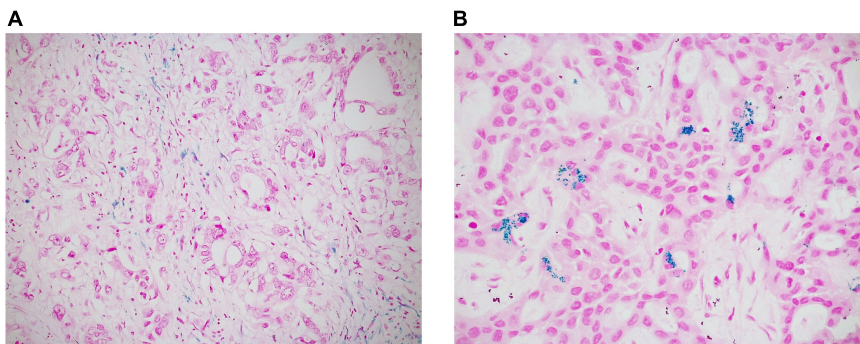


**FIGURE 3 |** Intratumoral iron deposits in ICC were evaluated as absent/present. Negative cases often showed iron deposition in peri-tumoral macrophages, but not in neoplastic cells [**(A)** original magnification 20x]; in positive cases, iron deposits appear as intracytoplasmic blue granules in tumor cells [**(B)** original magnification 40x].

features of all the patients are summarized in **Table 1**. Age, sex, the presence of underlying chronic liver diseases, and the administration of adjuvant chemotherapy were not related to patients' prognosis. Furthermore, overall and disease-free survivals were not different between patients with or without cirrhosis, probably because of the low number of cirrhotic patients in our cohort.

## Histological Features and Clinical-Pathological Correlations

Macroscopically, all cases were mass forming, while, histologically, there were 82 cases (73.2%) of small duct type ICC and 30 cases (26.8%) of large duct type ICC. We observed vascular invasion in 78 out of 112 patients (69.6%), while perineural invasion was present in 50/112 cases (44.6%), as reported in **Table 2**. Among the 33 patients who underwent lymphadenectomy, 15 had lymph node metastases. Forty-seven patients out of the 90 who underwent surgery (52.2%) had a complete surgical excision of the tumor (R0 cases), while the remaining 43 patients (47.8%) showed a microscopic neoplastic infiltration of the resection margin (R1 cases).

As expected, grading and the presence of perineural and vascular neoplastic invasion were all related to worse overall

($p = 0.009$, $p < 0.0001$, and $p = 0.004$, respectively) and disease-free survivals ($p = 0.01$, $p = 0.002$, and $p = 0.001$, respectively). An advanced T stage (i.e., T stage 3 and 4) was related to a reduced overall survival ($p = 0.06$). We failed to find any correlation between patients' prognosis and histotype or resection margin status. We did not observe differences in survival times between cases with or without lymph node metastases.

## Immunohistochemical Features and Clinical-Pathological Correlations

In our cohort, most of the cases showed GPX4 expression, either 1 + (43/112; 38.4%) or 2 + (46/112; 41.1%), while only 23 cases (20.5%) were completely negative. Twenty-two patients out of 112 (19.6%) showed 1 + TFR1 expression, while 7 cases showed a 2 + TFR1 positivity (6.3%). Intratumoral iron deposits were observed in only 2 cases (1.8%); among them, one showed a focal and one a diffuse deposition. Interestingly, the case that showed diffuse iron deposits had both a strong TFR1 expression and a completely negative GPX4 stain (**Supplementary Figure 1**). Taken together, these findings suggest an inhibition of the ferroptotic cascade in ICC. Most of our cases showed either a 1 + (56/112; 50%) or 2 + (51/112; 45.5%) STAT3 expression, with only 5 cases (4.5%) showing a completely negative reaction, suggesting an activation of the inflammatory pathway in our ICC

**TABLE 1 |** Clinical and laboratory features of the patients.

| Feature | N = 112 |
|---|---|
| **Age [years]** | |
| Median (range) | 68 (34 − 92) |
| **Sex N (%)** | |
| Males | 58 (51.8) |
| Females | 54 (48.2) |
| **Ferritin [ng/ml]** | |
| Median (range) | 260.5 (53 − 943.5) |
| **Underlying diseases N (%)** | |
| HBV hepatitis | 6 (5.4) |
| HCV hepatitis | 11 (9.8) |
| Alcoholic hepatitis | 6 (5.4) |
| NAFLD/NASH | 6 (5.4) |
| Cryptogenic cirrhosis | 1 (0.9) |
| **Cirrhosis N (%)** | 17 (15.2) |
| **Adjuvant chemotherapy N (%)** | 36 (32.1) |
| **Recurrence N (%)** | 60 (53.6) |
| **Exitus N (%)** | 73 (65.2) |

*HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.*

**TABLE 2 |** Histological features of the patients.

| Feature | N = 112 |
|---|---|
| **Histotype** | |
| N (%) | |
| Small duct type | 82 (73.2) |
| Large duct type | 30 (26.8) |
| **Grade** | |
| N (%) | |
| G1 | 15 (13.4) |
| G2 | 49 (43.8) |
| G3 | 48 (42.8) |
| **T stage [N = 90]** | |
| N (%) | |
| T1a | 13 (14.4) |
| T1b | 11 (12.2) |
| T2 | 46 (51.1) |
| T3 | 13 (14.4) |
| T4 | 7 (7.9) |
| **Vascular invasion N (%)** | 78 (69.6) |
| **Perineural invasion N (%)** | 50 (44.6) |
| **Lymph node metastasis [N = 33] N (%)** | 15 (45.5) |
| **Resection margin status [N = 90]** | |
| N (%) | |
| R0 | 47 (52.2) |
| R1 | 43 (47.8) |

**TABLE 3 |** Histochemical and immunohistochemical expression of the different markers in ICC cases.

| Marker | N = 112 |
|---|---|
| **GPX4** | |
| N (%) | |
| 0 | 23 (20.5) |
| 1 + | 43 (38.4) |
| 2 + | 46 (41.1) |
| **TFR1** | |
| N (%) | |
| 0 | 83 (74.1) |
| 1 + | 22 (19.6) |
| 2 + | 7 (6.3) |
| **Intratumoral iron deposits** | |
| N (%) | |
| Absent | 110 (98.2) |
| Present | 2 (1.8) |
| **STAT3** | |
| N (%) | |
| 0 | 5 (4.5) |
| 1 + | 56 (50) |
| 2 + | 51 (45.5) |

association between the presence of the same unfavorable histological factors and STAT3 expression ($p = 0.001$, $p = 0.04$, and $p = 0.07$ for grading, perineural invasion, and vascular invasion, respectively), confirming what previously reported (4, 10). No associations were found between the same histological features and TFR1 expression, neither between them and the presence of iron deposits. No correlations were found between marker expression (and iron deposits) and tumor T stage. We observed a direct association between STAT3 and GPX4 expression ($p < 0.0001$) (**Supplementary Figure 2**), suggesting inhibition of ferroptosis in ICCs with an inflammatory background. Accordingly, we found an inverse correlation between STAT3 and TFR1 expression ($p = 0.04$). We failed to find any correlation between TFR1 and GPX4 expression, as well as between ferroptosis markers and STAT3 expression and the presence of intratumoral iron deposits.

