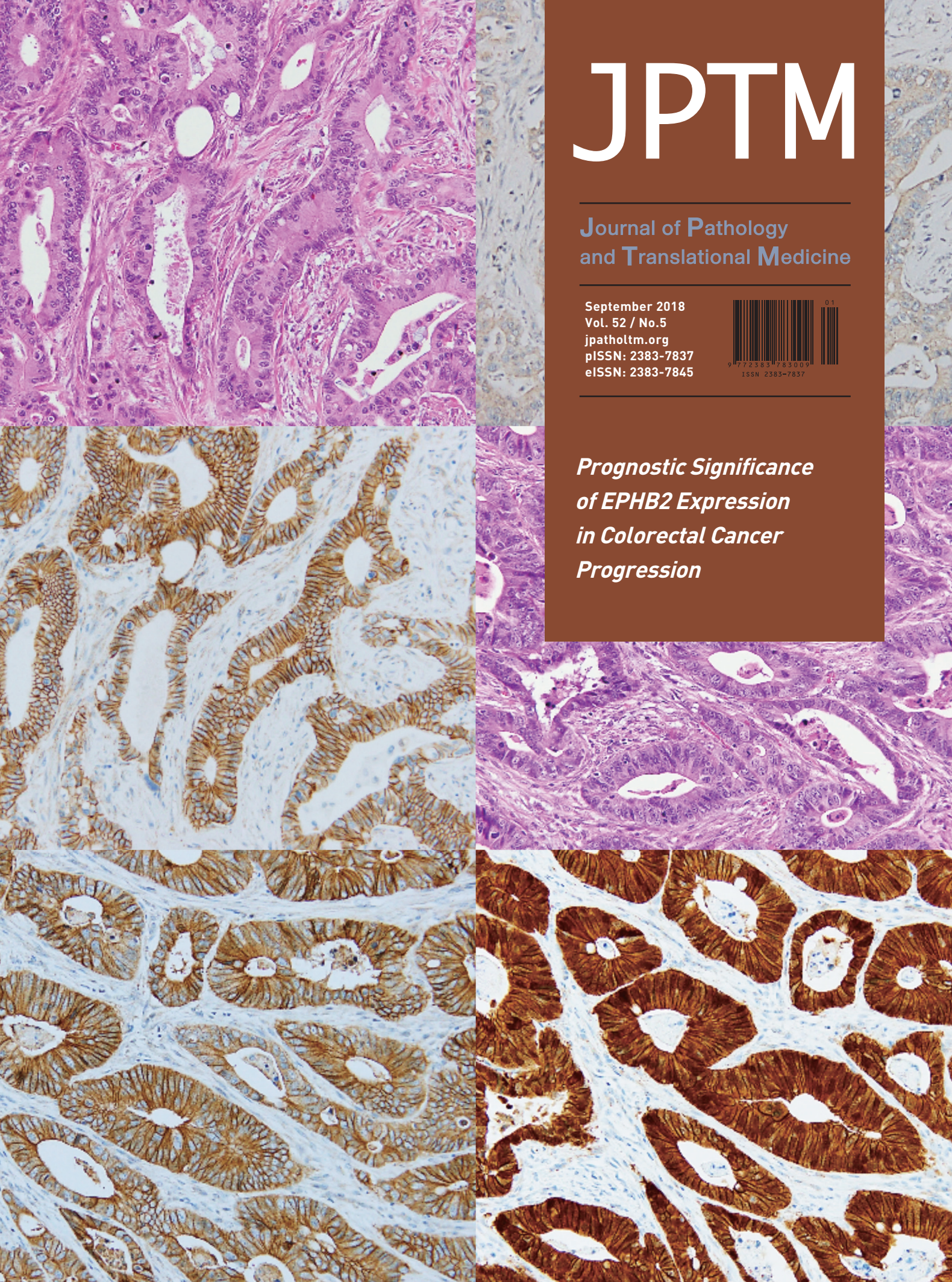


The cover features a collage of histological images. The top left shows a section of colorectal cancer tissue stained with hematoxylin and eosin (H&E), displaying glandular structures and cellular details. The bottom half of the cover is dominated by immunohistochemical (IHC) staining, showing brown chromogen (DAB) reaction in the nuclei of cells within the glandular structures, indicating positive expression of the EPHB2 protein. The background of the journal title and ISSN information is a solid dark brown color.

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*Prognostic Significance
of EPHB2 Expression
in Colorectal Cancer
Progression*

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The *Journal of Pathology and Translational Medicine* is an open venue for the rapid publication of major achievements in various fields of pathology, cytopathology, and biomedical and translational research. The Journal aims to share new insights into the molecular and cellular mechanisms of human diseases and to report major advances in both experimental and clinical medicine, with a particular emphasis on translational research. The investigations of human cells and tissues using high-dimensional biology techniques such as genomics and proteomics will be given a high priority. Articles on stem cell biology are also welcome. The categories of manuscript include original articles, review and perspective articles, case studies, brief case reports, and letters to the editor.

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Front cover image: Representative cases showing negative and positive EPHB2 and nuclear β -catenin expression in colorectal cancers (Fig. 4). p303.

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C-reactive Protein Overexpression in the Background Liver of Hepatitis B Virus–Associated Hepatocellular Carcinoma Is a Prognostic Biomarker

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Background: Chronic hepatitis B virus (HBV) infection is a leading cause of hepatocellular carcinoma (HCC). Peripheral blood C-reactive protein (CRP) concentration and CRP overexpression in HCC cells are proven to be prognostic markers for HCC, but the significance of CRP expression in non-neoplastic hepatocytes, which are the primary origin of CRP, has not been studied. This study was conducted to determine the clinicopathologic significance of CRP immunoreactivity in the background liver of HBV-associated HCC. **Methods:** CRP immunostaining was done on tissue microarrays of non-neoplastic liver tissues obtained from surgically resected, treatment-naïve HBV-associated HCCs (n=156). The relationship between CRP immunoreactivity and other clinicopathologic parameters including cancer-specific survival was analyzed. CRP immunoreactivity was determined using a 4-tier grading system: grades 0, 1, 2, and 3. **Results:** CRP was positive in 139 of 156 cases (89.1%) of non-neoplastic liver in patients with HCCs: grade 1 in 83 cases (53.2%); grade 2 in 50 cases (32.1%); and grade 3 in six cases (3.8%). The patients with diffuse CRP immunoreactivity (grade 3) had decreased cancer-specific survival ($p=.031$) and a tendency for shorter interval before early recurrence ($p=.050$). The degree of CRP immunoreactivity correlated with serum CRP concentration ($p<.001$). **Conclusions:** CRP immunoreactivity in non-neoplastic liver is a novel biomarker for poor cancer-specific survival of HBV-associated HCC and correlates with serum CRP concentration.

Key Words: Carcinoma, hepatocellular; C-reactive protein; Hepatitis B virus; Immunohistochemistry; Prognosis

Acute-phase reactant C-reactive protein (CRP) is primarily synthesized by hepatocytes in association with inflammation.¹ Pro-inflammatory cytokines interleukin (IL)-6 and IL-1 upregulate CRP transcription,^{2,3} and chronic inflammatory conditions of infectious (e.g., bacteria, virus) and noninfectious origins (e.g., toxins, obesity) provide the microenvironment for carcinogenesis primarily by inducing instability of the cellular genome.^{4,5} Inflammatory cellular infiltrates induce the pooling of pro-inflammatory cytokines and oxidative stress at the site of inflammation. Common molecules involved in inflammation-induced carcinogenesis include prostaglandins, nuclear factor κ B, and cytokines. The prognostic significance of CRP blood level has been shown in several human malignancies such as hepatocellular carcinoma (HCC).^{6,7} CRP-based risk assessment algorithms, combined with other parameters such as absolute neutrophil count and blood albumin level, also serve as prognostic indices.⁸⁻¹⁰

HCC is a viral infection-associated cancer posing a significant health-care burden worldwide.¹¹ We have shown that CRP immunoreactivity in HCC cells is a prognostic marker of treat-

ment-naïve HCC.¹² However, the clinicopathologic significance of CRP expression in non-neoplastic background liver, which should be the primary origin of CRP, has not been studied. We postulated that CRP expression in non-neoplastic liver would have pathobiological meaning because the liver is primarily responsible for CRP synthesis. Chronic infection by hepatitis B virus (HBV) is a leading cause of HCC, and this study was conducted to determine the clinicopathologic significance of CRP immunoreactivity in non-neoplastic liver tissue in HBV-associated HCC cases.

MATERIALS AND METHODS

Patients and tissue samples

A total of 156 cases of surgically resectable (R0) HBV-associated HCCs was retrieved from the files of the Department of Pathology, Asan Medical Center, Seoul, Republic of Korea. A tumor recurrence within 2 years after surgical resection was defined as early recurrence. The patients have provided written informed con-

sent, and this study was approved by the Institutional Review Board of Asan Medical Center, Seoul, Republic of Korea (S2011-0931).

Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs) were generated using paraffin blocks of formalin-fixed liver tissues. The hematoxylin and eosin-stained slides were reviewed to select the regions for TMA construction, and 2-mm-thick tissue cores were obtained in duplicates from the donor blocks and transferred onto the recipient blocks. From each TMA, 4- μ m-thick sections were obtained, and subsequent immunohistochemistry was done. For CRP immunohistochemical staining, a rabbit polyclonal anti-CRP antibody (Abcam, Cambridge, UK) was used at 1:1,000 dilution. The sections were placed on silanized slides, and heat-induced epitope retrieval was performed by treating the slides with Cell Conditioning 1 buffer for 32 minutes in a BenchMark XT automatic immunostainer (Ventana Medical Systems, Tucson, AZ, USA). An OptiView DAB IHC Detection Kit (Ventana Medical Systems) was used for the detection of signal. Evaluation of the immunoreactivity was done by a pathologist (C.J.K.), who was blinded to clinical information, using a 4-tier grading system: negative as grade 0; positive in less than 10% of tumor cells as grade 1; positive in less than 50% of tumor cells as grade 2; and diffusely positive in more than 50% of tumor cells as grade 3.

Statistical analysis

For statistical evaluation of the significance of the clinicopathologic parameters, the analyses of noncontinuous categorical variables were done using linear by linear association (chi-square test for trend). We also used logistic regression analysis for multivariate analysis of statistically significant variables in univariate analysis. The relationship between serum CRP level and liver tissue CRP immunoreactivity was analyzed using Spearman correlation analysis and displayed using dot plot. The Kaplan-Meier method and Cox regression analysis were used in the survival analysis. SPSS ver. 18.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

CRP immunoreactivity in liver tissues

CRP expression was observed as cytoplasmic immunoreactivity. Immunoreactive CRP was found in 139 of 156 cases (89.1%), and 17 cases (10.9%) were negative. Among CRP-positive cases ($n = 139$), more than half ($n = 83$; 59.7%) showed grade 1 immu-

noreactivity, followed by the cases with grade 2 ($n = 50$, 36.0%) and grade 3 ($n = 6$, 4.3%) immunoreactivity (Fig. 1). The patient demographics and the relationship between CRP immunoreactivity and other clinicopathologic parameters are summarized in Tables 1 and 2. Among the clinicopathologic parameters analyzed, tumor size and serum CRP concentration significantly varied according to CRP immunoreactivity ($p < .001$ and $p < .005$, respectively). Serum CRP concentration (median, 0.135 mg/dL; range, 0.03 to 6.95 mg/dL) showed a positive linear correlation with CRP immunoreactivity ($p < .001$; $\rho = 0.513$) (Fig. 2). The number of tumors ($p = .058$), serum α -fetoprotein level ($p = .056$), and Edmondson-Steiner histologic grade ($p = .055$) also tended to be different depending on the grade of CRP immunoreactivity.

Of note, CRP immunoreactivity grade 3 was exclusively associated with the presence of satellite nodules ($p < .005$). Furthermore, the patients with CRP immunoreactivity grade 3 showed significantly shorter cancer-specific survival (median, 89.3 months; range, 5 to 104 months) compared with CRP-negative or nondiffuse immunopositive cases (median, 60.7 months; range, 8 to 100 months; $p < .05$) (Fig. 3A). Early recurrence occurred in 63 cases (40.4%). Recurrence-free survival tended to be different between strong and diffuse immunopositive cases (grade 3) and CRP-negative or nondiffuse immunopositive cases (grades 0, 1, and 2). The CRP grade 3 group tended to have shorter time before recurrence (median, 37.8 months; range, 2 to 100 months) than CRP-negative or nondiffuse immunopositive cases did (median, 67.4 months; range, 2 to 103 months; $p = .050$) (Fig. 3B).

Multiple logistic analysis showed serum CRP level greater than 1 mg/dL to be independently correlated with high CRP grade ($p = .017$; odds ratio, 12.229; 95% confidence interval, 1.573 to 95.087), whereas tumor size greater than 5 cm and satellite nodule presence are not (Table 3). Kaplan-Meier survival curves demonstrated shorter survival period in satellite nodule-present group (median, 34.0 months; range, 17 to 64 months; $p < .001$) (Fig. 3C) and vascular invasion-present group (mean, 77.4 months; range, 67 to 88 months; $p = .001$) (Fig. 3D). Cox regression analysis results suggest that the presence of satellite nodule is an independent factor affecting cancer-specific survival (Table 4). Vascular invasion and high-grade CRP immunoreactivity might have independent influence on survival rate; however, statistical significance was not observed.

DISCUSSION

The primary findings of this study are as follows: (1) CRP expression is relatively common in the background liver tissues

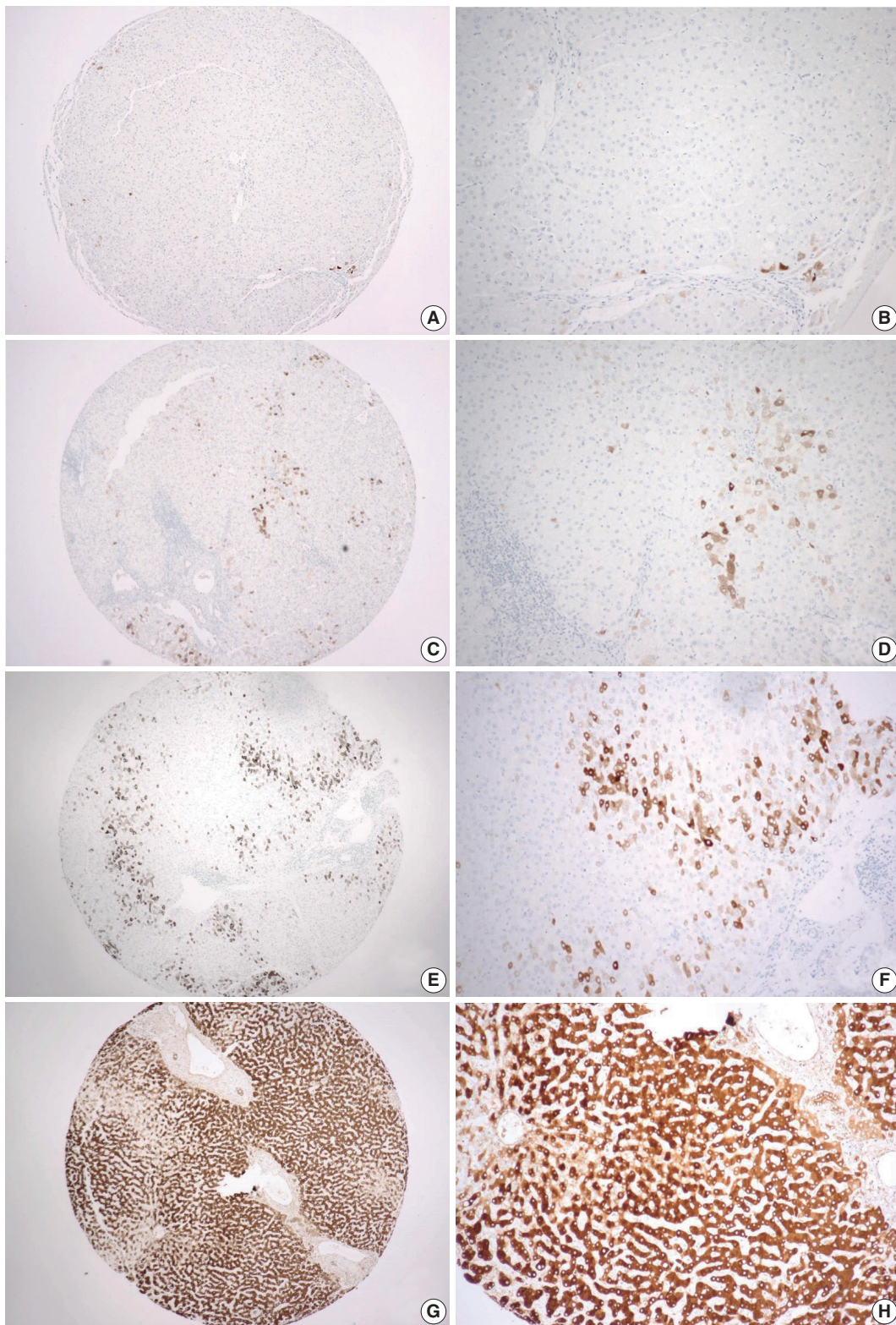


Fig. 1. Cytoplasmic C-reactive protein immunoreactivity in non-neoplastic hepatocytes in treatment-naïve, surgically resected HBV-associated hepatocellular carcinoma cases. Immunoreactivity was determined using a 4-tier grading system: A–B, grade 0; C–D, grade 1; E–F, grade 2; and G–H, grade 3.

Table 1. Clinical parameters and their relationship with CRP immunoreactivity

Clinical parameter	CRP grade 0	CRP grade 1	CRP grade 2	CRP grade 3	p-value
Sex					.590
Male	10 (6.4)	63 (40.4)	37 (23.7)	4 (2.6)	
Female	7 (4.5)	20 (12.8)	13 (8.3)	2 (1.3)	
Age (yr)					.764
<60	14 (9.0)	60 (38.5)	36 (23.1)	5 (3.2)	
≥60	3 (1.9)	23 (14.7)	14 (9.0)	1 (0.6)	
Tumor size (cm)					.000
<5	17 (10.9)	67 (42.9)	31 (19.9)	3 (1.9)	
≥5	0	16 (10.3)	19 (12.2)	3 (1.9)	
Serum AFP (ng/mL)					.056
<400	40 (25.6)	29 (18.6)	26 (16.7)	16 (10.3)	
≥400	24 (15.4)	11 (7.1)	5 (3.2)	5 (3.2)	
Serum CRP (mg/dL)					.002
<1	12 (7.7)	43 (27.6)	28 (17.9)	2 (1.3)	
≥1	0	3 (1.9)	5 (3.2)	3 (1.9)	
BCLC stage					.497
A	17 (10.9)	78 (50)	46 (29.5)	6 (3.8)	
B	0	5 (3.2)	4 (2.6)	0	
Fibrosis stage (Batts–Ludwig)					.773
Stage 1, 2	3 (1.9)	13 (8.3)	7 (4.5)	1 (0.6)	
Stage 3, 4	14 (9.0)	70 (44.9)	43 (27.6)	5 (3.2)	
Microvascular invasion					.890
Not identified	14 (9.0)	57 (36.5)	33 (21.1)	4 (2.6)	
Present	3 (1.9)	26 (16.7)	17 (10.9)	2 (1.3)	
Tumor number					.058
Less than 3	17 (10.9)	80 (51.3)	43 (27.6)	6 (3.8)	
3 or more	0	3 (1.9)	7 (4.5)	0	
Satellite nodule					.099
Absent	17 (10.9)	78 (50)	48 (30.8)	4 (2.6)	
Present	0	5 (3.2)	2 (1.3)	2 (1.3)	
Edmondson–Steiner grade (worst)					.169
Grade 1, 2	7 (4.5)	29 (18.6)	14 (9.0)	1 (0.6)	
Grade 3, 4	10 (6.4)	54 (34.6)	36 (23.1)	5 (3.2)	
Edmondson–Steiner grade (most)					.055
Grade 1, 2	14 (9.0)	57 (36.5)	30 (19.2)	3 (1.9)	
Grade 3, 4	3 (1.9)	26 (16.7)	20 (12.8)	3 (1.9)	
Capsular invasion					.292
Absent	17 (10.9)	65 (41.7)	40 (25.6)	5 (3.2)	
Present	0	18 (11.5)	10 (6.4)	1 (0.6)	
Early recurrence					.415
Absent	38 (24.4)	27 (17.3)	18 (11.5)	10 (6.4)	
Present	26 (16.7)	13 (8.3)	13 (8.3)	11 (7.1)	

Values are presented as number (%).

CRP, C-reactive protein; AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver Cancer.

in HBV-associated HCC cases (89.1%); and (2) there is an inverse relationship between diffuse and strong CRP immunoreactivity and cancer-specific patient survival ($p = .031$). Notable variables significantly associated with CRP immunoreactivity also included tumor size, presence of satellite nodules, and serum CRP con-

centration.

Hepatocellular carcinogenesis represents a classic model of viral etiology, and HCC is among the major causes of cancer-related mortalities.¹³ The prevalence of hepatitis virus infection is relatively higher in East Asian countries. Chronic HBV infection

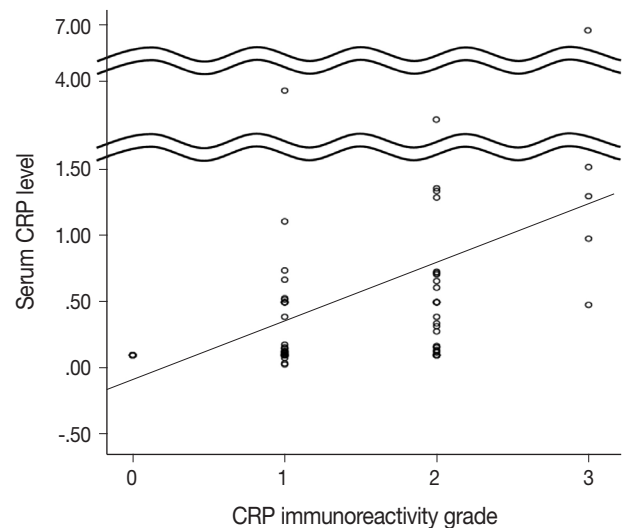
Table 2. Clinical parameters and their relationship with CRP immunoreactivity

Clinical parameters	CRP grade 0, 1, 2	CRP grade 3	p-value
Sex			.719
Male	110 (70.5)	4 (2.6)	
Female	40 (25.6)	2 (1.3)	
Age (yr)			.586
<60	110 (70.5)	5 (3.2)	
≥60	40 (25.6)	1 (0.6)	
Tumor size (cm)			.137
<5	115 (73.7)	3 (1.9)	
≥5	35 (22.4)	3 (1.9)	
Serum AFP (ng/mL)			.585
<400	95 (60.9)	16 (10.3)	
≥400	40 (25.6)	5 (3.2)	
Serum CRP (mg/dL)			.000
<1	83 (53.2)	2 (1.3)	
≥1	8 (5.1)	3 (1.9)	
BCLC stage			.538
A	141 (90.4)	6 (3.8)	
B	9 (5.8)	0	
Fibrosis stage (Batts–Ludwig)			.929
Stage 1, 2	23 (14.7)	1 (0.6)	
Stage 3, 4	127 (81.4)	5 (3.2)	
Microvascular invasion			.890
Not identified	104 (66.7)	4 (2.6)	
Present	46 (29.5)	2 (1.3)	
Tumor number			.515
Less than 3	140 (89.7)	6 (3.8)	
3 or more	10 (6.4)	0	
Satellite nodule			.003
Absent	143 (91.7)	4 (2.6)	
Present	7 (4.5)	2 (1.3)	
Edmondson–Steiner grade (worst)			.395
Grade 1, 2	50 (32.1)	1 (0.6)	
Grade 3, 4	100 (64.1)	5 (3.2)	
Edmondson–Steiner grade (most)			.379
Grade 1, 2	101 (64.7)	3 (1.9)	
Grade 3, 4	49 (31.4)	3 (1.9)	
Capsular invasion			.570
Absent	122 (78.2)	5 (3.2)	
Present	28 (17.9)	1 (0.6)	
Early recurrence			.182
Absent	91 (58.3)	2 (1.3)	
Present	59 (37.8)	4 (2.6)	

Values are presented as number (%).

CRP, C-reactive protein; AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver cancer.

is more common than infection by hepatitis C virus (HCV) is, and more than half of HCCs are associated with HBV infection worldwide.¹⁴ Hence, we focused our analysis of the clinicopathologic significance of CRP immunoreactivity on non-neoplastic

**Fig. 2.** Positive correlation between serum C-reactive protein (CRP) level and CRP immunoreactivity grades of non-neoplastic hepatocytes ($p < .001$; $\rho = 0.513$).

hepatocytes of HBV infection–associated HCCs. HBV infection increases the carcinogenic potential of hepatocytes, either by inducing chronic inflammation or direct integration into the host cell genome.^{11–15} The latter mechanism would explain HBV-associated HCCs in the absence of chronic hepatitis in the background liver. Chimeric integration of HBV genome into several host genes such as cyclin E1 and myeloid/lymphoid or mixed-lineage leukemia 4 has been documented by sequencing studies. The detection and sequencing of high-throughput viral integration done by Zhao *et al.*¹⁶ revealed 4,225 HBV integration events in neoplastic and non-neoplastic liver tissues from 426 patients. Among HCCs with grade 3 hepatocytic CRP immunoreactivity in this study ($n = 6$), three cases were with mixed macronodular and micronodular cirrhosis, two cases were with chronic hepatitis, and one case was without significant background inflammation or cirrhosis.

We previously reported significant correlation between CRP immunoreactivity in HCC cells and patient survival. We looked at the relationship between HCC and corresponding non-neoplastic background liver in this study (data not shown) and found a significant correlation between the two, which indicated that both neoplastic and non-neoplastic hepatocytes are under a similar pro-inflammatory microenvironment in terms of CRP expression.¹² Serum CRP has been consistently shown to be a key component of inflammation-based prognostication of HCC.^{17–19} The molecular mechanisms involved in the prognostic significance of CRP in HCC remain uncertain. However, a recent experimental investigation on CRP knockdown HepG2 cell proteome sug-

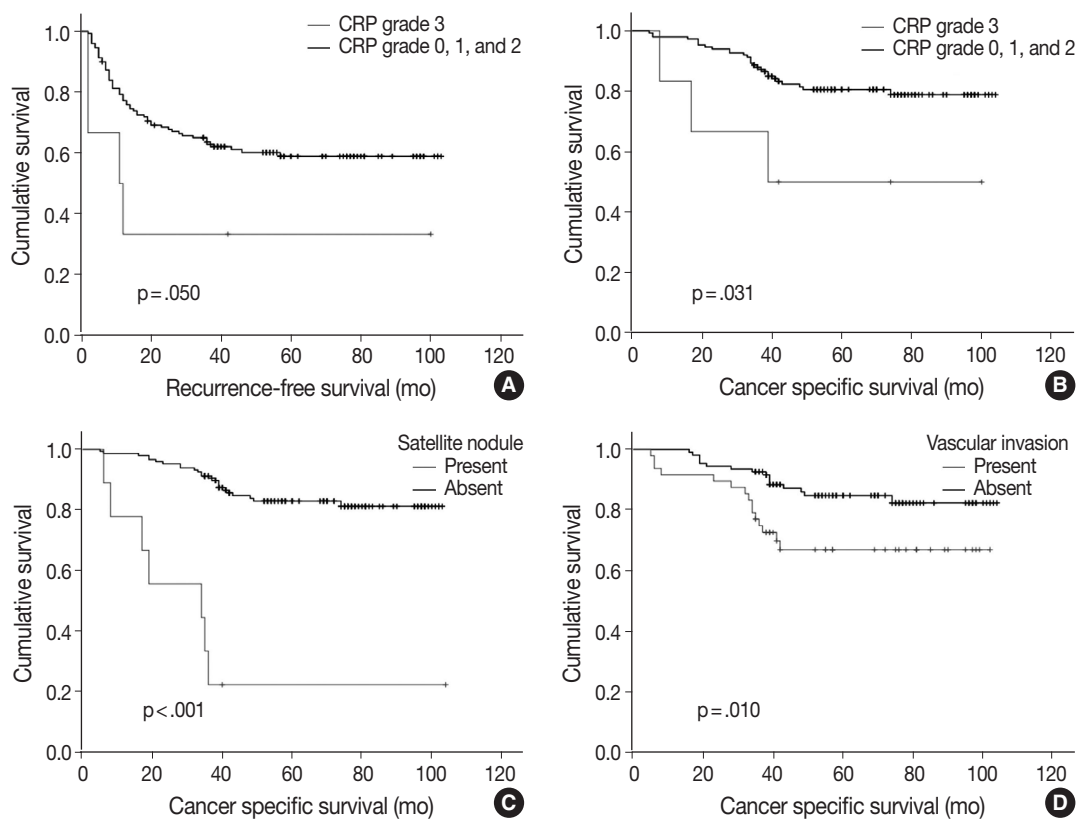


Fig. 3. Relationships between C-reactive protein (CRP) immunoreactivity of hepatocytes and patient outcomes. (A) There is a significant difference in cancer-specific survival between patients with CRP grade 3 immunoreactivity and those with CRP grade 0, 1, or 2 immunoreactivity ($p = .031$). (B) There was a marginal difference in early recurrence of cancer between the 2 groups ($p = .050$). (C, D) The presence or absence of satellite nodule (C) and vascular invasion (D) shows significant difference in cancer-specific survival period ($p < .001$ and $p = .001$, respectively).

Table 3. Factors affecting CRP immunoreactivity by multiple logistic regression analysis

Clinical parameters	OR	95% CI	p-value
Serum CRP	12.229	1.573–95.087	.017
Tumor size			.496
Satellite nodule			.282

CRP, C-reactive protein; OR, odds ratio; CI, confidence interval.

gested that CRP plays a role in promoting the migration, invasion, and metastasis of HCCs via activation of MEK/ERK and phosphoinositide 3-kinase/AKT signaling pathways. Notably, cathepsin D was among the proteins the expression of which was decreased in CRP knockdown cells.²⁰ Other studies have also shown promotion of cell proliferation and antiapoptotic effects in different cell types such as renal tubular epithelial cells and myeloma cells.^{21,22}

Although it is difficult to determine the relative contribution of HCC cells and non-neoplastic hepatocytes to serum CRP level, significant correlation between hepatocytic CRP immunoreactivity and blood CRP concentration supports the clinicopathologic significance of CRP as a surrogate prognostic marker of HBV-

Table 4. Cox regression analysis of parameters affecting cancer-specific survival in patients with HBV-associated hepatocellular carcinoma

Clinical parameters	HR (95% CI)	p-value
Satellite nodule	7.457 (3.004–18.514)	< .001
Vascular invasion		.077
CRP immunoreactivity		.063

HBV, hepatitis B virus; HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein.

associated HCC. Of note, Hu *et al.*²³ proposed that HCC cells are not primarily responsive to serum hepatocyte growth factor, IL-6, and CRP based on the fact that the levels of these proteins go up after resection of HCCs, but this does not rule out the possibility of the significant contribution of HCC cells in affecting blood CRP level.

Regarding the association with tumor size, serum CRP level and tumor size were shown to be a marker of efficacy of transcatheter arterial chemoembolization (TACE) in HCCs.²⁴ TACE reduced blood CRP level in HCC patients.²⁵ Liu *et al.*²⁶ reported

an association between preoperative serum CRP level and HCC tumor size and also found significantly decreased overall survival and recurrence-free survival in HCC patients with elevated serum CRP compared with those with normal serum CRP level. Another independent study by Hu *et al.*²³ demonstrated a relationship between tumor size and preoperative serum CRP level in HCC patients. A significant association with the presence of satellite nodule suggests the possibility of CRP expression and tumor growth characteristics. Kim *et al.*²⁷ reported that CRP is an independent predictor of tumor recurrence in HCC cases with portal vein invasion.

The findings in this study indicate that CRP immunoreactivity in non-neoplastic hepatocytes is a novel prognostic biomarker of HBV-associated HCCs and is associated with certain tumor growth characteristics. The use of immunohistochemistry has merit in that the methodology is easily applicable to routine diagnostic pathology services. Further studies are needed to determine the significance of hepatocytic CRP immunoreactivity in cases with HCV-associated HCCs or nonvirally induced HCCs.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Differential MicroRNA Expression between *EGFR* T790M and L858R Mutated Lung Cancer

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Background: MicroRNAs (miRNAs) are short, non-coding RNAs that mediate post-transcriptional gene regulation. They are commonly deregulated in human malignancies, including non-small cell lung cancer (NSCLC). The aim of this study is to investigate miRNA expression in T790M-mutated NSCLC resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors. **Methods:** Six cases of resected NSCLC harboring the T790M mutation were examined. We performed miRNA time polymerase chain reaction (PCR) array profiling using *EGFR* T790M-mutated NSCLC and L858R-mutated NSCLC. Once identified, miRNAs that were differentially expressed between the two groups were validated by quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** miRNA PCR array profiling revealed three up-regulated miRNAs whose expression levels were altered 4.0-fold or more in the *EGFR* T790M mutation group than in the L858R group: miR-1 (fold change, 4.384), miR-196a (fold change, 4.138), and miR-124 (fold change, 4.132). The three differentially expressed miRNAs were validated by qRT-PCR, and they were found to be overexpressed in the T790M group relative to L858R group. In particular, expression levels of miR-1 and miR-124 were significantly higher in the T790M group (p-value of miR-1 = .004, miR-124 = .007, miR-196a = .096). **Conclusions:** MiR-1, miR-124, and miR-196a are overexpressed in *EGFR* T790M mutated NSCLC.

Key Words: MicroRNAs; Lung neoplasms; *EGFR* T790M mutation

Lung cancer is the most common cause of cancer death worldwide, and advanced-stage non-small cell lung cancer (NSCLC) is considered an incurable disease.¹ Recently, the identification of genetic alterations present in lung cancer has resulted in the development of new treatment options for NSCLC and improved survival of NSCLC patients.² Among these genetic alterations, the epidermal growth factor receptor (*EGFR*) activating mutation is the most widely used target for therapy. However, most patients who initially benefit from the *EGFR* tyrosine kinase inhibitor (TKI) experience subsequent disease progression, and in more than 50% of cases, the mechanism of resistance is the T790M point mutation in the *EGFR* gene.³⁻⁵ As treatment with *EGFR*-TKIs has become routine for advanced lung cancer, the need to better understand the *EGFR* T790M mutation has increased.

MicroRNAs (miRNAs) are a class of short, non-coding RNAs that mediate post-transcriptional gene regulation. They

are involved in nearly all biologic processes, and deregulation of miRNA is correlated with many diseases, including cancer.⁶ Multiple previous studies have observed distinct patterns of miRNA expression across tumor types and revealed that up- or down-regulation of miRNA expression is indicative of a specific cancer.^{7,8} Moreover, a growing number of evidence indicates that certain miRNA profiles distinguish poor-prognosis cancers, and specific miRNA signatures can predict the clinical outcomes of tumors.⁹⁻¹¹ Recent research has suggested that miRNAs have therapeutic capacities and can be used in cancer treatment.¹²⁻¹⁴ All things considered, miRNAs are clinically useful biomarkers and potential therapeutic agents.

The aim of this study is to investigate the expression of miRNAs in *EGFR*-TKI resistant T790M mutation-positive lung cancer. For this purpose, we performed miRNA array profiling and compared miRNA expression between (1) NSCLC with the *EGFR*-TKI sensitive mutation (L858R) and (2) NSCLC with

the EGFR-TKI resistant mutation coexisting with the EGFR-TKI sensitive mutation (T790M/L858R). Using this method, we identified three differentially expressed miRNAs between the two groups. In this paper, we report on these differentially expressed miRNAs with a prediction of common target genes and discuss the possible role of each miRNA in the biology of lung cancer containing the *EGFR* T790M mutation.

MATERIALS AND METHODS

Sample collection

Pathology files from three institutions (Pusan National University Hospital, Pusan National University Yangsan Hospital, and Inje University Haeundae Paik Hospital) and from the time period between January 2011 and June 2016 were reviewed to identify NSCLC harboring the *EGFR* T790M mutation. After the exclusion of biopsy samples due to insufficient tumor material, six out of 1,445 lung cancer patients who had undergone surgical resection were enrolled (Pusan National University Hospital, 4 cases; Pusan National University Yangsan Hospital, 1 case; and Inje University Haeundae Paik Hospital, 1 case). All the included cases were adenocarcinomas harboring pre-existing *EGFR* T790M mutations before exposure to EGFR-TKI. All patients had coexisting TKI sensitive *EGFR* L858R point mutations, and no patients were known to have coexisting exon 19 deletion mutations. All patients underwent curative resection as their first treatment and did not have a history of *EGFR*-targeted therapy, neo-adjuvant chemotherapy, or radiation therapy. *EGFR* mutation testing was conducted as follows: Direct sequencing of the *EGFR* gene was performed in three patients (Pusan National University Hospital, 1 case; Pusan National University Yangsan Hospital, 1 case; Inje University Haeundae Paik Hospital, 1 case). At the Pusan National University Hospital, pyrosequencing was used in the case of one patient, and the peptide nucleic acid-mediated polymerase chain reaction (PCR) clamping method was used to detect the mutation in two patients. All six patients had an EGFR-TKI resistant T790M mutation and a coexisting TKI sensitive L858R mutation. For the control group, *EGFR* L858R mutant adenocarcinoma tissues from eight patients who underwent lung resection surgery were randomly selected. Additionally, four cases of *EGFR* wild-type adenocarcinoma and three non-neoplastic lung tissues were randomly selected and used as control in the miRNA array profiling. This study was approved by the institutional review board of Pusan National University Hospital (C1608-003-001), and informed consent from patients was waived.

miRNA extraction and cDNA synthesis

Hematoxylin and eosin (H&E) stained slides were prepared from routinely processed tissue sections using 10% buffered formalin and then reviewed to confirm the diagnosis. Five 10- μ m sections were cut from a representative paraffin block of each tumor and mounted on glass slides. The tumor area intended for tissue dissection was marked on the unstained slides using the matched H&E stained slide; therefore, only the tumor portion was subjected to miRNA analysis. Manually dissected tissue samples were placed in 1.5 mL microcentrifuge tubes and deparaffinized in xylene. After washing with ethanol, the total miRNA of the tissue was extracted and purified using a miRNeasy formalin-fixed paraffin-embedded (FFPE) kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of each isolated miRNA was measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the samples that showed optimal purity and quantities were subjected to further analysis. Then, miRNA was processed using an miScript II reverse transcription kit (Qiagen) and cDNA was synthesized.

miRNA array profiling

The synthesized cDNA was used as a template for subsequent real-time PCR. The reaction mix was prepared using a miScript SYBR Green PCR Kit (Qiagen). The miRNA PCR array method was employed for comprehensive miRNA expression profiling. We used the miScript miRNA PCR Array Human Cancer Pathway Finder (MIHS-102Z, Qiagen), which is composed of a 96-well plate containing 84 human cancer specific miRNA primers and 12 controls. The selected miRNAs are known to be associated with the diagnosis, staging, progression, or prognosis of various cancers. The primers used for miRNA array profiling are described in Supplementary Table S1. Using the RT² PCR array loading reservoir (Qiagen), equal amounts of the reaction mix were added to each well of the array plate. Then, real-time PCR was performed in the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and 84 miRNAs were simultaneously quantified in each sample. For miRNA PCR array profiling, we used four cases of *EGFR* T790M- and L858R-mutated adenocarcinoma as test samples and five cases of *EGFR* L858R-mutated adenocarcinoma as control samples. Additionally, four cases of *EGFR* wild-type adenocarcinoma and three non-neoplastic lung tissues were also used as controls in this array profiling.

