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*Rosette-forming Epithelioid Osteosarcoma* 

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#### Aims & Scope

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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Editors-in-Chief: Jung, Chan Kwon, MD, Park, So Yeon, MD
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Editorial Office
Room 1209 Gwanghwamun Officia, 92 Saemunan-ro, Jongno-gu, Seoul 03186, Korea
Tel: +82-2-795-3094 Fax: +82-2-790-6635 E-mail: office@jpatholtm.org
#1508 Renaissancetower, 14 Mallijae-ro, Mapo-gu, Seoul 04195, Korea
Tel: +82-2-593-6943 Fax: +82-2-593-6944 E-mail: office@jpatholtm.org

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Manuscript Editing by InfoLumi Co. 210-202, 421 Pangyo-ro, Bundang-gu, Seongnam 13522, Korea Tel: +82-70-8839-8800 E-mail: infolumi.chang@gmail.com

Front cover image: Macroscopic and microscopic findings of epithelioid osteosarcoma of the rib (p407).

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# Post-mortem assessment of vimentin expression as a biomarker for renal tubular regeneration following acute kidney injury

Juan Carlos Alvarez Moreno<sup>1</sup>, Hisham F. Bahmad<sup>1</sup>, Christopher A. Febres-Aldana<sup>1</sup>, Andrés Pirela<sup>2</sup>, Andres Azuero<sup>2</sup>, Ali Salami<sup>3</sup>, Robert Poppiti<sup>1,4</sup>

<sup>1</sup>Arkadi M. Rywlin M.D. Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, Miami Beach, FL;
 <sup>2</sup>Department of Internal Medicine, Mount Sinai Medical Center, Miami Beach, FL, USA;
 <sup>3</sup>Department of Mathematics, Faculty of Sciences, Lebanese University, Nabatieh, Lebanon;
 <sup>4</sup>Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

**Background:** Acute kidney injury (AKI) is a common cause of morbidity and mortality. It mainly targets the renal tubular epithelium with pathological changes, referred to as acute tubular injury. The latter is followed by a regenerative response that is difficult to visualize on routine hematoxylin and eosin (H&E) stains. In this study, we examined the regenerative capacity of renal tubules by correlating vimentin (VIM) immunohistochemical (IHC) expression and pathological findings of AKI and renal tubular regeneration (RTR) on H&E. **Methods:** We reviewed 23 autopsies performed in the clinical setting of AKI and RTR. VIM expression was scored in the renal cortical tubular epithelium using a statistical cutoff  $\ge$  3% for high expression and <3% for low expression. **Results:** Of the 23 kidney tissues examined, seven (30.4%) had low VIM expression, and 16 (69.6%) had high VIM expression. Kidney tissues with evidence of AKI and RTR had significantly higher VIM expression. Renal peritubular microenvironment features showing regenerative changes on H&E were associated with high VIM expression. In the univariate model, kidney tissues with RTR were 18-fold more likely to have high VIM expression. **Conclusions:** In conclusion, our findings suggest that VIM could serve as an IHC marker for RTR following AKI. However, correlation with H&E findings remains critical to excluding chronic tubular damage. Collectively, our preliminary results pave the way for future studies including a larger sample size to validate the use of VIM as a reliable biomarker for RTR.

Key Words: Acute kidney injury; Acute tubular injury; Renal tubular regeneration; Vimentin; Biomarker; Immunohistochemistry

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Corresponding Author: Juan Carlos Alvarez Moreno, MD, Arkadi M. Rywlin M.D. Department of Pathology and Laboratory Medicine, Mount Sinai Medical Center, 4300 Alton Rd, Miami Beach, FL 33140, USA

Tel: +1-786-280-9320, Fax: +1-305-674-2999, E-mail: Juan.Alvarez@msmc.com

Acute kidney injury (AKI) is a clinical scenario in which there is sudden loss of renal function. The term AKI recently has replaced "acute renal failure" (ARF), which was first described by William Heberden in 1802 as "ischuria renalis" [1,2]. It is characterized by oliguria and rapid development of azotemia [3]. This clinical entity usually is initiated and caused by hypovolemia and/or hypotension and frequently is associated with multiorgan failure [4]. In the United States, numerous studies have focused on analysis of data from the International Classification of Disease coding to measure the incidence of AKI in different clinical settings, with results showing increased incidence over time. The "Kidney Disease: Improving Global Outcomes" (KDIGO)-based criteria used to analyze the epidemiology of AKI might yield a much higher estimated incidence of AKI than the code-classified criteria [5,6]. Due to the absence of standardized classification criteria for AKI, there are many variations in the reported incidence and prevalence rates among hospitalized patients, ranging from < 1% to 66% [7].