Age, sex, the presence of any underlying chronic liver disease, the presence of cirrhosis, ferritin levels, tumor histotype, and the presence of nodal metastases were not related to the expression of any of the markers, neither to the presence of iron deposits.

As already reported in the literature, STAT3 expression was related to a worse overall ($p = 0.02$) and disease-free survival ($p = 0.001$) (**Figure 4**). We found significantly reduced overall ($p = 0.06$) and disease-free survivals ($p = 0.04$) in cases with a higher GPX4 expression, as shown by the Kaplan–Meyer curves (**Figure 5**). Considering GPX4 1 + and 2 + positive cases together, the association becomes even stronger for both overall and disease-free survival ($p = 0.03$ and $p = 0.01$, respectively) (**Figure 5**), suggesting that mild GPX4 presence is enough to inhibit ferroptosis and reduce survival times. TFR1 expression and the presence of iron deposits were not related to survival times.

cohort. Histochemical and immunohistochemical expressions of the different markers are summarized in **Table 3**.

Statistical analyzes showed a direct correlation between GPX4 expression and the presence of poor prognostic histological parameters, that is grading ($p < 0.0001$) and perineural ($p = 0.03$) and vascular invasion ($p < 0.0001$). We also found a direct
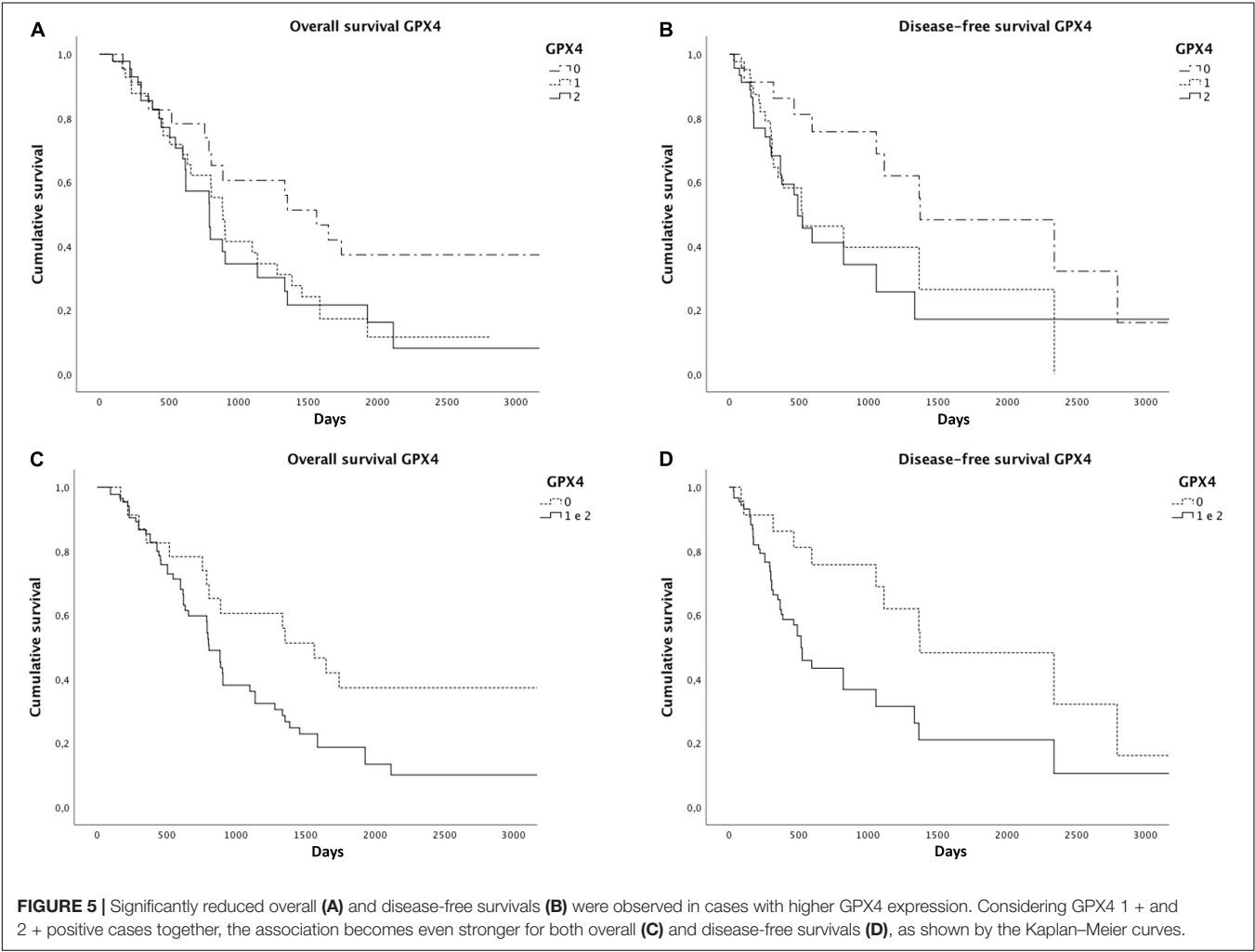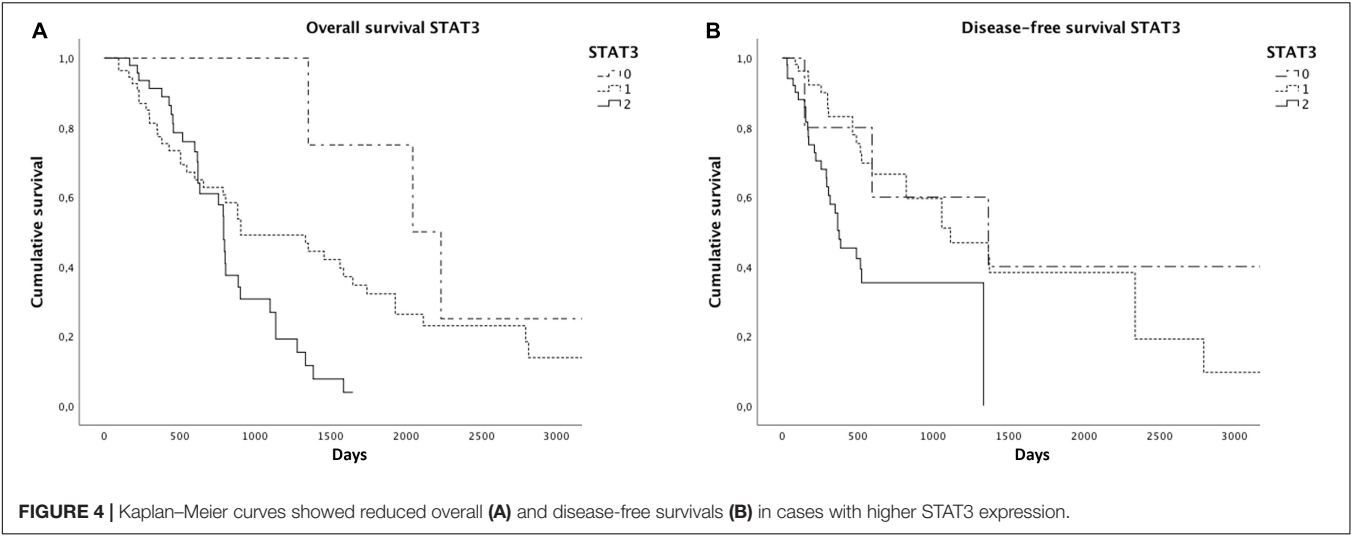
**FIGURE 4 |** Kaplan–Meier curves showed reduced overall **(A)** and disease-free survivals **(B)** in cases with higher STAT3 expression.