Data analysis

The miRNA profiling data were analyzed by web-based miRNA PCR Array Data Analysis software (<http://pcrdata-analysis.sabiosciences.com/mirna/arrayanalysis.php>) to identify differentially expressed miRNAs between the groups. This software automatically performs fold-change calculations from the user's uploaded Ct values obtained in miRNA PCR.

Validation of differentially expressed miRNAs

We validated the differentially expressed miRNAs selected from miRNA array profiling results with a larger sample group that included six cases of T790M/L858R-mutated adenocarcinoma and eight cases of L858R-mutated adenocarcinoma. In the T790M-positive group, four out of six cases were the same cases included in the microarray profiling. In the control group without the T790M mutation, all eight cases were different from those of microarray profiling. miRNA was processed using the miRNeasy FFPE (Qiagen) and TaqMan MicroRNA reverse transcription (Applied Biosystems) kits. For the targeted quantification of up- or down-regulated miRNAs, preformulated primers included in the TaqMan MicroRNA Assays (Applied Biosystems) were used. The PCR reaction mix was prepared with TaqMan Universal Master Mix II (Applied Biosystems) and the ABI 7500 Real-Time PCR system (Applied Biosystems) was used for quantitative real-time PCR (qRT-PCR) analysis. The miRNA expression level was normalized to endogenous control gene RNU48. Following the calculation of the Δ Ct values, miRNA expressions of T790M/L858R-mutated and L858R-mutated samples were compared by t-test using the statistical program R. A $p < .05$ was considered statistically significant.

Target gene prediction and gene set enrichment analysis

We evaluated common target genes of differentially expressed miRNAs using MicroRNA.org (<http://www.microrna.org>).¹⁵ Gene set enrichment analysis was done with the top 10%-ranked genes in the common target gene list using MSigDB v6.0.¹⁶

RESULTS

Clinicopathologic factors of the study population

Six cases of *EGFR* T790M-positive and eight cases of T790M-negative pulmonary adenocarcinoma were included in the study. The clinicopathologic information of the two patient groups are described in Supplementary Tables S2 and S3. In the T790M-positive group, all patients were female (age range, 47

to 72; mean, 63). Tumor size ranged from 2.0 to 3.6 cm (mean, 2.78). Five adenocarcinomas exhibited an acinar pattern, while 1 was an adenocarcinoma with a papillary pattern. In the control group without the *EGFR* T790M mutation, six patients were female and two were male. The patients' ages ranged from 44 to 72 years (mean, 61). Mean tumor size was 2.29 cm (range, 1.4 to 4.0 cm). The predominant histologic patterns were acinar (5 cases), lepidic (2 cases), and papillary (1 case). A representative microphotograph of T790M mutated adenocarcinoma (case 5) is shown in Fig. 1.

miRNA array profiling analysis

We identified three up-regulated miRNAs whose expression levels were altered by 4.0-fold or more in *EGFR* T790M/L858R mutated lung cancer tissues compared to L858R mutated tissues (Table 1). These were miR-1 (fold change, 4.3841), miR-196a (fold change, 4.1380), and miR-124 (fold change, 4.1321). There were no down-regulated miRNAs in the T790M-positive lung cancer group. Compared to *EGFR* wild-type lung cancer, T790M/L858R mutated lung cancer was found to have 20 up-regulated miRNAs, including miR-1 and miR-196a (Table 2). No significantly differentially expressed miRNAs were identified between the L858R and *EGFR* wild-type groups. When non-neoplastic lung tissue was used as a control sample, there were seven overexpressed miRNAs in the T790M/L858R group, one of which was miR-196a (Table 3).

Comparison of miRNA expression between the T790M/L858R and L858R groups (miR-1, miR-196a, miR-124)

The expression of miR-1, miR-196a, and miR-124, which

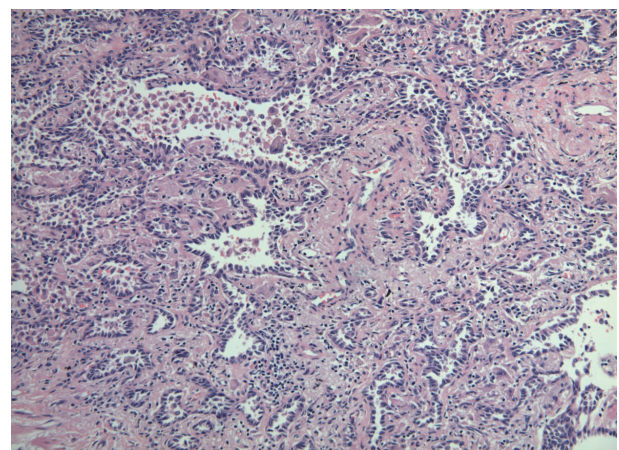


Fig. 1. Representative microphotograph of *EGFR* T790M mutated adenocarcinoma (predominantly acinar pattern of case 5). *EGFR*, epidermal growth factor receptor.

were found to be differentially expressed in array profiling, was validated by qRT-PCR. The three miRNAs were overexpressed in the T790M/L858R group when compared to the L858R group. In particular, the expression of miR-1 and miR-124 was

Table 1. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs L858R mutation)

miRNA	Fold change
miR-196a	4.1380
miR-124	4.1321
miR-1	4.3841

EGFR, epidermal growth factor receptor.

Table 2. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs *EGFR* wild type)

miRNA	Fold change
miR-196a	5.9922
miR-148b	5.0038
miR-146b	5.0653
miR-183	7.0982
miR-148a	5.7195
miR-218	6.0127
miR-29b	4.2675
miR-135b	8.4407
miR-21	7.1416
miR-10b	4.9795
miR-155	4.2678
miR-1	7.1204
miR-27b	5.0205
miR-7	6.0372
miR-9	7.0287
let-7f	5.9577
miR-10a	5.8620
miR-16	4.2105
miR-98	5.0532
miR-126	5.0032

EGFR, epidermal growth factor receptor.

significantly higher in the T790M group (p-value of miR-1 = .004, miR-124 = .007) (Fig. 2A, B). Expression of miR-196a was higher in the T790M group than in the L858R group, but not significantly so (p = .096) (Fig. 2C).

Prediction of common target genes of miR-1, miR-124, and miR-196a

We investigated putative common target genes of these three miRNAs using microRNA.org, and found 2,010 candidate genes. Then, the MSigDB web tool was used to conduct gene set enrichment analysis of the top 10%-ranked genes in the common target gene list. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that 18 gene sets were significantly enriched (false discovery rate < 0.05), including the sets of several cancer-related and signaling pathways (Supplementary Table S4).

DISCUSSION

Lung cancer is the leading cause of cancer mortality worldwide. Despite extensive efforts to improve the survival rate, the 5-year survival for NSCLC is only 21%.¹ Moreover, 57% of

Table 3. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs non-neoplastic lung tissue)

miRNA	Fold change
miR-196a	17.5784
miR-96	4.4977
miR-183	7.3777
miR-135b	9.3675
miR-21	6.1286
miR-9	9.7106
miR-210	4.2718

EGFR, epidermal growth factor receptor.

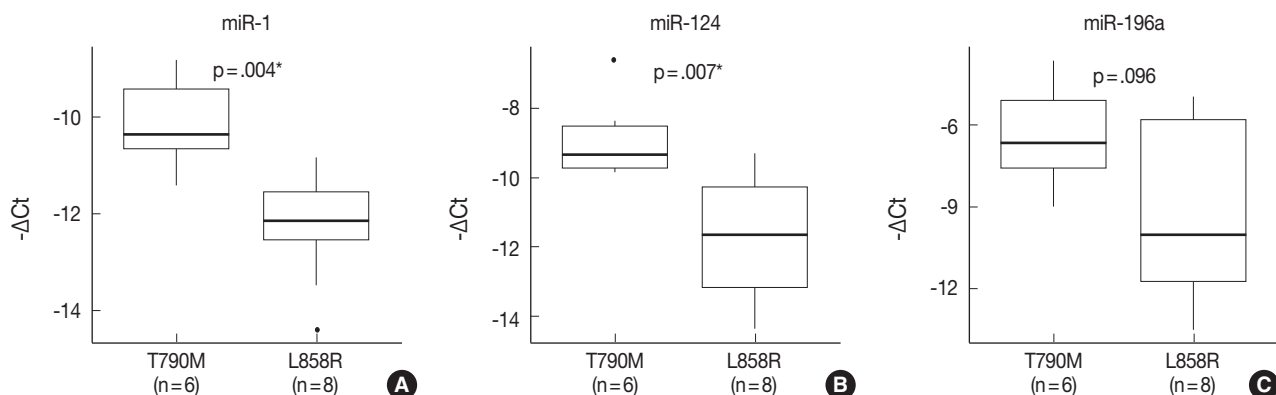


Fig. 2. Expression of miRNAs (A, miR-1; B, miR-124; and C, miR-196a) in *EGFR* T790M and L858R groups. EGFR, epidermal growth factor receptor.

lung cancers are diagnosed at an advanced stage, with a 5-year survival rate of 4%.¹ Over the last few decades, the development of targeted agents has provided marked survival benefits to lung cancer patients whose tumors harbor specific genomic alterations.¹⁷ In particular, agents targeting *EGFR*-activating mutations are the most widely used targeted therapy, and *EGFR* mutation analysis of NSCLC is now routine in standard clinical practice. It is well known that patients carrying a point mutation in exon 21 (L858R) or a deletion in exon 19, which account for approximately 90% of *EGFR*-activating mutations, have significant survival benefit when treated with EGFR-TKI.¹⁸ However, the majority of patients who initially respond to EGFR-TKIs acquire resistance to the drug, with the most important mechanism of resistance being a T790M gatekeeper mutation in the *EGFR* gene.⁴ This mutation leads to decreased drug binding through steric hindrance and increased binding affinity with ATP.^{19,20} The *EGFR* T790M mutation is a current focus of study for many investigators attempting to overcome EGFR-TKI resistance.^{21,22} Osimertinib, which was recently developed to treat patients with *EGFR* T790M mutation-positive NSCLC, is being tested in clinical trials.²³ However, the complex biology of EGFR-TKI resistance is not thoroughly understood, and new approaches are being explored.²⁴ We speculated that altered miRNA expression of T790M-mutated NSCLC might provide us with new insights into EGFR-TKI resistance.

miR-1 is known to be a muscle-specific miRNA that is preferentially involved in muscle development. However, many studies have also demonstrated that miR-1 functions as a tumor suppressor, regulating several oncogenic pathways in human cancers.^{25,26} According to previous research, the expression of this miRNA was significantly down-regulated in several cancers, and restoration of miR-1 in cancer cells resulted in inhibition of proliferation, migration, and invasion, indicating its therapeutic potential.²⁷ miR-124 has also been reported to act as a tumor suppressor in human cancers. Several studies have demonstrated that this miRNA is frequently silenced by DNA methylation, and thus, plays a pivotal role in carcinogenesis. Moreover, down-regulation of miR-124 was correlated with tumor progression and its reinstatement was suggested to be a novel therapeutic strategy.²⁸⁻³⁰ In the present study, these tumor suppressive miRNAs (miR-1, miR-124) were overexpressed in the T790M/L858R mutation group relative to the L858R mutation group. This result corresponds with previous studies indicating that the natural clinical behavior of the T790M mutated tumor is more indolent except for the poor response to EGFR-TKI therapy.³¹ Some studies revealed that patients who acquired the

EGFR T790M mutation after TKI therapy had longer survival, suggesting that the T790M mutation may be related to indolent biology and slow tumor growth.^{32,33} In the EURLAC (EU-Ropean TARceva vs Chemotherapy) study, the survival of patients with a pre-existing T790M mutation was superior to that of patients without it.³⁴ Fujita *et al.*³⁵ found that presence of the T790M mutation correlated with better outcomes even in patients treated with TKI. Taken together, these data suggest that T790M mutant tumors may have indolent clinical course and the tumor suppressive miRNAs identified in the present study may contribute to this.

Most studies of miR-196a have reported that this miRNA is overexpressed in a variety of human malignancies, especially in pancreatic cancer.^{36,37} It has a dominant effect on the inhibition of tumor suppressor genes, finally acting as oncogenes.³⁸ In this study, we found that miR-196a was up-regulated in T790M/L858R-positive tumors when compared to tumors that were only L858R-positive. We also observed that miR-196a was highly overexpressed in T790M-positive tumor tissue compared to non-neoplastic lung tissue, a finding that is consistent with the previous reports suggesting that miR-196a has an oncogenic function.

In our investigation, we also analyzed the common target genes of 3 miRNAs (miR-1, miR-124, and miR-196a) by gene set enrichment analysis. KEGG pathway enrichment analysis showed that the predicted targets were mainly enriched in cancer-related gene sets that included renal cell carcinoma, chronic myeloid leukemia, endometrial cancer, and non-small cell lung cancer, as well as in signaling pathway gene sets that included JAK-STAT, ErbB, and RIG-I-like receptor signaling pathways. Four genes (*SOS1*, *PIK3CG*, *NRAS*, and *CRKL*) were common to most of these gene sets. A review of previous studies revealed that these pathways or genes were reported to be associated with EGFR-TKI resistance. In a study using T790M-mutated NSCLC cell lines, the authors demonstrated that activation of the IL-6R/JAK1/STAT3 pathway induced *de novo* resistance to irreversible EGFR-TKIs.³⁹ In another study, oncogenic CRKL was shown to activate the SOS1-RAS-RAF-ERK and SRC-C3G-RAP1 pathways, and overexpression of CRKL in *EGFR*-mutant cells induced resistance to EGFR-TKI.⁴⁰ Wu *et al.*⁴¹ reported that targeted regulation existed between miR-124 and STAT3, and up-regulation of miR-124 suppresses STAT3. It is also noteworthy that miR-124 was observed to directly target the 3'-untranslated regions of *SOS1* mRNA and regulate the behavior of tumor cells.⁴² These findings indicate an association between resistance to EGFR-TKI and the up-regulated miRNAs

examined in this study, and we conjecture that miR-124 regulates the expression of STAT3 or SOS1 and ultimately targets the EGFR pathway. However, miRNAs target numerous genes, and their mechanism of action is extremely complicated. Therefore, further study is needed to validate the function of these miRNAs.

In this study, we used cases in which the T790M mutation pre-existing, rather than acquired. Acquired *EGFR* T790M mutations occur in 50%–60% of patients who initially benefited from EGFR-TKIs and are the primary mechanism of resistance to therapy. In some patients, however, *EGFR* T790M mutations may exist before EGFR-TKI exposure, and these pre-existing mutations are associated with *de novo* resistance to the drugs.⁴³ We used samples with pre-existing T790M mutations because repeat biopsies were rarely performed for lung cancer patients whose disease progressed after treatment with EGFR-TKIs. In cases where the biopsy was repeated, only small tissue fragments or cytology specimens were included, so even after confirming the presence of an acquired T790M mutation, the biopsy tissue was inadequate for further miRNA analysis. Therefore, all the available tissues for this study were of pre-existing T790M mutants before EGFR-TKI therapy. We assumed that the fundamental action of the *EGFR* T790M mutation does not differ between cases of acquired and pre-existing mutations. According to one previous study, a minor clone with a pre-existing T790M mutation is present at a low frequency in NSCLC, and selective pressure from EGFR-TKI therapy causes expansion of these mutant cells.⁴⁴ This selection results in the emergence of a resistant phenotype in the entire tumor cell population. Therefore, the acquired T790M mutation can be considered an enriched pre-existing T790M mutation. In a certain point of view, an investigation with a *de novo* T790M mutation sample might be better than with the samples of acquired T790M mutation, especially when the researcher wants to know the innate nature of *EGFR* mutation types, since the acquired T790M mutation may be accompanied by additionally accumulated genetic alterations during EGFR-TKI therapy.

We performed miRNA PCR array profiling using NSCLC tissue, and the results showed that 3 miRNAs (miR-1, miR-124, and miR-196a) were up-regulated in the T790M mutation group compared to the L858R mutation group. The miRNA expression levels obtained with qRT-PCR were in accordance with the array profiling results. The results from this study may yield insights into the mechanisms of EGFR-TKI resistance.

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Electronic Supplementary Material

Supplementary materials are available at Journal of Pathology and Translational Medicine (<http://jpatholtm.org>).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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p40 Immunohistochemistry Is an Excellent Marker in Primary Lung Squamous Cell Carcinoma

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Background: Lung cancer is the third most common cancer worldwide. With major advances in the molecular testing of lung cancers and the introduction of targeted therapies, the distinction between adenocarcinoma and squamous cell carcinoma as well as pathologic subtyping has become important. Recent studies showed that p40 is highly specific for squamous and basal cells and is superior to p63 for diagnosing lung squamous cell carcinoma. The aim of this study was to evaluate the use of p40 immunohistochemical stain in the diagnosis of non-small cell lung carcinoma and its potential to replace current p63 antibody as the best immunohistochemical squamous marker. **Methods:** Seventy formalin-fixed paraffin-embedded cases previously diagnosed as primary lung squamous cell carcinoma (n=35) and lung adenocarcinoma (n=35) from January 2008 to December 2016 were retrieved. The results of tumour cell immunoreactivity for p40 and p63 antibodies in lung squamous cell carcinoma and lung adenocarcinoma were compared. **Results:** p40 was expressed in 27 cases of lung squamous cell carcinoma (77.1%). All cases of lung adenocarcinoma (35/35, 100%) were negative for p40. p63 expression was positive in 30 cases of lung squamous cell carcinoma (85.7%) and 13 cases of lung adenocarcinoma (37.1%). Reactivity for both p40 and p63 in lung squamous cell carcinoma was strong and diffuse, whereas variable reactivity was observed in lung adenocarcinoma. **Conclusions:** p40 is an excellent marker for distinguishing lung squamous cell carcinoma from adenocarcinoma, and p40 expression is equivalent to p63 expression in lung squamous cell carcinoma.

Key Words: Non-small cell lung carcinoma; Immunohistochemistry; Lung neoplasms; p40; p63

Lung cancer is the most common cancer among males and the third most common cancer in the general population of Malaysia as well as worldwide. Worldwide, there was an estimated 1.8 million new cases of lung cancer in 2012.¹ In Malaysia, there were 10,608 cases of lung cancer diagnosed between 2007 to 2011, comprising 7,415 males and 3,193 females.² Lung cancer is also the most common cause of cancer deaths and is estimated to be responsible for 1.59 million mortalities.¹ Most lung cancers are detected at a late stage, and 60% of cases were detected at stage IV.¹

With recent advances in molecular testing of lung cancers and the introduction of targeted therapies, distinction between adenocarcinoma and squamous cell carcinoma, as well as pathologic subtyping, has become increasingly important. Specific therapies can be offered to patients depending on the histologic diagnosis and molecular status of the tumour. For example, patients with lung adenocarcinoma harbouring epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*) rearrangement are given first-line treat-

ment with tyrosine kinase inhibitors and crizotinib, respectively, while patients with squamous cell carcinoma are contraindicated for bevacizumab therapy, as it is associated with life-threatening haemorrhage.^{3,4} Therefore, it is crucial to establish the accurate histologic subtype to guide patient selection for further molecular testing.⁵

Patients with advanced disease are usually inoperable, so they often receive targeted molecular therapies.³ In such situations, a small biopsy and cytology preparations are often the only specimens available for histologic diagnosis and molecular testing.³ This has led to major changes in the latest pathologic classification by the World Health Organization (WHO) with particular emphasis on tumour classification and strategic tissue management for handling non-resected specimens.⁴ The diagnosis of adenocarcinoma and squamous cell carcinoma can be made based on morphology alone in 50%–70% of non-resected specimens.⁴ However, immunohistochemistry and special stains are often required in the setting of poorly differentiated tumours that do not show definite morphology by routine light microscopy.

The recommended markers for adenocarcinoma are thyroid transcription factor 1 (TTF1) and napsin A, whereas accepted antibodies for squamous differentiation include p63, p40, and cytokeratin (CK) 5/6.⁴ To preserve as much tissue as possible for molecular studies, algorithms recommend a maximum use of two antibodies in each case.^{4,6,7} Most tumours can be classified using a single adenocarcinoma marker and a single squamous marker.^{4,6}

p63 is a homologue of the p53 tumour suppressor gene that is responsible for proliferation and differentiation of epithelial progenitor cells.⁸ The p53 gene contains two promoters that produce two isoforms; one isoform contains the N-terminal transactivation domain (TAp63) and the other lacks this domain (Δ Np63).⁸ p63 is normally expressed in the nuclei of basal and progenitor cells of stratified epithelia such as skin, esophagus, tonsil, urothelium, ectocervix, and vagina, and in the basal cells of glandular structures of the thymus, prostate, breast, and bronchi.⁸ Both TAp63 and Δ Np63 show overlapping distribution in some epithelial tissue. However, TAp63 is more expressed in differentiated cells while Δ Np63 is seen in the stem-like cell populations.⁸

Anti-p63 (4A4) is a well-accepted immunohistochemical marker for lung squamous cell carcinoma in most laboratories. Despite having extremely high sensitivity, as antibodies for p63 (4A4) recognize both p63 and p40 proteins, studies have shown it has a lack of specificity in a subset of lung cancers, particularly lung adenocarcinoma.^{4,9} Focal or weak p63 staining can be seen in 30% of lung adenocarcinoma and has been misinterpreted to favour squamous differentiation.^{3,10} Interpretation of p63 is also dependent on TTF1 expression because only diffuse p63 staining in the absence of TTF1 is considered to favour squamous cell carcinoma.⁴ An incorrect diagnosis of squamous cell carcinoma could be made with unforeseen faulty TTF1 immunostaining.⁸ Moreover, additional squamous markers such as CK5/6 may be required, which compromises tissue preservation for molecular studies.^{8,11}

Antibody p40, which identifies Δ Np63, has been available for several years but its use for distinction of lung squamous cell carcinoma and adenocarcinoma was only recently studied. p40 is consistently the predominant isoform expressed in squamous cell carcinoma; thus, it offers improved specificity for diagnosing squamous cell carcinoma.⁸ Studies by Bishop *et al.*⁹ and Nonaka¹⁰ showed that p40 has 100% sensitivity and specificity in lung squamous cell carcinoma. Another study by Tacha *et al.*¹² reported an 85% sensitivity and 98% specificity.

In local Malaysian diagnostic histopathology laboratories,

selection of immunohistochemistry studies is highly dependent on the morphologic features present on hematoxylin and eosin sections and the availability of markers. Currently, there is no standardized diagnostic immunohistochemical workup in the diagnosis of non-small cell lung carcinoma (NSCLC). However, a panel of TTF1, CK5/6, and p63 is the most commonly used set of immunohistochemistry studies for distinguishing adenocarcinoma from squamous cell carcinoma.

In this study, we investigated the expression of p40 in lung squamous cell carcinoma and lung adenocarcinoma in comparison to p63 expression. In addition, the present study was performed to evaluate the utility of p40 and p63 in arriving at an accurate diagnosis in NSCLC.

MATERIALS AND METHODS

This study was approved by the Ethical Committee of Universiti Kebangsaan Malaysia Ref No. UKM 1.5.3.5/244/JEP-2016-089 and was exempted from informed consent by the institutional review board (IRB). All cases diagnosed as primary lung squamous cell carcinoma and adenocarcinoma between January 2008 and December 2016 were reviewed. Cases that fulfilled the inclusion and exclusion criteria with sufficient tissue samples were selected for this study. Hematoxylin and eosin stained slides were reviewed by two pathologists to confirm the diagnosis. Tumours were graded on the basis of WHO classification. The tissues were obtained from routine formalin-fixed paraffin-embedded blocks of small biopsies and resected specimens of 35 lung squamous cell carcinoma and 35 lung adenocarcinoma cases. Information on patient details was retrieved from the Computerized Medical Record System. Patient identity remained anonymous and each subject was coded accordingly.

Primary antibodies

Two antibodies were used in this study, mouse monoclonal p63 antibody (Clone DAK-p63, Ready-To-Use, Code IR662, Dako, Glostrup, Denmark) and mouse monoclonal p40 antibody (1:100, clone BC28, Code Ab172731, Abcam, Cambridge, UK). Normal skin and laryngeal squamous cell carcinoma tissues were used as the positive controls for p63 and p40, respectively.

Immunohistochemical staining method

Immunohistochemical staining was performed on tissue sections using the protocol from EnVision FLEX+, Mouse, High pH (Code No. K8012, Dako). Primary antibody was diluted to

optimal concentration using Antibody Diluent, Dako REAL (Code No. S2022, Dako).

The tissue blocks were sectioned to approximately 3 µm thickness. The tissue slides were incubated on hot plates at 60°C for 1 hour and initial deparaffinization and pre-treatment was performed in the Decloaking Chamber NxGen (Ref. No: DC2012-220V, Biocare Medical, Pacheco, CA, USA) using EnVision FLEX Target Retrieval Solution, High pH (Code No. DM828, Dako) at 110°C for 30 minutes. Slides were incubated with the primary antibodies for 20 minutes at room temperature, followed by incubation with EnVision FLEX/HRP (Code No. DM822, Dako) for 20 minutes. Sections were then incubated with 1× DAB-containing Substrate Working Solution for 10 minutes. Slides were then counterstained with Hematoxylin 2 (REF 7231, Thermo Scientific, Rockford, IL, USA).

Immunohistochemical staining analysis

The staining intensity of both p40 and p63 markers were scored as 0, 1+, 2+, or 3+ (Fig. 1) and the percentage of immunoreactive cells were recorded.⁹ Cases were considered positive if 5% or more of the tumour cells showed brown nuclear staining. Cases with less than 5% staining or no areas of positive staining were regarded as negative.

Statistical analysis

Data analysis was performed using the SPSS software program ver. 20.0 (IBM Corp., Armonk, NY, USA). Pearson chi-square test was used to assess the association between positivity of each marker and tumour diagnosis. Here, p-values less than .05 were considered statistically significant.

RESULTS

Epidemiologic and clinico pathologic data

A total of 70 cases of NSCLC were investigated. More than half of the cases presented with distant metastasis at diagnosis (64.2%). For squamous cell carcinoma, 32 (91.4%) cases were males and three cases (8.6%) were females. The mean age at squamous cell carcinoma diagnosis was 68 years, ranging from 36 to 98 years. Adenocarcinoma had a more similar sex distribution, with 19 cases (54.3%) in males and 16 cases (45.7%) in females. The mean age for adenocarcinoma was 59 years, ranging from 25 to 90 years. Squamous cell carcinomas comprised seven well, 11 moderately, and 15 poorly differentiated tumours. Among the adenocarcinomas, there were nine well, 14 moderately, and 12 poorly differentiated carcinomas. The clinical information is summarised in Table 1.

p40 immunostaining

Twenty-seven cases of primary lung squamous cell carcinoma (77.1%) were positive for p40 (Table 2). The reactivity of p40 nuclear staining in lung squamous cell carcinoma was strong and diffuse; 71.4% of cases had intensity score of 3+ in more than 50% of tumour cells (Table 3, Fig. 2). Remarkably, eight cases that were previously diagnosed as primary lung squamous cell carcinoma showed negative staining. Of these, six cases (17.1%) had intensity score of 0 and two cases (5.7%) showed minimal reactivity (less than 5% of tumour cells) with intensity score of 1+. The original slides were reviewed and all of these cases are poorly differentiated tumours with focal squamous-like morphology including solid growth pattern, abundant eosinophilic cytoplasm (resembling cytoplasmic keratinization), sharp cell borders, and intercellular bridges (Fig. 2). Six cases

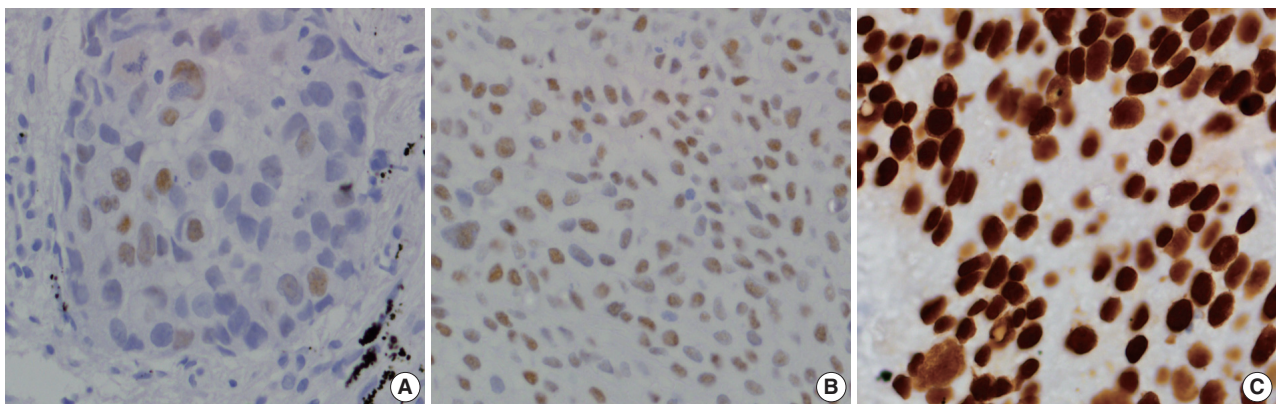


Fig. 1. Intensity score for positive p40 and p63 nuclear staining in tumour cells. (A) Score 1+ for weak positivity. (B) Score 2+ for moderate positivity. (C) Score 3+ for strong positivity.

had prior immunohistochemistry and the diagnosis of squamous cell carcinoma was made on the basis of negative TTF1 and p63 positivity ($n = 3$). The other two cases were diagnosed as squamous cell carcinoma based on morphology alone. In con-

Table 1. Demographic and clinicopathologic data of patients diagnosed with lung squamous cell carcinoma and lung adenocarcinoma

	Squamous cell carcinoma	Adenocarcinoma
Sex, n (%)		
Male	32 (91.4)	19 (54.3)
Female	3 (8.6)	16 (45.7)
Age at diagnosis, mean $\pm$ SD (yr)	68 $\pm$ 12.8	59 $\pm$ 14.2
Ethnicity		
Malay	23	20
Chinese	11	14
Indian	0	0
Others	1	1
Histologic grade		
Well differentiated	7	9
Moderately differentiated	12	14
Poorly differentiated	16	12

SD, standard deviation.

Table 3. Immunoreactivity for p40 and p63 in lung SCC and lung ADC

	No.	Cases with the following intensity score				Cases with the following proportion of immunoreactive cells			
		0	1	2	3	0%–4%	5%–25%	26%–50%	>50%
SCC									
p40	35	6 (17.1)	3 (8.6)	1 (2.9)	25 (71.4)	2 (5.7)	2 (5.7)	0	25 (71.4)
p63	35	4 (11.4)	2 (5.7)	3 (8.6)	26 (74.3)	1 (2.9)	4 (11.4)	0	26 (74.3)
ADC									
p40	35	33 (94.3)	2 (5.7)	0	0	2 (5.7)	0	0	0
p63	35	13 (37.1)	9 (25.7)	8 (22.9)	5 (14.3)	9 (25.7)	6 (17.1)	5 (14.3)	2 (5.7)

Values are presented as number (%).

SCC, squamous cell carcinoma; ADC, adenocarcinoma.

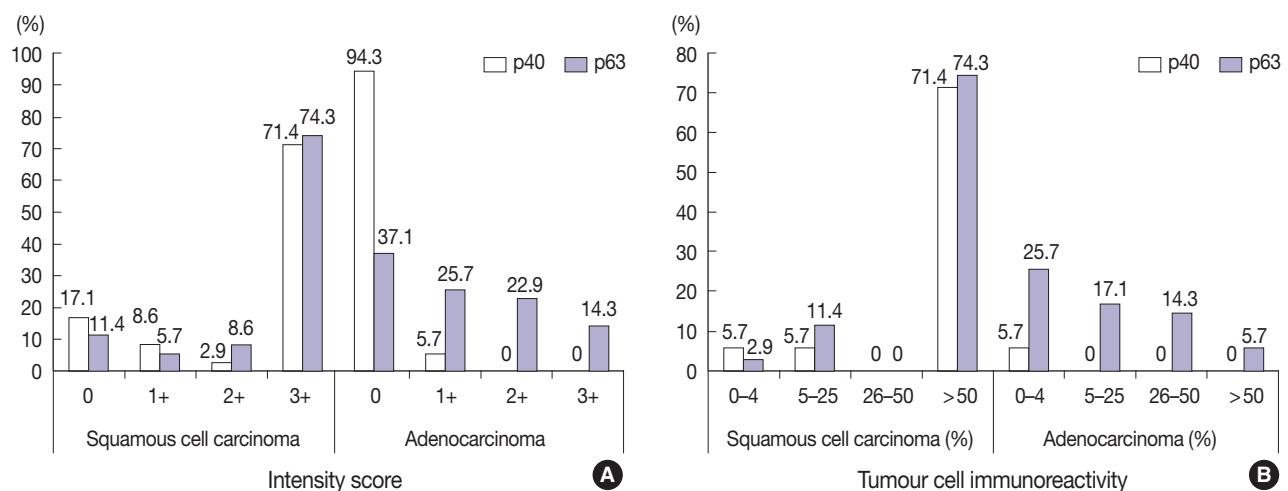


Fig. 2. Analysis of p40 and p63 expression in lung squamous cell carcinoma and adenocarcinoma. (A) Intensity score. (B) Tumour cell immunoreactivity.

trast, all (100%) lung adenocarcinomas were negative for p40.

Two cases of lung adenocarcinoma showed weak p40 expression (1+). However, the immunoreactive cells were minimal (in less than 5% of tumour cells) and scattered with no specific pattern. Immunopositivity of p40 and p63 according to tumour differentiation is shown in Table 4.

Table 5 summarizes immunohistochemical profile of p40-negative squamous cell carcinoma and histologic grading.

p63 immunostaining

Thirty cases (85.7%) of lung squamous cell carcinoma were

Table 2. Expression of p40 and p63 in lung squamous cell carcinoma and lung adenocarcinoma

	Squamous cell carcinoma	Adenocarcinoma
p40		
Positive	27 (77.1)	0
Negative	8 (22.9)	35 (100)
p63		
Positive	30 (85.7)	13 (37.1)
Negative	5 (14.3)	22 (62.9)

Values are presented as number (%).

positive for p63 (Table 2). The expression of p63 in lung squamous cell carcinoma was also strong and diffuse, equivalent to that of p40, and 74.3% of cases had intensity score of 3+ in more than 50% of tumour cells. Among lung adenocarcinomas, 13 cases (37.1%) showed p63 positivity. These cases demonstrated a vast range of immunoreactivity, with minimal reactivity (less than 5% of tumour cells) in nine cases, focal reactivity (5 to 50% of tumour cells) in 11 cases, and diffuse reactivity (more than 50% of tumour cells) in two cases (Table 3, Fig. 2).

All p63 negative cases were also negative for p40. Conversely, three p40 negative cases were p63 positive (Table 5). In their benign counterparts, both p40 and p63 showed nuclear staining in basal bronchial cells.

As shown in Table 6, the specificity of p40 for lung squamous cell carcinoma was 100% compared to 62.9% for p63. The sensitivity of p40 and p63 was 77.1% and 85.7%, respec-

tively. p40 has a higher positive predictive value for lung squamous cell carcinoma than p63 (100% vs 69.8%). Both markers had similar negative predictive values.

DISCUSSION

NSCLC, constituting tumours other than small cell carcinoma, accounts for 85% of all lung cancers; lung adenocarcinoma and squamous cell carcinoma are the most frequent histologic subtypes.^{4,13} Morphologically, adenocarcinoma is identified by glandular differentiation and mucin production. Squamous differentiation is recognized by keratinization, keratin pearls, and intercellular bridges.⁴ However, distinguishing between the two can be challenging, particularly in poorly differentiated tumours. In such cases, immunohistochemical markers are recommended for precise histologic subtyping.⁴ To date, the WHO recommends that cases should be classified as NSCLC, favouring adenocarcinoma when they are positive for an adenocarcinoma marker (TTF1 or mucin) with a negative squamous marker (p63 or p40). Conversely, cases with at least moderate diffuse staining for a squamous marker and negative adenocarcinoma marker should be classified as NSCLC, favouring squamous cell carcinoma.

Although most lung tumours can be accurately subtyped by co-expression of TTF1 and p63, a small percentage of cases showed overlapping (TTF1+/p63+) or indeterminate staining patterns (TTF1/weak p63+), leading to misinterpretation and

Table 4. Immunopositivity of p40 and p63 according to tumour differentiation

Tumour differentiation	No.	p40	p63
Squamous cell carcinoma			
Well differentiated	7	7/7 (100)	7/7 (100)
Moderately differentiated	12	12/12 (100)	12/12 (100)
Poorly differentiated	16	8/16 (50)	11/16 (68.8)
Adenocarcinoma			
Well differentiated	9	0/9 (0)	3/9 (33.3)
Moderately differentiated	14	0/14 (0)	8/14 (57.1)
Poorly differentiated	12	0/12 (0)	2/12 (16.7)

Values are presented as number (%).

Table 5. Histologic findings and IHC profile of p40-negative lung squamous cell carcinoma

Case No.	Nature of specimen	p40	p63	Histologic grade	Squamoid morphology	Other IHC
2	Lung biopsy	1+ (in <5%)	1+	Poorly differentiated	-	CK7+, TTF1-
3	Lung biopsy	0	0	Poorly differentiated	Focal intercellular bridging and keratinization	-
4	Lung biopsy	0	0	Poorly differentiated	Intercellular bridging and keratinization	-
9	Lung biopsy	0	2+ (in >50%)	Poorly differentiated	Abundant eosinophilic cytoplasm	CK+, TTF1+ (focal)
21	Lung biopsy	0	2+ (in 5%-25%)	Poorly differentiated	Focal intercellular bridging and keratinization	TTF1-, p63+
22	Lung biopsy	0	0	Poorly differentiated	Abundant eosinophilic cytoplasm	TTF1-, CK5/6-
33	Lung biopsy	0	0	Poorly differentiated	Abundant eosinophilic cytoplasm	CK7+ (focal), p63+ (focal), TTF1-
34	Lung biopsy	1+ (in <5%)	2+ (in 5%-25%)	Poorly differentiated	Focal intercellular bridging	CK7+, p63+, TTF1-

IHC, immunohistochemistry; CK, cytokeratin; TTF1, thyroid transcription factor 1.

Table 6. Sensitivity and specificity of p40 and p63 in lung squamous cell carcinoma

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	p-value
p40	77.1	100	100	81.4	<.001
p63	85.7	62.9	69.8	81.5	<.001

PPV, positive predictive value; NPV, negative predictive value.

incorrect diagnosis.^{7,11} p63 is a sensitive marker for squamous differentiation, but its specificity is only 60%–82%.^{9,10} Frequent p63 reactivity, particularly in adenocarcinoma and lymphoma, is a significant pitfall in diagnosis of lung cancer.⁹

Accurate histologic diagnosis and subtyping has major therapeutic implications.¹⁴ Current clinical practice guidelines recommend patients with lung adenocarcinoma or NSCLC favouring adenocarcinoma should undergo molecular testing.¹⁵ Incorrect diagnosis of squamous cell carcinoma may exclude patients from receiving targeted therapy, or worse, it may endanger patients to complications of the treatment.^{3,4}

In studies conducted on mainly resected specimens, p40 demonstrated 98%–100% specificity with comparable sensitivity to that of p63, indicating its reliability for diagnosing squamous cell carcinoma.^{9,10,12} Our study was conducted to investigate the expression of p40 and p63 in predominantly small biopsies and few resected specimens of lung squamous cell carcinoma and adenocarcinoma. The present study was in line with previous reports in concluding that p40 is more specific than the standard p63 antibody for diagnosing lung squamous cell carcinoma. However, our study demonstrated a lower sensitivity for p40 compared to prior studies.