Different etiologies contribute to the development of AKI, mainly leading to a rapid decline in glomerular filtration rate. Those include a decrease in renal blood flow in the case of prerenal AKI [4] or other causes in the settings of intrinsic renal or postrenal AKI. The kidneys are most vulnerable to hypoperfusion when autoregulation is impaired. This is seen frequently in elderly patients; patients with atherosclerosis, hypertension, diabetes, or early chronic kidney disease, where arterial and arteriolar nephrosclerosis are present; and patients who are receiving angiotensin receptor blockers or angiotensin-converting enzyme inhibitors [8,9]. The syndrome of AKI is most frequently due to prerenal failure as a result of volume depletion, where renal parenchymal injury is seen not commonly, or intrinsic causes reflected by acute tubular necrosis (ATN) secondary to ischemic or toxic insults [10]. Since necrosis is not evident always, there have been calls to substitute the term "acute tubular injury" (ATI).

The morphologic changes seen in ATI vary according to the severity of tubular damage. Mild changes include tubular dilation, loss of the proximal tubular brush border, and apical blebbing, particularly in the S3 segment of the proximal tubule. Indeed, the proximal tubule is the most susceptible site to injury [11]. The severe forms of ATI manifest from individual cells to confluent necrosis. Detachment from the basement membrane and shedding of proximal tubular epithelial cells with luminal accumulation of necrotic debris in distal tubular segments are characteristic of this entity [4,12]. However, renal tubules have a remarkable capacity to regenerate lost cells, usually within less than a week [13]. It is suggested in the literature that restoration of lost renal tubular cells is secondary to regeneration of the tubular epithelium [14]. Histopathological analysis of the kidney tissue distinguishes four stages of the renal tubular regeneration (RTR) process [15]. In the first stage, there is inflammation and death of the tubular epithelium via apoptosis, necrosis, or other death mechanisms. In the second stage, tubular epithelial cells undergo changes, such as a loss of brush border, tubular flattening, and rapid loss of cell polarity [16,17]. In this stage, cells change from the epithelial to mesenchymal phenotype in a process known as epithelial-to-mesenchymal transition (EMT) and overexpress vimentin (VIM) [18,19]. Regenerating cells are characterized by cytoplasmic basophilia, karyomegaly, and nuclear crowding along the affected tubule segment [20]. In the third stage, enhanced proliferation of most kidney cells occurs through increased levels of growth factors, including insulin like growth factor 1, hepatocyte growth factor, and fibroblast growth factors [21]. Lastly, maturation of epithelial cells occurs with restoration of nephron function [22].

The regenerative features of AKI/ATI are difficult to appreciate on routine light microscopy. The present study expands the assessment of the regenerative capacity of renal tubules in patients with AKI/ATI. We correlated the expression of VIM, a marker of RTR, with clinical and morphologic variables in individuals with varying degrees of AKI. Our results indicate that VIM expression is observed consistently in both AKI and chronic kidney disease (CKD). However, VIM labeling, in areas devoid of fibrosis and other signs of chronic damage, can serve as an immunohistochemical (IHC) biomarker to estimate AKI-associated regenerative responses in autopsies and potentially in surgical biopsies.