**FIGURE 5 |** Significantly reduced overall **(A)** and disease-free survivals **(B)** were observed in cases with higher GPX4 expression. Considering GPX4 1 + and 2 + positive cases together, the association becomes even stronger for both overall **(C)** and disease-free survivals **(D)**, as shown by the Kaplan–Meier curves.

## Molecular Features and Clinical-Pathological Correlations

Molecular analysis results are reported in **Table 4**. As shown, they revealed *IDH1* point mutations in 15 out of 76 tested cases (19.7%), of which 14 involving codon 132 and one involving codon 105. Only one case showed a point mutation in *IDH2* codon 172 (1.3%). In 9 cases (11.8%) molecular tests described a single nucleotide polymorphism (SNP) in codon 105 of the *IDH1* gene, causing a change in the nucleotide sequence from GGC [Gly] to GGT [Gly] (C > T). Among them, two cases showed both a point mutation in *IDH1* (codon 132) and the $IDH1^{105GGT}$ SNP. No relationships were found between the presence of IDH1 point mutations and the presence of the $IDH1^{105GGT}$ SNP.

Statistical analyzes showed an inverse correlation between the presence of $IDH1^{105GGT}$ SNP and tumor grading ($p < 0.0001$). On the contrary, a direct relationship was found between tumor grading and the wild type status of *IDH1-2* genes ($p = 0.004$). No associations between tumor grading and the presence of point mutations in *IDH1-2* genes were observed. We also failed to find any correlation between *IDH1-2* status and age, sex, the presence of any underlying chronic liver disease, the presence of cirrhosis, ferritin levels, tumor histotype, the presence of perineural and vascular invasion, T stage and the presence of nodal metastases.

Furthermore, GPX4 expression was inversely correlated to the presence of $IDH1^{105GGT}$ SNP ($p = 0.001$) (**Supplementary Figure 3**), while it was directly related to the presence of an *IDH1-2* point mutation ($p = 0.06$) or wild type *IDH1-2* ($p = 0.04$). No associations were found between *IDH1-2* status and STAT3 and TFR1 expression, neither between *IDH1-2* status and intratumoral iron deposits.

Finally, cases with $IDH1^{105GGT}$ SNP showed a better overall survival than cases with wild type *IDH1-2* (1,648 days vs. 887 days; $p = 0.04$) and cases with *IDH1-2* point mutations (1,648 days vs. 1,333 days; $p = 0.09$) (**Figure 6**). We did not find any relationship between *IDH1-2* status and disease-free survival times. The multivariate Cox regression analysis, including
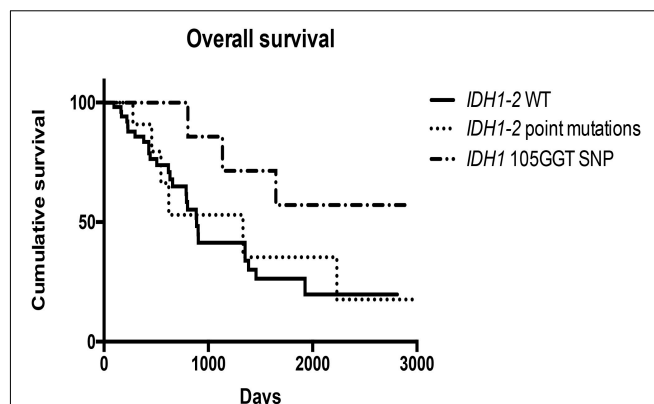


**FIGURE 6 |** Kaplan–Meier curves showed different overall survivals in cases bearing $IDH1^{105GGT}$ SNP, *IDH1-2* point mutations and wild type *IDH1-2*.

variables that were identified as significant on univariate survival analyzes (grading, perineural and vascular neoplastic invasion, T stage, STAT3 and GPX4 expression, and $IDH1^{105GGT}$ SNP) showed that only the presence of perineural invasion is an independent predictor of a worse overall survival [$p < 0.0001$, HR = 3.64 (95% CI: 1.86–7.11)], while the multivariate Cox regression analysis including grading, perineural and vascular neoplastic invasion, and STAT3 and GPX4 expression found that STAT3 expression and vascular invasion are independent predictors of a reduced disease-free survival [$p = 0.03$, HR = 1.81 (95% CI: 1.05–3.14), and $p = 0.05$, HR = 1.97 (95% CI: 1.02–3.84), respectively] (**Supplementary Table 1**).

## DISCUSSION

In this study, we describe for the first time that in most inflammatory ICCs ferroptosis seems to be not active, and that its activation may depend on *IDH1-2* status. In our cohort, GPX4 was overexpressed in almost 80% of the cases, suggesting an inhibition of the ferroptotic cascade in most of our patients. GPX4 expression was found to be related to the presence of histological poor prognostic features, namely high grade and vascular and perineural invasion, and to reduced overall and disease-free survivals, implying that ferroptosis inhibition confers a worse prognosis, as expected.