Based on a literature review, a case with negative p40 is less likely to be squamous cell carcinoma. A study by Tacha *et al.*¹² described six cases of poorly differentiated lung squamous cell carcinoma (5.6%) that were negative for p40; subsequent staining with other squamous markers were also negative. Kadota *et al.*⁵ reclassified 31 cases (6.5%) of lung tumours that were originally diagnosed as squamous cell carcinoma into other histologic subtypes based on negative p40 staining. Rekhtman *et al.*¹⁶ reclassified three tumours with an initial diagnosis of squamous cell carcinoma and lack of p40 expression as solid adenocarcinoma. In the present study, eight cases originally diagnosed as squamous cell carcinoma were negative for p40. Based on the review of histology sections of these cases as well as the p40 findings,

these cases were reclassified as poorly differentiated or solid type adenocarcinoma. Thus, p40 is highly recommended in cases of poorly differentiated carcinomas to confirm or exclude the line of squamous differentiation.

Three discrepant cases with p63 positivity, p40 negativity, and TTF1 negativity were initially diagnosed as squamous cell carcinoma. These cases were poorly-differentiated tumours with focal areas displaying squamoid morphology such as intercellular bridging and abundant eosinophilic cytoplasm (Fig. 3). TTF1 is not exhibited in 25% of lung adenocarcinomas, and the three discrepant cases in this study could be categorized as adenocarcinoma. Since there are few discrepant cases, future studies involving larger sample sizes of poorly differentiated cases are recommended to determine the sensitivity and specificity of p63 and p40 markers in defining subtypes of NSCLC. A three-panel approach of using p63, p40, and TTF1 in cases of carcinoma with complex tumour differentiation would assist in reaching a specific diagnosis.

The majority of cases (92.9%) in the current study were small biopsy tissues (n = 65) with only five resected specimens (7.1%). As addressed by Travis *et al.*,³ small biopsy specimens may not be representative of the total tumour due to histologic heterogeneity of lung cancers. Also, the distinguishable morphologic features of squamous or adenocarcinoma differentiation are only identifiable if the solid tumour component was sampled.¹⁷

Two p40 antibodies are available for current laboratory use, rabbit polyclonal p40 (RPP40) and mouse monoclonal p40 (MMP40, BC28). RPP40 was used in the above-mentioned studies.^{5,9,10} Nonaka¹⁰ reported variable background staining that did not hinder the staining interpretation. RPP40 has been demonstrated to stain macrophages in lung tissue and non-specific cytoplasmic staining in neoplastic cells while MMP40 has clear nuclear staining without non-specific background staining.¹² Mouse monoclonal antibody was used in the present

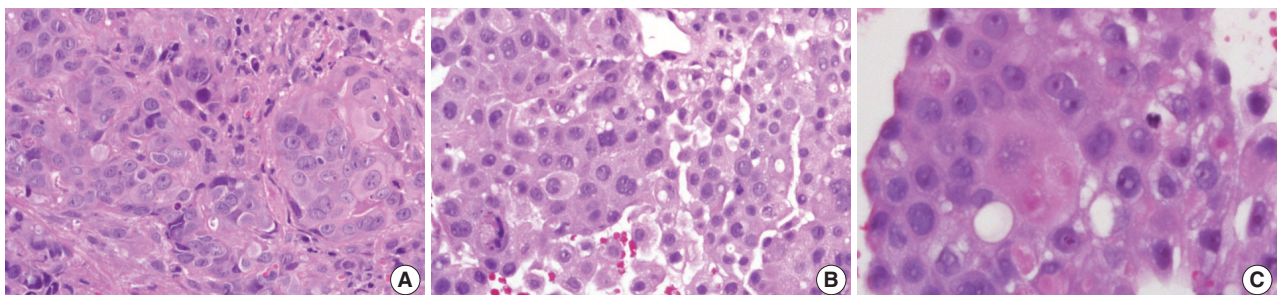


Fig. 3. Poorly differentiated tumour with squamous-like morphology. Tumour cells with abundant eosinophilic cytoplasm and distinct cell borders (A), intercellular bridges (B), and keratinization features (C).

study, and distinctive nuclear staining with a clean background was observed.

In summary, we demonstrated strong and diffuse p40 expression in the majority of lung squamous cell carcinomas and absence of p40 expression in all lung adenocarcinomas. Expression of p63 is similar to that of p40 in lung squamous cell carcinoma, but there was variable p63 immunoreactivity in lung adenocarcinoma. In poorly differentiated cases, a three-panel approach of p63, p40, and TTF-1 may help to distinguish adenocarcinoma from squamous cell carcinoma. Thus, p40 is an excellent marker for distinguishing lung squamous cell carcinoma from adenocarcinoma, and that its expression is equivalent to that of p63 in lung squamous cell carcinoma.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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Chronic Placental Inflammation as a Risk Factor of Severe Retinopathy of Prematurity

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Background: Chronic placental inflammation (CPI) has been implicated in the pathogenesis of diseases in premature infants, whereas retinopathy of prematurity (ROP) is a major complication primarily affecting preterm and very low-birth-weight (VLBW) infants. This study aims to investigate the association between CPI and ROP in VLBW infants. **Methods:** We performed a retrospective review of clinical records of VLBW infants born between 2013 and 2016. Placental pathology findings including CPI cases were analyzed using logistic regression to study infants' morbidities and other clinical characteristics. **Results:** A total of 402 infants with a mean (standard deviation) gestational age of 28.5 (2.8) weeks and birth weight of 1,027.2 (304.4) g were included. The incidence of ROP was 24.1%. CPI was found in 90 infants (22.4%), among which 28.9% (26 of 90) developed ROP, and 21.1% (19 of 90) underwent laser photocoagulation. Lower gestational age, lower birth weight, longer duration of oxygen supply, and presence of CPI were associated with the development of ROP. After adjustment for gestational age, birth weight, sex, duration of oxygen supply, and other overlapping placental pathology, CPI was associated with the odds for type 1 ROP that required laser photocoagulation (adjusted odds ratio, 2.739; 95% confidence interval, 1.112 to 6.749; $p = .029$). **Conclusions:** CPI was associated with severe ROP requiring treatment with laser photocoagulation in VLBW infants.

Key Words: Chronic chorioamnionitis; Villitis; Deciduitis; Retinopathy of prematurity; Infant, very low birth weight

Preterm birth is associated with an increased risk of perinatal mortality and long-term morbidity.¹ The pathological findings of the placenta often provide valuable information regarding the clinical outcomes of preterm infants because the changes in the placenta mirror intrauterine environments affecting the fetus.^{2,3} Acute and chronic placental inflammations are common pathology of preterm birth and are significantly associated with preterm birth and neonatal morbidity.¹⁻⁴ While acute placental inflammation (API) (acute chorioamnionitis and acute funisitis) is caused by the infiltration of neutrophils into the placental compartments, chronic placental inflammation (CPI) (chronic chorioamnionitis, chronic villitis, and chronic deciduitis) is characterized by the infiltration of lymphocytes, plasma cells, and/or macrophages.^{5,6} API accompanies elevation of proinflammatory cytokines such as interleukin (IL)-6 and IL-8 in the amniotic fluid and fetal plasma. On the other hand, CPI is associated with elevation of antiangiogenic T-cell chemokines CXCL9, CXCL10, and CXCL11 in the amniotic fluid and fetal plasma.⁶ Therefore, it

is very plausible that API and CPI affect the systemic biology of preterm newborns.

Illnesses associated with prematurity include respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), bronchopulmonary dysplasia (BPD), and retinopathy of prematurity (ROP). The prevalence of ROP has increased because of progressive improvement in the survival of preterm infants. The overall incidence of ROP has been reported to be 66% for any severity of ROP, 18% for moderately severe ROP, and 6% for severe ROP in preterm infants born weighing $\leq 1,250$ g.⁷ Lower gestational age (GA), lower birth weight, and longer oxygen supply in the postnatal period are known major risk factors for the development of ROP.⁸ Recent studies have suggested an association between chorioamnionitis and ROP.^{8,9} They reported that the incidence of ROP has increased more in infants born to mothers with histologic chorioamnionitis than in infants born to mothers without chorioamnionitis.^{1,8,9} An exposure of the immature retina to changing levels of oxygen leading to dysregulated expression of

retinal growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) seems to play a major role in the development of ROP.⁹ Maternal systemic inflammation, as in acute histologic chorioamnionitis, affects the development of ROP by decreasing levels of IGF-1,^{9,10} which causes abnormal vascularization of the retina. In view of the relationship between acute chorioamnionitis and ROP, we postulated that CPI representing maternal antifetal rejection with derangement in antiangiogenic chemokines can be associated with the development of ROP. This study was conducted to determine if there is a relationship between CPI and ROP in very low-birth-weight (VLBW) infants who were born less than 1,500 g.

MATERIALS AND METHODS

Study population

This was a retrospective cohort study of VLBW infants born at Asan Medical Center Children's Hospital, a tertiary academic center, and admitted to neonatal intensive care unit between January 2013 and December 2016. Infants with major congenital malformations or chromosomal abnormalities who died within 72 hours after birth, transferred to other hospitals, or did not have placental biopsies were excluded from analysis (Fig. 1).

Study design

Perinatal and neonatal clinicopathologic data on each infant including placental biopsies and presence of ROP were retrieved from the electronic medical records. The Institutional Review Board of Asan Medical Center (2017-1058) approved the collec-

tion and use of the clinical information for research purposes before the investigation was started and waived the requirement for informed consent.

Placental pathology

Placental examinations were routinely performed after delivery of VLBW infants. Hematoxylin and eosin–stained sections of the amnion, choriondecidua, umbilical cord, and chorionic plate were examined by pathologists at the same hospital. Histopathological screening of the placenta was performed according to the diagnostic criteria recommend by the Perinatal Section of the Society for Pediatric Pathology.¹¹ The placental pathological findings were grouped into four categories: amniotic fluid infection/inflammation (ACA), maternal vascular underperfusion (MVU), fetal vascular thrombo-occlusive disease (FVTOD), and CPI. The subtypes of CPI included chronic chorioamnionitis (CCA), chronic deciduitis, and villitis of unknown etiology (VUE).^{12,13}

Examination and treatment of retinopathy of prematurity

We had been performing routine eye examinations of neonates whose GA was < 32 weeks or whose birth weight was < 1,800 g. Eye examinations were performed at postnatal day 28 or at 32 weeks in postmenstrual age by an ophthalmologist. Performance of follow-up eye examinations depended on the disease severity. Diagnoses of ROP were based on the international classification.¹⁴ Laser treatment was performed for eyes reaching type 1 ROP. Type 1 ROP was divided into zone I, any stage ROP with plus disease; zone I, stage 3 ROP without plus disease; and zone II, stage 2 or 3 ROP with plus disease.¹⁵ We also included se-

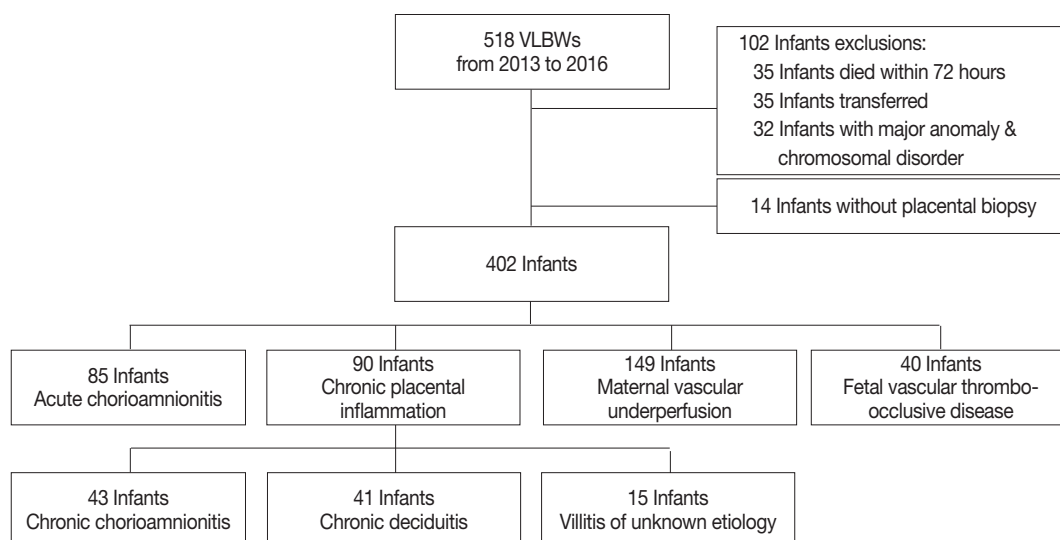


Fig. 1. Study population. VLBW, very low-birth-weight.

vere ROP as type 1 ROP that required laser photocoagulation.

Clinical data on neonates

Demographic characteristics such as GA, birth weight, sex, history of multiple births, incidence of maternal preeclampsia, type of delivery, antenatal steroid use, Apgar scores at 1 and 5 minutes, presence of major morbidities such as RDS, patent ductus arteriosus (PDA), BPD, pulmonary arterial hypertension (PAH), significant neurological injury (SNI), necrotizing enterocolitis (NEC), and late-onset sepsis (LOS), duration of hospital stay, and mortality were obtained for each subject. Significant PDA was defined as a condition requiring pharmacological and/or surgical treatment for the mitigation of hemodynamic disturbance. PAH was diagnosed based on the use of medication according to echocardiographic results. SNI included IVH severer than grade III or periventricular leukomalacia. LOS was defined as bacterial infection confirmed by a positive blood or cerebrospinal fluid culture after postnatal day 7.

Statistical analysis

Statistical analysis was performed using SPSS ver. 23.0 (IBM Corp, Armonk, NY, USA). Correlations between CPI and clinical outcomes were determined by performing the chi-square test or Fisher exact test for categorical data and the *t* test for continuous data. Logistic regression analysis was performed to estimate the independent effects of the main explanatory variables of interest. Statistical significance was defined as $p < .05$.

RESULTS

Clinical characteristics of the study population

A total of 518 VLBW infants were eligible for the study. Of these 518 infants, 35 infants who died within 72 hours, 35 infants who were transferred to other hospitals, 32 infants with major anomalies and chromosomal disorders, and 14 infants without placental biopsies were excluded from analysis (Fig. 1). A total of 402 VLBW infants were included in the analysis. The mean (standard deviation) GA, birth weight, and percentage of males were 28.5 (2.8) weeks, 1,027.2 (304.4) g, and 51.2%, respectively. The mean maternal age was 33.6 (17.1) years. The incidence of preeclampsia and intrauterine growth retardation (IUGR) were 16.9% and 33.1%, respectively. Regarding neonatal morbidity, the incidence of RDS, significant PDA, moderate to severe BPD, PAH, SNI, NEC $\geq$ stage 2, and LOS was 59.2%, 41.5%, 26.1%, 3.0%, 7.8%, 4.7%, and 21.9%, respectively. The incidence of any stage ROP and type 1 ROP requiring laser photo-

coagulation was 24.1% and 11.4%, respectively (Table 1). The mean duration of hospital stay of the infants was 67.4 (35.0) days, and the mortality rate was 3.9%.

Placental pathology

Of the cases included in this study, 122 did not show any placental pathological finding. Among the remaining 280 cases, 85 cases showed findings consistent with ACA, 90 cases showed CPI, 149 cases showed MVU, and 40 cases showed FVTOD (Fig. 1). Two or more placental pathological findings were superimposed in 82 cases, and one placental pathological finding was superimposed in 198 cases (Fig. 2). Of the 90 cases with CPI, 43 were with CCA, 41 were with chronic deciduitis, 15 were with VUE, and 16 showed overlapping patterns (Fig. 1).

Table 1. Baseline characteristics and morbidity of the study cohort

Variable	Value (n = 402)
Neonatal characteristics	
Gestational age (wk)	28.5 $\pm$ 2.8
Birth weight (g)	1,027.2 $\pm$ 304.4
Male	206 (51.2)
Multiple births	94 (23.4)
Maternal age (yr)	33.6 $\pm$ 17.1
Pre-eclampsia	69 (16.9)
Cesarean section	305 (75.1)
Antenatal steroid use	363 (90.3)
Apgar score at 1 min	5.0 (3–7)
Apgar score at 5 min	7.0 (1–9)
IUGR	133 (33.1)
Neonatal morbidity	
RDS	238 (59.2)
Significant PDA	167 (41.5)
Moderate to severe BPD	105 (26.1)
Postnatal steroid therapy	96 (23.9)
Duration of oxygen supply (day)	40.4 $\pm$ 2.1
Pulmonary hypertension	12 (3.0)
Severe neurologic injury (IVH $\geq$ grade 3 or PVL)	43 (7.8)
NEC ($\geq$ stage 2)	19 (4.7)
ROP (any stage)	97 (24.1)
Type 1 ROP	46 (11.4)
Anti-VEGF therapy	14 (2.7)
LOS	88 (21.9)
Hospital stay (day)	67.4 $\pm$ 35.0
Mortality	16 (3.9)

Values are presented as mean $\pm$ SD, number (%), or mean (IQR).

IUGR, intrauterine growth retardation; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia; NEC, necrotizing enterocolitis; ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor; LOS, late-onset sepsis; SD, standard deviation; IQR, interquartile range.

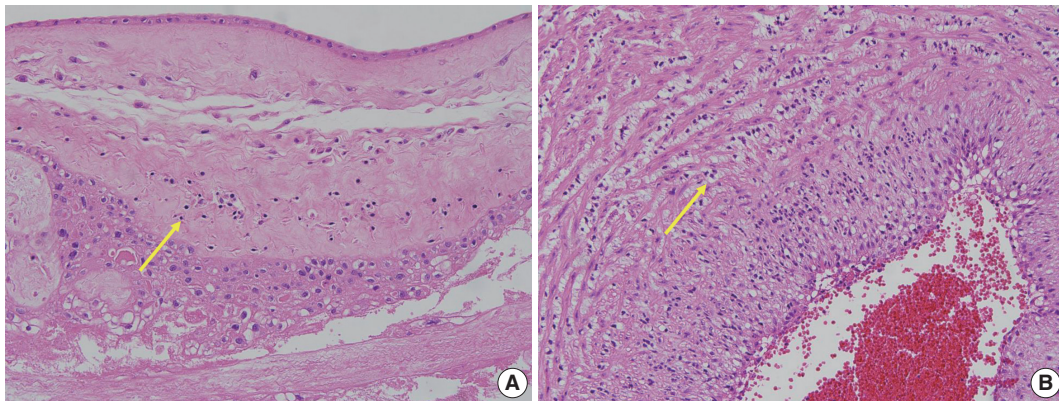


Fig. 2. Microscopic features of chronic chorioamnionitis and acute funisitis (representative case). (A) Lymphocytes (arrow) infiltrate into the chorionic trophoblast layer of the chorioamniotic membrane. (B) Neutrophils (arrow) infiltrate into the smooth muscle layer of the umbilical artery.

Table 2. Characteristics of infants on the basis of CPI

Variable	Non-CPI (n=312)	CPI (n=90)	p-value
Gestational age (wk)	29.1 ± 2.9	28.4 ± 3.2	.007*
Birth weight (g)	1,064.7 ± 286.9	1,012.5 ± 309.1	.589
Sex			.506
Male	154 (49.3)	48 (53.3)	
Female	158 (50.6)	42 (46.7)	
Pre-eclampsia	78 (25.0)	9 (10.0)	.031*
Antenatal steroid use	284 (91.0)	79 (87.8)	.398
IUGR	107 (34.3)	26 (28.9)	.338
RDS	177 (56.7)	61 (67.8)	.068
Significant PDA	127 (40.7)	40 (44.4)	.531
Moderate to severe BPD	79 (25.3)	26 (28.9)	.497
Postnatal steroid therapy	72 (23.1)	24 (26.7)	.496
Duration of oxygen supply (day)	41.4 ± 38.2	41.0 ± 38.1	.163
Severe neurologic injury (IVH ≥ grade 3 or PVL)	34 (10.9)	9 (10)	.051
NEC (≥ stage 2)	15 (4.8)	4 (4.4)	.882
LOS	65 (20.8)	23 (25.6)	.164
ROP (any stage)	71 (22.8)	26 (28.9)	.021*
Type 1 ROP	27 (8.6)	19 (21.1)	.003*
Hospital days (day)	66.5 ± 36.0	72.6 ± 36.1	.154

Values are presented as mean ± SD or number (%).

CPI, chronic placental inflammation; IUGR, intrauterine growth retardation; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia; NEC, necrotizing enterocolitis; LOS, late-onset sepsis; ROP, retinopathy of prematurity; SD, standard deviation.

* $p < .05$.

Association of CPI and retinopathy of prematurity

Of the 90 cases with CPI, 28.9% (26 of 90) developed any stage ROP, and 21.1% (19 of 90) underwent laser photocoagulation (Table 2). The infants with CPI showed a significantly lower GA ($p = .007$) and had a higher incidence of any stage ROP and history of laser photocoagulation treatment than the infants without CPI did ($p = .021$ and $p = .003$). Other characteristics were not significantly different between the two groups (Table 2).

Lower GA, lower birth weight, severe BPD, and longer dura-

tion of oxygenation treatment were associated with greater increases in the incidence of type 1 ROP. According to the pathological findings of the placenta, CPI and ACA statistically correlated with any stage ROP (odds ratio [OR], 2.050; 95% confidence interval [CI], 1.170 to 3.592; $p = .038$ and OR, 2.079; 95% CI, 1.177 to 3.671; $p = .012$) and type 1 ROP for laser photocoagulation (OR, 2.825; 95% CI, 1.487 to 5.367; $p = .002$ and OR, 1.977; 95% CI, 1.012 to 3.862; $p = .046$) in univariate analysis (Table 3).

Table 3. Logistic regression analysis to identify risk factors for any stage ROP and type 1 ROP

Variable	Any stage ROP				Type 1 ROP			
	No ROP (n=305)	ROP (n=97)	OR (95% CI)	p-value	No type 1 ROP (n=356)	Type 1 ROP (n=46)	OR (95% CI)	p-value
Gestational age (wk)	30.0±2.5	25.7±1.7	0.872 (0.847–0.897)	<.001*	30.4±2.8	25.1±1.5	0.882 (0.853–0.911)	<.001*
Birth weight (g)	1,152.5±239.5	740.6±212.9	0.993 (0.992–0.995)	<.001*	1100±267.7	680.8±198.7	0.993 (0.991–0.995)	<.001*
Male	157 (51.5)	49 (50.5)	1.000 (0.329–1.126)	.339	179 (50.3)	23 (50.0)	1.000 (0.504–1.305)	.219
Pre-eclampsia	62 (20.3)	7 (7.2)	0.919 (0.383–2.205)	.851	66 (18.5)	3 (6.5)	0.295 (0.089–0.981)	.046*
Antenatal steroid	271 (88.9)	92 (94.8)	1.547 (0.484–4.942)	.461	321 (90.2)	42 (91.3)	1.145 (0.388–3.382)	.807
IUGR	114 (37.3)	19 (19.6)	0.369 (0.323–1.265)	.199	125 (35.1)	8 (17.4)	0.389 (0.176–0.860)	.020*
Moderate to severe BPD	40 (13.1)	65 (67.0)	0.503 (0.361–0.508)	<.001*	67 (18.8)	38 (82.6)	0.467 (0.281–0.410)	<.001*
Postnatal steroid therapy	34 (11.1)	62 (63.9)	0.512 (0.380–0.531)	<.001*	60 (16.8)	36 (78.3)	0.399 (0.382–0.606)	<.001*
Duration of oxygen treatment (day)	27.9±26.1	81.2±42.7	1.054 (1.042–1.066)	<.001*	33.5±31.3	98.6±38.4	1.044 (1.031–1.057)	<.001*
Placental pathology								
CPI	64 (21.0)	26 (26.8)	2.050 (1.170–3.592)	.038*	71 (19.9)	19 (46.3)	2.825 (1.487–5.367)	.002*
CCA	33 (10.8)	10 (10.3)	0.159 (0.691–3.152)	.159	36 (10.1)	7 (15.2)	1.590 (0.663–3.816)	.299
Chronic deciduitis	29 (9.5)	12 (12.4)	0.244 (0.874–3.872)	.244	33 (9.3)	8 (17.4)	2.061 (0.887–4.784)	.093
VUE	13 (4.3)	2 (2.1)	0.139 (0.042–2.501)	.139	15 (4.2)	0	0.237 (0.013–4.413)	.335
ACA	53 (17.4)	32 (33.0)	2.079 (1.177–3.671)	.012*	70 (19.7)	15 (32.6)	1.977 (1.012–3.862)	.046*
MVU	118 (38.7)	31 (32.0)	0.744 (0.452–1.344)	.744	134 (37.6)	15 (32.6)	0.802 (0.417–1.540)	.507
FVTOD	29 (9.5)	11 (11.3)	0.806 (0.325–2.000)	.642	36 (10.1)	4 (8.7)	0.847 (0.287–2.498)	.763

Values are presented as mean±SD or number (%).

ROP, retinopathy of prematurity; OR, odds ratio; CI, confidence interval; IUGR, intrauterine growth retardation; BPD, bronchopulmonary dysplasia; CPI, chronic placental inflammation; CCA, chronic chorioamnionitis; VUE, villitis of unknown etiology; ACA, amniotic fluid infection/inflammation; MVU, maternal vascular underperfusion; FVTOD, fetal vascular thrombo-occlusive disease; SD, standard deviation.

*p<.05.

Table 4. Placental histology associated with any stage ROP and type 1 ROP

Placental pathology	No ROP		Any stage ROP		Type 1 ROP	
	Adjusted OR (95% CI)	p-value ^a	Adjusted OR (95% CI)	p-value ^a	Adjusted OR (95% CI)	p-value ^a
CPI	1.008 (0.492–2.068)	.982	1.647 (0.702–3.992)	.246	2.739 (1.112–6.749)	.029*
ACA	0.811 (0.429–1.940)	.811	1.267 (0.531–3.023)	.594	0.833 (0.319–2.173)	.708
MVU	0.630 (0.315–1.261)	.630	2.036 (0.865–4.792)	.103	0.570 (0.211–1.539)	.267
FVTOD	0.815 (0.298–2.226)	.690	1.080 (0.360–3.240)	.126	0.424 (0.141–1.272)	.126

Chronic placental inflammation was statistically associated with type 1 ROP by multiple logistic regression analysis.

ROP, retinopathy of prematurity; OR, odds ratio; CI, confidence interval; CPI, chronic placental inflammation; ACA, amniotic fluid infection/inflammation; MVU, maternal vascular underperfusion; FVTOD, fetal vascular thrombo-occlusive disease.

*p<.05.

^aAdjusted for gestational age, birth weight, sex, duration of oxygen treatment, pre-eclampsia, IUGR and the overlapping placental pathology.

Table 4 compares the placental pathological findings in terms of their effect on any stage ROP and type 1 ROP. Multiple logistic regression analysis adjusted for GA, birth weight, sex, incidence of preeclampsia and IUGR, duration of oxygen supply, and overlapping placental pathology was performed. CPI was found to be independently associated with type 1 ROP (adjusted OR, 2.739; 95% CI, 1.112 to 6.749; p=.029). ACA was significantly associated with type 1 ROP in the univariate analysis, but not with type 1 ROP after adjustment analysis. Any stage ROP was not significantly associated with any placental finding after adjustment analysis.

DISCUSSION

Many investigators have studied the relationship between placental lesions in VLBW infants and clinical complications because examinations frequently provide information valuable for the management of neonates.^{2,4-6} For example, the presence of acute chorioamnionitis and funisitis indicates in utero fetal systemic inflammation as a consequence of ACA.¹⁶ In this study, we showed a significant correlation between CPI and severe ROP that required laser photocoagulation (type 1 ROP) in VLBW infants for the first time.

A prominent cause of visual impairment and blindness in children, ROP is a developmental perturbation of the retina.^{9,17,18}

The structural characteristics of ROP include the initial cessation of retinal vascularization and subsequent proliferation of retinal vessels.^{17,19} ROP is caused by impaired autoregulation of the retinal blood vessels and sudden postnatal oxygen tension. Retinal neovascularization in ROP cases involves angiogenic factors such as VEGF in preterm infants and VLBW infants.²⁰⁻²² Recent studies have reported that lower GA, lower birth weight, and excessive oxygen exposure are major risk factors for ROP.²⁰⁻²³ Other studies demonstrated that both antenatal and neonatal exposures to inflammation are consistently associated with an increased risk of ROP as well as cerebral white matter damage and BPD in preterm infants.^{9,24,25} The formation of new blood vessels from existing vessels, angiogenesis, is affected not only by normal physiological growth, but also by pathological events such as inflammation.^{24,26} Inflammation is a host defense mechanism serving to protect tissues from infection and injury, and studies have shown that pro-inflammatory cytokines such as tumor necrosis factor α and IL-1 function as proangiogenic factors.^{24,27-29}

Although oxygen supply is far better managed by using non-invasive ventilators today, ROP remains a major issue because survival rates of infants with extremely low GA and birth weight and immature retinas at high risk of ROP have substantially increased. The lack of in utero angiogenic factors such as VEGF, IGF-1, and erythropoietin significantly increases the risk of ROP.^{20,30} Omega long-chain polyunsaturated fatty acids, which are provided by maternal and fetal interaction, are also important for retinal development.³⁰⁻³² Therefore, the risk of ROP may increase in cases of intrauterine insufficiency of angiogenic factors in infants with lower GA and birth weight.

It has been recently proposed that idiopathic CPI is a feature of maternal antifetal rejection mimicking allograft rejection.¹² The placenta and fetus are semiallografts to the mother, and fetal (paternal) human leukocyte antigen-specific antibodies are detected in the sera of subsets of pregnant women.^{6,12} CPI is characterized by the infiltration of maternal CD8⁺ T cells into fetal placental tissues and associated with the overexpression of C-X-C-motif chemokines such as CXCL9, CXCL10, and CXCL11 in placental tissues.^{6,12} Also a marker of CPI coupled with maternal-fetal rejection,^{33,34} elevation of CXCL10 level has been shown to occur in the event of rejection of allografts such as the liver and kidneys.³⁵

The findings in this study suggest that CPI may be at least partially responsible for the increased incidence of severe ROP that required laser photocoagulation after adjustment for GA, birth weight, sex, duration of oxygen supply, and other overlapping placental pathologies. We did not adjust for BPD because the condition is a confounding variable overlapping with the

duration of oxygen supply. The association between CPI and severe ROP suggests that maternal-fetal rejection associated with increased fetal plasma antiangiogenic CXCL10 level contributes to abnormal angiogenesis of the retina in VLBW infants. A relationship between the subtypes of ROP and the intrauterine environment that triggers preterm birth also has been proposed.³⁶ Likewise, acute chorioamnionitis may increase the risk of ROP by directly sensitizing the developing retina to oxygen-induced changes in VEGF availability and subsequent vascular development or by causing systemic hypotension resulting in retinal hypoperfusion or ischemia.^{8,9,25}

Decreased production of placental growth factors (PGF), 45- to 50-kDa dimeric glycoproteins with 53% sequence homology with VEGF, plays a key role in placental angiogenesis.³⁷⁻⁴⁰ PGF may have therapeutic potential in cases of disorders associated with problems of angiogenesis such as cancer, retinopathy, and certain inflammatory disorders.⁴⁰ This is another piece of evidence indicating the importance of placenta-derived growth factors for developmental angiogenesis. Therefore, it is plausible that CPI-mediating antiangiogenic effects lead to deregulated angiogenesis of ROP in VLBW infants.

There are some limitations of our study. First, it was a retrospective study of a relatively small number of inborn infants at a single hospital; thus, the findings might not extrapolate to a larger population. Second, multiple pathological diagnoses were given in some cases, which can interfere with the strength of the association. Third, in the association between CPI and type 1 ROP, we had a wide confidence interval (1.112–6.749) because many factors may play a contributory role in ROP development. Despite these limitations, this study demonstrates an association between CPI and severe ROP in VLBW infants. Future studies that examine the direct causality of CPI in the development of retinal damage and ROP are needed.

In conclusion, the pathological findings of the placenta are important in predicting the outcomes of preterm infants and VLBW infants. This study shows for the first time that CPI may affect the development of type 1 ROP requiring treatment of laser photocoagulation in VLBW infants.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Prognostic Significance of EPHB2 Expression in Colorectal Cancer Progression

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Background: A receptor tyrosine kinase for ephrin ligands, EPHB2, is expressed in normal colorectal tissues and colorectal cancers (CRCs). The aim of this study was to investigate EPHB2 expression over CRC progression and determine its prognostic significance in CRC. **Methods:** To measure EPHB2 mRNA and protein expression, real-time polymerase chain reaction and immunohistochemistry were performed in 32 fresh-frozen and 567 formalin-fixed paraffin-embedded CRC samples, respectively. We further investigated clinicopathological features and overall and recurrence-free survival according to EPHB2 protein expression. **Results:** The EPHB2 level was upregulated in CRC samples compared to non-cancerous tissue in most samples and showed a strong positive correlation with AXIN2. Notably, CD44 had a positive association with both mRNA and protein levels of EPHB2. Immunohistochemical analysis revealed no difference in EPHB2 expression between adenoma and carcinoma areas. Although EPHB2 expression was slightly lower in invasive fronts compared to surface area ($p < .05$), there was no difference between superficial and metastatic areas. EPHB2 positivity was associated with lymphatic ($p < .001$) and venous ($p = .001$) invasion, TNM stage ($p < .001$), and microsatellite instability ($p = .036$). Kaplan–Meier analysis demonstrated that CRC patients with EPHB2 positivity showed better clinical outcomes in both overall ($p = .049$) and recurrence-free survival ($p = .015$). However, multivariate analysis failed to show that EPHB2 is an independent prognostic marker in CRCs (hazard ratio, 0.692; $p = .692$). **Conclusions:** Our results suggest that EPHB2 is overexpressed in a subset of CRCs and is a significant prognostic marker.

Key Words: EPHB2; Colorectal neoplasms; Immunohistochemistry; Prognosis

The receptor tyrosine kinases (RTKs) are transmembrane glycoproteins that are important regulators of cell growth, differentiation, and migration. The 16 vertebrate EPH receptors form the largest subgroup of RTKs and are subdivided into EPHA and EPHB kinases depending on homology and binding specificity to their cell-bound ligands called ephrins.¹ In cooperation with membrane-bound ephrins, EPHs organize tissue patterning, cell positioning, and organ patterning and regulate cell growth during normal and tumor development.^{2–4} Studies on EPHs and ephrins initially focused on the patterning of embryonic tissue, but have since also shown important roles in adult tissue and organ maintenance, regeneration, and a variety of other processes, including synaptic plasticity and response to nerve damage, bone remodeling, stem cell location and proliferation, and cancer development.⁵

Numerous studies have implicated a role for EPH–ephrin signaling in cancer progression. In recent years, abnormal expression of EPH genes has been reported in a wide range of human malignancies such as neuroblastoma⁶ and carcinomas of breast,⁷

lung,⁸ gastric,⁹ prostate,¹⁰ ovary,¹¹ colon,¹² and skin.¹³ Many researchers have suggested a role for EPHs and ephrins in colorectal cancers (CRCs), in particular EPHB2 is the most expressed EPH receptor in the normal intestine and is mainly produced in epithelial cells.¹⁴ Analysis of EPHB2 and EPHB3 in colon tumor mouse models demonstrated that upregulated expression resulted in dysregulated epithelial cell migration in the crypt villus junction, indicating a role for EPH receptors in colon cancer.¹⁵ In this study, we investigated the expression of EPHB2 in human CRC samples and assessed its expression profile during CRC progression. In addition, we examined the prognostic significance of EPHB2 positivity in a large cohort of human CRC patients.

MATERIALS AND METHODS

Subjects

CRCs were collected from 567 patients who underwent surgical resection at Seoul National University Hospital (SNUH) between

2004 and 2006. Clinicopathological data of patient age, sex, tumor location, size, histological subtype, differentiation, presence of lympho-vascular invasion, American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) cancer stage (seventh edition), time of death, tumor recurrence, and follow-up time were obtained by reviewing the clinical and pathological reports. Fifty-six CRC samples were obtained from patients at Jeju National University Hospital (JNUH); 22 cases were CRCs arising from preexisting adenomas, and 34 cases were conventional CRCs with lymph node metastasis. The histopathologic features were evaluated by two gastrointestinal pathologists (J.M.B and G.H.K) for CRCs from SNUH and by one pathologist (B.G.J) for CRCs from JNUH. In addition, 32 paired, fresh-frozen CRC tissues and matched normal tissues were obtained from the Jeju National University Hospital Biobank, a member of the National Biobank of Korea, for which informed consent was obtained from all subjects. All procedures were in accordance with the Helsinki Declaration of 1964 and later versions, and this study was approved by the Institutional Review Boards of SNUH (IRB No. 1502-029-647) and JNUH (IRB No. 2017-04-001).

Tissue microarray construction

Thirteen tissue microarrays (TMAs) containing 642 CRCs from SNUH were generated as previously described.¹⁶ In brief, through histologic examination, the representative cancer areas

containing more than 70% of cells were marked in each case. For each CRC, core tissue biopsies (2 mm in diameter) were obtained from individual formalin-fixed paraffin-embedded samples (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea). With CRCs from JNUH, two TMAs including 22 pairs of adenoma and carcinoma areas and five TMAs including 34 ulcerofungating CRCs with lymph node metastasis were constructed with 4 mm cores. For ulcerofungating CRCs, superficial and invasive portions, and metastatic cancers in the lymph node were included.