# MATERIALS AND METHODS

#### Study design and setting

We conducted a retrospective 10-year review (2010-2020) of all adult autopsies performed at Mount Sinai Medical Center of Florida, with cause of death related to hypoperfusion. Exclusion criteria were (1) death related to trauma, (2) death during surgery, and (3) complete autolysis of kidney tissues. Clinicopathological parameters of the patients were retrieved from electronic pathology and medical records. The variables collected were age, gender, cause of death (cardiac, respiratory, stroke, sepsis, among others), CKD, CKD stage, fold estimated glomerular filtration rate (eGFR), fold creatinine, chronic hemodialysis, diabetes mellitus, hypertension, shock during hospitalization, clinical evidence of AKI/ATI, AKI type, arteriosclerosis, arteriolosclerosis, arteriolosclerosis severity, glomerulosclerosis, glomerulosclerosis severity, significant interstitial fibrosis, interstitial fibrosis severity, tubular atrophy, tubular atrophy severity, pathologic evidence of AKI, tubulorrhexis, pathologic evidence of ATN, evidence of regenerating tubular epithelium, and renal peritubular microenvironment features on hematoxylin and eosin (H&E) stains (cortical) and on periodic acid-Schiff (PAS) stains (cortical).

#### Histopathologic evaluation

Tissue samples were fixed in neutral buffered formalin and embedded in paraffin for histologic processing. Slides of the kidney sections were stained with H&E and PAS reaction and evaluated in a blinded fashion by two independent pathologists with experience in renal pathology. No significant interobserver disagreement was noted in the interpretation between the two pathologists. Although no quantitative criteria were used to assess AKI and RTR, AKI was defined by the presence of tubular dilation, loss of the proximal tubular brush border, tubulorrhexis, and apical blebbing [4], and RTR was defined by cytoplasmic basophilia, karyomegaly, and nuclear crowding along the affected tubule segment [20]. The collective presence of those features was necessary to define AKI and RTR and was achieved by assessing tissue slides systematically and observing renal tubules within at least 10 high power fields (HPFs). Arteriosclerosis was described as thickening of the intima or media of the arteries. Arteriolosclerosis was characterized by hyalinosis of the afferent or efferent vessels and arterioles [23]. Chronic damage was

estimated by evaluating tissue fibrosis including glomerulosclerosis [24], interstitial fibrosis, and tubular atrophy, defined as reduced size of tubules with attenuation of the luminal epithelium [25]. Autolysis was characterized by ghost cells with pyknotic or absent nuclei [26]. Fragmentation/duplication of the basement membrane and thickened basement membrane were recorded.

IHC staining for VIM (Ventana Medical Systems Inc., Tucson, AZ, USA) protein expression was performed using standard processing techniques. Appropriate positive and negative controls were run simultaneously. VIM expression was assessed by positive gold-brown cytoplasmic stain only in the tubular cells. Screening of the kidney tissue sections was performed in a systematic manner. In areas without chronic changes or acute tubulointerstitial inflammation, an average of approximately 30 tubules was counted randomly within 10 HPFs. Scoring was performed at 400x magnification. In non-pathological conditions, glomeruli normally stain positive for VIM [27], serving as internal positive controls in our study. A positive tubule was defined as immunoreactivity of more than 50% of the tubular epithelial cells. The percentage of VIM expression was calculated by dividing the number of tubules with  $\geq 50\%$  VIM-positive cells by the total number of tubules (approximately 30), multiplied by 100. This percentage is referred to as the VIM score. We defined our VIM positivity threshold by setting a cutoff of 3% (Fig. 1B). Evaluation was restricted to areas without advanced/obvious acute or chronic changes as VIM is expressed ubiquitously in tubular atrophy and areas of severe fibrosis. In fact, persistence of VIM+ epithelial cells in CKD likely is responsible for activating fibrogenic pathways, that is, those not part of RTR.

#### Statistical analysis

The VIM score was categorized into less than 3% and greater than or equal to 3%. This cutoff of 3% was assigned based on the VIM score distribution, where 95.7% of the total population (22/23) clustered in the "less than or equal to 20% VIM score." Multiple cutoffs have been explored to determine the optimal stratification of patients. The optimal cutoff point for VIM that provided the greatest statistical significance was 3%. Data retrieved from medical charts were entered into a Microsoft Excel spreadsheet that was designed specifically for this study. Then, the data were transferred into the Statistical Package of Social Science (IBM Corp., Released 2013, SPSS Statistics for Windows ver. 22.0, Armonk, NY, USA), which was used for data cleaning, management, and analyses. Descriptive statistics were carried