We also observed that STAT3 was overexpressed in more than 90% of cases, indicating that they can be classified as inflammation-related ICC, according to the molecular classification proposed by Sia et al. (9). We confirm the poor prognostic role of STAT3 already reported in the literature (4, 10). In fact, we found a correlation between its expression and the presence of histological poor prognostic features, and worse overall and disease-free survivals in cases with higher STAT3 expression. Since this subclass of ICC is characterized by a pro-inflammatory and anti-apoptotic molecular milieu, and that pro-apoptotic drugs demonstrated only mild improvements in patients' prognosis and a frequent development of chemoresistance (4, 7, 11), the induction of other

**TABLE 4 |** *IDH1* and *IDH2* gene status in ICC cases.

| Adequacy | N = 90 |
| --- | --- |
| Inadequate samples | 14/90 (15.6%) |
| Adequate samples | 76/90 (84.4%) |
| **Molecular alterations** | **N = 76** |
| ***IDH1* point mutations\*** | 15/76 (19.7%) |
| *Arg132Cys* | 6/76 (7.9%) |
| *Arg132His* | 2/76 (2.6%) |
| *Arg132Val* | 1/76 (1.3%) |
| *Arg132Ser* | 1/76 (1.3%) |
| *Arg132X* | 4/76 (5.2%) |
| *Gly105Asn* | 1/76 (1.3%) |
| **$IDH1^{105GGT}$ SNP\*** | 9/76 (11.8%) |
| **$IDH2$ Arg172X** | 1/76 (1.3%) |
| ***IDH1-2* WT** | 53/76 (69.7%) |

*\*2 cases showed both an IDH1 point mutation in codon 132 (Arg132Cys and Arg132His) and $IDH1^{105GGT}$ SNP. SNP, single nucleotide polymorphism; WT, wild type.*

different cell death types, including ferroptosis, is a possible alternative way to kill resistant tumor cells. In line with this hypothesis, the high GPX4 levels found in our patients suggest that ferroptosis could be pharmacologically induced, by acting directly on GPX4 inhibition.

Molecular analyses on our cohort demonstrated *IDH1* (codon 132 or 105) or *IDH2* (codon 172) point mutations in nearly 20% of the cases, in line with the literature (3, 4, 14–17). Surprisingly, we found a SNP on codon 105 of the *IDH1* gene in 12% of our cases, which was never reported in ICC before. Patients bearing this molecular feature had a good histological profile, namely low histological tumor grade, and longer overall survival times. Synonymous SNPs are point mutations that cause a nucleotide change, which do not alter the amino acid sequence of the protein. However, sometimes they may lead to a protein defect and have functional consequences (24). The *IDH1*$^{105GGT}$ SNP we described in our cohort was previously reported in acute myeloid leukemia, gliomas, and thyroid tumors, and have a poorly understood role in tumorigenesis (24–27). Indeed, it was linked to an adverse prognosis in acute myeloid leukemia and glioblastomas, while it seemed to confer longer survivals in patients with grade II or III gliomas (28, 29), in line with our results. The explanation of this finding is not clear, since the biologic consequences of this SNP remain speculative. It has been hypothesized that *IDH1*$^{105GGT}$ SNP may alter *IDH1* mRNA stability or increase mRNA levels, leading to altered NADPH production (24, 28, 30), but further studies are needed to elucidate this issue. In line with previously reports (24, 28, 30), in two of our cases we described the concomitant presence of the *IDH1*$^{105GGT}$ SNP and a point mutation in *IDH1* (codon 132). It is known that, even if *IDH1*$^{105GGT}$ SNP is very close to codon 132, no correlation between the two molecular alterations exists (24, 30), as we confirm in our cohort.

The inverse correlation we observed between the presence of *IDH1*$^{105GGT}$ SNP and GPX4 levels may suggest an activation of the ferroptotic cascade in ICCs bearing this molecular feature, and eventually explains the longer overall survivals observed in these patients. On the other side, we failed to confirm the association between *IDH1*$^{R132C}$ mutation and reduced GPX4 levels reported by Wang et al. (21), but this might be explained by the different experimental conditions we worked under (cell lines versus ICC tissue and *in vitro* versus *in vivo*). Considering our survival data, it is possible that both *IDH1*$^{105GGT}$ SNP and *IDH1*$^{R132C}$ mutation act on GPX4 in different ways or with different intensity, since *IDH1*$^{R132C}$ mutation-bearing cases showed overall survival times shorter than those seen in *IDH1*$^{105GGT}$ SNP cases, but longer than those of patients with wild type *IDH1-2*. However, molecular mechanisms explaining how *IDH1*$^{105GGT}$ SNP act on GPX4 level reduction are unknown and impossible to infer basing on our data. So additional *in vitro* studies are indispensable to address this key issue. Moreover, the number of patients overall bearing the *IDH1*$^{105GGT}$ SNP in our cohort is low, and this limits the strength of our data, so additional studies based on larger cohorts may be of help to confirm our results. It is of interest to note that patients bearing *IDH1*$^{105GGT}$ SNP are not expected to respond to GPX4-inhibitor drugs, such as RSL3, since GPX4 levels are already

very low in these cases. However, as Wang et al. reported on *IDH1*$^{R132C}$-mutated cell lines (21), *IDH1*$^{105GGT}$ SNP-bearing ICCs may respond to ferroptosis-inducers acting on GSH levels, such as erastin. Therefore, knowing the molecular background in ICC patients is fundamental to choose the appropriate pharmacological therapy to induce tumor cell death, particularly in cases developing drug-resistance.

In conclusion, our study demonstrates for the first time that in most inflammatory ICCs ferroptosis seems to be not active, and that its triggering may be related to some molecular features of the tumor. *IDH1-2* status is essential to determine whether (and which type of) ferroptosis-inducer drugs might be useful in ICC patient treatment, especially in drug-resistant cases.

## DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for Clinical Research—University Hospital of Padova, Italy. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

## ACKNOWLEDGMENTS

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.886229/full#supplementary-material

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# Digital pathology – Rising to the challenge

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Digital pathology has gone through considerable technical advances during the past few years and certain aspects of digital diagnostics have been widely and swiftly adopted in many centers, catalyzed by the COVID-19 pandemic. However, analysis of requirements, careful planning, and structured implementation should to be considered in order to reap the full benefits of a digital workflow. The aim of this review is to provide a practical, concise and hands-on summary of issues relevant to implementing and developing digital diagnostics in the pathology laboratory. These include important initial considerations, possible approaches to overcome common challenges, potential diagnostic pitfalls, validation and regulatory issues and an introduction to the emerging field of image analysis in routine.

KEYWORDS

digital pathology, scanner acquisition, validation, image analysis, artificial intelligence

## Introduction

In many parts of the world, at least part of pathologists' work has become "digital," i.e., conventional routine replaced by digital images. However, many pathology institutes that pursue the path to digital pathology realize that this is not always as straightforward as it seems. As technical aspects and possibilities advance at nearly dizzying speed, it must be emphasized that a digital approach will affect many fundamental aspects of daily routine in a pathology lab. Careful planning and certain practical considerations can be paramount for a smooth transition to an efficient digital workflow. The objective of this article is to provide a broad overview of digital pathology for the general pathologist and address real-world issues which may be underrepresented in the literature. Select review articles, white papers and consensus guidelines are recommended for further in-depth reading (1–5).

What constitutes digital pathology? Broadly interpreted, this can entail any sort of work involving a digital image. For instance, digital photography to capture and store macroscopic images can be considered one of the first widespread aspects of digital pathology. Real-time sharing of pathology images with a common viewer (telepathology) has been favored for some time by hospital systems with multiple sites

and increasingly implemented during the COVID-19 pandemic ([6]). The most complex application is the digitization and storage of whole slides images (WSI), either for use in diagnostic routine or for educational or research purposes.