Immunohistochemistry

Immunohistochemistry was performed on 4- μ m TMA sections using a Ventana BenchMark XT Staining systems (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. The primary antibodies used were anti-EPHB2 (1:700, R&D Systems, Minneapolis, MN, USA), anti-CD44 (1:100, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), and anti- β -catenin (1:800, 17C2, Novocastra Laboratories). The expression of EPHB2 and CD44 was determined by examining the tumor cell membrane. For each tumor, the intensity and percentage of tumor cells expressing EPHB2 ($n = 567$) or CD44 ($n = 87$) were evaluated. Histscores (H-scores) were calculated by multiplying the intensity score (0, negative; 1, weak; 2, moderate; and 3, strong) and percentage of positive tumor cells (range, 0 to

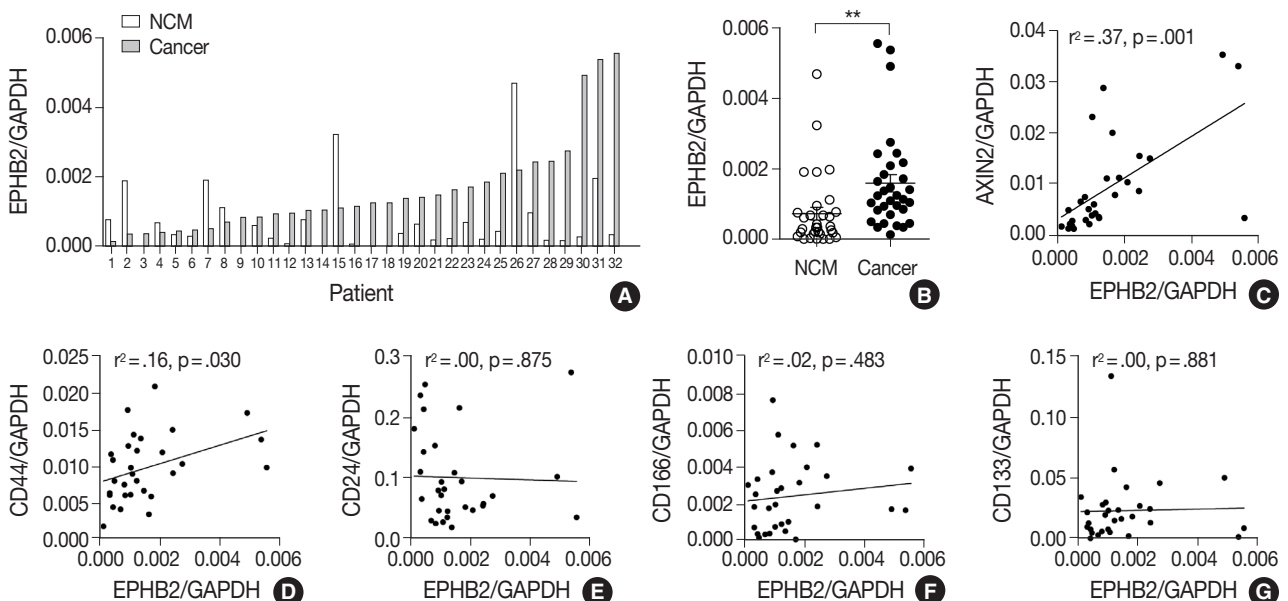


Fig. 1. EPHB2 expression in colorectal cancers (CRCs) and matched normal colon tissues. (A, B) Real-time polymerase chain reaction analysis of EPHB2 mRNA level in CRCs and corresponding non-cancerous mucosa (NCM). (C) A correlation between EPHB2 and AXIN2 expression. (D–G) Correlations between EPHB2 and candidate cancer stem cell markers.

100), ranging from 0 to 300. For statistical analyses for EPHB2, we used a cutoff of 40 on the basis of the distribution of the H-scores (median value, 40). CRCs with H-score of 40 or lower were classified as negative, while cases with H-score higher than 40 were classified as positive. β -Catenin staining was considered positive when more than 10% of tumor cell nuclei were strongly stained.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from 32 paired fresh-frozen CRCs and matched non-cancerous colon tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μ g) was subjected to reverse-transcription with oligo-dT primers and the GoScript

reverse transcription system (Promega, Madison, WI, USA). Complementary DNA was used to conduct real-time polymerase chain reaction (PCR) with Premix EX Taq (Takara Bio, Shiga, Japan) following the manufacturer's instructions. The cycling conditions were as follows: initial denaturation for 30 seconds at 95°C, followed by 40 cycles of 95°C for 1 second and 60°C for 5 seconds in a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The TaqMan expression assays were performed for the following genes: Hs00362096_m1 (EPHB2), Hs00610344_m1 (AXIN2), Hs02379687_s1 (CD24), Hs01075684_m1 (CD44), Hs01009250_m1 (PROM1/CD133), Hs00233455_m1 (ALCAM/CD166), and Hs02786624_g1 (GAPDH). GAPDH served as the endogenous control.

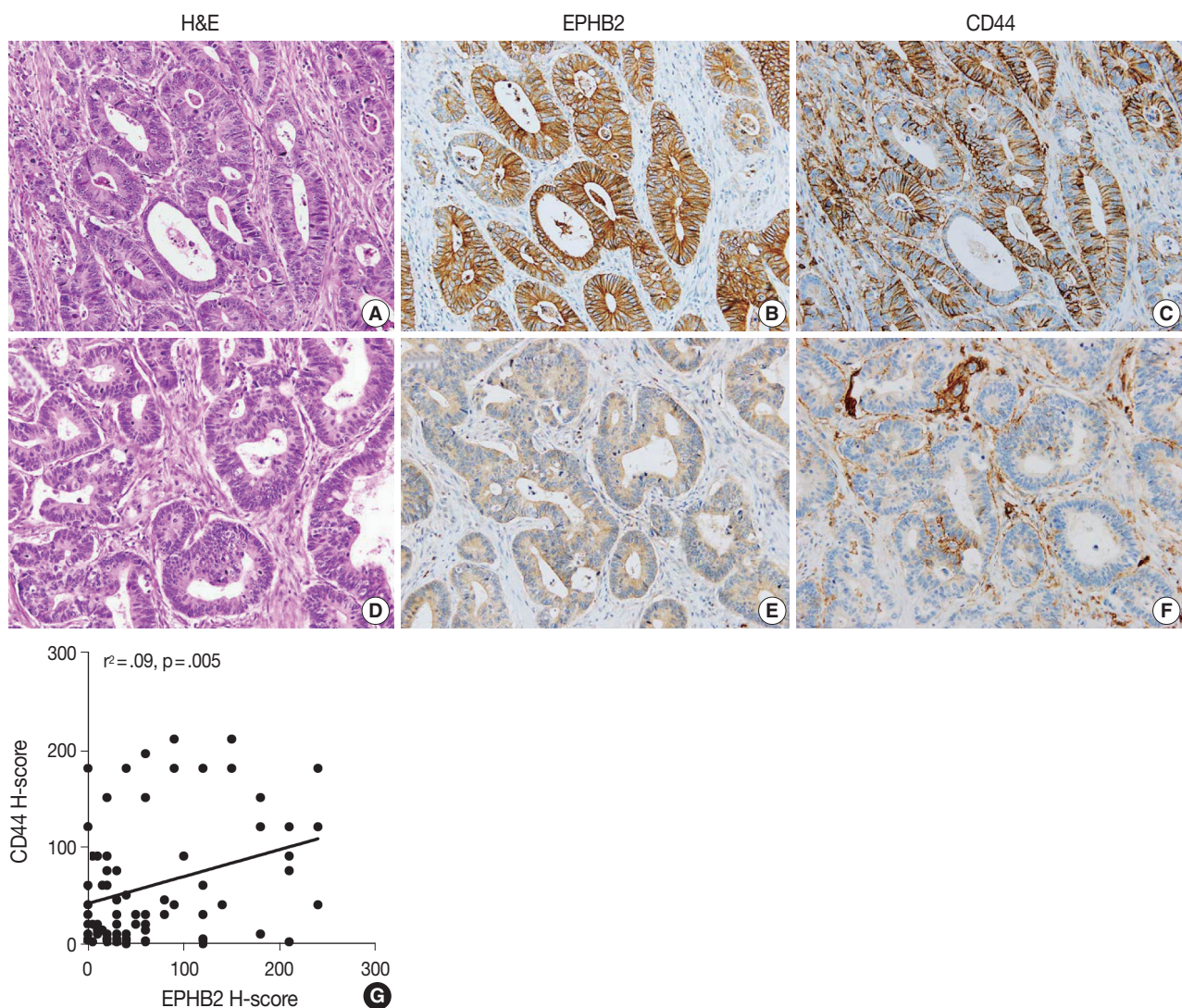


Fig. 2. Representative cases showing high EPHB2 and CD44 expression (A–C) and low EPHB2 and CD44 expression (D–F) in colorectal cancers. (G) Scatter plot with a regression line showing a positive correlation between EPHB2 and CD44 expression. H-scores, Histoscores.

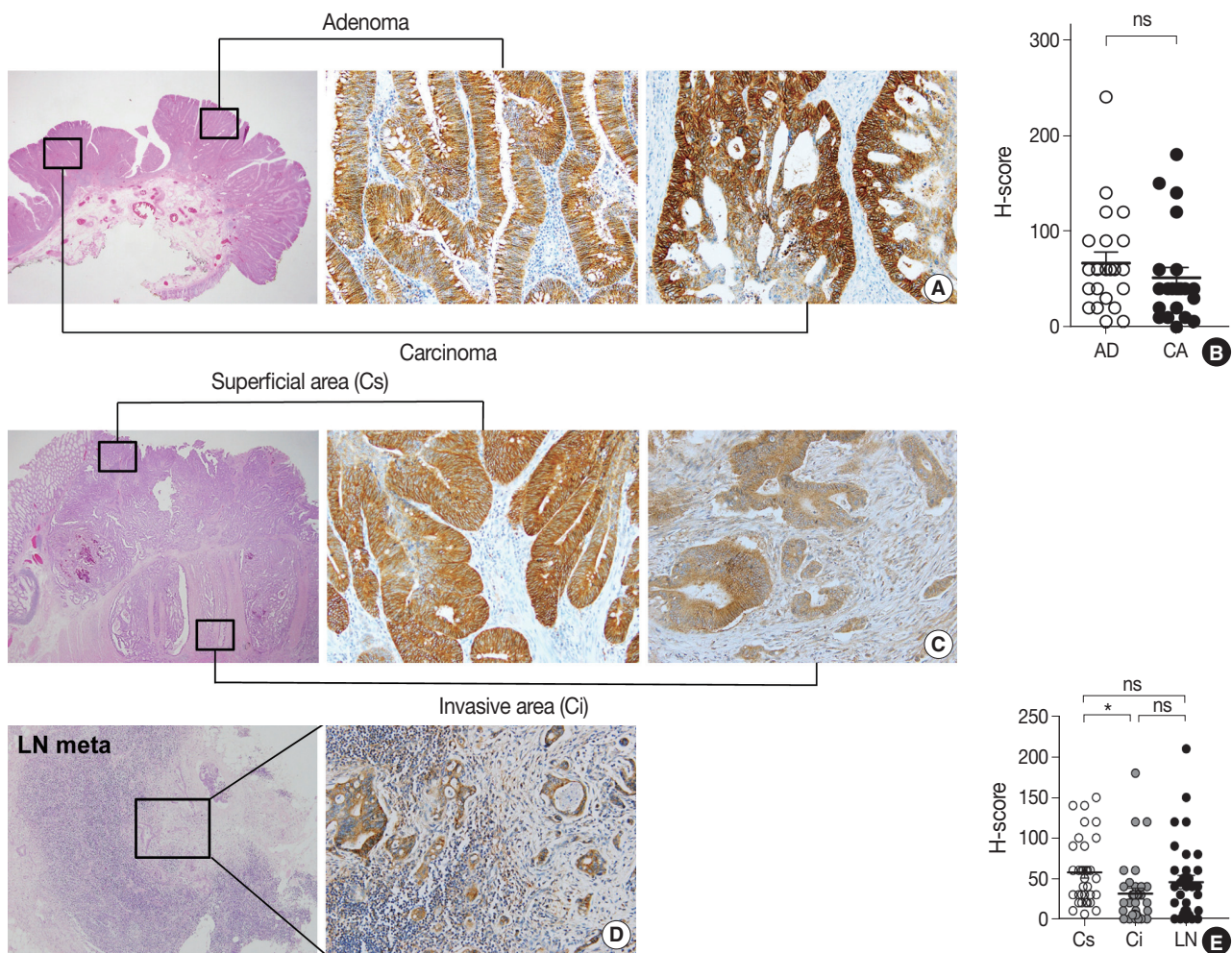


Fig. 3. Expression profile of EPHB2 during colorectal cancer (CRC) progression. (A, B) EPHB2 expression in adenoma and carcinoma portions in CRCs arising in pre-existing adenomas. (C–E) EPHB2 expression in three spots of ulcerofungating CRCs; superficial area, invasive fronts, and metastatic cancers. * $p < .05$. ns, not significant; AD, adenoma; CA, carcinoma; LN, lymph node metastasis; Cs, superficial cancer; Ci, invasive cancer.

BRAF and KRAS mutation and microsatellite instability analysis

Thorough microscopic examination, representative cancer areas in each case were marked, microdissected, and then incubated at 55°C with lysis buffer and proteinase K for 2 days. *KRAS* codons 12 and 13 and *BRAF* codon 600 mutations were determined by PCR-restriction fragment length polymorphism and direct sequencing techniques. Among the 567 CRCs, 29 and five samples were excluded from the *KRAS* and *BRAF* mutation analyses, respectively, due to an insufficient DNA amount. The microsatellite instability (MSI) status of each cancer tissue was determined using five National Cancer Institute recommended markers (BAT25, BAT26, D2S123, D5S346, and D17S250). The MSI status of each case was classified into three groups: MSI-high (two or more unstable markers among the five markers), MSI-

low (one unstable marker), or microsatellite stable (no unstable marker).

CpG island methylator phenotype analysis

CpG island methylator phenotype (CIMP) status was determined by MethyLight assay. Sodium bisulphite modification of genomic DNA samples was conducted for all 567 CRC tissues, as previously described.¹⁷ The methylation statuses of eight CIMP-specific CpG islands (*MLH1*, *NEUROG1*, *CRABP1*, *CACNA1G*, *CDKN2A [p16]*, *IGF2*, *SOCS1*, and *RUNX1*) were quantified. CIMP-high was defined as having five or more hypermethylated markers, and CIMP-low was defined as having four or fewer hypermethylated markers out of eight. CIMP-negative was defined as having no hypermethylated marker.

Table 1. Associations between EPHB2 and clinicopathological characteristics

	Total	EPHB2		p-value
		Negative	Positive	
No. of patients	567 (100)	259 (46)	308 (54)	
Age (yr)				
≥ 60	319 (56)	127 (40)	192 (60)	.002 ^a
< 60	248 (44)	132 (53)	116 (47)	
Sex				
Male	343 (61)	161 (47)	182 (53)	.491 ^a
Female	224 (39)	98 (44)	126 (56)	
Location				
Proximal	148 (26)	65 (44)	83 (56)	.632 ^a
Distal	419 (74)	194 (46)	225 (54)	
Differentiation				
WD	44 (8)	16 (36)	28 (64)	.064 ^b
MD	504 (89)	230 (46)	274 (54)	
PD	19 (3)	13 (68)	6 (62)	
Lymphatic invasion				
Negative	323 (57)	110 (34)	213 (66)	<.001 ^a
Positive	244 (43)	149 (61)	95 (39)	
Venous invasion				
Negative	493 (87)	212 (43)	287 (57)	.001 ^a
Positive	74 (13)	47 (64)	27 (36)	
T category				
T1	20 (3)	4 (20)	16 (80)	<.001 ^b
T2	90 (16)	25 (28)	65 (72)	
T3	408 (72)	197 (48)	211 (52)	
T4	49 (9)	33 (67)	16 (33)	
N category				
N0	298 (53)	111 (37)	187 (63)	<.001 ^b
N1	151 (27)	68 (45)	83 (55)	
N2	118 (21)	80 (68)	38 (32)	
M category				
M0	473 (83)	205 (43)	268 (57)	.013 ^a
M1	94 (17)	54 (57)	40 (43)	
Tumor stage ^c				
I	91 (16)	24 (26)	67 (74)	<.001 ^b
II	186 (33)	76 (41)	110 (59)	
III	196 (35)	105 (54)	91 (46)	
IV	94 (17)	54 (57)	40 (43)	
β-catenin				
No nuclear stain	212 (37)	110 (52)	102 (48)	.024 ^a
Nuclear stain	355 (63)	149 (42)	206 (58)	

Values are presented as number (%).

^aFisher's exact test; ^bPearson chi-square test; ^cAmerican Joint Committee on Cancer 7th edition.

Statistical analysis

All data were analyzed using SPSS statistical software ver. 18.0 (SPSS Inc., Chicago, IL, USA) and Prism ver. 5.0 (Graph-Pad Software, San Diego, CA, USA). The correlations between EPHB2 and cancer stem cell markers or CD44 were evaluated by the Spearman correlation test. Between-group comparisons

of the real-time PCR data were performed using Student's t-test. The correlations between EPHB2 positivity and clinicopathological parameters were tested using Pearson's chi-square test or Fisher exact test. Survival curves were estimated using the Kaplan-Meier method, and log-rank test was used to compare groups. Using Cox proportional hazards model, multivariate analyses were performed to identify independent prognostic factors. A p-value of <.05 was considered statistically significant.

RESULTS

EPHB2 is upregulated in human CRCs

To measure the expression level of *EPHB2* in CRCs, we performed real-time PCR with 32 pairs of fresh-frozen human CRC samples and adjacent normal colon tissues. Comparing individual cases, *EPHB2* mRNA expression was higher in CRC samples than in non-cancerous tissues in most samples (77%, 25 out of 32 cases) (Fig. 1A), and the mean *EPHB2* level was also significantly higher in CRCs than in matched colon tissues ($p < .01$) (Fig. 1B). *EPHB2*, a Wnt signaling target gene, showed a strong positive correlation with *AXIN2*, a direct target of the Wnt pathway ($p < .001$), confirming enhanced Wnt signaling activity in CRCs (Fig. 1C). As *EPHB2*-high cancer cells have been suggested as a cancer stem cell population in CRCs,¹⁸ we investigated the correlations between *EPHB2* expression and expression of other candidate cancer stem cell (CSC) markers *CD24*, *CD44*, *CD133*, and *CD166*. Notably, we found that only *CD44* showed a positive association with *EPHB2* ($p = .030$) (Fig. 1D–G), which was confirmed at the protein level by a positive correlation between EPHB2 and CD44 H-scores in CRCs ($n = 87$) (Fig. 2).

EPHB2 expression during CRC progression

To explore the expression profile of EPHB2 expression during CRC progression, we collected 22 cases of CRCs arising from pre-existing adenomas and measured the H-scores of EPHB2 in adenoma and carcinoma portions (Fig. 3A). However, no difference in H-scores was observed between the adenoma and carcinoma areas (Fig. 3B). Next, we collected 34 ulcerofungating CRCs with lymph node metastasis and examined the H-scores in three spots for each cancer: the superficial fungating area, invasive fronts, and metastatic cancer cells (Fig. 3C, D). EPHB2 expression was slightly lower in invasive fronts compared to surface area ($p < .05$), but did not differ between superficial and metastatic areas (Fig. 3E).

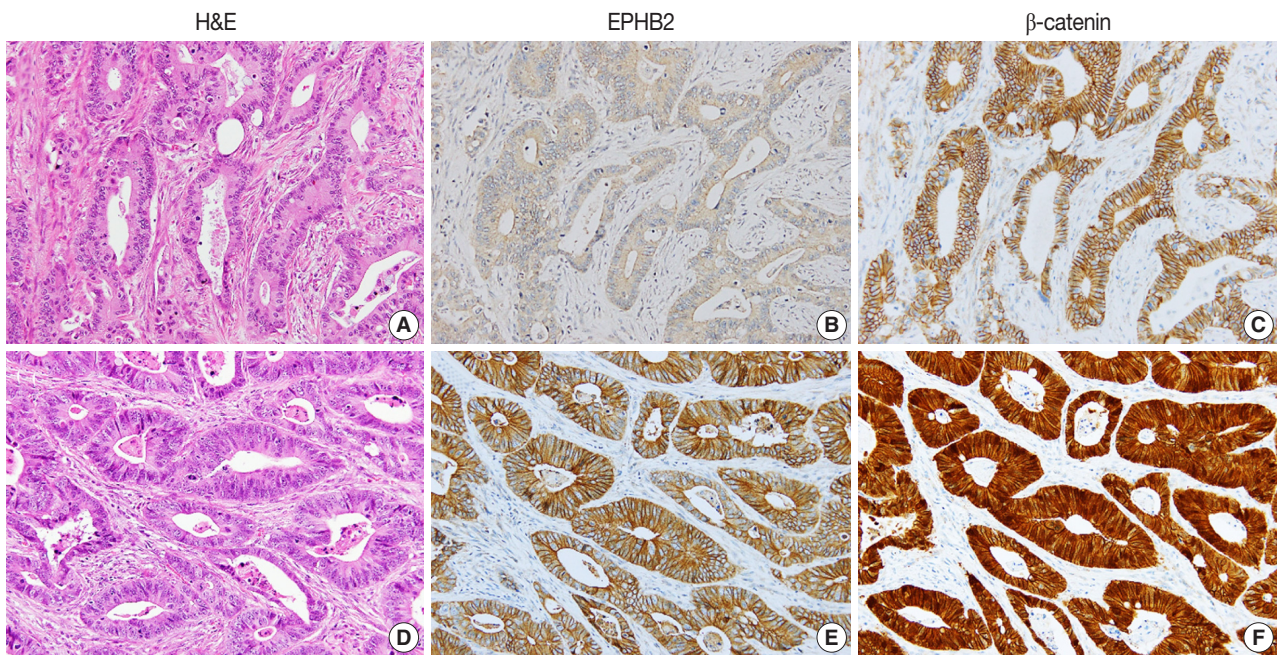


Fig. 4. Representative cases showing negative EPHB2 and nuclear β -catenin expression (A–C) and positive EPHB2 and nuclear β -catenin expression (D–F) expression in colorectal cancers.

Table 2. Associations between EPHB2 expression and molecular characteristics

	Total	EPHB2		p-value
		Negative	Positive	
No. of patients	567 (100)	259 (46)	308 (54)	
CIMP				
Negative/Low	528 (93)	237 (45)	291 (55)	.163 ^a
High	39 (7)	22 (56)	17 (44)	
MSI				
Negative/Low	519 (91)	244 (47)	275 (53)	.036 ^a
High	48 (9)	15 (31)	33 (69)	
KRAS (n=538)				
Wt	390 (73)	181 (46)	209 (54)	.347 ^a
Mt	148 (27)	62 (42)	86 (58)	
BRAF (n=562)				
Wt	531 (95)	243 (46)	288 (54)	.443 ^a
Mt	31 (5)	12 (39)	19 (61)	

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; Wt, wild type; Mt, mutation.

^aPearson chi-square test.

Clinicopathological and prognostic significance of EPHB2 expression in CRCs

To investigate the prognostic value of EPHB2 in CRCs, immunohistochemical staining was performed on 12 TMAs, and 567 cases were included for the analysis. The clinicopathological relevance of EPHB2 positivity is summarized in Table 1. EPHB2 expression was significantly higher in CRCs from old patients ($p = .002$). EPHB2 positivity exhibited negative correlations with lymphatic ($p < .001$) and venous ($p = .001$) invasion, T category (p

$< .001$), N category ($p < .001$), M category ($p = .013$), and tumor stage ($p < .001$). Nuclear β -catenin staining was observed more frequently in EPHB2-positive CRCs (Table 1, Fig. 4). EPHB2 positivity was higher in CRCs with MSI-high than in those with MSI-negative/low, whereas EPHB2 expression had no correlation with mutation status of *BRAF*, *KRAS*, or *CIMP* (Table 2). Kaplan-Meier analysis demonstrated that CRC patients with EPHB2 positivity showed better clinical outcomes in both overall ($p = .048$) (Fig. 5A) and recurrence-free survival ($p = .015$)

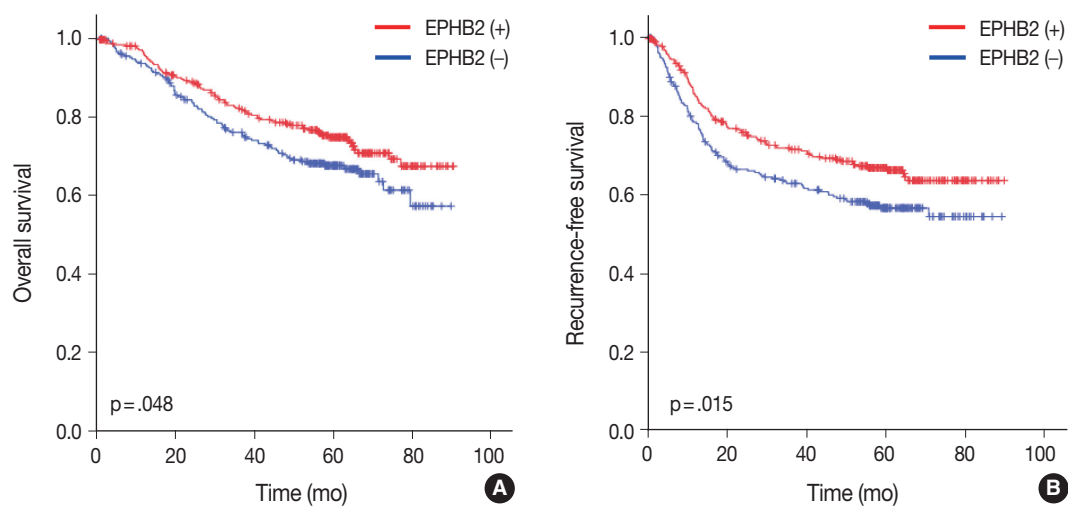


Fig. 5 . Prognostic significance of EPHB2 expression in overall (A) and recurrence-free (B) survival in colorectal cancers.

Table 3. The results of multivariate analysis for survival rate in colorectal cancers

Variable	HR	95% CI	p-value ^a
Age (>60 yr/<60 yr)	1.720	1.247–2.374	.001
Site (distal/proximal)	0.741	0.536–1.025	.070
Differentiation (poor/moderate/well)	1.405	0.849–2.326	.186
Lymphatic invasion (positive/negative)	1.058	0.754–1.485	.742
Venous invasion (positive/negative)	1.766	1.230–2.535	.742
Tumor stage (IV/III/II/I)	2.789	2.248–3.460	<.001
EPHB2 (positive/negative)	0.692	0.679–1.293	.692

HR, hazard ratio; CI, confidence interval.

^aCox proportional hazard model.

(Fig. 5B). However, multivariate analysis failed to show EPHB2 as an independent prognostic marker in CRCs (hazard ratio, 0.692; $p = .692$) (Table 3).

DISCUSSION

A number of studies have reported that a direct correlation is present between loss of EPHB2 and CRC progression,^{12,19–21} and EPHB2 decline was shown to be attributed to promoter methylation.²² To verify downregulation of EPHB2 with CRC progression, we attempted to find the difference in EPHB2 expression between adenoma and carcinoma portions and between superficial, invasive fronts, and metastatic cancers in the lymph nodes. However, we only observed a slight reduction in EPHB2 level in invasive fronts compared to superficial tumor cells. On the other hand, EPHB2 expression remained persistent during adenoma-carcinoma transition and lymph node metastasis. Our results suggest that enhanced EPHB2 level in CRCs tends to persist during cancer progression. The discrepancy may be in part explained by the difference in CRC samples and methods,

and further studies are required to clarify the alterations in EPHB2 expression during CRC progression and metastasis.

The prognostic significance of EPHB2 expression in CRCs has been controversial. Some reports have shown that EPHB2 overexpression is associated with prolonged survival in CRCs.^{19,23} On the contrary, Merlos-Suarez *et al.*¹⁸ have suggested that EPHB2-high expression identifies CRC stem cells and increased risk of recurrence. With a large cohort of human CRCs in this study, we demonstrated that membranous EPHB2 expression is negatively correlated with lympho-vascular invasion and was a prognostic marker associated with better overall and recurrence-free survival. A tumor suppressor role for EPHB2 has been described in various cancers including prostate, breast cancers, and CRC. In particular, it has been suggested that EPH2 compartmentalizes the expansion of CRC cells through a mechanism dependent on E-cadherin-mediated adhesion; in turn, this compartmentalization restricts the spread of EPHB2-expressing CRC cells.²⁴ Therefore, it is reasonable to hypothesize that EPHB2-mediated signaling prevents cancer progression by suppressing cancer migration and invasion, leading to better clinical outcomes for EPHB2-positive

CRC patients.

In the intestinal epithelium, EPHB2 signaling controls the positioning of cell types along the crypt-villus axis.¹⁵ In addition, EPHB2 belongs to the signature genes enriched in the intestinal stem cells, one of the β -catenin and Tcf4 target genes regulated by the Wnt signaling pathway. The most common mutation in CRCs is inactivation of the adenomatous polyposis coli gene, resulting in abnormal Wnt activation. Thus, it is possible to speculate that enhanced Wnt signaling activity might be responsible for elevated EPHB2 expression in CRCs, which was supported by our finding of a strong correlation of EPHB2 mRNA expression with AXIN2, another Wnt pathway target gene. Moreover, we observed that nuclear β -catenin expression, a hallmark of abnormal Wnt activity, was significantly associated with EPHB2 expression, suggesting that the Wnt pathway is likely to be responsible for EPHB2 upregulation in CRCs.

EPHB2-high cells were used to isolate CRC cells with intestinal stem cell-like phenotype positioned at the bottom of tumor structures reminiscent of crypts, which displayed robust tumor-initiating capacity as well as long-term self-renewal potential, indicating a CSC population.¹⁸ As several genes such as CD24, CD44, CD133, and CD166 have also been proposed as CSC markers in CRCs, we investigated whether EPHB2 expression is associated with any of these candidate CSC markers and found that only CD44 has a positive correlation with EPHB2 mRNA expression, and this finding was consistent with the immunohistochemical analysis showing a positive correlation between H-scores of EPHB2 and CD44. It would be worthwhile to explore the interactions between CD44 and EPHB2 in CRCs through double staining or an *in vitro* study.

In conclusion, upregulated EPHB2 expression persists during adenoma-carcinoma transition and lymph node metastasis, but slightly declines in the invasive fronts of CRCs. EPHB2 positivity is negatively correlated with lymphatic and venous invasion and TNM stage, but positively correlated with MSI-high. In addition, EPHB2 is significantly associated with better clinical outcomes in CRCs. Taken together, our findings suggest that EPHB2 can be used as a prognostic marker for CRC patients.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Interleukin-31, Interleukin-31RA, and OSMR Expression Levels in Post-burn Hypertrophic Scars

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Background: Although several studies have shown the role of interleukin-31 (IL-31) and its receptors in inducing pruritus in certain skin disorders, knowledge of its role in post-burn hypertrophic scars is insufficient. Therefore, the histopathological expression levels of IL-31, IL-31 receptor alpha (IL-31RA), and oncostatin M receptor (OSMR) in post-burn hypertrophic scar tissues were investigated and compared with normal tissue expression levels. **Methods:** Samples of hypertrophic scar tissue were obtained from 20 burn patients through punch biopsy. Normal samples were obtained from areas adjacent to the burn injury site of the same patients. Samples were placed in 10% neutral buffered formalin, embedded in paraffin, and processed into serial 5- μ m sections. Immunohistochemistry results were semi-quantitatively evaluated for IL-31, IL-31RA, and OSMR. By hematoxylin and eosin staining, epidermal and dermal thickness were assessed with a microscope and digital camera. Intensities were rated on a scale of 1 to 4. **Results:** Percentages for IL-31, IL-31RA, and OSMR in the epidermal basal layer cell cytoplasm were significantly greater in the burn scar tissue compared to normal skin, as well as the dermal and epidermal thickness ($p < .05$). There was a significant difference in IL-31 epidermal basal layer intensity in burn scar tissue compared to normal skin ($p < .05$). Besides the OSMR basal layer intensity, IL-31 and IL-31RA intensities between the burn scar and normal tissues were not significant. However, correlations were significant, indicating that the greater the infiltration percentage, the higher the intensity ($p < .05$). **Conclusions:** IL-31, IL-31RA, and OSMR expression levels are increased in hypertrophic scars compared with normal tissue.

Key Words: Burns; Cicatrix; Interleukin-31; IL31RA protein; OSMR protein

Burns are a severe and traumatic injury to the skin. Although approximately 300,000 deaths still occur annually due to burns, mortality rates have been reduced due to advancements in acute burn care. Unfortunately, burn recovery remains a challenging process due to abnormal scarring of the skin, which has an adverse impact on quality of life.¹ Following a burn injury, the incidence of hypertrophic scar development is reported to be as high as 77%.²

Normal wound healing of the human skin occurs through a balance of deposition and removal of structural proteins and glycoproteins at an optimal speed for the development of a healthy, functional scar.³ However, this balance is rarely maintained in post-burn hypertrophic scars, causing an over-growth of collagen in the dermis, proteoglycans, fibronectin, and tissue water.⁴⁻⁷ Dermal fibroblasts may have a large influence on increased matrix production, through continuously high amounts

of fibrogenic cytokines and other changes in dermal structure.⁸⁻¹⁰ The thin collagen fibers with increased synthesis and crosslinks found in hypertrophic scars result in scars that are raised, thick, red, firm, and do not grow beyond the original wound margins.¹¹ Many burn survivors who develop hypertrophic scars complain of functional disability, cosmetic dysfunction, pain, and itchiness, also known as pruritus.² For many patients, the most distressing symptom of hypertrophic scar formation is the associated pruritus.¹²

Pruritus affects burn survivors' quality of life by causing sleep disturbances, daily activity impairment, and psychological problems. Pruritus induces scratching, which can lead to wound infections and disrupt the proper wound healing process.¹³ After a burn injury, the wound healing process may last up to a year. Wound healing consists of three phases: early inflammatory, intermediate proliferative, and late remodeling.¹⁴ Acute itching eventually disappears in the remodeling phase. Chronic itching

occurs at relatively low intensities and is associated with injury depth, although it may persist for more than 2 years.¹⁵ Post-burn pruritus is regarded as a complex disease that is combined with pruritogenic, neuropathic, neurogenic, and psychogenic mechanisms, especially in chronic cases.¹⁶ Various existing treatment methods are not completely satisfactory since the exact mechanism of post-burn pruritus is unclear.¹⁴

Interleukin (IL)-31 appears to have a role in pruritus induction in disorders such as atopic dermatitis (AD) and prurigo nodularis.¹⁷⁻¹⁹ IL-31 is a fairly novel cytokine derived from T-cells and is included in the IL-6 cytokine family. It signals through a heterodimeric receptor composed of IL-31 receptor alpha (IL-31RA) and Oncostatin M receptor (OSMR).

Patients who commonly experience pruritus are those affected by AD. Studies have shown an association between pruritic diseases and IL-31. Sonkoly *et al.*²⁰ examined human IL-31 and its role in pruritic and non-pruritic inflammatory skin diseases.²⁰ The study found significant overexpression of IL-31 in atopic skin with pruritus compared to psoriasis, which rarely itches and is thus considered a non-pruritic inflammatory disease. The highest levels of IL-31 were detected in prurigo nodularis, an intense form of pruritic chronic inflammation of the skin. *In vivo*, rapid production of IL-31 was reported with the staphylococcal superantigen in atopic subjects. Therefore, when IL-31 levels are elevated in lesions, they may amplify the inflammation of the skin through chemokine induction, subsequently causing T-cell recruitment. As a result, IL-31 may be produced by activated skin-infiltrating T cells, worsening the inflammation and pruritus.²⁰

Murine neuronal tissue studies have found that IL-31RA plays a significant role in itching and that OSMR is also recruited by IL-31.^{21,22} According to Le Saux *et al.*,²³ IL-31 needs to bind to IL-31RA before OSMR because of a conformational transformation that occurs in IL-31. This change is essential for the binding of IL-31 to OSMR.²³ Therefore, IL-31RA and OSMR should also be included in the discussion on pruritus.

Based on the reports of elevated IL-31 and its receptors in the previously mentioned pruritic conditions, potentially important but unidentified roles of IL-31, IL-31RA, and OSMR in the development of pruritus in post-burn hypertrophic scars may exist. Although several studies have demonstrated the role of IL-31 in inducing pruritus in certain skin disorders, knowledge of its role in post-burn hypertrophic scars is insufficient. This appears to be the first study to examine this cytokine and its receptors in post-burn hypertrophic scars. Therefore, the purpose of this study was to examine the expression levels of IL-31, IL-31RA, and OSMR in hypertrophic scar tissue versus normal tissue.

MATERIALS AND METHODS

Patient selection

Twenty samples of hypertrophic burn scars were obtained from patients who had visited the Hallym University Hangang Sacred Heart Hospital in South Korea. Subjects were included in the study if they were at least seven years of age at the time of examination. Subjects were excluded if they (1) had a pre-existing disease known to frequently cause pruritus, such as chronic systemic or dermatologic disease, (2) were pregnant, (3) were unable to specifically describe their pruritus, (4) were on concurrent systemic medications that may affect their pruritic symptoms such as antihistamines, morphine, or systemic steroids, (5) had any psychotic diseases, (6) had cancer, (7) had an active infection, or (8) had been treated with immunomodulators, UV irradiation, or hydrogen peroxide within three months before surgery. All subjects provided informed consent. The protocols of this study were approved by Hallym University Hangang Sacred Heart Hospital Institutional Review Board (IRB approval No. 2011-186).

Histopathological analysis

Skin samples were obtained through punch biopsy (6 mm) during surgery from twenty patients between 2011 and 2012. Control tissue samples were obtained from non-burned skin areas adjacent to the burn injury site from the same patients. Tissue samples from hypertrophic scars were taken from the burn lesions of the same patients. The tissue samples were placed in 10% neutral buffered formalin for 18 hours. Paraplast (Sigma-Aldrich, St. Louis, MO, USA) was used for paraffin embedding. Serial sections 5- μ m in thickness were processed.²⁴ The IL-31 antibody (Novus Biologicals, Littleton, CO, USA), IL-31RA antibody (Abnova, Taipei, Taiwan), and OSMR antibodies (Novus Biologicals) were used for staining purposes and sections were assessed by a pathologist. Immunohistochemistry results were semi-quantitatively evaluated for IL-31, IL-31RA, and OSMR based on the estimated epidermal positive basal cell percentage. Cells were counted in the stroma using a built-in 10 $\times$ 10 grid within the Nikon microscope (Plan-Apo, Nikon, Tokyo, Japan) with each section measuring 25 μ m (0.025 mm) at $\times$ 400 magnification and the upper edge of the grid placed at the epidermal junction.²⁵ Three areas with the greatest density of inflammatory cells with the most distinct inflammation were counted per visual field and the cells were regarded as either immunoreactive or non-immunoreactive.¹⁹ The IL-31RA epidermis, IL-31 epidermis basal layer, and OSMR epidermis basal layer staining intensities

were rated as 1 when the positivity could be seen at 400, 2 at 200, 3 at 100, and 4 at 40-fold magnification with use of a Nikon microscope (Plan-Apo) with a digital camera (Nikon, DS-Ri2, Nikon digital SLR camera FX-format CMOS sensor optimized for microscopy).²⁴ One pathologist and one dermatologist assessed the epidermal thickness, dermal thickness, and IL-31, IL-31RA, and OSMR infiltration through hematoxylin eosin staining through use of a Nikon microscope (Plan-Apo) with a digital camera (Nikon, DS-Ri2, Nikon digital SLR camera FX-format CMOS sensor optimized for microscopy), and the average values were obtained.

Statistical analysis

SPSS ver. 21.0 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Normal skin and burn scar tissue comparisons were performed using the paired *t* test and the Wilcoxon signed-rank test. Pearson and Spearman rho was used to examine the correlation between IL-31, IL31-RA, and OSMR percentages and intensities. Values were regarded as significant if the *p*-value was < .05.

RESULTS

This study included tissue samples from twenty subjects. The characteristics of the burn subjects are presented in Table 1.