Fig. 1. Representative hematoxylin and eosin (H&E) (upper panels) and immunohistochemistry (lower panels) images of kidney tissues. (A) Kidney tissues showing diffuse and strong vimentin (VIM) expression in areas of chronic tubular damage (patients with chronic kidney disease). (B) Kidney tissues showing low and high VIM expression. Slides were stained with H&E and VIM stains, and images were examined at ×400.

out and reported as frequencies and percentages for categorical variables and as means±standard deviations for continuous variables. Characteristics of patients and other clinicopathological parameters among the two studied groups (VIM < 3%, low, and  $\geq$  3%, high) were tabulated. Baseline comparisons between the two studied groups were performed using the Mann-Whitney U test for continuous variables. The chi-square test was used to assess any significant association between the categorical variables. Thereafter, univariate logistic regression was used to determine the associations between VIM (low/high) as a dependent variables. The level of significance was set at p < .05 for all statistical analyses.

### RESULTS

#### VIM expression and its correlation with clinical characteristics

A total of 23 patients was selected based on the inclusion criteria. From this group, high VIM expression ( $\geq 3\%$ ) was observed in 16 patients (Fig. 2). Among our patient cohort, 13 (56.5%) were females and 10 (43.5%) were males. Patients were divided into two age groups,  $\leq 65$  years (7 patients, 30.4%) and > 65years (16 patients, 69.6%). The patients were distributed according to cause of death: cardiac (4 patients, 17.4%), respiratory (6 patients, 26.1%), stroke (2 patients, 8.7%), sepsis (7 patients, 30.4%), and other (3 patients, 13.0%). There were 16 patients (69.6%) with no CKD and seven patients (30.4%) with CKD. Fold eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation [28]. Also, fold creatinine was estimated using the lowest value recorded during or before hospitalization and the highest value recorded during hospitalization. Chronic hemodialysis was found in one patient (4.3%). Sixteen patients (69.6%) did not have diabetes mellitus, and 16 patients (69.6%) had hypertension. Shock during hospitalization was present in 10 patients (43.5%). Clinical evidence of acute kidney injury was documented in 13 patients (56.5%) (Table 1). No statistically significant difference was found between patients regarding age, gender, cause of death, CKD, CKD stage, chronic hemodialysis, diabetes mellitus, hypertension, shock during hospitalization, fold eGFR, fold creatinine, or clinical evidence of acute kidney injury with respect to VIM expression.

#### VIM expression is significantly correlated with AKI and RTR

We next sought to assess the correlation between VIM expression sion and other histopathologic variables. High VIM expression was significantly associated with arteriosclerosis, arteriosclerosis severity, and glomerulosclerosis (p = .005, p = .002, and p = .033, respectively) (Table 1). Kidney tissues with pathologic evidence of ATN and RTR had significantly higher VIM expression (p = .036 and p = .007, respectively) (Table 1, Fig. 2). Also, renal peritubular microenvironment features showing regenerative changes on H&E were associated with high VIM expression (p = .009) (Table 1). In univariate models, kidney tissues with evidence of AKI and RTR were 15.00-fold more likely to have high VIM expression (odds ratio [OR], 7.500; 95% confidence interval [CI],



Fig. 2. Correlation between vimentin (VIM) expression on one hand and pathologic evidence of acute kidney injury (AKI) (A) and renal tubular regeneration (RTR) (B) on the other hand. Kidney tissues with evidence of AKI and RTR had significantly higher VIM expression (p=.065 and p=.017, respectively). Chi-square test was used to assess significant association between the two variables (\*p<.05).