## Advantages and challenges of digital pathology

There are various potential advantages of digital pathology, including:

- Patient safety-related: Provided storage is reliable and data secure, a scanned slide cannot be lost or broken. Most viewer programs also have annotation functions, which enable the documentation of exact measurements (for instance, the exact infiltration depth of a tumor or distance to resection margins).
- Access to slides: One of the most obvious drivers of digital pathology in the past 2 years has been pandemic-related to enable remote work. The retrieval of archived slides can also be considerably simplified and does not require staff assistance. Depending on the laboratory setup, scanned cases can also improve workflow by reducing physical slide distribution.
- Sharing cases: Digitized scans can be easily shared for second opinions, interdisciplinary tumor conferences and educating students and residents.
- Research: Established archives of scanned WSI or tissue microarrays can be mined for research projects and enables centralized, standardized repositories of research data (including patient data, tissue-related data etc.).
- Implementation of automated algorithms: WSI are prerequisite for the vast possibilities of additional image analysis software for diagnostic assistance (see below).

## Initial considerations

The benefits of digital are certainly met with their share of challenges, both from a technical and organizational point of view. First, it should be clear that there is no "one-size-fits-all" solution and that needs of individual institutions will differ. Prior to making large investments, current system requirements should be specified and future ones anticipated. Important considerations may be:

- Scope of application: Is the aim to go fully digital or only scan a certain amount of slides? The amount of expected slides to be scanned and the scanning speed will influence the type and number of scanners to purchase.

- What are system requirements and IT resources? How can storage space be ensured and how much is needed? How can graphics processing units/central processing units (GPUs/CPUs) be accessed? This will highly depend on the planned scope of application. For instance, assuming a biopsy slide output of 1,600/day and an average of 2 GB/slide, 1 PB/year should be calculated if all scans are to be archived.
- System compatibility: How is the interface with the laboratory information system (LIS) and how compatible is the scanner with barcodes from different manufacturers?
- Requirements for access to scans and image management system (IMS): Should scans be available *via* web browser? How can slides be shared? What are requirements for annotation and measurement tools? How can patient metadata from the LIS be integrated to the IMS?
- Workflow integration: How does scanning harmonize with other lab processes? Is continuous loading/unloading possible?
- Openness of system for application of plugin software: Does the scan and proposed IMS enable the use of desired third party software?
- Regional differences in regulatory issues: Is FDA or equivalent approval required for a scanner to be used in a diagnostic setting?
- Financial aspects: Transitioning to a digital pathology workflow requires considerable short-term investments and additional personnel. However, well-planned implementation may result in increased productivity in the long-term, and potentially counteract shortages in trained pathologists who are faced with an increased case load ([5], [7]). The business case and potential financial gains of a particular pathology institute highly depend on the scope of the project and pre-existing efficiency of the laboratory setup ([7]).

Some points can only be adequately addressed in a trial period. At the beginning, scanner acquisition is typically one of the first large investments in implementing digital pathology. For instance, a possible approach would be to test several scanners and submit them to a performance test (**Table 1**), including ([8]):

- Technical aspects: Speed, file size, interruptions, rescan rates, focus and tissue identification issues; what is the proportion of poor quality scans (blurriness, incomplete scans).
- Workflow-related aspects: Handling and user-friendliness, continuous scanning, LIS integration, openness of file format.
- Medical aspects: How do MDs rate the quality of scans including special stains and the usability of the viewer? What is the general impression among different scanners?

# Getting started

The implementation of digital pathology is complex and interdisciplinary. Key players include IT, laboratory staff, MDs and institute management. Although the strategic decision to use digital pathology is traditionally made at the management level, it is important to involve all units at an early stage of development. Our digital pathology team includes a project manager, 2 pathologists, a specialized technician, an IT specialist responsible for our LIS and a LEAN specialist, and meets regularly with the head of the pathology lab and representatives of our LIS and IMS providers. The digital pathology group also works closely with our computational pathology researchers. However, the main goal is the continuous development of digital pathology for diagnostic routine in the department.

Our histology lab is organized according to Lean principles (9). As many processes in the lab including the physical transfer of histology slides already revolve around a Lean workflow we were faced with the challenge of integrating an additional step. Since scanning needs to match the batch-based distribution of slides, continuous scanning is necessary to keep our workflow running. Also, space restrictions and an ideal position of the scanner need to be taken into consideration (Figure 1). However, a Lean approach to digitizing cases is not just limited to the scanner itself. Automated processes starting with accession can help to minimize waste and save time. For instance, if correctly identified, a particular case can be processed so that slides are scanned and algorithms already run before the scan reaches the pathologist. In addition, standardized sampling and measurements of gross specimens enable the automated labeling of scanned slides and necessary elements directly transferred to synoptic reports.

Some practical aspects of tissue processing will influence digitizing slides. For example, small biopsies very close to the edges of the slide may not be identified by the scanner, which can lead to relevant patient safety issues (see discussion on quality control). Therefore, this must be taken into account at the microtome. Also, some scanners will accept racks directly from some stainers, but it must be ensured that slides are dry, as slides with wet mounting medium tend to stick to the rack and will not be scanned. The use of barcodes for specimen labeling is

TABLE 1  Example performance test for scanners under consideration for purchase.