Comparison of burn scar and normal skin tissue characteristics are presented in Table 2. There was a significant difference in dermal thickness in burn scar tissue compared with normal skin ($4,826.85 \pm 1,955.34 \mu\text{m}$ and $1,809.50 \pm 745.54 \mu\text{m}$, respectively; $p < .001$) and the epidermal thickness was signifi-

cantly thicker in the burn scar tissue compared with normal skin ($5,112.25 \pm 2,522.42 \mu\text{m}$ vs. $1,729.88 \pm 806.55 \mu\text{m}$, respectively; $p < .001$). The epidermal basal layer percentage of IL-31 was significantly greater in burn scar tissue compared with normal skin (22.4% and 12.7%, respectively; $p < .001$) (Fig. 1). However, there was no significant difference in IL-31 epidermal basal layer intensity in burn scar tissue compared with normal skin ($p > .05$). The IL-31RA epidermal percentage was significantly greater in burn scar tissue compared with normal skin (29.8% and 23.9%, respectively; $p < .001$) (Fig. 1). The comparison of IL-31, IL-31RA, and OSMR percentage levels between control and scar tissue is shown in Fig. 2. There were no significant differences in IL-31 epidermis basal layer intensity ($p > .05$). The OSMR epidermal basal layer cytoplasm percentage was greater in burn scar tissue compared with normal skin (46.50%

Table 1. Characteristics of burn subjects (n=20)

Variable	Value
Sex (male:female)	14:6
Side (right:left)	10:10
Age at time of examination (yr)	27.53 $\pm$ 18.73
Age at burn incident (yr)	18.04 $\pm$ 20.05
Scar age (mo)	46.42 $\pm$ 97.97
No. of burn sites	Both arms (4), head (11), both legs (1), foot (4)
No. of burn sources	Hot water (8), friction (1), iron (2), flame (4), electricity (1), chemical (1), steam (3)
No. of burn treatments	CT (7), STSG (5), FTSG (5), STSG+Alloderm (3)

Age at time of examination and scar age values are presented as mean $\pm$ standard deviation.

CT, conservative treatment; STSG, superficial thickness skin graft; FTSG, full-thickness skin graft.

Table 2. Histopathological comparison of normal tissue and scar tissue characteristics

Variable	Normal tissue (n=20)	Scar tissue (n=20)	t/z	p-value
Exam age (yr)	21.80 $\pm$ 18.10	21.80 $\pm$ 18.10	-1.000	.325
Sex (male:female)	14:6	14:6		
Burn age (yr)		10.20 $\pm$ 16.36		
Scar age (mo)		115.60 $\pm$ 145.15		
Dermal thickness (μm)	1,809.50 $\pm$ 745.54	4,826.85 $\pm$ 1,955.34	-7.802	< .001***
Epidermal thickness (μm)	114.05 $\pm$ 30.91	174.10 $\pm$ 51.56	-6.528	< .001***
IL-31 epidermis basal layer percentage (%)	12.00 $\pm$ 12.39	17.50 $\pm$ 16.81	-3.425	< .001***
IL-31 epidermis basal layer intensity			-1.941	.052
IL-31RA epidermis percentage (%)	38.50 $\pm$ 24.54	56.00 $\pm$ 28.72	-4.148	< .001***
IL-31RA epidermis intensity			-1.694	.900
OSMR epidermis basal layer cytoplasm (%)	25.00 $\pm$ 17.91	46.50 $\pm$ 27.96	-4.747	< .001***
OSMR epidermis basal layer intensity			-2.841	.005**
No. of mast cells	4.17 $\pm$ 3.20	8.00 $\pm$ 8.00	-2.504	.022*

Values are presented as mean $\pm$ standard deviation.

%, percentage of infiltration; Intensity, intensity of the microscopic expression.

Significant * $p < .05$, ** $p < .01$, *** $p < .001$.

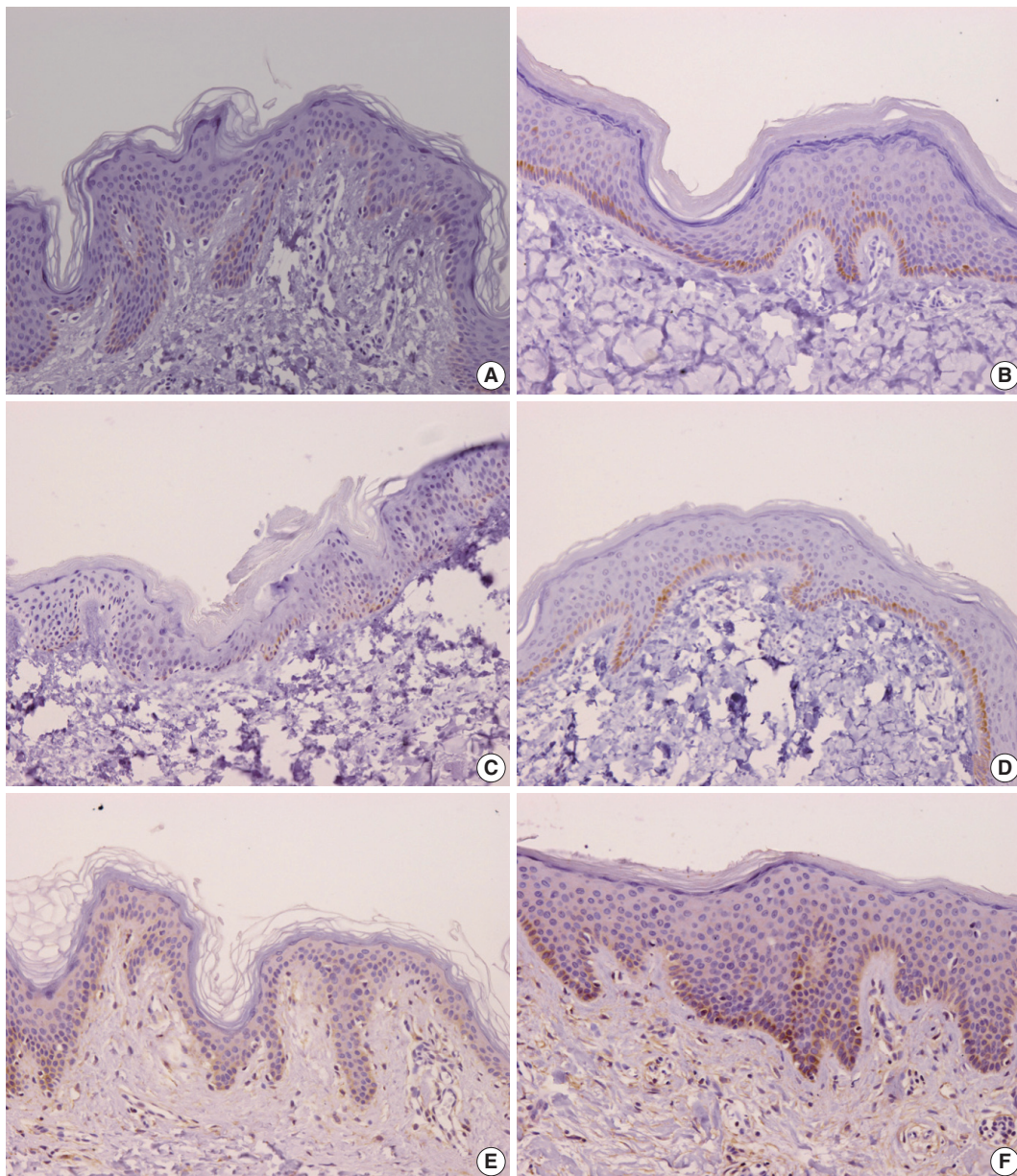


Fig. 1. (A) Interleukin 31 (IL-31) control. (B) IL-31 scar. (C) IL-31 receptor alpha (IL-31RA) control. (D) IL-31RA scar. (E) Oncostatin M receptor (OSMR) control. (F) OSMR scar. IL-31, IL-31RA, and OSMR infiltration.

and 25.00%, respectively; $p < .001$) (Fig. 1). There was also a significant difference in OSMR epidermis basal layer intensity in burn scar tissue compared with normal skin ($p < .005$). Pearson and Spearman rho analyses showed that the correlations between the percentages and intensities of IL-31, IL-31RA, and OSMR in burn scar tissue were significant ($p < .05$), indicating that the greater the infiltration percentage, the higher the intensity, based on a scale of 1 to 4 (Table 3).

There was also a significant difference in the number of mast cells, which was greater in burn scar tissue compared with normal

skin ($p < .05$) (Table 2).

DISCUSSION

The objective of this study was to examine the expression of IL-31, IL-31RA, and OSMR in hypertrophic scar tissue and compare the results with those from normal tissue. IL-31 is a T-cell cytokine which acts through a heterodimeric receptor such as IL-31RA and OSMR, which are expressed by epithelial cells.²⁶

Our study has found that IL-31, IL-31RA, and OSMR are

expressed by epidermal basal cells from both normal and burn scar tissue samples. However, IL-31 and IL-31RA expression was significantly higher in the burn scar epidermal basal cells ($p < .001$) compared with normal skin tissue. The OSMR epidermal basal layer cytoplasm percentage was also significantly greater than in normal skin. Our findings are relatable to previous studies that have found greater levels of IL-31 and its receptors in subjects with various pruritic skin conditions compared with non-pruritic and normal skin.^{17-20,23} Nobbe *et al.*¹⁹ reported that the majority of the immunoreactivity stainings for IL-31RA and OSMR were cytoplasmic staining patterns.

Cutaneous T-cell lymphoma patients also commonly suffer from pruritus and have recently been found to have elevated IL-31 expression in the epidermis and dermis and increased IL-31RA and OSMR expression in the epidermis only.²⁷

It appears that IL-31 and its receptors have their respective roles in pruritus induction. IL-31 is postulated to stimulate the keratinocytes and infiltrating cells to release other mediators

involved in the induction of pruritus. Moreover, IL-31 appears to cause pro-inflammatory cytokines to be released from macrophages, eosinophils, and monocytes.^{26,28}

Expression of IL-31RA and OSMR has been found in keratinocytes and the dorsal root ganglia, which are the locations where the cutaneous sensory neuron soma reside and their sensory fibers are directed towards the skin.²⁹ These sensory neurons may be involved in the itch sensation and are thought to be stimulated by IL-31. IL-31 may serve as a possible connection between the immune and sensory nervous system.³⁰ Therefore, although the presence of elevated IL-31 and its relation to pruritus is significant, the roles of IL-31RA and OSMR should not be neglected and should be taken into consideration in the discussion of pruritus.

This study found that burn scar thickness and epidermal thickness was significantly increased compared with normal tissue. When a burn injury occurs on the skin, the skin barrier function is damaged or destroyed.³¹ Similarly, for patients with AD, the skin-barrier function is most commonly impaired, indicating that the epithelial defense system serves a significant role in the pathogenesis of AD.³² When the skin barrier is altered or damaged in AD patients, this is partially associated with the distribution of the stratum corneum liquid composition, which allows harmful substances to penetrate cutaneously and subsequently signals for proliferation and differentiation of the epidermis.³¹ Singh *et al.*³³ found that intradermal injection of recombinant mouse IL-31 (rIL-31) produced epidermal thickness and that elevated levels of IL-31 by T cells subsequently induced thickening of the epidermis and inflammatory infiltrates.³³

There was no significant difference in intensity levels for all variables, except for the OSMR basal layer intensity. This discrepancy may be attributed to the small sample size, but could show significance in future studies with larger sample sizes.

Although this study did not specifically look for pruritus in those with hypertrophic scars, the results show characteristics similar to pruritic skin conditions, such as AD, in which there

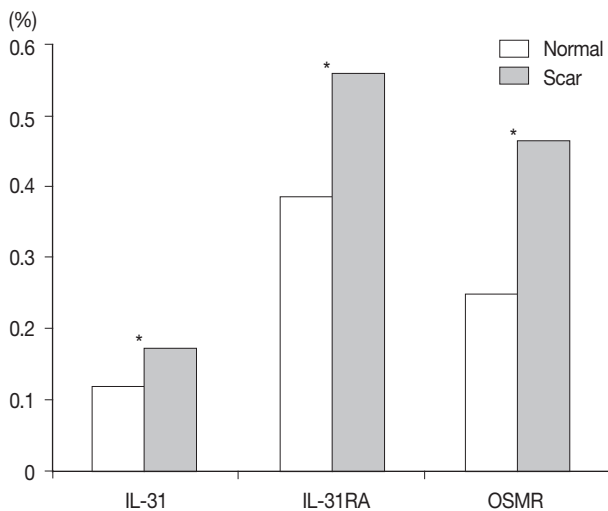


Fig. 2. Comparison of interleukin 31 (IL-31), IL-31 receptor alpha (IL-31RA), and oncostatin M receptor (OSMR) percentage values between the normal and scar tissue ($n=20$). * $p < .05$.

Table 3. Correlation between IL-31RA, IL-31, OSMR percentage (Pearson) and intensity (Spearman rho) in burn scar ($n=20$)

	IL-31RA epidermis (%)	IL-31RA epidermis intensity	IL-31 epidermis basal (%)	IL-31 epidermis basal intensity	OSMR epidermis basal cytoplasm (%)	OSMR epidermis basal intensity
IL-31RA epidermis %	1	0.745***	0.534*	0.566**	0.545*	0.447*
IL-31RA epidermis intensity		1	0.675**	0.834***	0.641**	0.597**
IL-31 epidermis basal %			1	0.953***	0.786***	0.650**
IL-31 epidermis basal intensity				1	0.808***	0.683**
OSMR epidermis basal cytoplasm					1	0.814***
OSMR epidermis basal intensity						1

Intensity rated as 1 when the positivity could be seen at 400, 2 at 200, 3 at 100, and 4 at 40-fold magnification with use of a Nikon microscope with a digital camera. 1=weak intensity, 4=strong intensity. The greater the infiltration percentage, the higher the intensity based on a scale of 1 to 4.

IL-31, interleukin 31; IL-31RA, IL-31 receptor alpha; OSMR, oncostatin M receptor.

* $p < .05$, ** $p < .01$, *** $p < .001$.

are significantly greater IL-31, IL-31RA, and OSMR expression levels in the skin.

There was no significant difference in intensities between the control and burn scar tissues, aside from the OSMR basal layer intensity. However, Pearson and Spearman rho results showed significant correlations between infiltration percentages and intensities in burn scar tissues, where the greater the percentage of infiltration, the greater the grade in intensity, based on the scale of 1 to 4. As stated earlier, the IL-31RA epidermis, IL-31 epidermis basal layer, and OSMR epidermis basal layer staining intensity was rated as 1 when the positivity could be seen at 400, 2 at 200, 3 at 100, and 4 at 40-fold magnification with use of a Nikon microscope. In this case, an intensity grade of 4 is equal to 40-fold magnification; this indicates that the intensity is strong with low magnification because if the percentage of infiltration is high enough, a smaller amount of magnification is required to observe the infiltration through the microscope.

The mean value of the mast cells was significantly greater in the burn scar compared with the normal tissue samples. This finding may be significant for several reasons. Antimicrobial peptide proteins (AMPs), such as human β -defensins (hBDs), provide innate immunity and defense against microbial invasion, which is a common risk factor with burn injury, and are formed in the deep portions of burned skin.^{34,35} Mast cells are located within the dermis and have been found to be another source of IL-31.³⁴ AMPs have been found to cause mast cells to release IL-31 mRNA, and may lead to protein production and release.³⁴ Niyonsaba *et al.*³⁶ has also stated that mast cells are involved in the pathological process of other disorders of the skin in which AMP concentrations are enhanced.³⁶ In the case of wounding and lichen planus, direct contact between skin-derived AMPs and mast cells occurs, which subsequently activates due to basal membrane impairment between the dermis and epidermis. Sites of infection and inflammation show elevated amounts of hBDs and leucine-leucine 37-amino acid peptide (LL-37) in the human epithelium. LL-37 is induced in the keratinocytes of humans during contact dermatitis³⁷ and hBDs may contribute to skin inflammatory responses by causing mast cells to secrete IL-31 along with other factors that are pruritogenic. These findings may support the link between increased IL-31 levels and post-burn hypertrophic scar pruritus in the present study.

This study had some limitations. The sample size was small and subject complaints of pruritus were not fully assessed. In addition, no other techniques were used to confirm our findings. However, this study has shown that there are pathological similarities between post-burn pruritus and other skin diseases known

to cause pruritus, such as AD, prurigo nodularis, and cutaneous T-cell lymphoma, all of which exhibit increased expression levels of IL-31.

Therefore, the findings of this study may enlighten the understanding of pruritic events occurring in post-burn hypertrophic scars. Further studies with larger sample sizes that investigate the relationship between IL-31 and receptor expression levels with subjective and objective measurements of pruritus are warranted.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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An Immunohistochemical and Polarizing Microscopic Study of the Tumor Microenvironment in Varying Grades of Oral Squamous Cell Carcinoma

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Background: Invasion of epithelial cells into the connective tissue brings about massive morphological and architectural changes in the underlying stroma. Myofibroblasts reorganize the stroma to facilitate the movement of tumor cells leading to metastasis. The aim of this study was to determine the number and pattern of distribution of myofibroblasts and the qualitative and quantitative change that they cause in the collagen present in the stroma in various grades of oral squamous cell carcinoma (OSCC). **Methods:** The study was divided into two groups with group I (test group, 65 cases) consisting of 29 cases of well-differentiated squamous cell carcinoma, 25 moderately differentiated SCC, and 11 poorly differentiated SCC, and group II (control group) consisting of 11 cases of normal mucosa. Sections from each sample were stained with anti- α -smooth muscle actin (α -SMA) antibodies, hematoxylin and eosin, and Picrosirius red. Several additional sections from each grade of OSCC were stained with Masson's trichrome to observe the changes in collagen. For the statistical analysis, Fisher's exact test, Tukey's *post hoc* honest significant difference test, ANOVA, and the chi-square test were used, and $p < .05$ was considered statistically significant. **Results:** As the tumor stage progressed, an increase in the intensity α -SMA expression was seen, and the network pattern dominated in more dedifferentiated carcinomas. The collagen fibers became thin, loosely packed, and haphazardly aligned with progressing cancer. Additionally, the mean area fraction decreased, and the fibers attained a greenish yellow hue and a weak birefringence when observed using polarizing light microscopy. **Conclusions:** Myofibroblasts bring about numerous changes in collagen. As cancer progresses, there is increase in pathological collagen, which enhances the movement of cells within the stroma.

Key Words: Myofibroblast; Collagen; Oral squamous cell carcinoma; α -Smooth muscle actin; Picrosirius red; Polarizing microscope

In India, 130,000 people succumb to oral cancer annually, which translates to approximately 14 deaths per hour.¹ It accounts for over 30% of all cancers in India.² In the United States, oral cancer represents approximately 13% of all cancers with 30,000 new cases every year.¹

The tumor stroma plays a critical role during carcinogenesis as it is required for the tumor to grow beyond a minimal size of 1–2 mm.³ Inappropriate synthesis or degradation of any extracellular matrix (ECM) component can alter cell physiology and aid in the progression of disease.⁴ The mechanical quality of the ECM is mainly dependent on its collagen content because collagen is the main barrier to be cleared away during invasion.⁵

The ECM mainly consists of type I collagen (approximately 90%) with 8%–10% type III collagen. Electron microscopic studies have shown that type I collagen fibers are coarse and are composed of closely packed, thick fibrils, whereas type III collagen fibers are fine and are composed of loosely dispersed, thin

fibrils.⁶ Collagen fibers exhibit changes in type, diameter, color, orientation, density, and amount with tumor progression.⁷

Myofibroblasts are cancer-induced host cells of the microenvironment and are believed to be derived from normal fibroblasts during times of tissue stress or altered homeostasis. They are defined immunohistochemically by the presence of α -smooth muscle actin (α -SMA).⁸ Additionally, they produce inflammatory mediators and growth factors that aid in ECM reorganization and stimulate epithelial cell proliferation.⁹

Picrosirius red (PSR) stain is a highly specific and selective stain for collagen fibers due to its ability to differentiate between pathological and normal collagen.¹⁰ Using this stain, collagen fibers can be evaluated both qualitatively and quantitatively, and this information can provide useful clues to the various aspects of tumor progression.⁷

The aim of this study was to determine the number and pattern of distribution of myofibroblasts and the qualitative and quan-

titative changes that they cause in the collagen present in the stroma in various grades of oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS

After obtaining permission from the Institutional Scientific and Ethical Committee (approval no. 811516/OP/EC), the present study was carried out on tissues archived in the department. Medical records, including informed consent and pathology reports, were reviewed and the patient's age, sex, tumor location, tumor size, differentiation, invasion depth of tumor, lymph node metastasis, and clinical stage were recorded. Patients who had received preoperative chemotherapy and/or radiotherapy were excluded from the study.

The study comprised 76 cases, which were divided into two groups. Group I consisted of 65 cases of histologically proven OSCC with 29 cases of well-differentiated squamous cell carcinoma (WDSCC), 25 of moderately differentiated SCC (MDSCC), and 11 of poorly differentiated SCC (PDSCC). Group II was the control and consisted of 11 cases of normal mucosa obtained following an operculectomy or frenectomy. Smooth muscle cells surrounding the blood vessels were used as a positive internal control for the α -SMA stained slides.

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μ m, and three sections from each sample were prepared. The first section was mounted on a poly-L-lysine coated slide and stained with antibodies against α -SMA. The second section was stained with hematoxylin and eosin, observed under a light microscope, and graded according to the Broder classification. The third section was stained with PSR and observed under a polarizing light microscope. Several additional sections from each grade of OSCC were obtained and stained with Masson's Trichrome to observe the morphological changes in collagen.

For immunohistochemistry (IHC), the sections were first deparaffinized, hydrated and washed in distilled water for 5 minutes. Antigen retrieval was then performed by immersing the slides in pre-warmed 1 M citrate buffer (pH 6.0) and heating in a microwave oven at 95–98°C for 20 minutes. Blocking of the endogenous peroxidase was performed by immersing the slides in a mixture of 50 mL of methanol with 1.5 mL of hydrogen peroxide for 30 minutes in a humidified chamber followed by washing in distilled water for 5 minutes. The slides were then cooled to room temperature and washed in 1 M Tris buffer (pH 7.2). Primary mouse anti-human α -SMA monoclonal antibody clone 1A4 (Thermo Scientific, Waltham, MA, USA) at a dilution of 1:50–100 was added to the sections, and the slides were incu-

bated for 30 minutes at room temperature. The slides were then washed in 1 M Tris buffer (pH 7.2), and "horse radish peroxidase-conjugated goat anti-mouse secondary antibody" was added to the sections and incubated for 30 minutes. The slides were washed again in 1 M Tris buffer (pH 7.2) followed by the addition of diaminobenzidine (DAB) chromogen in DAB buffer (Thermo Scientific). The slides were incubated for 3 minutes then counterstained with hematoxylin.¹¹ The anti- α -SMA antibody-labelled cells were identified by the strong, dark brown cytoplasmic staining of the myofibroblasts. The samples were then assessed for the intensity and pattern of myofibroblastic proliferation.

For PSR staining, paraffin-embedded tissue sections were dehydrated and de-waxed then stained with Weigert's hematoxylin for 8 minutes. The slides were then washed for 10 minutes under running tap water and stained with PSR stain for 1 hour followed by washing in 2 changes of acidified water. The sections were dehydrated in three changes of 100% alcohol and cleared by dipping in xylene followed by mounting using DPX mounting media and a cover slip. Using light microscopy, the nuclei appear black and collagen appears red. Using polarizing microscopy, collagen fibers produce orange red (OR), yellowish orange (YO), or green/greenish yellow (G/GY) birefringence.¹¹

Intensity and pattern of α -SMA expression

Intensity score for α -SMA

The percentage of cells positive for α -SMA in the tumor stroma were classified into the following categories¹²: absent/0, no positive cells; mild/1+, 1%–33% positive cells; moderate/2+, 34%–66% positive cells; and intense/3+, 67%–100% positive cells.

Pattern of α -SMA expression

The distribution and arrangement of positively stained myofibroblast cells were classified into three groups: focal, spindle, and network.¹³ Focal indicates no specific arrangement of the myofibroblasts. In the spindle groups, myofibroblasts were arranged in one to three rows in a regular order in the periphery of the neoplastic islands or in the connective tissues with distinctive cell margins around the myofibroblasts and malignant tissue. A "network" classification includes myofibroblasts with vesicular nuclei and abundant cytoplasm arranged in multiple rows with an interwoven network of cytoplasmic extensions forming a network in the stroma of the connective tissue.

Collagen evaluation

The parameters used for the evaluation of the collagen were

thickness, arrangement and orientation, packing, mean area fraction, and hue and birefringence exhibited by the fibers.

For measuring fiber thickness, images of PSR-stained slides were obtained at 400× magnification and processed using image analysis software (Image J, ver. 1.46 r, NIH, Bethesda, MD, USA). In samples from normal tissues, collagen fibers from the lamina propria were studied, and in OSCC samples, collagen fibers around tumor islands were used. For each section, two separate high-power fields with at least 50 fibers of each size (25 each of thick and thin fibers) were examined.¹⁴ Collagen fibers with a thickness of 2–10 µm were considered thick, type I fibers and fibers 0.5–1.5 µm in diameter were considered thin, type III fibres.¹⁵

The arrangement and orientation of collagen fibers were categorized as either parallel or haphazard based on their appearance in relation to the tumor islands. The evaluation was performed in five selected fields at 100× magnification.⁷

The packing of the collagen fibers was categorized as either dense or loose based on their appearance in 5 selected fields at 100× magnification in the immediate vicinity of the tumor islands.⁷

For measuring the mean area fraction, images of the sections at 400× magnification were evaluated using the image analysis software. The percentage of the area occupied by collagen fibers in a given field was calculated for each grade of OSCC and also for normal mucosa.⁷

For determining hue and birefringence, five random high-power fields for each slide of the connective tissue stroma at 400× magnification were evaluated. In samples from normal tissues, collagen fibers from the lamina propria were studied, and in OSCC samples, collagen fibers around tumor islands were used. The predominant hue exhibited by the collagen fibers was classified as either OR, YO, or G/GY, and the birefringence was classified as either strong or weak.^{6,7,16-18}

Categorical variables, such as α -SMA expression, pattern, collagen fiber orientation and packing, and hue and birefringence, are expressed as a percentage, and continuous variables, such as collagen fiber thickness and mean area fraction, are expressed as the mean and standard deviation. For the categorical variable, the

chi-square test was used to test the significance of the association. When more than 20% of the individual cells had an expected value of less than five Fisher exact test was used. For the continuous variables, the mean and SD of the subgroups were tested for significance using Analysis of Variance (ANOVA). Since ANOVA does not comment on significant differences in subgroups, Tukey's *post hoc* honest significance difference testing was used for further analysis if the ANOVA results were significant. For the collagen fiber thickness and mean area fraction, all four statistical tests were used. For all tests, $p < .05$ was considered statistically significant.

RESULTS

Expression of α -SMA was not observed in the stroma of normal oral mucosa except for the blood vessels. The α -SMA expression in OSCC samples is shown in Table 1 and Figs. 1, 2, and 3. Among the different degrees of differentiation, a statistically significant increase in the intensity of α -SMA expression was found in MDSCC compared to WDSCC and in PDSCC compared to MDSCC ($p < .001$). A variation in the pattern of α -SMA expres-

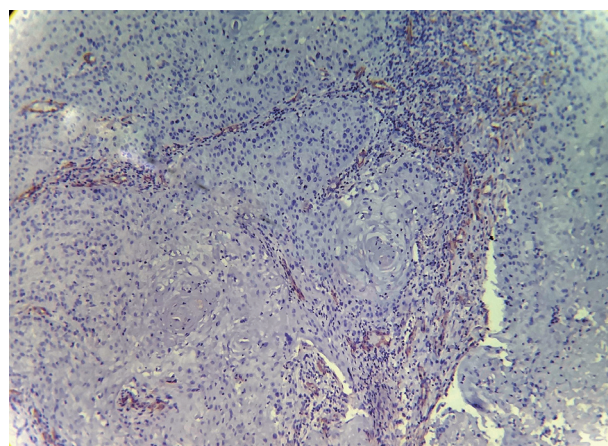


Fig. 1. Light microscopy image of well-differentiated squamous cell carcinoma showing mild α -smooth muscle actin positivity in the stroma. Myofibroblasts can be seen distributed in a focal pattern.

Table 1. Level of α -SMA expression in malignant lesions

Type of lesion	No. of cases	α -SMA intensity score (%)			
		0	1+ (mild)	2+ (moderate)	3+ (intense)
WDSCC	29	4 (13.8)	17 (58.6)	8 (27.6)	0
MDSCC	25	0	7 (28)	13 (52)	5 (20)
PDSCC	11	0	0	3 (27.3)	8 (72.7)

Values are presented as number (%).

Fisher exact test $p < .001$.

α -SMA, α -smooth muscle actin; WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

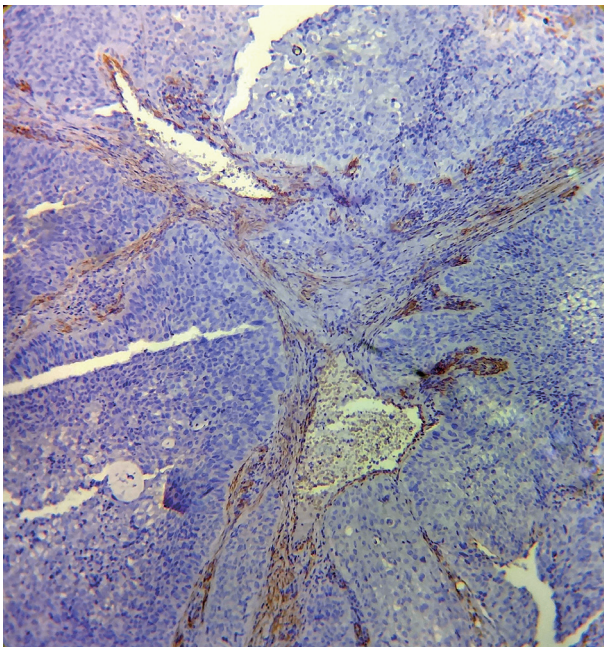


Fig. 2. Light microscopy image of moderately differentiated squamous cell carcinoma showing intense α -smooth muscle actin positivity in the stroma. Myofibroblasts can be seen distributed in a spindle pattern.

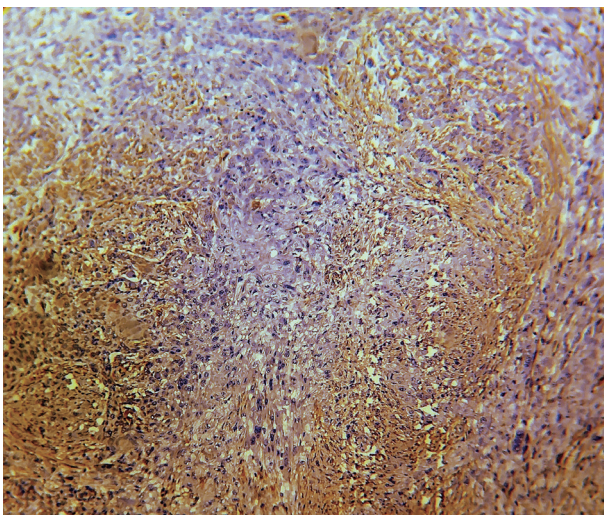


Fig. 3. Light microscopy image of poorly differentiated squamous cell carcinoma showing intense α -smooth muscle actin positivity in the stroma. Myofibroblasts can be seen distributed in a network pattern.

Table 2. Pattern of α -SMA expression in malignant lesions

Type of lesion	No. of cases	Negative staining	Focal pattern	Spindle pattern	Network pattern
WDSCC	29	4 (13.8)	19 (65.5)	6 (20.7)	0
MDSCC	25	0	3 (12)	8 (32)	14 (56)
PDSCC	11	0	0	2 (18.2)	9 (81.8)

Values are presented as number (%).

Fisher exact test $p < .001$.

α -SMA, α -smooth muscle actin; WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

sion was noted among the malignant lesions, which included focal, spindle, and network patterns of stromal myofibroblast positivity as shown in Table 2. Statistically significant differences were found in the expression pattern among the malignant lesions ($p < .001$). The network pattern was significantly dominant in carcinomas with less differentiation.

The mean, SD, and range of fiber thickness (measured in μm) of different grades of OSCC are presented in Table 3. In WDSCC and normal mucosa, the collagen fibers appeared predominantly as bundles of thick fibers. The thickness gradually decreased as the carcinomas progressed from well to poorly differentiated. Statistically significant differences were seen between WDSCC and MDSCC ($p < .001$), WDSCC and PDSCC ($p < .001$), MDSCC and normal cells ($p = .041$), and PDSCC and normal cells ($p = .002$). The collagen fibers predominantly exhibited a parallel orientation in WDSCC, which gradually changed to a haphazard pattern with the progression towards poorly differentiated carcinoma as shown in Figs. 4, 5, and 6. These changes were statistically significant ($p = .002$) as shown in Table 4. The collagen fibers were densely packed around the tumor islands in most cases of WDSCC but loosely packed in most of the cases of MDSCC and in all cases of PDSCC. These differences were statistically significant ($p = .001$) as shown in Table 5. The mean area fraction occupied by the collagen fibers gradually decreased as the OSCC progressed from well to poorly differentiated. Statistically significant

Table 3. Collagen fiber thickness (μm) in varying grades of oral squamous cell carcinoma

Group	No.	Mean $\pm$ SD	Minimum–Maximum
WDSCC	29	2.9 ± 1.1	1.0–5.3
MDSCC	25	1.9 ± 0.5	1.0–3.4
PDSCC	11	1.3 ± 0.4	1.0–2.2
Normal mucosa	11	2.7 ± 1.0	1.1–4.2

ANOVA test results: $F(3,72) = 13.423$, $p < .001$. Significant at $p < .01$.

Post-hoc Tukey's honest significant difference testing reveals significant differences between WDSCC and MDSCC ($p < .001$); WDSCC and PDSCC ($p < .001$); MDSCC and normal ($p = .041$); and; PDSCC and normal ($p = .002$).

SD, standard deviation; WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

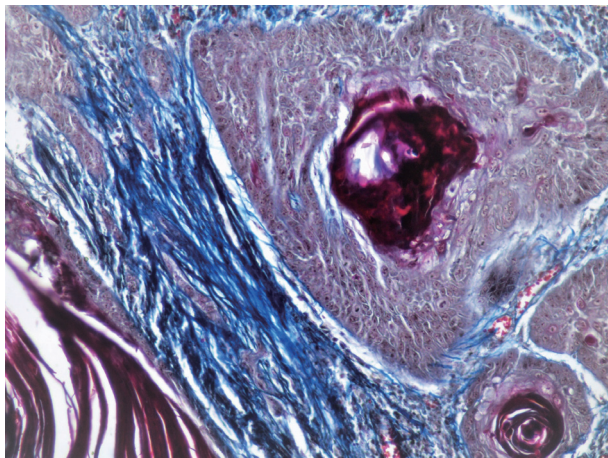


Fig. 4. Light microscopy image of a Masson's trichrome-stained section of well-differentiated oral squamous cell carcinoma showing densely packed collagen fibers exhibiting a parallel arrangement.

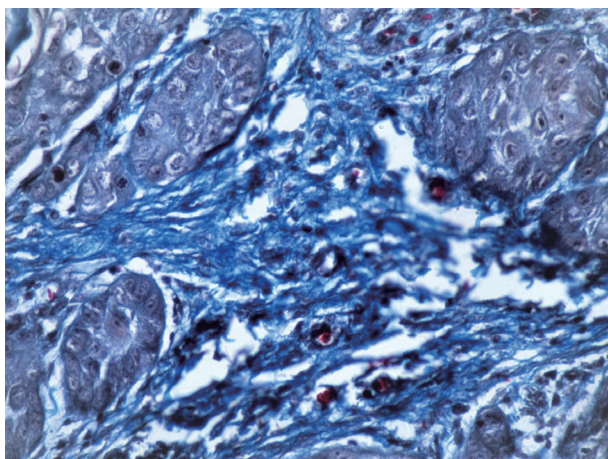


Fig. 5. Light microscopy image of a Masson's trichrome-stained section of moderately differentiated oral squamous cell carcinoma showing haphazardly arranged collagen fibers.

differences were seen between the groups as shown in Table 6.

In a majority of the samples of normal mucosa and WDSCC, the collagen fibers predominantly exhibited an OR hue with strong birefringence as seen in Fig. 7. In most of the cases of MDSCC, the fibers predominantly exhibited a YO hue with strong birefringence as seen in Fig. 8, and in a majority of the PDSCC cases, the fibers predominantly exhibited a GY hue with weak birefringence as seen in Fig. 9. These groupings were highly significant ($p < .001$) as shown in Tables 7 and 8.

DISCUSSION

In our study, OSCC was more common in males aged 40–59

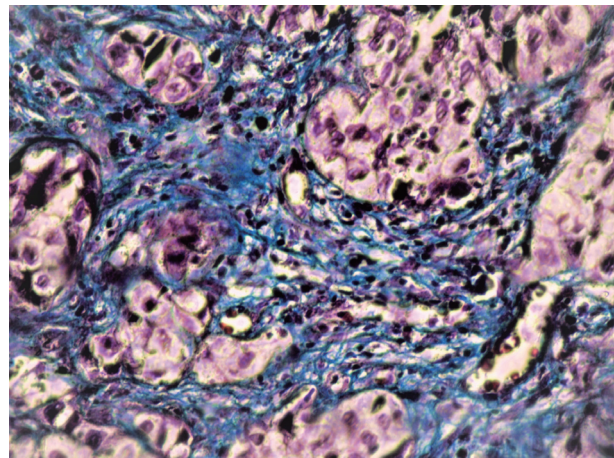


Fig. 6. Light microscopy image of a Masson's trichrome-stained section of poorly differentiated oral squamous cell carcinoma showing haphazardly arranged, loosely packed collagen fibers.

Table 4. Orientation of collagen fibers around tumor islands in varying grades of oral squamous cell carcinoma

Group	Fibre orientation		Total
	Haphazard	Parallel	
WDSCC	13 (44.8)	16 (55.2)	29 (100)
MDSCC	19 (76)	6 (24)	25 (100)
PDSCC	11 (100)	0 (0)	11 (100)
Total	43 (66.2)	22 (33.82)	65 (100)

Values are presented as number (%).

Chi-square = 12.6, $df = 2$, $p = .002$. Significant at $p < .05$.

WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

Table 5. Packing of collagen fibers around tumor islands in varying grades of oral squamous cell carcinoma

Group	Fibre arrangement		Total
	Dense	Loose	
WDSCC	19 (65.5)	10 (34.5)	29 (100)
MDSCC	10 (40)	15 (58.8)	25 (100)
PDSCC	0	11 (100)	11 (100)
Total	29 (44.6)	36 (55.4)	65 (100)

Values are presented as number (%).

Chi-square = 14.2, $df = 2$, $p = .001$. Significant at $p < .05$.