Table 1. Correlation between vimentin expression and acute kidney injury

Verieble	Tatal	Vimentin expression		n voluo
Variacie	IOLAI	Low < 3%	High ≥3%	p-value
Age (mean±SD)	$69.00 \pm 15.03$	$57.86 \pm 15.96$	$73.88 \pm 12.09$	.015
Age (yr)				.066
≤65	7 (30.4)	4 (57.1)	3 (42.9)	
>65	16 (69.6)	3 (18.8)	13 (81.3)	
Sex				.382
Female	13 (56.5)	3 (23.1)	10 (76.9)	
Male	10 (43.5)	4 (40.0)	6 (60.0)	
Cause of death				.777
Cardiac	4 (17.4)	1 (25.0)	3 (75.0)	
Respiratory	6 (26.1)	2 (33.3)	4 (66.7)	
Stroke	2 (8.7)	1 (50.0)	1 (50.0)	
Sepsis	7 (30.4)	2 (28.6)	5 (71.4)	
Other	3 (13.0)	0	3 (100)	
CKD	× ,			.266
No	16 (69.6)	6 (37.5)	10 (62.5)	
Yes	7 (30.4)	1 (14.3)	6 (85.7)	
CKD stage	· · · ·		( )	.792
1	-	-	-	
2	1 (14.3)	0	1 (100)	
3	5 (71.4)	1 (20.0)	4 (80.0)	
4	1 (14.3)	0	1 (100)	
5	-	-	-	
Fold eGFB				952
<-0.5	7 (38 9)	2 (28 6)	5 (71 4)	1002
>_0.5	11 (61 1)	3 (27.3)	8 (72 7)	
Fold creatinine	11 (01.1)	0 (21.0)	0 (12.1)	457
	12 (66 7)	1 (33 3)	8 (66 7)	.407
>20	6 (33 3)	1 (16.7)	5 (83 3)	
Chronic hemodialysis	0 (00.0)	1 (10.7)	0 (00.0)	100
No	22 (05 7)	7 (21 9)	15 (69.2)	.499
Voc	1 (4 2)	0 (0 0)	1 (100)	
Teo Diabatao maliitua	1 (4.3)	0 (0.0)	1 (100)	066
Na	10 (00 0)	C (07 E)	10 (C0 E)	.200
NO	7 (20, 4)	1 (14.0)	10 (02.3)	
Tes	7 (30.4)	1 (14.3)	0 (65.7)	000
Hypertension	7 (00 4)	0 (00 0)		.898
NO	7 (30.4)	2 (28.6)	5 (71.4)	
Yes	16 (69.6)	5 (31.3)	11 (68.8)	
Shock during hospitalization		4 (22.2)	0 (00 0)	.968
NO	13 (56.5)	4 (30.8)	9 (69.2)	
Yes	10 (43.5)	3 (30.0)	7 (70.0)	
Clinical evidence of AKI	- ()	- />	_ /	.493
No	9 (39.1)	2 (22.2)	7 (77.8)	
Yes	14 (60.9)	5 (35.7)	9 (64.3)	
AKI type				.603
No	9 (39.1)	2 (22.2)	7 (77.8)	
Prerenal	10 (43.5)	3 (30.0)	7 (70.0)	
Renal	4 (17.4)	2 (50.0)	2 (50.0)	
Arteriosclerosis				.005
No	3 (13.0)	3 (100.0)	0	
Yes	20 (87.0)	4 (20.0)	16 (80.0)	

(Continued to the next page)