**Technical aspects**

| | |
|---|---|
| File size | File sizes can vary considerably (up to over fourfold) among different scanners and will therefore have a major impact on the storage space needed. |
| Scan time at 40× magnification and for dayload | The scanning speed can be a bottleneck in the diagnostic workflow depending on the number of slides, the type of specimen (biopsies vs. resections) and the number of pathologists to be signing out cases digitally. In our experience, scan times may vary up to a minute per slide on different scanners at the same magnification. |
| Interruptions | The vulnerability to interruptions is one of the most important aspects of scanner performance and can have an especially profound impact on overnight scanning. Software, hardware and slide-related issues may all contribute to interruptions. |
| Rescan rate | After quality control check, establish the proportion of slides that need to be rescanned (e.g., due to focus issues and missing tissue). |
| Focus | Tissue can be either entirely or only partially out of focus. The technology of continuous autofocusing can result in different areas of the slide being out of focus but lower rates of WSI completely out of focus in comparison to scanners using focus points. |
| Tissue identification | Scanners use their own algorithms to detect tissue on a slide and therefore variability in tissue detection may be seen among different scanners. For patient safety reasons it is crucial that all tissue is scanned and this must be ensured for each slide. Faintly stained tissue (e.g., myxoid substance or fat) can be missed and only partially scanned by some scanners. |
| Openness of file format | The scan format should ideally be open for the integration for an independent choice of image analysis tools and convertible into other formats. |
| **Workflow-related aspects** | |
| User friendliness | The laboratory staff should be involved in evaluating the usability of scanner software and hardware. |
| Loading of racks | There are several possibilities: manual loading of slides one by one, overturning of racks from staining machines in one step and direct loading of racks from stainers. However, slides with wet mounting medium tend to stick to the rack and will not be scanned. |
| Continuous scanning | Some scanners stop to open, others continue with the process even if opened to reload, which can have a considerable effect on case distribution depending on the workflow. |
| LIS integration | This is crucial, especially for work in the remote setting in terms of work lists and case management. Cooperation with LIS providers is important to ensure that the requirements of the institute are met. |
| **MD-related aspects** | |
| IMS | The IMS may be from a different provider than the scanner itself, but is essential for digital sign-out and should be considered during scanner testing. Pathologists should feel comfortable using the IMS, which should provide certain tools (measurement, area calculation, regions of interest, snapshot etc.). |
| Pathologist evaluation | Pathologists should compare their impression of WSI in terms of quality of the scans and their level of diagnostic confidence. A case set should including potentially difficult cases (e.g., special stains containing microorganisms). Side by side comparisons of WSI from different can be especially helpful in the decision making process. |
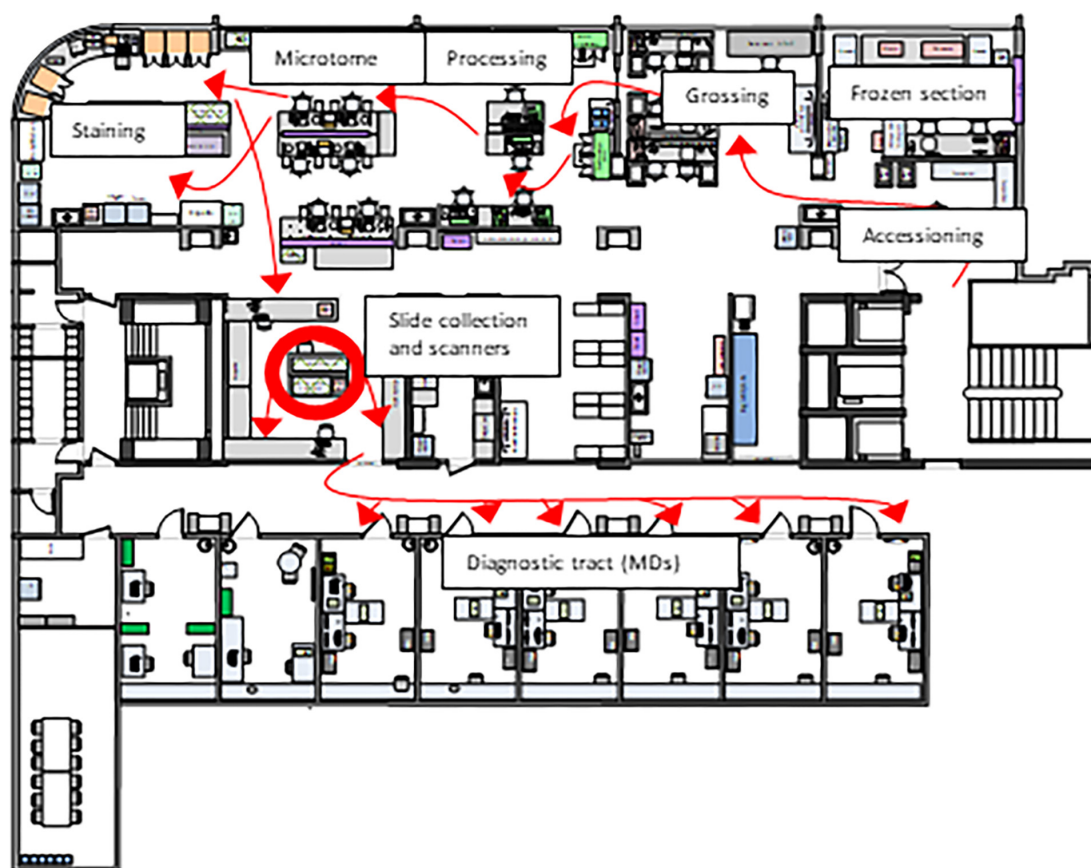
**FIGURE 1**

Layout of our histology laboratory according to Lean principles. The red arrows that represent the path taken by a specimen from acquisition to the MD are unidirectional ("LEAN biopsy/resection street"). Therefore, the Lean solution was to place scanners in the slide sorting area (red circle), which minimizes waste both for a hybrid solution (digital and conventional sign-out) and fully digital sign-out.

used in many laboratories and is required for a digital workflow. Regardless of whether slides are scanned or not, barcodes are the basis of a digital tracking system, which can drastically improve patient safety and also be used as a management tool (e.g., measurement of turnaround times and performance indicators). Robust barcode reading is also essential for the scanning process and there are several types of barcode printers which vary in terms of price, print quality and scanner readability (10). The highest quality barcodes are produced by thermal printers which are more expensive than other types of printers, but may save time and effort by drastically reducing failure rates in barcode reading. Alternatively, training laboratory staff to recognize poor quality barcodes may also yield acceptable barcode scanning rates.

## The MD team

Prior to routine scanning in our department (also before the COVID-19 pandemic), MDs were involved in rating of several

tested scanners in terms of handling issues, comparison of side by side scans and scan quality and asked about their general attitude toward digital pathology in general. Most pathologists were open to the digital future, for instance 75% felt they would be able to make diagnoses on scanned images (8). This is in concordance to a national survey performed in Switzerland in 2019, where the majority of pathologists (66%) also reported feeling comfortable rendering primary diagnoses on scanned slides (11). Inevitably, the importance of digital pathology has risen significantly, leading to increased use digital pathology in daily routine. Unsurprisingly, a more recent poll of Swiss pathologists at the height of the pandemic revealed the use of digital pathology for primary routine diagnostics had more than doubled since onset of the pandemic (30 vs. 13.5%) (12).

In the midst of the rise in digital pathology, it cannot be forgotten that currently, the vast majority of pathologists have completed their training and accumulated years if not decades of experience using conventional light microscopy. Therefore, most pathologists still currently consider conventional microscopy as gold standard not only when it comes to

diagnostic confidence, but also speed and efficiency in routine. In our experience, when faced with a high diagnostic workload and the option between digital and conventional work, many pathologists will still prefer to work with glass slides. The optimization of digital pathology in this regard is truly challenging on many levels and should not be underestimated. Even if performance issues as frequently encountered in the setting of remote work can be eliminated, each extra mouse click will add up to a considerable and noticeable amount of extra time spent on sign-out over the course of a busy workday. Investing time to achieve optimal user interface in the LIS and IMS are especially important and require close cooperation between the MDs and technical team.

The perceived ease of digital pathology is an important factor for everyday use in diagnostic routine. Speed, accuracy, ease of use and ergonomics should all be taken into consideration. However, although crucial from a practical point of view, reports in the literature on such aspects of digital sign-out are rare. Certain input devices such as a conventional mouse may be straining, especially when using the wheel for zooming over a prolonged period of time. Here, other types of devices may be preferred, especially in terms of comfort (13).