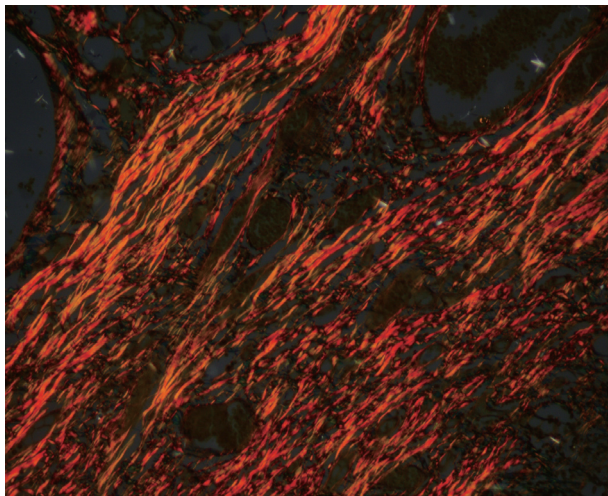
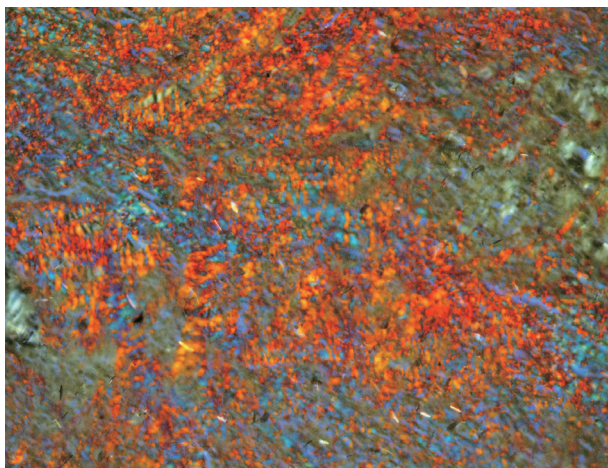
WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

years, and the most common site for oral cavity lesions was the floor of the mouth. In previous studies, oral cancer was also most common in middle-aged and older individuals.^{19,20} Increased exposure to risk factors, such as smoking and tobacco or betel chewing, in males makes them more prone to develop oral cancer.^{21,22} Due to the habitual placement of tobacco or a betel quid

Table 6. Mean area fraction of collagen fibers in varying grades of oral squamous cell carcinoma

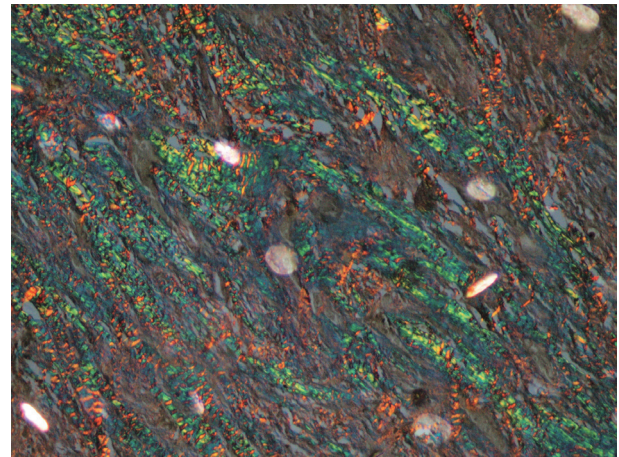
Group	No.	Mean $\pm$ SD (%)	Minimum–Maximum
WDSCC	29	25.1 $\pm$ 5.5	17.7–37.0
MDSCC	25	18.9 $\pm$ 3.7	11.5–24.1
PDSCC	11	10.6 $\pm$ 3.0	7.0–16.3
Normal mucosa	11	31.5 $\pm$ 3.5	27.4–36.2

ANOVA test results. $F(3,72)=51.2$, $p<.001$. Significant at $p<.05$.
Post-hoc Tukey's honest significant difference (HSD) testing reveals significant differences between all of the groups with each other ($p<.001$).
 SD, standard deviation; WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

**Fig. 7.** Polarizing light microscopy image of a Picrosirius red-stained section of well-differentiated oral squamous cell carcinoma showing densely packed collagen fibers (arrow) exhibiting a parallel arrangement and orange-red birefringence.**Fig. 8.** Polarizing light microscopy image of a Picrosirius red-stained section of moderately differentiated oral squamous cell carcinoma showing haphazardly arranged, loosely packed collagen fibers (arrow) exhibiting yellowish-orange birefringence.

on the floor of the mouth, this site appears to be at increased risk of developing carcinoma.

In our study, we found a significant increase in the number of

**Fig. 9.** Polarizing light microscopy image of a Picrosirius red-stained section of poorly differentiated oral squamous cell carcinoma showing haphazardly arranged, loosely packed collagen fibers (arrow) exhibiting greenish-yellow birefringence.**Table 7.** Colors observed via polarizing light microscopy in varying grades of oral squamous cell carcinoma

Group	Hue			Total
	OR	YO	GY	
Normal mucosa	6 (54.6)	4 (36.4)	1 (9.1)	11 (100)
WDSCC	18 (62)	8 (27.5)	3 (10.3)	29 (100)
MDSCC	9 (35.3)	13 (52)	3 (12)	25 (100)
PDSCC	0 (0)	3 (27.3)	8 (72.7)	11 (100)
Total	33 (43.4)	28 (36.8)	15 (19.7)	76 (100)

Values are presented as number (%).

Fisher's exact test, $p<.001$. Significant at $p<.01$.

OR, orange red; YO, yellowish orange; GY, greenish yellow; WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

Table 8. Nature of birefringence observed in varying grades of oral squamous cell carcinoma

Group	Birefringence		Total
	Strong	Weak	
Normal mucosa	10 (90.9)	1 (9.1)	11 (100)
WDSCC	26 (89.6)	3 (10.3)	29 (100)
MDSCC	19 (76)	6 (24.0)	25 (100)
PDSCC	3 (27.3)	8 (72.7)	11 (100)
Total	60 (81.1)	14 (18.9)	76 (100)

Values are presented as number (%).

Chi-square = 19.2, $df=3$, $p<.001$. Significant at $p<.01$.

WDSCC, well differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

myofibroblasts in OSCC compared to normal tissue, and more myofibroblasts were seen in more dedifferentiated carcinomas. Previous studies have demonstrated that factors derived from aggressive tumor cells are able to diffuse through the basement membrane and stimulate myofibroblast transformation.^{23,24} OSCC-derived tumor growth factor $\beta 1$ promotes fibroblast–myofibroblast trans-differentiation, and factors released from these myofibroblasts induce tumor cellular proliferation.^{25–27} Additionally, hyaluronan is implicated in myofibroblast formation and maintenance. Reduced turnover of hyaluronan has been linked to differentiation of myofibroblasts, and there is a reciprocal relationship between the amount of hyaluronan in the matrix and the ability of myofibroblasts to deposit fibrillar matrix components.²⁸

We found large variations in the pattern of α -SMA expression among the malignant lesions ranging from focal to spindle to network patterns of stromal myofibroblast positivity. In general, the network pattern of α -SMA was seen more often in more dedifferentiated carcinomas. It is likely that neoplastic lesions show more severe invasive behavior and a poorer prognosis because of the higher number of network-arranged myofibroblasts.^{13,25} In a similar study, IHC was used to detect α -SMA–positive myofibroblasts in gastric cancer stromata and in non-neoplastic mucosa. Because more myofibroblasts were seen in gastric cancer than in the non-neoplastic mucosa, it was concluded that an increased number of collagen-producing myofibroblasts may be a crucial cause of increased collagen deposition in gastric cancer.²⁹

In our study, the collagen fibers predominantly appeared as bundles of thick fibers in WDSCC. Previous studies have shown that a collagen-rich microenvironment can promote invasion and metastasis.³⁰ Similar to previous findings, the thickness of the collagen fibers decreased with progressing OSCC.^{6,31} As cancer progresses, changes in the thickness of collagen fibers occurs with a decrease in the existing type I collagen and a simultaneous increase in type III collagen due to enzymatic degradation brought about by myofibroblasts.^{7,32} Matrix metalloproteinases (MMPs) and lysosomal enzymes, particularly acidic cathepsin, are important proteolytic enzymes responsible for connective tissue dissolution.^{6,33} Hyaluronan synthase 2 is one of the key regulators responsible for myofibroblast-mediated OSCC progression and acts by modulating the balance of MMP1 and tissue inhibitor of metalloproteinases (TIMP1).³⁴

Several changes occurred in the arrangement of the collagen fibers with the progression of OSCC. First, the orientation of collagen fibers changed from parallel to haphazard as seen in other studies.^{7,18,35} Several studies have reported that collagen fibers are realigned with respect to the tumor border to promote cell inva-

sion by enabling cells to migrate along the collagen fibres.^{36–40} The packing of the fibers also changed from dense to loose as the carcinoma progressed, which was similar to a previous study.⁷ The dense arrangement of collagen fibers is due to increased synthesis and increased cross-linking of fibrillar collagen by myofibroblasts.²⁹ With the progression of cancer, there is increased degradation of the stroma making it loosely packed.^{6,33} Finally, the mean area fraction occupied by collagen fibers decreased gradually with progression from WDSCC to PDSCC as in previous studies.^{41,42} This could be attributed to MMP-1, which causes degradation of type I collagen leading to a decrease in the mean area fraction.⁴²

Differences in interference colors and birefringence intensity can be due to distinct patterns of physical aggregation, the degree of polymerization, and the three-dimensional organization of collagen fibres.^{15,43} The strong birefringence and OR hue of the control and WDSCC samples appear to be related to the higher amount of thick type I collagen fibers. The weak birefringence and greenish-yellow hue of the PDSCC samples could be either be due to an increased number of thin fibers (i.e., type III collagen fibers that were identified as reticulin fibers) or the result of abnormal or pathological collagen formed by the tumor cells or stroma. Regardless of the cause, our results were in accordance with those of other studies.^{3,6,7,16,17,31}

It was recently reported that pharmacological inhibition of NOX4 slows tumor growth in vivo by targeting myofibroblasts.⁴⁴ Lysyl oxidase-like 2 (LOXL2) antibodies disrupt the orientation and width of collagen fibers, ultimately resulting in decreased tumor growth.⁴⁵ Our data revealed that collagen fibers were quantitatively and qualitatively reorganized in the stroma of OSCC. We believe that the increase in activated fibroblasts (i.e., myofibroblasts) was a crucial contributor to collagen reorganization. Initially, there is an attempt to restrict the movement of tumor cells, but as the cancer progresses, there is an increase in pathological collagen, which enhances movement of the cells within the stroma. Therefore, targeting myofibroblasts and collagen cross-linking enzymes may be a promising treatment for oral cancer.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Significance of Intratumoral Fibrosis in Clear Cell Renal Cell Carcinoma

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Background: Intratumoral fibrosis (ITF) is a frequent histologic finding in solid organ tumors. Renal cell carcinoma (RCC) is a highly vascularized tumor with different shapes and degrees of ITF and inflammation. ITF is a poor prognostic factor, especially in breast cancer, and is related to intratumoral necrosis (ITN) and intratumoral inflammation (ITI). However, the significance of ITF in RCC has not been fully studied. In this study, we evaluate the relationships between ITF and other clinicopathologic parameters associated with RCC prognosis. **Methods:** ITF was evaluated in 204 clear cell renal cell carcinoma (CCRCC) specimens according to presence and grade of fibrosis, degree of ITI, and presence of ITN. Lysyl oxidase (LOX) expression in tumor cells was also evaluated with clinicopathologic parameters. **Results:** Among 204 CCRCC cases, 167 (81.7%) showed ITF, 71 (34.8%) showed ITI, 35 (17.2%) showed ITN, and 111 (54.4%) showed LOX expression. ITF correlated with Fuhrman nuclear grade ($p=.046$), lymphovascular invasion (LVI) ($p=.027$), and ITN ($p=.036$). Patients with ITF had a poor five-year overall survival rate ($p=.104$). **Conclusions:** ITF is related to other poor prognostic factors in CCRCC, such as Fuhrman nuclear grade, ITN, and LVI, but ITF itself had no significant correlation with prognosis of CCRCC.

Key Words: Carcinoma, renal cell; Fibrosis; Inflammation; Protein-lysine 6-oxidase

Renal cell carcinoma (RCC) is a common tumor and is one of the main causes of death by cancer in Western countries.¹ In Korea, the number of patients diagnosed with RCC has also been increasing recently.² Clear cell renal cell carcinoma (CCRCC) is the most common variant of RCC and represents an aggressive histologic subtype.³ CCRCC is a good example of a heterogeneous tumor with different clones in different areas of the same tumor at the genetic level.^{4,5} This may be why it has been difficult to develop targeted or personalized therapies for RCC.⁶ RCC is also resistant to chemotherapy and radiotherapy. The recently increased survival rate of RCC is probably due to early diagnosis and improved surgical techniques.⁷

Metastases are involved in more than 90% of patients who die from cancer. Many studies have shown that microenvironment and extracellular matrix (ECM) remodeling have crucial roles in accelerating tumor proliferation and metastasis.⁸⁻¹¹ Lysyl oxidase (LOX) is a copper enzyme with important functions in remodeling of the ECM.¹² LOX oxidizes the lysine residues of structures that compose the ECM, thereby catalyzing the crosslinks of collagen and elastin fibers to form a mature structure.^{13,14}

Intratumoral fibrosis (ITF) and intratumoral inflammation (ITI) are frequent histologic findings in solid organ tumors. Many

studies have shown that ITF is a poor prognostic factor, especially in breast cancer, and is related to intratumoral necrosis (ITN) and inflammation.^{15,16} RCC is also a highly vascularized tumor with different shapes and degrees of ITF and ITI. As far as we know, there are nearly no studies of the significance of ITF in RCC.

The goal of our study is to determine the relationships between ITF, ITI, LOX expression, and other prognostic factors of RCC. If ITF, ITI, or LOX is related to poor prognosis of RCC, these findings could be used to identify potential targets for personalized therapies.

MATERIALS AND METHODS

Patients and materials

This study was performed on 224 RCC samples diagnosed from 2003 to 2015 at Daegu Catholic University Medical Center. The specimens included 163 total nephrectomies and 61 partial nephrectomies. Follow-up data were started at the time of diagnosis until August 31, 2016. The average follow-up period was 66.3 months, excluding patients lost to follow-up. Two pathologists (H.K. Oh and J.W. Joung) reviewed all slides to evaluate the diagnosis, histologic type, Fuhrman nuclear grade, tumor

diameter, lymphovascular invasion (LVI), ITF, ITI, and ITN. This study was approved by the Institutional Review Board of Daegu Catholic University Medical Center (CR-18-009-RES-001-R) and performed in accordance with the Helsinki declaration. Because this is a retrospective study, informed consent was waived. All patient information was anonymized prior to analysis.

Tissue microarray and immunohistochemistry

Tissue microarrays that contained representative tumor cores (2 mm in diameter) were used to evaluate LOX expression. Immu-

nohistochemistry was performed using polyclonal rabbit LOX antibody (dilution 1:500, Novus Biologicals, Littleton, CO, USA) in the Leica Bond-Max automated immunostainer (Leica Biosystems, Wetzlar, Germany).

Grading of intratumoral fibrosis, intratumoral inflammation, and LOX expression

ITF is composed of fibroblasts admixed with various volumes of collagen surrounded by tumor cells and located within the tumor boundary. Like many studies in breast cancer, ITF is defined when

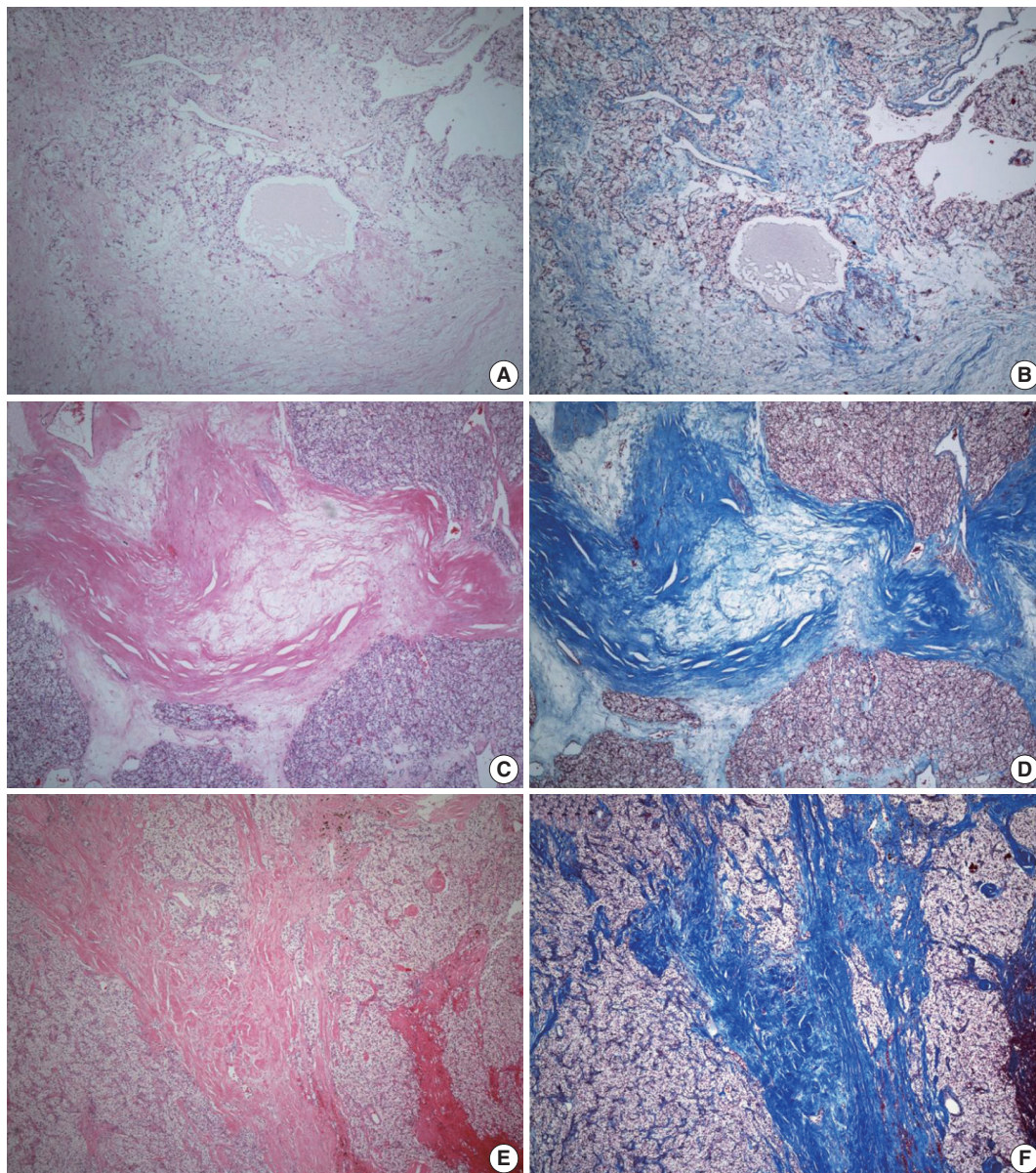


Fig. 1. Intratumoral fibrosis (ITF). Hematoxylin and eosin staining is compared with trichrome staining. (A, B) Grade 1 ITF mainly composed of loose connective tissue. (C, D) Grade 2 ITF composed of collagen fibers and loose connective tissue. (E, F) Grade 3 ITF mainly composed of sclerotic collagen fibers.

the size is more than 0.1 cm.^{15,16} In this study, ITF was classified into grades 0 to 3. Grade 0 ITF was defined as no fibrosis within tumor. Grade 1 ITF was mainly composed of loose connective tissue, grade 2 ITF was composed of collagen fibers and loose connective tissue, and grade 3 ITF was mainly composed of thick sclerotic collagen fibers (Fig. 1).

To investigate the relationship between ITF and ITI, ITI was graded from 0 to 3 according to the amount of inflammatory cells within the tumor boundary. Grade 0 ITI had no inflam-

matory cell infiltration, while grade 1 ITI had a few scattered inflammatory cells. Grade 2 ITI was characterized by focal aggregation of inflammatory cells, and grade 3 ITI was defined as diffuse or nodular aggregation of inflammatory cells (Fig. 2). ITF grade 0 was classified as negative, and grades 1, 2, and 3 were classified as positive. ITI of 0 or 1 was classified as negative, and ITI grade 2 or 3 was classified as positive.

LOX expression level was graded as 0 to 3 according to the degree of intensity and proportion of stained tumor cells: LOX

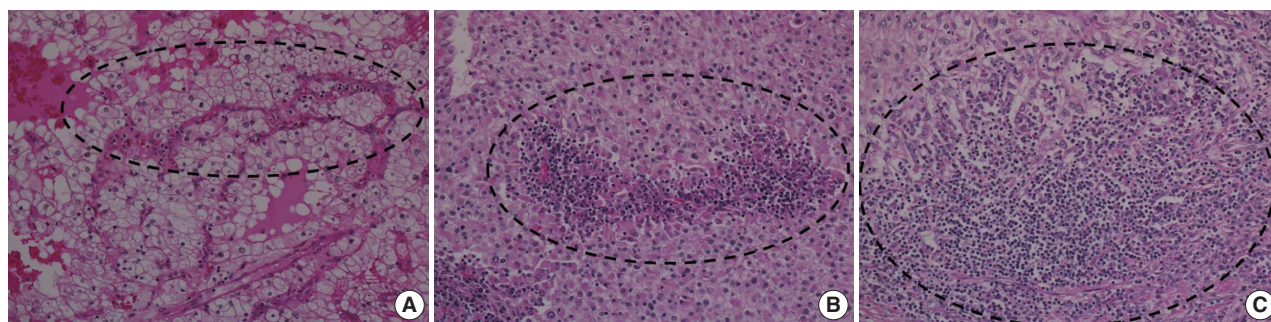


Fig. 2. Intratumoral inflammation (ITI). (A) Grade 1 ITI with scattered inflammatory cells. (B) Grade 2 ITI with focal aggregation of inflammatory cells. (C) Grade 3 ITI with diffuse or nodular aggregation of inflammatory cells.

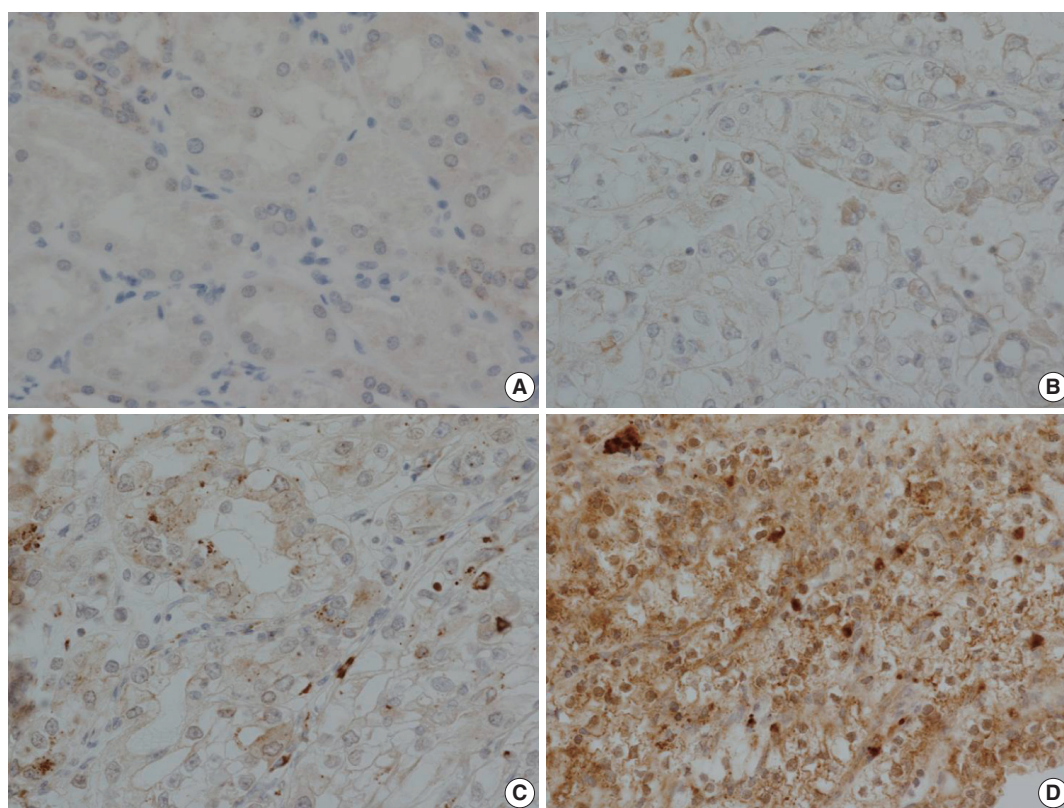


Fig. 3. Immunohistochemical staining of lysyl oxidase (LOX). (A) Control staining of normal kidney. Tubular cells show no reactivity. (B) LOX expression 1, with focal and weak staining in tumor cell cytoplasm. (C) LOX expression 2, with moderate cytoplasmic staining in nearly 50% of tumor cells. (D) LOX expression 3, with strong and diffuse staining of tumor cell cytoplasm.

expression is scored as 0 (no staining), 1 (minimal intensity, < 10% of cells), 2 (moderate intensity, 10%–49% of cells), or 3 (strong intensity, ≥ 50% of cells). LOX expression was then classified as negative (0 and 1) or positive (2 and 3). Every tissue microarray contained normal kidney as a control group. In normal kidneys, tubular cells show no reactivity to LOX (Fig. 3).

Statistical analysis

All statistical analyses were performed using SPSS software program ver. 19.0 for Windows (SPSS Inc., Chicago, IL, USA). The relationships between ITF, ITI, LOX expression, and clinicopathologic parameters were analyzed using the chi-square test. The 5-year overall survival rate according to presence of ITF was analyzed using the Kaplan-Meier method. The clinicopathologic parameters evaluated were patient age, sex, histologic type, Fuhrman nuclear grade, tumor size, pathologic staging, LVI, and ITN.

RESULTS

Clinicopathologic characteristics of patients

In 224 cases of RCC, the three most common tumor types were CCRCC (204 cases), papillary RCC (11 cases), and chromophobe RCC (5 cases). Other types include mucinous RCC (one case), mucinous tubular and spindle-cell carcinoma (one case), oncocytoma (one case), and unclassified RCC (one case). CCRCC cases represented 91.1% of the total RCC cases (204/224).

Of the 204 patients with CCRCC, 139 (68.1%) were male and 65 (31.9%) were female. The average age (mean ± standard deviation [SD]) at diagnosis was 59.7 ± 11.8 years and ranged between 27 and 87 years. Follow-up data were obtained from 191 patients (93.6%) with average follow-up period of 50.4 months. Based on the follow-up data available on patients, 11 patients (5.8%) died of disease, and 25 patients (13.1%) had metastasis to other organs.

Average tumor diameter (mean ± SD) was 4.48 ± 3.01 cm (range 0.5 to 19 cm). Fuhrman nuclear grade distribution was 12 G1 (5.9%), 118 G2 (57.8%), 55 G3 (27.0%), and 19 G4 (9.3%). A total of 130 cases (63.7%) were low grade (grade 1 and 2), and 74 cases (36.3%) were high grade (grade 3 and 4). There were 154 cases of pT1 (75.5%), 19 cases of pT2 (9.3%), 30 cases of pT3 (14.7%), and one case of pT4 (0.5%). A total of 173 cases (84.8%) were low stage (stages 1 and 2), and 31 cases (15.2%) were high stage (stages 3 and 4). When performing statistical analysis, Fuhrman nuclear grade and pathologic T stage

were divided into low (nuclear grades 1 and 2 or T stages 1 and 2) and high (nuclear grades 3 and 4 or T stages 3 and 4) groups. The clinicopathologic characteristics are listed in Table 1.

Associations between ITF and clinicopathologic characteristics

ITF was found in 167 cases (81.7%), indicating that ITF is a relatively common finding in CCRCC. Clinicopathologic parameters that are strongly associated with ITF are Fuhrman nuclear

Table 1. Clinicopathologic characteristics of patients

Clinicopathological parameter	No. (%)
Age (yr)	
Mean ± SD	59.7 ± 11.8
Range	27–87
Tumor size (cm)	
Average ± SD	4.48 ± 3.01
Range	0.5–19
Sex	
Male	139 (68.1)
Female	65 (31.9)
Fuhrman nuclear grade	
1	12 (5.9)
2	118 (57.8)
3	55 (27.0)
4	19 (9.3)
Pathologic stage	
1	154 (75.5)
2	19 (9.3)
3	30 (14.7)
4	1 (0.5)
Lymphovascular invasion	
Absent	175 (85.8)
Present	29 (14.2)
Tumor necrosis	
Absent	169 (82.8)
Present	35 (17.2)
Intratumoral inflammation	
0	25 (12.3)
1	108 (52.9)
2	59 (28.9)
3	12 (5.9)
Intratumoral fibrosis	
0	37 (18.1)
1	59 (28.9)
2	77 (37.7)
3	31 (15.2)
LOX	
0	11 (5.4)
1	82 (40.2)
2	82 (40.2)
3	29 (14.2)

SD, standard deviation; LOX, lysyl oxidase.

grade ($p = .003$), LVI ($p = .027$), and ITN ($p = .036$) according to presence (grades 1, 2 and 3) and absence (grade 0) of ITF. LOX expression status was not statistically associated with ITF (Table 2).

Associations between ITI and clinicopathologic characteristics

ITI was found in 71 (34.8%) cases. Clinicopathologic parameters that are strongly associated with ITI are tumor size ($p < .001$), Fuhrman nuclear grade ($p < .001$), pathologic stage ($p = .004$), LVI ($p < .001$), and ITN ($p < .001$). The LOX expression status was not statistically associated with ITI (Table 3).

Associations between LOX expression and clinicopathologic characteristics

LOX expression was found in 111 cases (54.4%). No clinicopathologic parameters evaluated were statistically associated with LOX expression (Table 4).

Table 2. Associations of intratumoral fibrosis with clinicopathologic characteristics

Clinicopathological parameter	Intratumoral fibrosis		p-value
	Absent	Present	
Age (yr)			
<60	21 (20.8)	80 (79.2)	.330
≥60	16 (15.5)	87 (84.5)	
Sex			
Male	26 (18.7)	113 (81.3)	.758
Female	11 (16.9)	54 (83.1)	
Tumor size (cm)			
≤7	34 (20.2)	134 (79.8)	.093
>7	3 (8.3)	33 (91.7)	
Fuhrman nuclear grade			
Low (grade 1 and 2)	31 (23.8)	99 (76.2)	.003**
High (grade 3 and 4)	6 (8.1)	68 (91.9)	
Pathologic stage			
Low (stage 1 and 2)	33 (19.1)	140 (80.9)	.295
High (stage 3 and 4)	4 (12.9)	27 (87.1)	
Lymphovascular invasion			
Absent	36 (20.6)	139 (79.4)	.027*
Present	1 (3.4)	28 (96.6)	
Tumor necrosis			
Absent	35 (20.7)	134 (79.3)	.036*
Present	2 (5.7)	33 (94.3)	
Intratumoral inflammation			
Absent	25 (18.8)	108 (81.2)	.738
Present	12 (16.9)	59 (83.1)	
LOX expression			
Absent	16 (17.2)	77 (82.8)	.752
Present	21 (18.9)	90 (81.1)	

Values are presented as number (%).

LOX, lysyl oxidase.

* $p < .05$, ** $p < .01$.

Associations between ITF and clinicopathologic characteristics

ITF size was on average 0.56 cm with a SD of 0.42 cm, ranging between 0.1 and 2.0 cm. The median value was 0.5 cm. ITF size was divided into two groups: small was defined as 0.5 cm or less, large was defined as over 0.5 cm. Large ITF size is associated with a higher Fuhrman nuclear grade ($p = .007$) (Table 5).

Association between ITF and patient prognosis

The 5-year overall survival rate of patients excluding loss to follow-up was 94.2% (180/191). Patients without ITF had 100% (36/36) 5-year overall survival rate, and patients with ITF had 92.9% (144/155) 5-year overall survival rate. The p -value was .104 (Fig. 4). Statistically, ITF and 5-year overall survival rate showed no significant correlation.

Table 3. Associations of intratumoral inflammation with clinicopathologic characteristics

Clinicopathological parameters	Intratumoral inflammation		p-value
	Absent	Present	
Age (yr)			
<60	67 (66.3)	34 (33.7)	.735
≥60	66 (64.1)	37 (35.9)	
Sex			
Male	85 (61.2)	54 (38.8)	.076
Female	48 (73.8)	17 (26.2)	
Tumor size (cm)			
≤7	120 (71.4)	48 (28.6)	<.001***
>7	13 (36.1)	23 (63.9)	
Fuhrman nuclear grade			
Low (grade 1 and 2)	102 (78.5)	28 (21.5)	<.001***
High (grade 3 and 4)	31 (41.9)	43 (58.1)	
Pathologic stage			
Low (stage 1 and 2)	120 (69.4)	53 (30.6)	.004**
High (stage 3 and 4)	13 (41.9)	18 (58.1)	
Lymphovascular invasion			
Absent	124 (70.9)	51 (29.1)	<.001***
Present	9 (31.0)	20 (69.0)	
Tumor necrosis			
Absent	124 (73.4)	45 (26.6)	<.001***
Present	9 (25.7)	26 (74.3)	
Intratumoral fibrosis			
Absent	25 (67.6)	12 (32.4)	.738
Present	108 (64.7)	59 (35.3)	
LOX expression			
Absent	60 (64.5)	33 (35.5)	.852
Present	73 (65.8)	38 (34.8)	

Values are presented as number (%).

LOX, lysyl oxidase.

** $p < .01$, *** $p < .001$.

DISCUSSION

There are many similar mechanisms between tumor development, tumor metastases, and formation of fibrosis. One of these mechanisms is ECM remodeling,^{17,18} which occurs predominantly while forming fibrosis and results in many changes in the ECM. These changes also activate other signaling pathways.^{10,11,19}

Inflammatory cell infiltration in tumors and the tumor micro-environment is a significant factor in tumor progression. Recent studies have shown that tumor-associated immune cells play a role in RCC initiation and development.^{20,21} Some inflammatory cells in RCC, such as T lymphocytes and macrophages, may affect tumor progression and invasion.²² Yang *et al.*²³ reported that infiltrating macrophages could accelerate cancer invasion by increasing the epithelial to mesenchymal transition. This ability of macrophages is thought to be associated with activation of the AKT/mammalian target of rapamycin signal. Further research on this pathway could be helpful in developing a new target

Table 4. Associations of LOX expression with clinicopathologic characteristics

Clinicopathological parameter	LOX expression		p-value
	Absent	Present	
Age (yr)			
<60	43 (42.6)	58 (57.4)	.392
≥60	50 (48.5)	53 (51.5)	
Sex			
Male	61 (43.9)	78 (56.1)	.475
Female	32 (49.2)	33 (50.8)	
Tumor size (cm)			
≤7	77 (45.8)	91 (54.2)	.879
>7	16 (44.4)	20 (55.6)	
Fuhrman nuclear grade			
Low (grade 1 and 2)	56 (43.1)	74 (56.9)	.209
High (grade 3 and 4)	37 (50.0)	37 (50.0)	
Pathologic stage			
Low (stage 1 and 2)	77 (44.5)	96 (55.5)	.295
High (stage 3 and 4)	16 (51.6)	15 (48.4)	
Lymphovascular invasion			
Absent	76 (43.4)	99 (56.6)	.128
Present	17 (58.6)	12 (41.4)	
Tumor necrosis			
Absent	80 (47.3)	89 (52.7)	.270
Present	13 (37.1)	22 (62.9)	
Intratumoral inflammation			
Absent	7 (5.3)	126 (94.7)	.911
Present	4 (5.6)	67 (94.4)	
Intratumoral fibrosis			
Absent	16 (43.2)	21 (56.8)	.752
Present	77 (46.1)	90 (53.9)	

Values are presented as number (%).
LOX, lysyl oxidase.

therapy for RCC.

There is increasing evidence that ITF is associated with poor prognosis in breast cancers.^{15,16} ITF is related to many factors,

Table 5. Associations of intratumoral fibrosis size with clinicopathologic characteristics

Clinicopathological parameter	Intratumoral fibrosis size (cm)		p-value
	≤0.5	>0.5	
Age (yr)			
<60	57 (71.3)	23 (28.7)	.523
≥60	58 (66.7)	29 (33.3)	
Sex			
Male	82 (72.6)	31 (27.4)	.135
Female	33 (61.1)	21 (38.9)	
Tumor size (cm)			
≤7	96 (71.6)	38 (28.4)	.118
>7	19 (57.6)	14 (42.4)	
Fuhrman nuclear grade			
Low (grade 1 and 2)	76 (76.8)	23 (23.2)	.007**
High (grade 3 and 4)	39 (57.4)	29 (42.6)	
Pathologic stage			
Low (stage 1 and 2)	97 (69.3)	43 (30.7)	.475
High (stage 3 and 4)	18 (66.7)	9 (33.3)	
Lymphovascular invasion			
Absent	100 (71.9)	39 (28.1)	.055
Present	15 (53.6)	13 (46.4)	
Tumor necrosis			
Absent	95 (70.9)	39 (29.1)	.253
Present	20 (60.6)	13 (39.4)	
Intratumoral inflammation			
Absent	78 (72.2)	30 (27.8)	.205
Present	37 (62.7)	22 (37.3)	
LOX expression			
Absent	50 (64.9)	27 (35.1)	.311
Present	65 (72.2)	25 (27.8)	

Values are presented as number (%).

LOX, lysyl oxidase.

**p<.01.

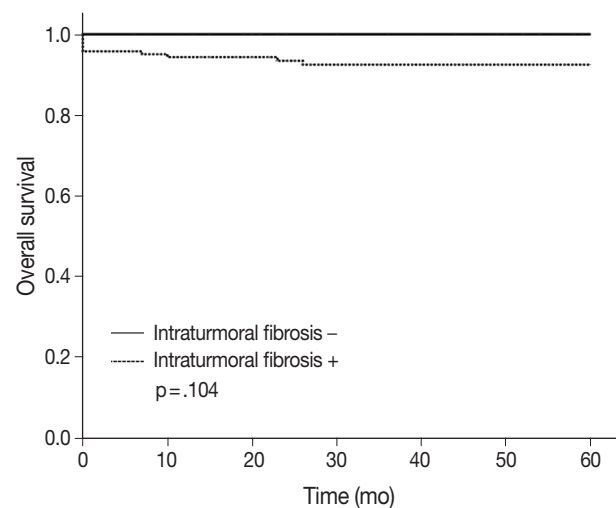


Fig. 4. 5-Year overall survival rate according to intratumoral fibrosis.

such as higher histologic grade, pathologic T stage, lymph node metastasis, and increased risk of recurrence in breast cancer. In many studies, ITF as a prognostic factor independently correlates with other prognostic factors.²⁴ In 1987, Delahunt and Nacey²⁵ studied prognostic factors of RCC with 102 cases. They accessed many clinicopathological parameters to find any relationship with prognosis. They categorized fibrosis into three groups: slight or absent, moderate, and severe. Their study showed that fibrosis was not correlated with prognosis of RCC.

In our study, ITF is related to higher histologic grade, LVI, ITN, and ITI in CCRCC. Frank *et al.*²⁶ reported that ITN is a prognostic factor according to the SSIGN (tumor stage, size, grade, and necrosis) scoring system. Our result shows that ITF and ITI are strongly associated with ITN. We statistically analyzed two groups of ITF to identify differences between the presence and grades of ITF. In group 1, ITF 0 was classified as negative, and ITF 1, 2, and 3 were classified as positive. In group 2, ITF 0 and 1 were considered negative, and ITF 2 and 3 were considered positive. Statistically, no significant differences were identified between these two groups, probably because the presence of ITF itself is a meaningful finding rather than ITF grade. The large ITF size is related to a higher Fuhrman nuclear grade. The 5-year overall survival rate tends to be better for patients who do not have ITF, but statistical significance has not been identified ($p = .104$).