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#### Table 1. Continued

Veriela	Total Nia of restingto	Vimentin expression			
Variable	Iotal No. of patients	Low < 3%	High ≥3%	p-value	
Arteriolosclerosis				.062	
No	13 (56.5)	6 (46.2)	7 (53.8)		
Yes	10 (43.5)	1 (10.0)	9 (90.0)		
Arteriosclerosis severity				.002	
Intimal thickening < media thickness	12 (52.2)	7 (58.3)	5 (41.7)		
Intimal thickening ≥ media thickness	11 (47.8)	0	11 (100)		
Glomerulosclerosis				.033	
<10% globally sclerotic glomeruli	12 (52.2)	6 (50.0)	6 (50.0)		
≥10% globally sclerotic glomeruli	11 (47.8)	1 (9.1)	10 (90.9)		
Glomerulosclerosis severity (%)				.571	
<10	17 (73.9)	6 (35.3)	11 (64.7)		
10–50	4 (17.4)	1 (25.0)	3 (75.0)		
>50	2 (8.7)	0	2 (100)		
Significant interstitial fibrosis				.066	
No	7 (30.4)	4 (57.1)	3 (42.9)		
Yes	16 (69.6)	3 (18.8)	13 (81.3)		
Interstitial fibrosis severity (%)				.247	
<10	18 (78.3)	7 (38.9)	11 (61.1)		
10–50	4 (17.4)	0	4 (100)		
>50	1 (4.3)	0	1 (100)		
Tubular atrophy (%)				.328	
<10	21 (91.3)	7 (33.3)	14 (66.7)		
≥10	2 (8.7)	0	2 (100)		
Tubular atrophy severity (%)				.619	
<10	21 (91.1)	7 (33.3)	14 (66.7)		
10–50	1 (4.3)	0	1 (100)		
>50	1 (4.3)	0	1 (100)		
Pathologic evidence of AKI				.036*	
No	9 (39.1)	5 (55.6)	4 (44.4)		
Yes	14 (60.9)	2 (14.3)	12 (85.7)		
Tubulorrhexis				.226	
No	17 (73.9)	4 (23.5)	13 (76.5)		
Yes	6 (26.1)	3 (50.0)	3 (50.0)		
Pathologic evidence of ATN				.221	
No	12 (52.2)	5 (41.7)	7 (58.3)		
Yes	11 (47.8)	2 (18.2)	9 (81.8)		
Evidence of regenerating tubular epithelium				.007	
No	10 (43.5)	6 (60.0)	4 (40.0)		
Yes	13 (56.5)	1 (7.7)	12 (92.3)		
Renal peritubular microenvironment features on H&E (cortical)				.009	
No changes	4 (17.4)	4 (100.0)	0		
Reactive/regenerative changes in AKI	11 (47.8)	2 (18.2)	9 (81.8)		
Consistent with early chronic changes (tubular atrophy/interstitial fibrosis)	3 (13.0)	0	3 (100)		
Areas with multinucleated cells	5 (21.7)	1 (20.0)	4 (80.0)		
Renal peritubular microenvironment features on PAS (cortical)	/			.416	
No changes	15 (65.2)	6 (40.0)	9 (60.0)		
lubulorrhexis	3 (13.0)	1 (33.3)	2 (66.7)		
Fragmentation/duplication of basement membrane	3 (13.0)	0	3 (100)		
I hickened basement membrane	2 (8.7)	0	2 (100)		

Values are presented as number (%) unless otherwise inidcated.

Significant p-value < .05.

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; AKI, acute kidney injury; ATN, acute tubular necrosis; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff.

	Univariate analysis		
Variable	OR	95% CI	p-value
Age (yr)			
≤ 65	1	-	-
> 65	5.778	0.819-40.760	.078
Sex			
Female	1	-	-
Male	0.450	0.074-2.741	.386
Cause of death			
Sepsis	1	-	-
Respiratory	0.800	0.076-8.474	.853
Cardiac	1.200	0.073-19.631	.898
Stroke	0.400	0.061-10.017	.577
Other	-	-	-
CKD			
No	1	-	-
Yes	3.600	0.345–37.616	.285
Fold eGFR			
<-0.5	1	-	-
≥ -0.5	1.067	0.129-8.793	.952
Fold creatinine			
< 2.0	1	-	-
>2.0	2.500	0.214-29.254	.465
Diabetes mellitus			
No	1	-	-
Yes	3.600	0.345-37.616	.285
Hypertension			
No	1	-	-
Yes	0.880	0.125-6.192	.898
Shock during hospitalization			
No	1	-	-
Yes	1.037	0.173-6.233	.968
AKI			
No	1	-	-
Yes	0.514	0.076-3.488	.496
AKI type			
No	1	-	-
Prerenal	0.667	0.084-5.301	.702
Benal	0.286	0.023-3.523	.328
Arteriolosclerosis	0.200	0.020 0.020	.020
No	1	_	_
Vac	7 714	0 746-79 771	087
Glomerulosolorosis		0.140 10.111	.007
< 10% globally seleratic glomeruli	1		
	10 000	0.957_10/./90	054
2 10% globally sciencic glotheruli	10.000	0.307-104.430	.004
	1		
< 10	1 626	0 129 10 297	-
\u0-00 > 50	1.000	0.100-19.007	.030
>UU Cignificant interatitial fibracia	-	-	-
Significant interstitial librosis	4		
NO Mar			-
res	5.//8	0.819-40.760	.078

Table 2. Results of the logistic regression analysis with vimentin expression as a dependent variable (univariate analysis)

(Continued to the next page)