Although several studies have demonstrated that digital pathology is non-inferior to conventional light microscopy in a wide spectrum of diseases and organ systems (14, 15), there are still some potential issues to be aware of. For instance, the detection of microorganisms and subtle morphological features such as nuclear details, including mitotic figures and chromatin patterns on scanned slides can be challenging. This can be due to several reasons. For instance, poor quality of scans can be a major source of uncertainty and discrepancies from glass slide diagnoses, in which case rescanning at a higher magnification or Z-stacking may increase diagnostic confidence (**Figure 2**) (14). However, it has also been suggested that many discrepancies are not due to limitations of diagnosis on WSI as a process but other factors, such as naturally occurring interobserver variability, inadequate tissue processing or lack of experience of the pathologist (16).

## Validation

As digital diagnostics represents a technique that significantly differs from conventional light microscopy, validation tests must encompass the entire digital system to ensure necessary standards are met. This includes the scanning process, the LIS and IMS interface and archiving of WSI. Good practice statements and recommendations of the College of American pathologists (CAP) (3) from which general principles have been adapted by most societies are summarized in **Table 2**.

Pathologists themselves are a crucial part of the validation process, especially since not all pathologists may be confident with working in a digital process and should be given the

possibility to acquire skills outside live reporting. There are several published recommendations for pathologist validation which are more or less extensive (3, 17, 18) but share the following principles:

- Training and validation cases should reflect real life work and include all types of stains used in diagnostic routine (including special stains and immunohistochemistry).
- Validation should include a comparison between diagnoses made by the same pathologist on glass slides and WSI after a washout period to minimize recall bias and eliminate interobserver variability. All pathologists using digital diagnostics in routine must complete the validation study.
- Documentation of the validation protocol including concordance and discordance of cases. The level of concordance between diagnoses on glass slides and WSI is generally expected to be over 95%.

In addition to diagnostic routine on WSI, all image analysis tools must be validated by each institution prior to clinical use, irrespectively of FDA- (or equivalent) approval status. As specific guidelines for validation studies and acceptance criteria for such tools are lacking, the approval of a validation study design is left up to the discretion of the medical director. However, the basic aspects as mentioned above should be applied: the validation study should be applicable to the intended clinical use in which the tool will be employed and encompass the spectrum and complexity expected to be encountered in diagnostic routine. Important considerations for in-house algorithm validation study design are listed in **Table 3**.

## Quality control of whole slides images

Suboptimal WSI can be due either to issues in tissue handling (e.g., folds, tears, poor staining, thick cuts etc.) or the scanning process (focus issues, tissue recognition, etc.). Quality control of WSI is important for troubleshooting and fixing the root of the cause. If left up to the pathologist to identify poor images, important time is lost in which the underlying problem could already have been solved. Therefore, quality control should ideally be performed in the pathology laboratory. Quality control of glass slides may be acceptable on random samples and certain issues picked up by the naked eye of experienced technicians, but quality control of WSI may require opening each scan, which is quite time-consuming and not feasible for many laboratories – especially considering the amount of extra work scanning may entail. Therefore, automated quality control tools are highly desirable to relieve the laboratory staff of this process. Several automated tools have been proposed, some of which merely identify focus quality (19, 20). However, a more comprehensive solution which addresses both laboratory- and
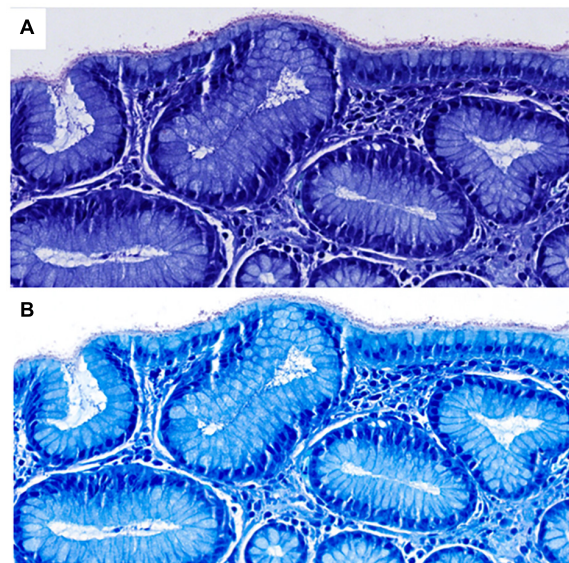
**FIGURE 2**
Scanned Giemsa stains from two different scanners **(A,B)** used in diagnostic routine at our institute in a case of H. pylori gastritis (both at 40×, images courtesy of Ursina Begré). Note the differences in color and brightness between scanners. In a survey of our MDs, scanner B provided a higher level of diagnostic security on Giemsa scans of gastric biopsies but both scanners performed similarly at 40×. Therefore, Giemsa stains for gastric biopsies are scanned at higher magnification per default.

**TABLE 2** Summary of good practice statements (GPS) of the College of American pathologists (3).

GPS 1: All pathology laboratories implementing digital pathology for diagnostic purposes should carry out their own validation studies.

GPS 2: Validation should be appropriate for and applicable to the intended clinical use and clinical setting of the particular application. Validation of WSI systems should involve specimen preparation types relevant to intended use. If a new application for WSI is desired and differs materially from the previously validated use, a separate validation for the new application should be performed.

GPS 3: Validation should closely simulate the real-world clinical environment in which the technology will be used.

GPS 4: Validation should encompass the entire WSI system. However, it is not necessary to validate each individual component (i.e., computer hardware, monitor, network, scanner) of the system or the individual steps of the digital imaging process.

GPS 5: Laboratories should have procedures in place to address changes to the digitized system that could impact clinical results.

GPS 6: Pathologists adequately trained to use the WSI system must be involved in the validation process.

GPS 7: The validation process should confirm all material on a glass slide to be scanned is included in the digital image.

GPS 8: Documentation should be maintained recording the method, measurements, and final approval of validation for the WSI system to be used in the laboratory.

GPS 9: Pathologists should review slides in a validation set in random order. This applies both to the review modality (glass slides or digital) and the order in which slides are reviewed within each modality.

scan-related quality control issues would be ideal, especially in a workflow where all slides are digitized. In 2019, the free open-source software HistoQC was introduced as potential tool for automated quality control (21). Although more of a prototype in its initial form, hampered by its complex configuration and limitations in interpreting calculated metrics (22), further developments may be expected for this tool to be used as a ready-to-use program for clinical application in the future.