LOX modifies collagen and elastin fibers in the ECM.²⁷ Collagen I is the most common substrate used by LOX. The most important factor in forming fibrosis is increased synthesis of ECM protein by fibroblast activation, and collagen type I is the most commonly synthesized protein during this process. When collagen I is present at a high level in vital organs, it sometimes causes severe morbidity and mortality. Many studies have shown that collagen I plays a major role in cancer development and metastases.²⁸ In breast cancers, the formation of fibrosis by remodeling of the ECM was also found to be associated with gene expression patterns related to adverse prognosis and cancer metastasis.²⁹⁻³² Jensen *et al.*³³ reported that, in recurrent breast cancer, an increased level of the collagen I precursor, procollagen I, could be detected in patient serum.

In this study, we investigated the relationship between LOX expression and ITF formation. Our hypothesis was that LOX participates in formation of ITF in CCRCC. However, our results show no correlation between LOX expression and ITF formation or other clinicopathologic features. Hence, ITF in CCRCC may be formed by other cytokines during ECM remodeling. Further study of other fibrogenic cytokines, such as transforming growth

factor β and tumor necrosis factor α , is needed.

Our results show no relationship of ITF or ITI with LOX expression; there may be some reasons for this result. First, LOX is not involved in forming ITF of CCRCC. Second, there could be other classifications for grading LOX expression. We assumed that all normal tubular cells show no reactivity to LOX, although some normal tubular cells showed grade 1 expression. If we adjust LOX grading, different results may be obtained. We also found relatively low rates of death by CCRCC because the follow-up period was short and there was a high proportion of low-grade patients (Fuhrman nuclear grade 1 or 2).

Our results show that ITF is a pathologic finding related to other adverse prognostic factors, including higher Fuhrman nuclear grade, ITN, and LVI. However, ITF itself shows no direct correlation with poor prognosis of CCRCC. LOX expression shows no correlation with ITF or any other clinicopathologic parameters in this study. Further evaluation of other fibrogenic cytokines in addition to LOX should be considered.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Prognostic Role of Metastatic Lymph Node Ratio in Papillary Thyroid Carcinoma

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Background: The aim of this study is to elucidate the clinicopathological significances, including the prognostic role, of metastatic lymph node ratio (mLNR) and tumor deposit diameter in papillary thyroid carcinoma (PTC) through a retrospective review and meta-analysis. **Methods:** We categorized the cases into high (≥ 0.44) and low mLNR (< 0.44) and investigated the correlations with clinicopathological parameters in 64 PTCs with neck level VI lymph node (LN) metastasis. In addition, meta-analysis of seven eligible studies was used to investigate the correlation between mLNR and survival. **Results:** Among 64 PTCs with neck level VI LN metastasis, high mLNR was found in 34 PTCs (53.1%). High mLNR was significantly correlated with macrometastasis (tumor deposit diameter ≥ 0.2 cm), extracapsular spread, and number of metastatic LNs. Based on linear regression test, mLNR was significantly increased by the largest LN size but not the largest metastatic LN (mLN) size. High mLNR was not correlated with nuclear factor κ B or cyclin D1 immunohistochemical expression, Ki-67 labeling index, or other pathological parameters of primary tumor. Based on meta-analysis, high mLNR significantly correlated with worse disease-free survival at the 5-year and 10-year follow-up (hazard ratio [HR], 4.866; 95% confidence interval [CI], 3.527 to 6.714 and HR, 5.769; 95% CI, 2.951 to 11.275, respectively). **Conclusions:** Our data showed that high mLNR significantly correlated with worse survival, macrometastasis, and extracapsular spread of mLN. Further cumulative studies for more detailed criteria of mLNR are needed before application in daily practice.

Key Words: Papillary thyroid carcinoma; Lymph node metastasis; Metastatic lymph node ratio; Prognosis; Meta-analysis

Papillary thyroid carcinoma (PTC) is a malignant tumor with favorable prognosis.¹ Neck level VI lymph node (LN) metastasis is associated with primary tumor size, tumor multifocality, and extrathyroidal extension.² However, neck level VI LN metastasis is frequently found in up to 80% of patients.^{3,4} The prognosis of patients with neck level VI LN metastasis is different from those without nodal disease.⁵ The new eighth American Joint Committee on Cancer (AJCC) staging has been introduced and applied in daily practice.⁶ An important change in pN stage in the eighth AJCC staging systems is that stage group is not different based on the difference between pN1a and pN1b.^{6,7} In the seventh AJCC staging, cases with pN1b disease are defined as stage IVA, regardless of pT stage in patients of 45 years and older.⁷ However, in the eighth AJCC staging, pN1 disease is classified as stage II in pT1 and pT2 in 55 years and older patients.⁶ Furthermore, pN1 disease is considered as a single group without distinction between pN1a and pN1b in risk assessment. Because the risk assessment models of the eighth AJCC staging are simpler com-

pared with the previous version, a more detailed risk stratification may be necessary.

To predict the prognosis of patients with nodal disease, pathological parameters, such as number of examined LNs and metastatic LNs (mLNs), mLN size, metastatic foci size within mLN, extracapsular spread, and metastatic LN ratio (mLNR), have been introduced and their prognostic roles evaluated.^{2,6} The mLNR is defined as the ratio of number of mLNs to number of LNs examined. The prognostic role of the mLNR has been used to study various malignant tumors, including PTCs.⁸⁻¹⁸ Positive correlation between high mLNR and worse survival has been reported for various malignant tumors, such as stomach, colorectal, and pancreatic cancers.^{9,13,15,18-21} However, detailed criteria for the high mLNR cut-off value and the minimal number of examined LNs required have not been fully elucidated.

The aim of this study is to elucidate the clinicopathological significances of mLNR and the correlations with pathological characteristics of LN through a retrospective review of PTC with

neck level VI LN metastasis. In addition, meta-analysis was conducted to evaluate the correlation between high mLNR and survival.

MATERIALS AND METHODS

Patients and evaluation of pathological features

The present study included 122 patients with conventional PTC who received neck level VI LN dissection. All PTCs were surgically resected at Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine (Seoul, Republic of Korea) from January 1 to December 31, 2010. Among 122 patients with PTC, central LN metastasis (level VI) was found in 64. To elucidate the clinicopathological significance of the mLNR, clinicopathological parameters, such as age, sex, tumor size, *BRAF*^{V600E} mutation, tumor multifocality, and extrathyroidal extension, a review of medical charts and pathological records was performed. All dissected LNs were embedded for microscopic examination, and LN characteristics were evaluated. pTNM staging was performed according to the eighth AJCC TNM classification system.⁷ All procedures performed in the current study were approved by the Institutional Review Board (IRB; approval No. KBSMC 2016-04-070). Formal written informed consent was waived by the IRB.

Evaluation of mLN characteristics

All embedded LNs were evaluated by reviewing glass slides. Evaluated characteristics were number of dissected LNs and mLNs, largest LN size, largest mLN size, and mean LN size, mLNR, and tumor deposit size of mLN. mLNR was defined as number of mLNs to total number of dissected LNs. We categorized the cases into high (≥ 0.44) and low mLNR (< 0.44) and into macrometastasis (tumor deposit size ≥ 0.2 cm) and micrometastasis (< 0.2 cm). In the present study, based on median cut-off value (mLNR, 0.44), eligible studies were divided into high and low mLNR subgroups.

Tissue array methods

Two array blocks containing 64 PTCs with neck level VI LN metastasis were prepared for immunohistochemistry. A tissue core was obtained from each primary tumor. Core tissue biopsies (2 mm diameter) were collected from individual paraffin-embedded donor PTC blocks and delivered in recipient tissue array blocks using a trephine apparatus. Each block contained non-neoplastic thyroid tissue as an internal control.

Immunohistochemical staining

Sections 4 μ m in size were cut, deparaffinized, and hydrated using routine xylene-alcohol procedures before incubation with 0.01 M citrate buffer (pH 6.0) for 5 minutes in a microwave oven for antigen retrieval and treatment with 3% H₂O₂ to quench endogenous peroxidase. Sections were treated with normal serum from the animal used to generate secondary antibodies to block nonspecific binding and then incubated with anti-nuclear factor κ B (NF- κ B) RelA (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin D1 (1:100, Thermo Fisher Scientific, Fremont, CA, USA), or anti-Ki-67 (1:50, Santa Cruz Biotechnology). A compact polymer method from Bond Intense detection kits (Leica Biosystems, Newcastle upon Tyne, UK) was used for immunohistochemical staining and 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) for visualization. To confirm reaction specificity, a negative control without primary antibody was included. For counterstaining, immunostained sections were lightly stained with Mayer's hematoxylin.

Nuclear expression of NF- κ B RelA, regardless of cytoplasmic expression, was considered as NF- κ B activation. In the present study, NF- κ B positivity was defined as NF- κ B stain in $\geq 5\%$ of tumor cell nuclei.²²⁻²⁴ Regarding interpretation for cyclin D1, patients showing nuclear staining in $\geq 10\%$ of the tumor cells were considered positive.²⁵ For Ki-67 staining, we evaluated at least 300 cells and counted cells with nuclear staining for each specimen. Proliferation index (%) was $100 \times \text{Ki-67-positive cells} / 300 \text{ cells}$.

Meta-analysis

Relevant articles were obtained by searching PubMed and MEDLINE databases up to May 15, 2018. Searches were performed using the keywords 'papillary thyroid carcinoma' and 'metastatic lymph node ratio or lymph node ratio.' The title and abstract of all searched articles were screened for exclusion. Review articles were also screened to find additional eligible studies. Search results were then scanned based on the following inclusion and exclusion criteria: (1) human thyroid tissue was investigated, (2) available information for the correlation between mLNR and survival in PTC with neck level VI LN metastasis, (3) case reports or non-original articles were excluded, and (4) non-English language publications were excluded. Data from all eligible studies were extracted by two authors. The following data were extracted from each of the eligible studies^{8,10-12,14,16,26}: first author's name, year of publication, number of patients analyzed, and correlation between mLNR and survival based on previous studies.^{27,28}

Statistical analysis

SPSS ver. 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. The correlations between mLNR and clinicopathological parameters, including the immunohistochemical results of primary tumor, were determined using either chi-square test or the Fisher exact test (two-sided). The relationships between mLNR and tumor size, LN size, mLN size, and tumor deposit diameter of LN were analyzed using a two-tailed Student's t-test. Linear regression analysis was conducted to investigate correlations between primary tumor size and tumor multifocality, number of tumors, and supplemental tumor size.

In the meta-analysis, data were analyzed using the Comprehensive Meta-Analysis software package (Biostat, Englewood, NJ, USA). The correlation between high mLNR and survival rate in patients with PTC was investigated using meta-analysis.

Table 1. The correlation between mLNR and clinicopathological features in PTCs

	High mLNR (≥ 0.44)	Low mLNR (< 0.44)	p-value
Total (n = 64)	34 (53.1)	30 (46.9)	
Age (yr)	45.2 $\pm$ 12.7	46.3 $\pm$ 12.8	.718
Sex			> .999
Male	9 (26.5)	7 (23.3)	
Female	25 (73.5)	23 (76.7)	
Tumor size (cm)	1.41 $\pm$ 0.76	1.42 $\pm$ 0.64	.991
Tumor size			.668
Overt PTC (> 1 cm)	21 (61.8)	21 (70.0)	
Microcarcinoma (≤ 1 cm)	13 (38.2)	9 (30.0)	
BRAF mutation			.884
Present	32 (94.1)	27 (90.0)	
Absent	2 (5.9)	3 (10.0)	
Multifocality of tumor			.726
Present	15 (44.1)	11 (36.7)	
Absent	19 (55.9)	19 (63.3)	
No. of tumors			.855
1	19 (55.9)	19 (63.3)	
2	9 (26.5)	7 (23.3)	
3	3 (8.8)	3 (10.0)	
4	2 (5.9)	1 (3.3)	
5	0 (0.0)	0	
6	1 (2.9)	0	
Extrathyroidal extension			.753
Present	25 (73.5)	24 (80.0)	
Absent	9 (26.5)	6 (20.0)	
Tumor stage (8th)			.712
I	26 (76.5)	25 (83.3)	
II	8 (23.5)	5 (16.7)	
III	0	0	
IV	0 (6.7)	0 (6.7)	

Values are presented as number (%) or mean $\pm$ SD.
mLNR, metastatic lymph node ratio; PTC, papillary thyroid carcinoma; SD, standard deviation.

We performed subgroup analysis based on follow-up period and cut-off value for high mLNR. Heterogeneity between studies was analyzed using the Q and I^2 statistics and presented as p-values. In addition, sensitivity analysis was conducted to assess the heterogeneity of eligible studies and the impact of each study on the combined effect. Because various cut-off values for high mLNR were used for various populations, the application of random-effect model rather than fixed-effect model was more suitable. The assessment of publication bias was performed using Begg's funnel plot and Egger's test. The results were considered statistically significant at $p < .05$.

RESULTS

Correlations between mLNR and clinicopathological parameters

Among 122 patients with PTCs, neck level VI LN metastasis was found in 64. We first investigated the clinicopathological significance of high mLNR (≥ 0.44) in 64 PTC patients with neck level VI LN metastasis. No significant correlation was observed between high mLNR and clinicopathological parameters, such as age, sex, tumor size, *BRAF*^{V600E} mutation, tumor multifocality, and pTNM stage (Table 1). Next, the correlations between mLNR and pathological statuses of examined and mLNs were evaluated. The tumor deposit diameters of mLNs were 0.43 ± 0.31 cm and 0.29 ± 0.41 cm in high and low mLNR, respectively ($p =$

Table 2. The correlation between mLNR and pathological status of LN in papillary thyroid carcinomas

	High mLNR (≥ 0.44)	Low mLNR (< 0.44)	p-value
Total (n = 64)	34 (53.1)	30 (46.9)	
mLNR	0.72 $\pm$ 0.21	0.22 $\pm$ 0.11	< .001
Largest tumor deposit diameter (cm)	0.43 $\pm$ 0.32	0.29 $\pm$ 0.41	.114
Macrometastasis (> 0.2 cm)			.006
Present	28 (82.4)	14 (46.7)	
Absent	6 (17.6)	16 (53.3)	
Extracapsular spread			.007
Present	13 (38.2)	2 (6.7)	
Absent	21 (61.8)	28 (93.3)	
Examined lymph node			
Number	6.53 $\pm$ 5.39	11.80 $\pm$ 8.72	.006
Mean size (cm)	0.32 $\pm$ 0.14	0.26 $\pm$ 0.10	.041
Largest LN size (cm)	0.54 $\pm$ 0.29	0.55 $\pm$ 0.36	.947
Metastatic lymph node			
Number	4.38 $\pm$ 3.37	2.27 $\pm$ 2.07	.003
Mean size (cm)	0.38 $\pm$ 0.19	0.41 $\pm$ 0.21	.547
Largest LN size (cm)	0.53 $\pm$ 0.29	0.48 $\pm$ 0.38	.597

Values are presented as number (%) or mean $\pm$ SD.
mLNR, metastatic lymph node ratio; LN, lymph node; SD, standard deviation.

.114) (Table 2). The rate of macrometastasis (> 0.2 cm) in high mLNR was significantly higher than in low mLNR (82.9% vs 44.8%, $p = .006$). In addition, extracapsular spread of LN was more frequently found in high mLNR than in low mLNR (37.1% vs 6.9%, $p = .007$). However, high mLNR was not significantly correlated with mean size or largest size of examined and mLNs. The mLNR was significantly increased by the largest LN size, but not the largest mLN size ($p = .001$ vs $p = .364$) (Fig. 1). In the high mLNR group, the largest examined LN and the largest mLN were equal to 94.1%; however, in the low mLNR group, the rate was 56.7%.

We previously reported that nuclear NF- κ B RelA of the primary

tumor significantly correlated with LN metastasis.²⁹ In the present study, the correlations between mLNR and the immunohistochemical expressions of primary tumor, such as NF- κ B RelA, cyclin D1, and Ki-67 labeling index, were investigated. High mLNR was not significantly correlated with nuclear NF- κ B RelA ($p = .271$) or cyclin D1 expression ($p > .999$). In addition, no significant correlation between high mLNR and ki-67 labeling index was observed ($p = .917$).

Meta-analysis of the correlation between high mLNR and survival

The database search identified 288 reports. Of these, 254 re-

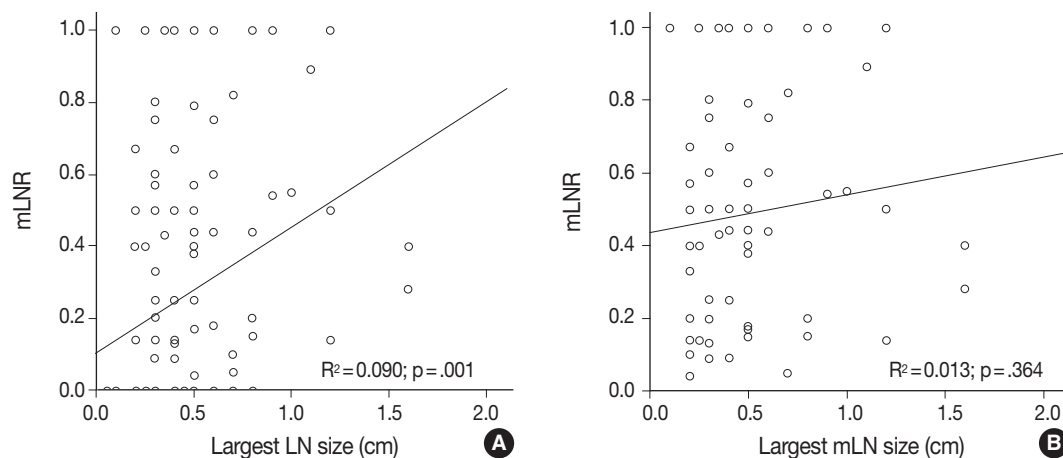


Fig. 1. Correlation between metastatic lymph node ratio (mLNR) and largest lymph node size (A) and largest metastatic lymph node (mLNR) size (B) based on linear regression.

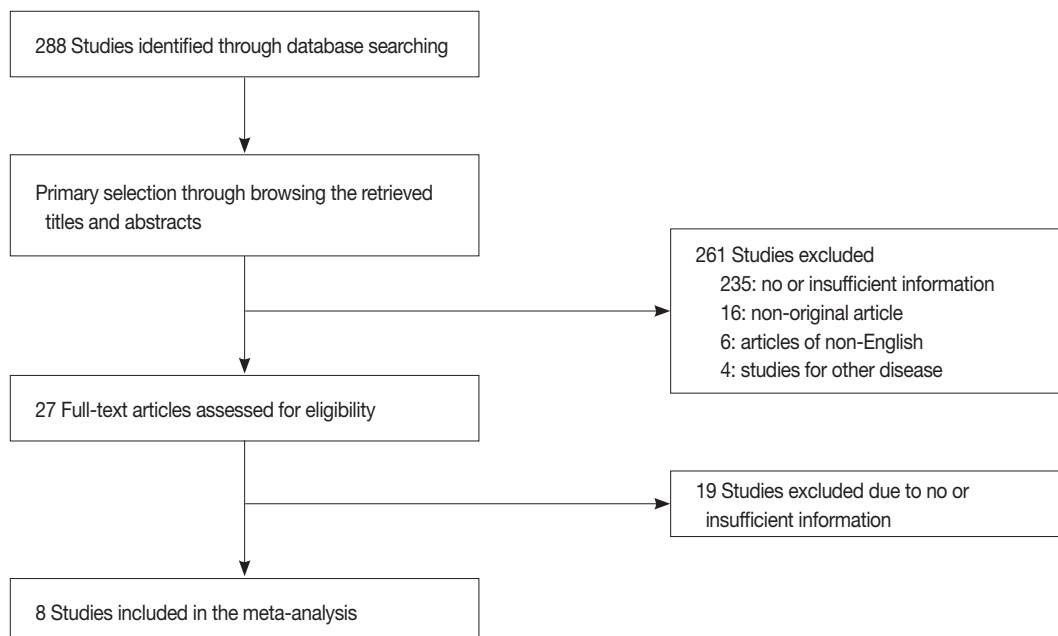


Fig. 2. Flow chart of study search and selection methods.

ports were excluded due to lack of sufficient information. Other studies were excluded because they reported the results of other diseases ($n = 4$), were non-English ($n = 6$), or were non-original articles ($n = 16$) (Fig. 2). After applying the inclusion and exclusion criteria, seven reports were finally included for meta-analysis.^{8,10-12,14,16,26} Eligible studies used various cut-off values for high mLNR ranging from 0.22 to 0.65. To elucidate the prognostic role of mLNR, the correlation between high mLNR and survival rate was investigated. High mLNR significantly correlated with worse disease-free survival at the 5-year and 10-year follow-up (hazard ratio [HR], 5.408; 95% confidence interval [CI], 3.189 to 9.170 and HR, 6.020; 95% CI, 3.483 to 10.404, respectively) (Fig. 3). To evaluate the optimal cut-off value for high mLNR, subgroup analysis based on a cut-off value of 0.44, was performed. High (≥ 0.44) and low mLNR cut-off (< 0.44) subgroups showed significant correlation between high mLNR and worse disease-free survival at the 5-year follow-up (HR, 5.088; 95% CI, 1.926 to 13.443 and HR, 5.570; 95% CI, 2.833 to 10.952; respectively) and 10-year follow-up (HR, 5.597; 95% CI, 1.806 to 17.350 and HR, 6.158; 95% CI, 2.863 to 13.245, respectively).

DISCUSSION

Involvement of neck level VI LN is common in PTC and occurs in up to 80% of patients.²⁹ The clinicopathological significance of neck level VI LN metastasis is of less concern because patients with PTC have favorable prognosis. The nodal disease is subdivided into pN1a and pN1b based on the location of involved LNs in the eighth AJCC staging.⁶ However, usefulness of pN1a disease stratification for predicting prognosis has not been fully elucidated. In the present study, the correlations between mLNR and pathological features of mLN and the prognostic role of mLNR in patients with PTC were evaluated using a retrospective study and meta-analysis.

In AJCC staging, pN stage is subdivided into pN1a and pN1b based on the location of the involved nodes in PTC. If involvement of neck level VI LN is identified without lateral neck LN involvement, pN stage is defined as pN1a. In a previous study, PTC with pN1a showed worse prognosis compared with pN0, regardless of pT stage.⁵ However, because higher rate of neck level VI LN metastasis is well known, stratification of pN1a may be needed for predicting the detailed prognosis of patients with

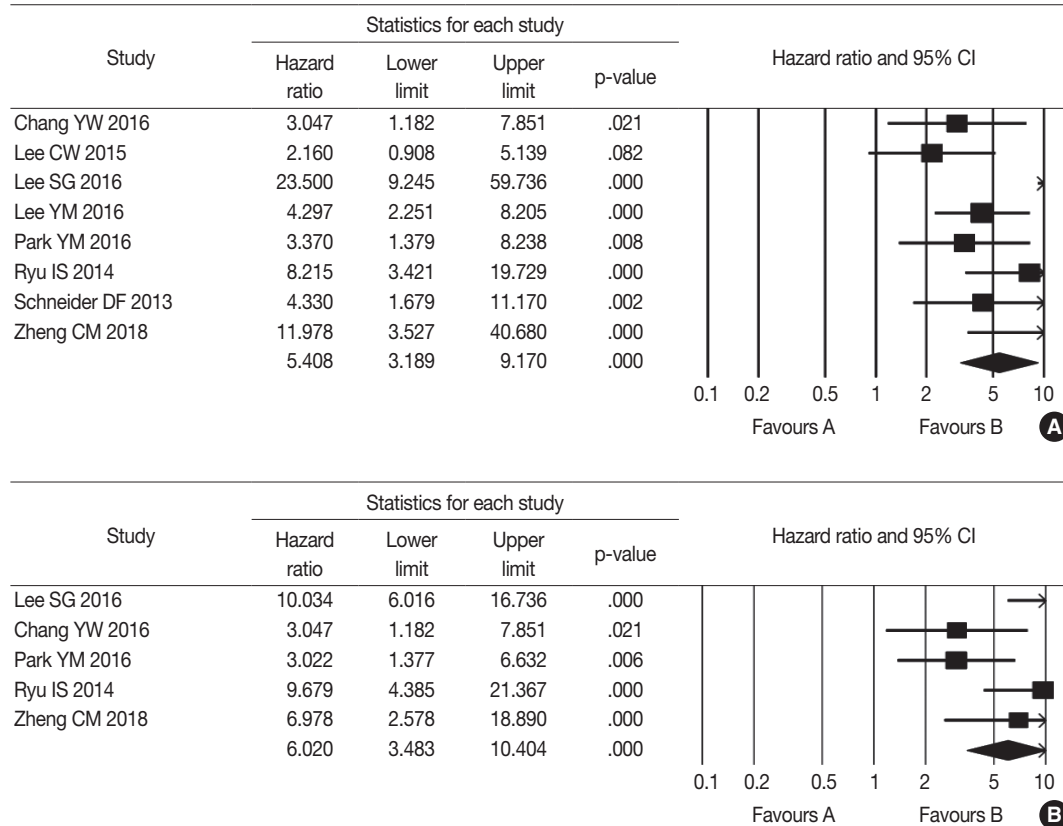


Fig. 3. Forest plot diagram for the correlation between high metastatic lymph node ratio and disease-free survival at the 5-year (A) and 10-year (B) follow-up.^{8,10-12,14,16,17,26} CI, confidence interval.

nodal disease. Various parameters, such as numbers of examined and mLN, mLN size, metastatic foci size within mLN, extracapsular spread, and mLNR, have been introduced.^{2,6} However, the prognostic role of various parameters has not been fully understood. In addition, the detailed criteria for evaluation of various parameters are neither elucidated nor introduced in the eighth AJCC staging.⁶

The introduction of mLNR is included in the eighth AJCC staging for various malignant tumors, but application of pN stage has not been implemented.⁶ Although the number of reports on the prognostic role of mLNR in various malignant tumors is increasing,^{9,13,15,18-21} conclusive information regarding the mLNR in PTCs is not available. The definition of mLNR is the ratio of number of mLN to number of examined LNs. Therefore, the number of examined LNs can affect the mLNR. In colon cancer, positive LNs were increased based on harvested LNs.³⁰ In addition, to evaluate the prognostic role of mLNR, the minimal number of examined LNs is required. However, the pertinent cut-off values for examined LNs have not been fully evaluated in PTC. In the eighth AJCC staging, the criteria for examined or mLN numbers has not been determined when evaluating pN stage.

In a previous study, the pathological characteristics of primary tumor showed an effect on LN metastasis.²⁹ However, in the present study, high mLNR was not significantly correlated with characteristics of the primary tumor. To date, the correlations between mLNR and pathological features of examined and mLN have not been fully understood. The present study showed that high mLNR significantly correlated with macrometastasis (> 0.2 cm), extracapsular spread, and largest LN size. However, significant correlation between high mLNR and largest or mean mLN size was not observed. On preoperative ultrasonography, several features such as node size, hyperechogenicity, echogenicity of hilum, calcification, and intranodal cystic necrosis, indicated suspicion of metastasis.²⁹ These are not absolute criteria because LNs detected on preoperative ultrasonography might not match postoperative pathological examinations.³¹ Therefore, the prediction of mLNR is preoperatively difficult before pathologic examination.

In a previous study using the Surveillance, Epidemiology, and End Results (SEER) Registry Data, high mLNR was not significantly correlated with worse overall survival (HR, 0.95; 95% CI, 0.58 to 1.57).³² However, because this report may have included PTCs with lateral neck LN metastasis, the prognostic role of mLNR in PTC with pN1a disease cannot be evaluated. To date, the prognostic role of high mLNR in PTC with pN1a disease has not been fully investigated due to higher incidence and relatively better prognosis of pN1a disease in patients with PTC.

In addition, usefulness of prognostic stratification in pN1a disease is unclear. In the current meta-analysis, high mLNR significantly correlated with worse disease-free survival. Based on our results, the prognostic stratification of pN1a is possible. Compared to other pathological parameters, mLNR is advantageous due to its simplicity and objectivity and lack of interobserver difference. In a previous study, the prognosis was different between patients with pN1a and pN0 disease.⁵ However, prognosis may be controversial based on the follow-up period. Our results showed that, regardless of follow-up period, mLNR was a predictor of worse prognosis in PTC with pN1a disease.

Eligible studies used various high mLNR cut-off values ranging from 0.22 to 0.65. In the current meta-analysis, subgroup analysis showed that high (≥ 0.44) and low mLNR cut-off (< 0.44) subgroups showed a correlation between high mLNR and worse disease-free survival at the 5-year and 10-year follow-up. Among eligible studies in the low mLNR cut-off subgroup (< 0.44), Lee *et al.*¹⁰ showed no significant correlation between high mLNR and worse disease-free survival at 5-year follow-up, regardless of higher HR (> 1.0). In the current meta-analysis, the optimal cut-off value could not be determined due to similar results obtained in the high and low mLNR cut-off subgroups. Additional studies are necessary to define the optimal cut-off value; however, the prognostic role of mLNR in PTC with pN1a disease was demonstrated, regardless of mLNR cut-off value.

In conclusion, our results showed that high mLNR significantly correlated with macrometastasis and extracapsular spread of mLN. In addition, a significant correlation between high mLNR and worse disease-free survival was found in meta-analysis. Therefore, in situations with simplified pN stage, mLNR may be important for prediction and stratification of the prognosis of patients with nodal disease. Further cumulative studies for the optimal high mLNR cut-off value and the minimal number of examined LNs required are needed before application in daily practice.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Bile Granuloma Mimicking Peritoneal Seeding: A Case Report

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Laparoscopic cholecystectomy is a widely used treatment method for most cholelithiasis and is a relatively safe procedure. Foreign body granulomatous reaction to bile or gallstone spillage during laparoscopic cholecystectomy has rarely been reported. We report a case of bile granuloma after laparoscopic cholecystectomy, which mimicked peritoneal seeding. A 59-year-old Korean man presented with right upper quadrant pain. He underwent laparoscopic cholecystectomy for acute cholecystitis with cholelithiasis. Pathologic examination revealed an incidental adenocarcinoma invading the lamina propria with acute cholecystitis and cholelithiasis. After 3 months, follow-up abdominal computed tomography revealed a subhepatic nodule, which showed hypermetabolism on positron emission tomography-computed tomography. Suspecting localized peritoneal seeding, wedge resection of the liver, wedge resection of the transverse colon, and omentectomy were performed. Pathologic examination of the resected specimens revealed multiple bile granulomas. Awareness of bile granuloma mimicking malignancy is noteworthy for patient management to reduce unnecessary procedure during postoperative surveillance.

Key Words: Bile; Granuloma; Cholecystectomy

Laparoscopic cholecystectomy is a widely used treatment method for acute cholecystitis that has many advantages over open cholecystectomy.¹ Complications following laparoscopic cholecystectomy are relatively insignificant; however, bile or gallstone spillage during laparoscopic cholecystectomy is not infrequent, occurring in 10% to 40% of cases.² In most cases, they generally have no detrimental consequences, but might lead to peritoneal granulomatous inflammation.³⁻⁶ The differential diagnoses include various infectious and noninfectious causes of peritoneal granuloma and may clinically result in misdiagnosis as malignancy.⁴ Here, we report a case of bile granuloma that mimicked peritoneal seeding following laparoscopic cholecystectomy.

CASE REPORT

A 59-year-old Korean man presented with right upper quadrant pain for 3 days. He had no previous medical history. Physical examination was unremarkable except for right upper quadrant tenderness. C-reactive protein level was 15.89 mg/dL (normal < 0.5 mg/dL). Total bilirubin (1.42 mg/dL; normal, < 1.2 mg/dL) and direct bilirubin (1.18 mg/dL; normal, < 0.4 mg/dL)

were slightly increased. Other laboratory findings, including carcinoembryonic antigen (CEA), CA19-9, liver enzyme, and kidney function test results, were within normal ranges. Abdominal computed tomography (CT) revealed a markedly distended gallbladder and a 32-mm polypoid mass in the neck of the gallbladder, suggesting acute calculous cholecystitis or gallbladder cancer. The patient underwent laparoscopic cholecystectomy. During surgery, gallbladder aspiration was performed due to distension. There was no perforation during gallbladder bed dissection with no gross bile contamination of the peritoneum or any conspicuous bile leakage. Gross examination revealed a 2.8 × 1.6 cm polypoid mass with several black stones up to 0.9 cm without perforation. Microscopic findings of the gallbladder showed adenocarcinoma and acute cholecystitis. The adenocarcinoma was limited to the polyp and invaded the lamina propria without lymphovascular invasion, perineural invasion, or involvement of the resection margin.

After 3 months, he had no clinical symptoms, such as abdominal pain or discomfort. Follow-up abdominal CT revealed an ill-defined mass lesion in the right subhepatic space, which showed hypermetabolism on positron emission tomography-computed tomography (PET-CT) (Fig. 1). Follow-up tumor markers were

within the normal range. Suspecting localized peritoneal seeding, exploratory laparotomy was performed. It revealed adhesion between the liver, omentum, transverse colon, and peritoneum. There were seeding mass-like lesions in the gallbladder bed and adhesion sites. Wedge resection of the liver, wedge resection of the transverse colon, and omentectomy were done. Gross finding of the resected specimens showed ill-defined yellow to brownish nodular lesions up to 1.1×1.0 cm in the liver and transverse colon without necrosis (Fig. 2). Microscopic findings of the liver, transverse colon, and omentum showed brown pigments and foreign-body type multinucleated giant cells with numerous lymphocytes, suggesting the diagnosis of granulomatous inflammation (Fig. 3). The brown pigment was compatible with bile

pigment and was greenish brown on Fouchet's staining. There was no evidence of malignant tumor cells, and stone and crystal deposits were not present. He had no complication or recurrence for 9 months after surgery. This study was approved by the Institutional Review Board of Keimyung University Dongsan Medical Center (IRB No. 2018-03-052) and informed consent was waived.

DISCUSSION

Although laparoscopic cholecystectomy has many advantages over standard open cholecystectomy, several intraoperative complications of laparoscopic cholecystectomy should be noted. Common complications are iatrogenic perforation of the gallbladder,

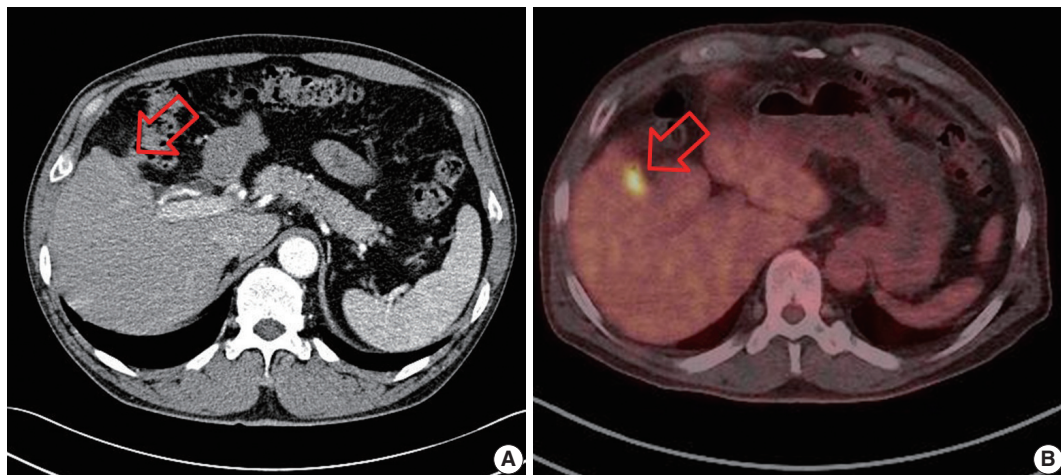


Fig. 1. Findings of abdominal computed tomography (CT) (A) and positron emission tomography-computed tomography (PET-CT) (B). Abdominal CT showed ill-defined mass lesions (arrow) in the right subhepatic space near the gallbladder bed. PET-CT demonstrated a focal hypermetabolic lesion (arrow) in the right subhepatic space.

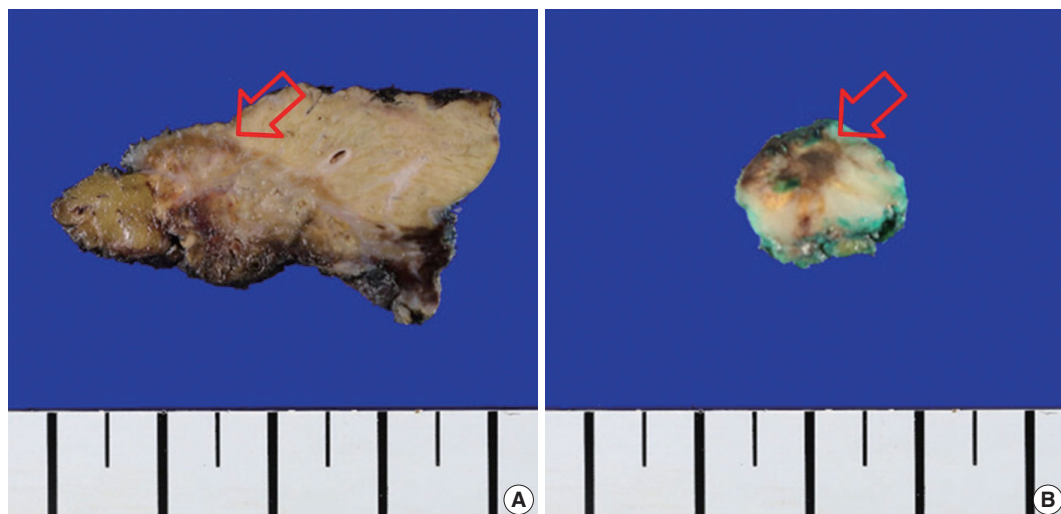


Fig. 2. Gross finding of the specimens showed ill-defined yellow to brownish, variegated, nodular lesions (arrows) without necrosis in the liver (A) and the transverse colon (B).