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#### Table 2. Continued

M. 2010	Univariate analysis			
variable	OR	95% CI	p-value	
Evidence of AKI				
No	1	-	-	
Yes	7.500	1.023-54.996	.047	
Tubulorrhexis				
No	1	-	-	
Yes	0.308	0.044-2.171	.237	
Evidence of ATN				
No	1	-	-	
Yes	3.214	0.474-21.800	.232	
Evidence of regenerating tubular epithelium				
No	1	-	-	
Yes	18.000	1.632-198.508	.018	
Microenvironment features on H&E (cortical)				
Reactive/regenerative in AKI	1	-	-	
Areas with multinucleated cells	0.889	0.061-12.885	.931	
Consistent with chronic changes (tubular atrophy/interstitial fibrosis)	-	-	-	
No changes	-	-	-	
Microenvironment features on PAS (cortical)				
No changes	1	-	-	
Tubulorrhexis	1.333	0.098-18.192	.829	
Fragmentation/duplication of basement membrane	-	-	-	
Thickened basement membrane	-	-	-	

OR, odds ratio; CI, confidence interval; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; AKI, acute kidney injury; ATN, acute tubular necrosis; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff. Significant p-value <.05.

1.023 to 54.996; p = .047 and OR, 18.000; 95% CI, 1.632 to 198.508; p = .018, respectively) (Table 2).

#### DISCUSSION

In this study, we showed a significant association between VIM expression by cortical tubular epithelia and RTR in the setting of AKI/ATN. First, VIM can be expressed by cortical tubular epithelia, but never by medullary epithelia, in diverse histologic landscapes. The cortical tubular epithelium of degenerated nephrons with patchy interstitial fibrosis, glomerulosclerosis, and tubular atrophy can display widespread VIM expression. For instance, severely impaired tubules with hyaline casts, so-called tubular thyroidization, exhibited diffuse and strong positivity for VIM (Fig. 1A). This aberrant VIM expression in CKD represented an obstacle to evaluating RTR in AKI. As an attempt to explore the relationship between VIM expression and RTR, we quantify VIM immunoreactivity in areas without chronic damage or tissue remodeling (Fig. 1B). Thus, any VIM expression by the cortical tubular epithelium of preserved nephrons can be considered a response to acute injury. In fact, the most severe cases of ATN displayed widespread VIM expression in tubular epithelial cells, a finding that has been observed in animal models [29]. Although the fold changes in creatinine and eGFR correlated with histologic evidence of AKI/ATN, they were not significantly associated with VIM expression in our cohort. This might be because the autopsies were performed at different stages of AKI. In fact, we hypothesize that, in some cases, VIM expression was low because there was not enough time to develop a regenerative response in the tubules.

The VIM evaluation criteria proposed in our study are based on the number of positive tubules (defined as more than 50% of the tubular epithelial cells showing positive gold-brown cytoplasmic stain within each tubule) in an average of 30 tubules (counted randomly within 10 HPFs) and dividing this number by the total number of tubules assessed, multiplied by 100. This percentage is referred to as the VIM score. A VIM score < 3% signifies low VIM expression; a VIM score  $\geq$  3% signifies high VIM expression. After systematic quantification, VIM was an independent predictor for RTR with 18-fold risk. There was no statistically significant difference between patients with regard to clinical characteristics. VIM, also known as fibroblast intermediate filament, is the major intermediate filament found in stromal cells [30]. These cell types include fibroblasts, endothelial cells, macrophages, melanocytes, Schwann cells, and lymphocytes [31]. This is in contrast to keratin, which is the intermediate filament found in epithelial cells. Studies have shown that undifferentiated cells of the metanephric mesenchyme express VIM but not cytokeratins [32]. Tubular epithelial cells, however, do not express VIM at any developmental stage [32]. This supports the notion that the presence of VIM-positive cells in the kidney exemplifies the mesenchymal phenotype where cells undergo EMT [33]. A study by Mezni et al. [33] demonstrated that tubular expression of VIM is a good marker of EMT and can predict long-term renal graft fibrosis.