## Image analysis — friend or foe?

One of the most obvious advantages of digital pathology is the implementation of automated algorithms to assist in diagnostics. Most tools rely on artificial intelligence, which is an umbrella term that encompasses machine learning and deep learning. Machine learning is a term for computer systems that learn and adapt without following explicit instructions by using algorithms and statistical models to analyze and draw interferences from patterns with data. Deep learning is a type of machine learning based on artificial neural networks, which are inspired by the understanding of biological neural networks (23). Image analysis tasks cover a wide range of potential applications. Basic tasks that might already be in use include quantitative analyses such as a count of objects (e.g., mitoses, tumor-infiltrating lymphocytes, immunohistochemical biomarkers like Ki-67 in breast cancer etc.) that are traditionally perceived as repetitive

**TABLE 3** Considerations for validation study design for image analysis tools.

| | |
|---|---|
| Ground truth definition | The algorithm output must be compared to a ground truth to establish precision and recall (precision addresses the proportion of positive identifications that was actually correct, i.e., a model with no false positives has a precision of 1.0; recall addresses the proportion of actual positives that were correctly identified, i.e., a model with no false negatives has a recall of 1.0) This can be done in several ways:<br>1. Manual annotation: most exact method for comparison to algorithm output but time-consuming<br>2. Eyeballing (if applicable): Region of interest can be pre-set, replicates real-life diagnostic setting<br>3. Comparison with previously reported values (derived from LIS): Least exact method, region of interest not standardized, but least time-consuming |
| Case selection | There are no published guidelines on the number of cases that should be included but the case mix should reflect the real life setting in terms of morphological heterogeneity and complexity (e.g., different histological variants/subtypes) |
| Acceptable range of output values | Define acceptable range of deviation to ground truth. This may depend on clinically relevant cutoffs that determine therapy (e.g., PD-L1, Ki67) |
| Possible confounding effects | If several scanners are used to run the algorithm on check whether the scanner has an effect on algorithm output |
| Identify discrepant cases and analyze reasons for discrepancy | Output values outside the defined acceptable range are discrepant to the ground truth. Can systematic reasons be identified (for example threshold of color detection, falsely identified tumor cells etc.)? Are the ground truth values really correct? In the case of substantial discrepancies, support of the provider may be warranted. |

and time-consuming in routine and may be prone to inter- and intraobserver variability (24).

Other applications may focus on evaluating diagnostic features, such as distinguishing diseased from normal tissue, grading cancers or differentiating between different cancer types. Some systems appear highly successful in tumor detection with reported near perfect accuracy rates (25). There are currently two approaches to implementing cancer-detection algorithms, either as a "first read," in which slides are analyzed prior to the pathologist, highlighting suspicious areas with the aim to improve diagnostic efficiency. In the "second read" approach, slides are evaluated in parallel to the pathologist and notifies in case of clinically relevant discrepancies (for instance a missed focus of cancer tissue) with the intention to minimize error rates. At present, FDA-approval for use in primary diagnostics is limited to only very few products (26) but will certainly be expanded in the near future.

More advanced applications include predictions that might not be evident to the human eye. Comprehensive image analysis for complex aspects such as different cell types in the tumor microenvironment or tumor heterogeneity is simply not feasible without some kind of computational method. Clinically relevant information such as survival, molecular classifications or response to therapy can possibly be predicted by a WSI. For instance, a published algorithm reported predicting HER2 amplification in breast cancer with an AUC of 0.70 (95% CI 0.63–0.77) and more favorable survival in trastuzumab-treated patients according to the automated prediction status (ERBB2 score) (27). In addition, a high ERBB2 score in CISH-HER2 negative patients was associated with unfavorable survival, indicating that the algorithm picked up HER2-cancer-like morphological features linked to poorer outcomes. Another potential tool aiming for a one-stop-shop workflow for pan-cancer image-based detection of clinically actionable genetic alterations based on H&E stained slides was able to significantly predict mutational status of at least some oncogenic genes in

14/14 cancer types, whereas the highest accuracy was achieved for lung, colorectal and breast cancer (AUC 0.60–0.78; 0.65–0.76 and 0.66–0.78, respectively) (28). Evidently, such tools are still of exploratory nature, but it can be assumed that they too will be used in the clinical setting at some point. However, several issues concerning validation in algorithms that could directly influence clinical decision-making will need to be addressed – especially if artificial intelligence (AI)-tools are considered to replace the current gold standard of molecular-based assays - underlining the need for investigation in randomized clinical trials.

Although many studies report high accuracy rates of AI algorithms, this does not necessarily translate to usability in routine (29). Algorithms themselves should undergo rigorous validation on multiple levels according to good practice guidelines (23), ensuring exposure to a wide variety of data sources (including external validation sets, different scanners etc.). However, even if all measures are taken to meet these standards, real-life data will still typically have many more sources of nuances and variation than the datasets used for training and validation. Additionally, it must be emphasized that algorithms are far from replacing the work of pathologists, which is to take a integrative approach incorporating medical knowledge, diagnostic experience and the particular circumstances of a certain case to either make a final diagnosis or decide if further work-up is needed. Therefore, for the foreseeable future a constructive strategy could be to view AI tools as a way of increasing efficiency and enhancing the quality of diagnoses in routine (30).

## Globally relevant issues

Clearly, digital pathology requires significant financial investments that might be affordable for high-income countries but exorbitant for many low-and middle-income countries. Although listed prices vary greatly depending on the product,

a single high-throughput scanner will cost between $100'000 to $400'000 (7). Further costs include hardware (high-resolution display screens and high-performance computers), additional software, IT infrastructure and personnel. In addition to financial issues, low-and middle-income countries tend to suffer from a general shortage of pathologists which often prefer to practice in urban agglomerations, whereas a large proportion of the population lives in rural areas (31). Therefore, unfortunately, the wealth of a particular region will influence potential applications of digital pathology. For instance, especially developing countries can benefit from educational aspects of digital pathology with increasing availability of virtual or hybrid courses, eliminating travel costs. Also, telepathology can aid in seeking second opinions from colleagues, increasing diagnostic quality. Algorithm-assisted diagnostics can also potentially be used to provide some relief to a high workload. Although traditionally, all of these aspects require some financial investment in terms of scanning machines, recent efforts have been made to develop a cost-efficient real-time microscope-based solution for deploying AI tools, eliminating the need for WSI (32).

## Conclusion

Digital pathology is a complex endeavor which requires careful planning in advance. The preparation phase should include detailed analysis of the requirements, starting point and goals of a particular institute as there is no one-size-fits-all solution. In our experience, it is important to assemble a team from the beginning consisting of key players involving all areas which will be affected by digital pathology (including MDs, lab technicians, researchers, etc.). Test phases with structured performance analysis are recommended prior to purchasing large equipment such as scanners. To ensure equivalent diagnostic quality to conventional routine diagnostics, validation studies on the process of digital pathology as a whole and add-ons such as image analysis tools must be performed by each laboratory according to guidelines or good practice standards. Acceptance of digital

pathology generally appears to be high, although affinity for digital work can vary among pathologists. Providing a user friendly, simple and ergonomic digital workspace requires effort but can ease the transition to routine digital diagnostics. At the moment, AI algorithms can be viewed as a possibility to aid and enhance certain aspects of routine diagnostic work rather than replacing the pathologist. Combining forces of human experience, research and AI is bound to help advance an efficient workflow and personalized healthcare treatment.

## Publisher's note

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