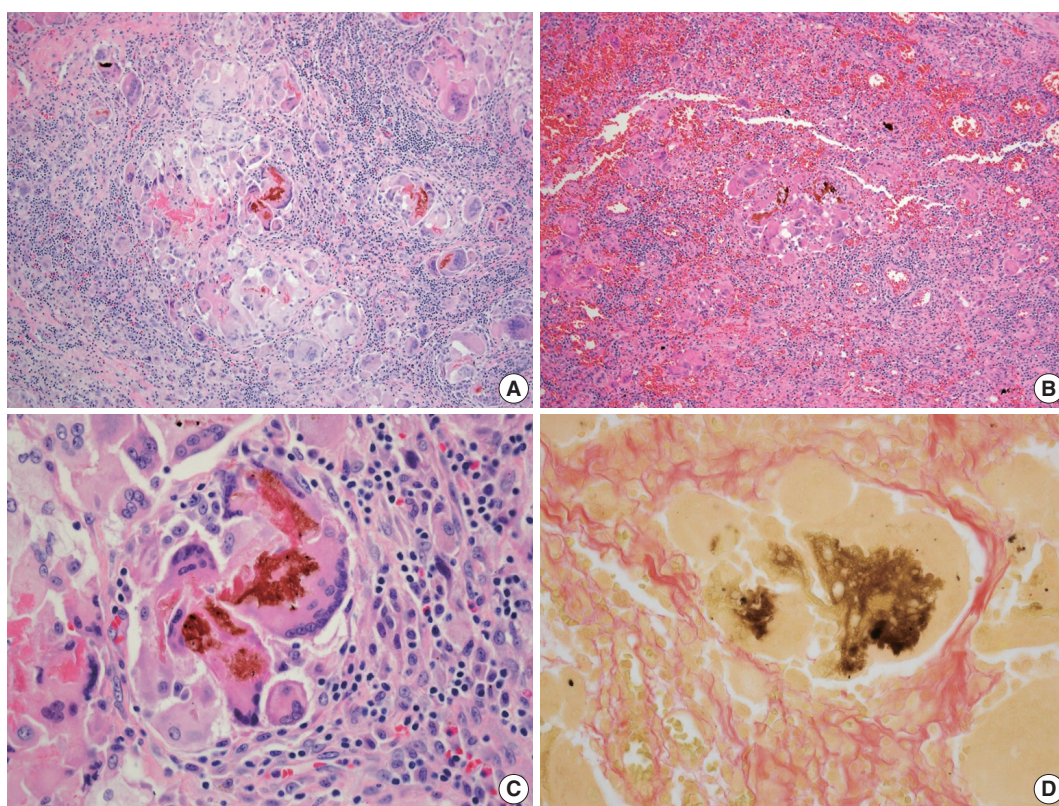


Fig. 3. Microscopic findings of the lesion. The liver (A) and transverse colon (B) showed granulomatous inflammation, which consists of brown pigments and multinucleated giant cells with numerous lymphocytes (C). The pigment was greenish brown on Fouchet's technique (D).

bleeding of the tissues near the gallbladder, and gallstone spillage into the peritoneal cavity.⁶ Bile or gallstone spillage during laparoscopic cholecystectomy is not infrequent, occurring in 10% to 40% of cases.^{2,7} Gallbladder aspiration is a technique to reduce the possibility of unintentional perforation during dissection by decreasing the gallbladder volume. It is controversial whether gallbladder aspiration has the effect of reducing operative time or decreasing the risk of perforation.⁸⁻¹⁰ In our patient, gallbladder aspiration was performed to reduce the volume. If there was no perforation or grossly obvious bile leakage during gallbladder bed dissection, a possibility of bile leakage through the aspiration site may be considered. Occurrence of gallstone spillage complications can range from a few days to several years.¹¹ Early complication of bile or stone spillage is peritonitis with acute symptoms. Acute symptoms may not occur if bile leakage is mild, or if the surgeon executes immediate irrigation or stone removal. If bile or stone spillage is not accompanied by acute symptoms, it is a silent event without detrimental sequelae.⁵ However, abscess formation and fistula formation are medium- to long-term complications.¹² Patients with gallstone spillage may be asymptomatic until complications develop. In our patient, if there was no follow-

up abdominal CT, he might not have known about the presence of bile granuloma because he had no symptoms.

Foreign body reaction to bile or gallstone spillage during laparoscopic cholecystectomy as the cause of intraperitoneal granulomas has been rarely reported.^{2,3,5,7,12,13} Foreign-body granulomas are generally asymptomatic.¹⁴ Given that most patients do not undergo follow-up abdominal imaging study after laparoscopic cholecystectomy, they may not know about granulomas if they are asymptomatic. Most cases were found during cesarean section or gynecological surgery.^{3,5,13} Here, we describe a case of multiple bile granulomas with clinical suspicion of peritoneal seeding after laparoscopic cholecystectomy. To our knowledge, three cases similar to our case have been reported. In the first case, a 37-year-old woman who underwent cholecystectomy due to perforated gallbladder showed multiple bile granulomas that mimicked disseminated malignancy during cesarean section.⁵ In the second case, a 59-year-old man presented with foreign body granuloma due to spilled gallstones after laparoscopic cholecystectomy that mimicked a retroperitoneal sarcoma.² In the third case, a 29-year-old woman who underwent laparoscopic cholecystectomy with stone spillage during procedure showed several hard nodules in

omentum that mimicked metastases from unknown primary tumor during cesarean section.¹³

18-Fluoro-2-deoxyglucose (FDG) is well known to accumulate in inflammatory cells, including neutrophils, lymphocytes, and macrophages due to elevated glucose requirements in various inflammatory conditions.¹⁵ Therefore, PET-CT may show false-positive FDG uptake in several benign conditions, including granulomatous disease. Furthermore, foreign body granulomas have been reported to masquerade as malignant tumors.^{16,17} In our case, an increased FDG uptake of the ill-defined mass lesion in the right subhepatic space was mistaken for localized peritoneal seeding.

The current case emphasizes four significant features. Granuloma due to spilled bile or gallstone is a rare complication following laparoscopic cholecystectomy and should be considered in a patient presenting with a peritoneal mass after laparoscopic cholecystectomy. Secondly, the aspiration itself may cause bile leakage without obvious perforation. Thirdly, the surgeon should make a note in the patient's medical record after laparoscopic cholecystectomy whether the gallbladder had been perforated and whether there had been any bile or stone leakage during the procedure for future reference. Finally, a biopsy or diagnostic laparoscopy with frozen section of the peritoneal nodule is preferred over open exploratory laparotomy in questionable cases.

In summary, bile leakage during laparoscopic cholecystectomy could lead to bile granuloma mimicking peritoneal seeding. This reminds the importance of caution when removing the gallbladder in case of acute calculous cholecystitis. In addition, the possibility of bile granuloma should be considered in patients with gallbladder cancer during follow-up after surgery.

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Conflicts of Interest

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Ovarian Gynandroblastoma with a Juvenile Granulosa Cell Tumor Component in a Postmenopausal Woman: A Case Report and Literature Review

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Gynandroblastoma is an extremely rare sex cord-stromal tumor with both female (granulosa cell tumor) and male (Sertoli-Leydig cell tumor) elements. Juvenile granulosa cell tumors are also very rare and are so named because they usually occur in children and adolescents. A 71-year-old woman with right upper quadrant abdominal pain visited our hospital. Pelvic computed tomography showed a large multilocular cystic mass, suspected to be of ovarian origin. We performed a total abdominal hysterectomy (total abdominal hysterectomy was performed) with bilateral salpingo-oophorectomy. A 13-cm multilocular cystic mass with serous fluid was observed in her right ovary. Upon microscopic examination, the solid component of the mass showed both Sertoli-Leydig cell and juvenile granulosa cell differentiation, which we diagnosed as gynandroblastoma. Gynandroblastoma with a juvenile granulosa cell tumor component is extremely rare and, until now, only six cases have been reported in the English literature. We report the first gynandroblastoma with a juvenile granulosa cell tumor component diagnosed in an elderly patient, along with a literature review.

Key Words: Ovarian gynandroblastoma; Juvenile granulosa cell tumor; Ovary; Postmenopause

Gynandroblastoma is a rare ovarian tumor that manifests with a combination of two histological elements: granulosa cell tumor (GCT) and Sertoli-Leydig cell tumor (SLT) differentiation. Robert Meyer was the first to describe this type of tumor in 1930.¹ Since then, only 28 cases have been reported in the English literature.^{2,3} Gynandroblastoma usually consists of adult-type granulosa cells. Cases of juvenile GCT (JGCT) are rarer and have only been reported in women of reproductive age. Herein, we report the first case of ovarian gynandroblastoma with JGCT in an elderly patient. This study was approved by the Institutional Review Board of Yeungnam University Hospital with a waiver of informed consent (IRB No. YUMC 2018-04-019).

CASE REPORT

A 71-year-old woman (gravida 6, para 4) with acute abdominal pain in the right lower quadrant visited our hospital's emergency room. Computed tomography revealed a large multiloculated

cystic mass in the pelvic cavity (Fig. 1A). Subsequent ultrasonography revealed a cystic mass of approximately 13 cm in the right ovary (Fig. 1B). The patient had no family history of genetic syndromes and no other underlying diseases. She experienced menarche at age 16 and menopause at age 53. She had no history of symptoms associated with endocrine manifestations, i.e., neither estrogenic nor androgenic changes, such as endometrial hyperplasia, abnormal endometrial bleeding, oligomenorrhea, infertility, or virilization.

Her preoperative serum carbohydrate antigen (CA) 19-9 (19.48 U/mL) and CA125 (7.45 U/mL) levels were within normal ranges. Taking into consideration the preoperative diagnosis of torsion of the right ovarian tumor, the patient underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy.

On gross examination of the right ovary, a 13.0×7.5×6.5-cm multilocular ovarian cyst with a smooth and glistening surface was observed (Fig. 2A). The cyst was filled with serosanguinous fluid, and the inner surface had a mainly smooth appearance. In

the cystic lumen, a 3.7×1.8 -cm solid yellowish mass was observed (Fig. 2B). There were no remarkable findings upon gross examination of the uterus, fallopian tubes, and left ovary.

Microscopically, the ovarian tumor consisted of a JGCT (40%) and a well-differentiated SLT (60%) (Fig. 3A). The JGCT displayed nodular growth patterns with follicles of various shapes and sizes. The follicles contained basophilic secretions resembling mucin (Fig. 3B). Cytologically, the tumor cells of the JGCT were round and had indistinct cell borders with abundant eosinophilic cytoplasm. The tumor cells' nuclei were round-to-oval with open chromatin, but nuclear grooves were less prominent than usually observed in adult-type GCT. There was no striking nuclear atypia, and Call-Exner bodies were not identified. The SLT showed well-formed tubules of Sertoli cells and fibrous band-like stroma with conspicuous Leydig cells (Fig. 3C). The Sertoli cells were cuboid-to-columnar with round nuclei and inconspicuous nucleoli. Nuclear atypia was absent. Leydig cells presented

with abundant eosinophilic cytoplasm. Some tumor cells had foamy and vacuolated cytoplasm, but Reinke crystals were not prominent (inset of Fig. 3C).

Immunohistochemically, the tumor cells of both tumor components were positive for calretinin and inhibin, and negative for epithelial membrane antigen (Fig. 3D–F).

The patient had no post-surgical complications, and there was no evidence of recurrence or metastasis during two years of follow-up.

DISCUSSION

Gynandroblastoma is a subtype of ovarian sex cord-stromal tumor and is usually observed in young women. It is identified by its admixture of ovarian and testicular tissues or cells. The most important clinical manifestation is hormonal dysfunction. Because this tumor is hormonally active, patients afflicted with it

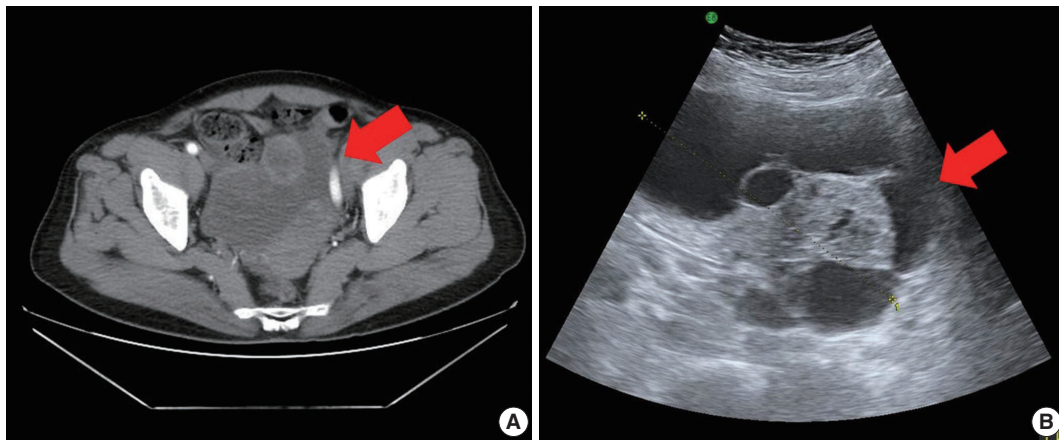


Fig. 1. Pelvic computed tomography (CT) and ultrasonography findings. (A) Pelvic CT reveals a large multilocular cystic mass (arrow) with a solid component. (B) Ultrasonography shows a cystic mass (arrow) of approximately 13 cm arising from the right adnexa.

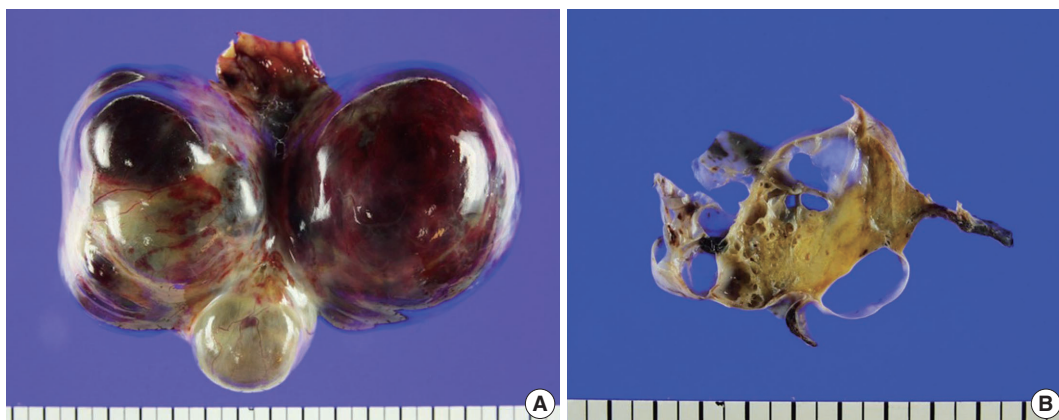


Fig. 2. Gross findings. (A) A $13.0 \times 7.5 \times 6.5$ -cm multilocular cystic and solid mass replaced the entire ovary. (B) The cysts were filled with serosanguinous fluid and approximately 30% of the mass was solid, hard, and yellow.

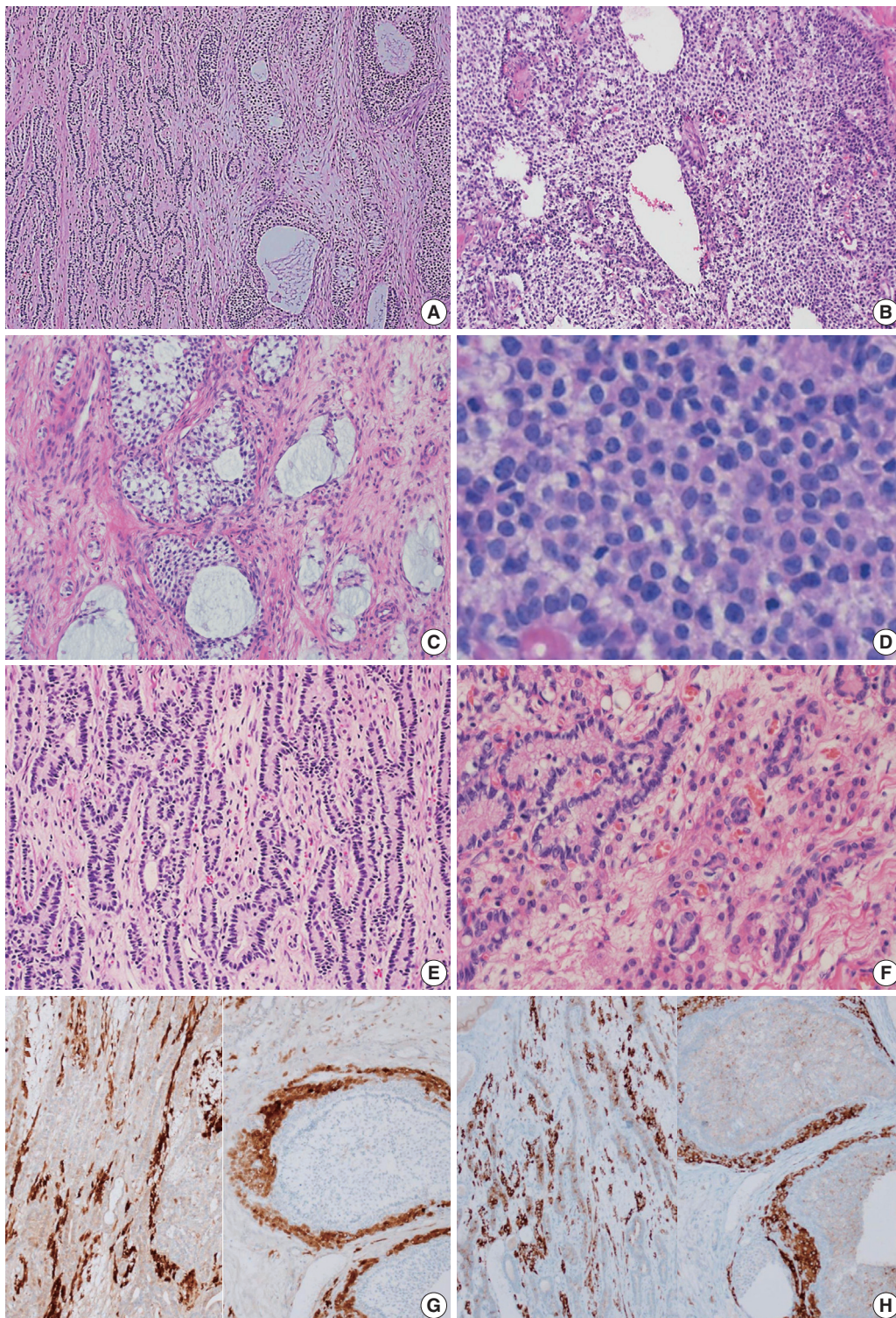


Fig. 3. Microscopic findings. (A) The tumor was composed of a Sertoli-Leydig cell tumor (SLT) and a juvenile granulosa cell tumor (JGCT). (B) The JGCT shows solid and nodular growth patterns with relatively uniform tumor cells. (C) Various sized follicles with mucin-like basophilic secretions are also frequently observed. (D) The tumor cells were rounded and had hyperchromatic nuclei without nuclear grooves, which are the characteristic features of adult granulosa cell tumors. (E) A well-differentiated SLT component is also identified. (F) Sertoli cells formed hollow tubules with delicate fibrous stroma. Small clusters of Leydig cells are observed in the fibrous stroma. (G, H) The immunohistochemical staining results confirms that the tumor was of sex cord-stromal origin. Both components stained positive for calretinin (G, left, SLT; right, JGCT) and inhibin (H, left, SLT; right, JGCT).

could present with either estrogenic or androgenic symptoms.⁴ Gynandroblastoma with JGCT is very rare with only 6 of 28 gynandroblastoma cases reported in the English literature to date.^{2,3,5-8} All reported cases occurred in premenopausal women between 15 and 39 years old. Our case is the first gynandroblastoma with JGCT diagnosed in a 71-year-old postmenopausal elderly woman. Approximately 97% of JGCTs occur during the first three decades of life. Only a few known cases of JGCT occurred in women > 30 years old; just two cases of JGCT as a component of gynandroblastoma have been reported in patients over the age of 30.^{2,7} Thus, our case of gynandroblastoma with JGCT in a postmenopausal woman is extremely unusual. However, the JGCT component of our patient's tumor showed typical histologic features, such as variously sized follicles that contained basophilic secretions.

The tumor diagnosis was based on histologic features. The diagnostic criteria for gynandroblastoma indicate that the minor tumor component (either Sertoli-Leydig cells in a GCT, or granulosa cells in an SLT) should account for at least 10% of the entire tumor. Sometimes, well-differentiated Sertoli-Leydig cells can be focally observed in GCTs and vice versa. Therefore, sufficient tumor sampling is essential for an accurate diagnosis. Although most reported GCT cases are of the adult-type, a juvenile-type has also been identified. The histology of adult-type and juvenile-type cells is the same for GCT; therefore, it is not difficult to diagnose these tumors. Several immunohistochemical markers exist to aid differential diagnosis, but these markers are used to diagnosis every type of ovarian sex cord-stromal tumor and they are not specific to gynandroblastoma. The representative sex cord-stromal cell markers include inhibin, calretinin, SF1, WT-1, and CD99.² MART-1/melan-A is a marker specific to SLT and steroid cell tumors.⁹ Additionally, the cell cycle regulatory protein 14-3-3 sigma was recently identified as a diagnostic marker for GCT and steroid cell tumors.¹⁰ In our case, both JGCT and SLT components were positive for inhibin and calretinin.

The histogenesis of gynandroblastoma is unknown, but it is generally assumed that it originates from a single progenitor cell that can differentiate into both female and male elements.¹¹ During embryogenesis, gonadal tissues develop from the mesoderm of the urogenital ridge and the ridge organizes the endocrinologically active tissues of the gonad, which includes granulosa cells, Sertoli cells, theca cells, ovarian stromal cells, and Leydig cells.¹² Gynandroblastoma and other sex cord-stromal tumors are considered to have their origin in the cells of the urogenital ridge.

It is difficult to characterize the biological behavior of gynandroblastoma due to its extremely low incidence. Based on the reported cases, it appears to have a benign course. The majority

of gynandroblastomas were stage I tumors and most patients with stage I gynandroblastoma were successfully treated by simple surgical resection with regular follow-up. Only one recurrence case has been reported, in which the tumor reportedly recurred 10 years after the original tumor was surgically removed.⁴ Likewise, our patient had a stage I tumor without extra-ovarian involvement. Currently, 26-month postsurgery, the patient has not shown any clinical evidence of recurrence. Given the evidence from previous reports and our current case, gynandroblastoma has a relatively benign clinical course, and our patient appears to have had a low-grade tumor.

Recently, several genetic studies of ovarian sex cord-stromal tumors focusing on cytogenetic alterations have been published.¹³ The most widely studied genetic change is the *FOXL2* mutation C134W (402 C > G) found in GCTs.¹⁴ *FOXL2* is a transcription factor that is restrictedly expressed in granulosa cells during development and adulthood.¹⁵ This mutation is widely observed in adult type-GCT and can be a diagnostic or prognostic marker.¹⁵⁻¹⁷ Approximately 30% of JGCT tumors harbor the *gsp* oncogene and 60% contain the *AKT1* gene mutation.^{18,19} These biological markers have the potential to be of therapeutic value. Regarding SLT, somatic or germline mutations of *DICER1*, which encodes an RNase III endonuclease, are well-known genetic alterations.¹³ Somatic hot spot mutations of *DICER1* have been observed in approximately 60% of SLTs.²⁰ These frequent genetic changes of sex-cord stromal tumors had been understudied with regard gynandroblastoma. Oparka *et al.*¹¹ evaluated the C134W (402 C > G) *FOXL2* mutation, a frequently observed mutation in adult-type GCT, in seven gynandroblastomas. This mutation was not detected in any GCT or SLT component of gynandroblastoma cases. However, other researchers observed *FOXL2* mutations in both GCT and SLT components of two gynandroblastomas and *DICER1* mutations were also observed in a few cases.¹³ The therapeutic or diagnostic value of these mutations for gynandroblastoma should be studied.

In conclusion, gynandroblastoma is an ovarian sex cord-stromal tumor with both male and female elements. Gynandroblastoma with JCGT is very rare and it typically occurs in women of reproductive age. However, it can also occur in postmenopausal women. The pathogenesis and biologic behavior of gynandroblastoma are still not well known. However, based on the limited number of previous studies, this tumor shares many clinicopathologic characteristics with other sex-cord stromal tumors, including GCT and SLT. More comprehensive histologic and genetic studies are needed.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Abrupt Dyskeratotic and Squamoid Cells in Poorly Differentiated Carcinoma: Case Study of Two Thoracic NUT Midline Carcinomas with Cytohistologic Correlation

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Cytologic diagnosis of nuclear protein in testis (NUT) midline carcinoma (NMC) is important due to its aggressive behavior and miserable prognosis. Early diagnosis of NMC can facilitate proper management, and here we report two rare cases of thoracic NMC with cytohistologic correlation. In aspiration cytology, the tumor presented with mixed cohesive clusters and dispersed single cells, diffuse background necrosis and many neutrophils. Most of the tumor cells had scanty cytoplasm and medium-sized irregular nuclei, which had fine to granular nuclear chromatin. Interestingly, a few dyskeratotic cells or squamoid cell clusters were present in each case. Biopsy specimen histology revealed more frequent squamous differentiation, and additional immunohistochemistry tests showed nuclear expression of NUT. Because this tumor has a notorious progression and has been previously underestimated in terms of its prevalence, awareness of characteristic findings and proper ancillary tests should be considered in all suspicious cases.

Key Words: BRD4-NUT fusion oncogene protein; NUT midline carcinoma; Lung; Cytology

Nuclear protein in testis (NUT) midline carcinoma (NMC) is one of the arduous differential diagnoses with histologic features of poorly differentiated or undifferentiated carcinoma.^{1,2} Fluorescence in situ hybridization (FISH) and a specific antibody to identify gene rearrangement allow rapid diagnosis of NMC.³ However, delays in diagnosis without any suspicion of NMC can affect proper management and survival due to the extremely aggressive behavior and dismal prognosis for this cancer.¹ Previous studies of cytologic findings in NMC are limited because of its rarity; herein, we present two interesting cases of NMC arising in the lung with cytohistologic findings. The Institutional Review Board (IRB) of Samsung Medical Center approved this case report (IRB file No. 2017-09-050) and informed consent was waived.

CASE REPORT

Case 1

A 45-year-old male presented with a 3-month history of

cough. Chest X-ray and computed tomography (CT) showed a 3.8-cm-sized mass in the right lower lobe and multiple enlarged mediastinal lymph nodes (Fig. 1A–C) in which ¹⁸F-fluorodeoxyglucose uptake was detected (Fig. 1D). For diagnosis and nodal staging, endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) and biopsy were performed. Aspirates from both lymph nodes and lung mass were highly cellular. Some tumor cells were cohesive enough to form 3-dimensional clusters, but others were dispersed in single cells (Fig. 1E). Frequent crush artifacts and naked nuclei were easily identified. Tumor cells were poorly to undifferentiated with scanty cytoplasm and irregular nuclear contour. Nuclear chromatin was fine to granular and nucleoli were small but occasionally identified (Fig. 1F). Unexpectedly, a few scattered dyskeratotic cells were noticed (Fig. 1E, F). Many neutrophils and necrosis were present in the background (Fig. 1F). Biopsy of mediastinal lymph nodes showed sheets of metastatic undifferentiated tumor cells with large area of necrosis and peritumoral neutrophilic

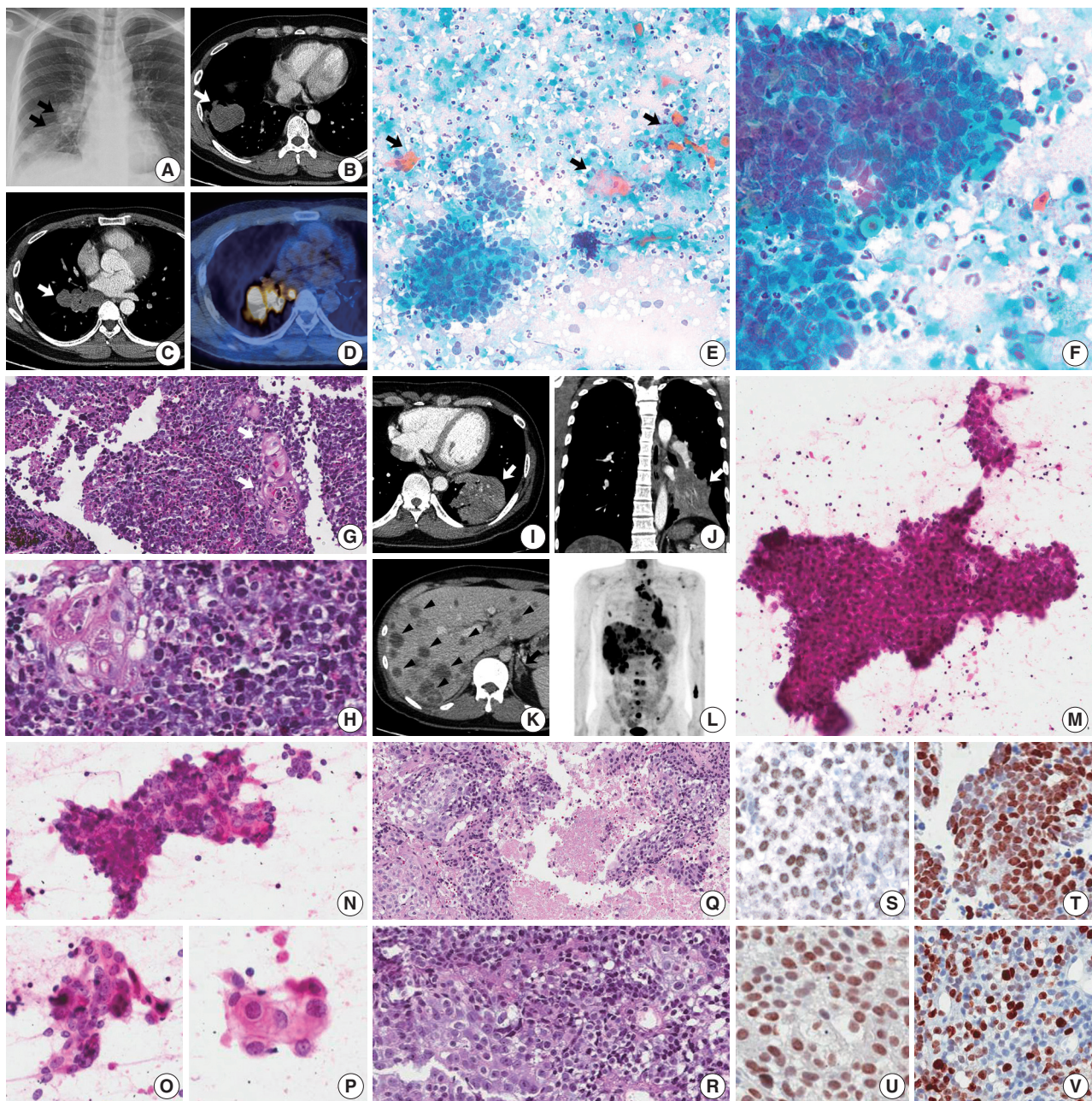


Fig. 1. Thoracic nuclear protein in testis (NUT) midline carcinoma and cytohistologic findings. (A–H) Case 1. (A–D) Chest radiograph and computed tomography (CT) shows 3.8-cm-sized mass in the right lower lobe with multiple enlarged lymph nodes (arrows). Positron emission tomography (PET) reveals ^{18}F -fluorodeoxyglucose uptake of the mass and lymph nodes. (E) Mediastinal lymph node aspiration smears are highly cellular with cohesive clusters and dispersed single cells. Scattered dyskeratotic cells and squamous differentiations are noticed (arrows). (F) Tumor cells have scanty cytoplasm, nuclear molding, irregular nuclear contours, and fine to granular nuclear chromatin. Nucleoli are small but occasionally identified. A few dyskeratotic cells are also identified. Background of the smear shows many neutrophils and necrosis. (G, H) Biopsy of a mediastinal lymph node shows monotonous tumor cells and hyperchromatic nuclei. Nuclei have fine to granular chromatin and the occasional small nucleolus, and the cytoplasm is scant and delicately amphophilic. Foci of squamous differentiation are more often identified than in cytology. (I–R) Case 2. (I–L) Chest and abdomen CT show a 5.5-cm-sized mass (white arrow) in the left lower lobe, and multiple metastases in both a lobe of the liver (arrowheads) and the adrenal glands (black arrow). PET highlights multiple hypermetabolic lesions in the whole spine, ribs, pelvic bone and scapulae, in addition to the lung and liver masses. (M–P) In aspiration cytology of a mediastinal lymph node, some tumor cells are medium-sized and poorly differentiated with scanty cytoplasm and hyperchromatic nuclei. However, others have more abundant amphophilic to eosinophilic cytoplasm and a lower nuclear to cytoplasmic ratio, which can be considered squamous differentiation. (Q, R) Histology of a mediastinal lymph node shows two different tumor cell components with extensive necrosis, poorly differentiated cells and squamous cells, which are similar to the findings of aspiration cytology. (S–V) Immunohistochemistry shows nuclear expression of NUT in case 1 (S) and case 2 (U). Tumor cells are positive for p63 (T, case 1) and there is a variable proliferation index of Ki-67 (V, case 2).

infiltration (Fig. 1G). Tumor cells had scant and delicate amphophilic cytoplasm. Nuclear chromatin and contour were similar to the findings of aspirate smears. Foci of abrupt squamous differentiation were identified more often than in cytology (Fig. 1H). Under additional immunohistochemistry (IHC) staining, these tumor cells showed a very high proliferation index of up to 70% in Ki-67 (1:200, Dako, Glostrup, Denmark) and were positive for pan-cytokeratin (1:500, Dako), p53 (1:4,000, Thermo Fisher, Rockford, IL, USA), p63 (1:200, Biocare, Concord, CA, USA), and NUT (1:100, C52B1, Cell Signaling Technology, Danvers, MA, USA), and up to 70% of tumor cells expressed programmed death-ligand 1 (PD-L1; RTU, 22C3, Dako) (Fig. 1S, T). Tumor cells were negative for Oct3/4 (1:50, Santa Cruz Biotechnology, Dallas, TX, USA), chromogranin (1:400, Dako), CD99 (1:50, Dako), CD45 (1:1000, Dako), CD56 (1:200, Leica Biosystems, Novocastra, Newcastle upon Tyne, UK), and thyroid transcription factor 1 (TTF-1; 1:50, Dako), which can help rule out the possibilities of other malignancies.

Case 2

A previously healthy 32-year-old male presented with a persistent cough for 3 months, blood-tinged sputum and chest pain. On chest CT, a 5.5-cm-sized consolidative lung mass accompanied by enlarged mediastinal lymph nodes was identified at the left lower lobe (Fig. 1I, J). Abdomen-pelvis CT showed multiple necrotic masses scattered throughout both lobes of the liver and bilateral adrenal glands (Fig. 1K), and positron emission tomography highlighted multiple hypermetabolic bony lesions in the whole spine, bilateral ribs, pelvic bones, sternum and both scapulae (Fig. 1L). Cytology of lymph node aspirates from EBUS-TBNA showed dispersed and clustered tumor cells in the background of necrosis (Fig. 1M). Some tumor cells had large vesicular nuclei with open chromatin, prominent nucleoli, scanty cytoplasm and a high nuclear to cytoplasmic (N/C) ratio. Naked nuclei were frequently identified in scattered individual cells. However, others exhibited more abundant amphophilic to eosinophilic cytoplasm and granular nuclear chromatin with a lower N/C ratio, which could be considered squamous differentiation (Fig. 1N–P). Biopsy of the mediastinal lymph nodes and liver showed metastatic carcinoma in a necrotic background composed of two morphologically different tumor cell components: poorly differentiated cells with hyperchromatic nuclei and scanty cytoplasm, and squamoid cells with less hyperchromatic nuclei, prominent nucleoli and ample cytoplasm (Fig. 1Q, R). By IHC, tumor cells were positive for p63, p53, and NUT, negative for TTF-1 and CD56, and had a relatively low Ki-67

proliferation index of approximately 25% (Fig. 1U, V). PD-L1 IHC test revealed low expression of about 1% of the tumor.

DISCUSSION

Diagnosis of NMC in cytology is challenging. Cytologic findings are characterized by high cellularity with medium-sized poorly to undifferentiated cells. Cohesiveness of these tumor cells is variable² and while clusters of tumor cells are usually patternless, some can be arranged in a pseudoglandular pattern in focal areas.⁴ Round to oval nuclei have irregular contours, but their size is relatively uniform and monotonous rather than pleomorphic. Nuclear chromatin is not coarse or condensed but fine to granular and vesicular. Nucleoli are usually single, small and prominent.⁵ Scant cytoplasm is amphophilic to eosinophilic and nuclear molding is frequent. The absence of cytoplasm makes nuclei naked and squeezing artifacts are common, especially in smears rather than liquid-based preparations. Mitosis is frequent, but atypical mitosis is rare. Necrotic debris, apoptotic bodies and nuclear dust with many neutrophils fill the background of the tumor cells. Previous reports have described difficulty in finding squamous differentiation in cytology specimens,^{2,5} but tumor cell clusters with a squamoid appearance or a few dyskeratotic cells were easily noticed in our cases. This finding can be variable, from subtle to very distinct with obvious keratinization.

The cytogenetic rearrangement of this tumor is unique and characteristic. Two-thirds of such tumors have chromosomal translocation t(15;19), which forms the *BRD4-NUT* fusion oncogene, and subsets have *BRD3-NUT* and other NUT-variant rearrangement.¹ In recent studies, knockdown of BRD-NUT induces rapid squamous differentiation with a halt in proliferation in patient-derived NMC cells.^{6,7} This result strongly suggests the role of BRD-NUT as a fusion protein that blocks differentiation, and is compatible with its characteristic cytologic and histologic findings. The tumor histology is usually made of sheets of undifferentiated cells. Nuclear size and shape, chromatin pattern, nucleoli and cytoplasm are similar to the cytology findings. Frequent areas of necrosis are common, and diffuse infiltrations of neutrophils among tumor cells are easily identified. Despite the simple and uniform chromosomal abnormality, some tumor cells might be out of influence from the cytogenetic alteration. Accordingly, there may be focal areas of abrupt squamous differentiation that are more commonly identified than in cytology specimens.

Differential diagnosis of NMC in cytology includes small cell carcinoma (SCC), basaloid squamous cell carcinoma (BSqCC), and small cell variant of squamous cell carcinoma (SCVSqCC).

The cytology of SCC shows predominantly single cells, but has some cell clustering with nuclear molding exhibiting fine, open chromatin, and was inconspicuous to small nucleoli.^{8,9} Scant cytoplasm, artifactually crushed and naked nuclei, and background necrosis are common, and all of these findings mimic NMC. However, keratinized squamous cells are rare, even if some aspirate smears have bronchial epithelial cells with squamous metaplasia.

BSqCC and SCVSqCC are more problematic diagnoses because both have small cells with some dyskeratosis and squamous differentiation. The cytology of BSqCC has small to medium-sized nuclei with fine chromatin, small to inconspicuous nucleoli, scant cytoplasm and nuclear molding with necrosis.⁹ SCVSqCC shows similar small cells but slightly more abundant, bluish cytoplasm, with vesicular nuclei and prominent nucleoli.¹⁰ Both basaloid and SCVSqCC have areas of necrosis, squamous differentiation and dyskeratosis, which is often noticed in NMC but usually composed of predominantly cohesive cell clusters rather than dispersed single cells.⁹ It is important to note that all these tumors have similar cytologic findings and aggressive behaviors but require different management.

In IHC, NMC tumor cells are positive for pan-cytokeratin, p53, p63 and NUT, and usually have a high but variable proliferation index by Ki-67. It is hard to differentiate NMC from possible mimickers without diagnostic markers, which are not routinely screened. If the cytomorphology is suspicious for NMC, a FISH assay and IHC test should be performed in all cases due to the underestimation of its prevalence.¹ A previous study examined NUT IHC expression using a monoclonal antibody in 114 cases of poorly differentiated carcinoma or unclassified malignancy in the mediastinum, and 3.5% of cases showed nuclear expression with a median age of 50.¹¹ Further studies are warranted in other anatomic sites, regardless of age.

Currently, conventional cytotoxic chemotherapy and radiation therapy are not effective in treating NMC. Unlike other usual carcinomas, this tumor has a typical chromosomal alteration and genetic stability. Novel targeted therapy using histone deacetylase inhibitors and BET inhibitors has been developed lately based on the idea that BRD-NUT fusion proteins specifically bind to acetylated histones.¹ These drugs are now in several clinical trials (ClinicalTrials.gov identifiers: NCT01587703, NCT01987362, NCT02711137, and NCT02307240). Not only doctors, but also patients, continue to struggle with NMC at this moment. To treat this disease properly, all pathologists should pay attention to this underestimated entity.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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