As we mentioned earlier, there is evidence in the literature of renal tubular epithelial regeneration post-injury. A study by Vogetseder et al. [34] and another by Fujigaki et al. [19] showed that dividing cells in the S3 segment of proximal tubules and in the distal tubules had a basolateral expression of Na-K-ATPase (a marker of terminal epithelial differentiation) at the same level as neighboring non-proliferating cells. These cells survived after injury carrying intact nuclei, actively proliferating, and expressing VIM. Such cells can be referred to as progenitor cells of the kidney [35]. In 2003, Maeshima et al. identified progenitor-like cells present throughout the renal tubules of adult rats via BrdU labeling [36]. Thereafter, more studies followed, proving the existence of progenitor/stem cell-like cells in the adult kidney [37,38]. Kitamura et al. [37], for instance, isolated a cell line (rKS56) with a high proliferative potential from adult rat kidneys. Interestingly, Bussolati et al. [38] recently discovered CD133+ progenitor cells within the adult human kidney and expressing the embryonic kidney marker PAX2. Those cells were capable of expansion and self-renewal in vitro.

Several studies have described this subpopulation of cells having stem cell/progenitor properties different from those of normal epithelial cells [29,38,39]. The main markers of this population were CD24, CD133, and VIM. These progenitor cells were scattered throughout the proximal tubule in the normal human kidney [29]. Compared to conventional epithelial cells, progenitor cells contain less cytoplasm, fewer mitochondria, and have no brush border [29]. Regenerating tubular epithelium is likely a collage of expanded progenitor cells with diverse stages of maturation/differentiation. VIM expression in regenerating tubules could be secondary to activated transitory EMT programs prior to full functional and morphologic recovery [29,40]. Reviewing the literature on RTR and nephrogenic markers of regeneration reveals some pre-clinical in vitro and in vivo studies and a few other studies conducted on humans. Hansson et al. [41] used transmission electron microscopy, immunoelectron microscopy, and immunofluorescence of the human kidney cortex

to explore progenitor-like cells following injury. Regenerating tubules demonstrated expression of progenitor-cell markers such as CD133, VIM, KRT19, and CLDN1 [41]. Vansthertem et al. [42] showed some regenerating cells in renal tubules after ischemia and expressing BrdU, CD44, and VIM could originate from an extrarenal source and reach the renal parenchyma via blood vessels [42].

Our study demonstrates, with statistically significant evidence (p = .018), the correlation between RTR and high VIM expression. This further supports what has been previously observed in other studies regarding the validity of VIM as a potential regenerative biomarker. In our study, we found correlation between VIM expression and different features of AKI other than histologic findings such as arteriosclerosis, arteriolosclerosis, and glomerulosclerosis. The meaning of VIM expression in CKD is more ambiguous. Interestingly, VIM-expressing epithelial cells are encountered, in addition to CKD, in many other chronic diseases characterized by parenchymal loss and tissue fibrosis, such as idiopathic pulmonary fibrosis, cirrhosis, and scleroderma, among others [40,43-45]. Persistence of VIM-expressing epithelia has been associated with organ dysfunction and fibrogenesis [46]. Further studies can elucidate the role of VIM expression in CKD.

We acknowledge that our study has several limitations. First, in the case of an autopsy, searching the entire kidney specimen and performing immunohistochemistry on a whole section slide is possible, although in practice, one representative section usually is submitted and retained for a specific time period (three months after the final report for wet tissues in non-forensic autopsies, according to the College of American Pathologists recommendations for the minimum requirements for retention of laboratory records and materials [47]). Hence, it is not possible to acquire further sections from the kidney specimens. Second, since we used kidney tissues from deceased patients, autolysis can alter the reactivity to IHC assays. In a few tested autolyzed tissues, VIM immunoreactivity decreases with autolytic changes. Interstitial and glomerular stromal cells served as positive internal controls. Third, due to the retrospective nature of our study, selection bias and performance bias were inevitable. Lastly, our patient cohort was selected from a single medical center and was a relatively small sample size; therefore, future studies are warranted from multiple centers to verify the results on a larger population of patients. Nevertheless, future studies would be of interest to evaluate the clinical significance of VIM expression in renal biopsies from living patients to assess adequately RTR.

#### Conclusion

There is a need to identify novel biomarkers for RTR. Our findings suggest that VIM could serve as an excellent potential IHC marker for RTR following AKI in the right clinical and morphologic context, especially important since regenerative responses are difficult to appreciate on routine light microscopy. Nonetheless, correlation with H&E findings remains critical to exclude chronic tubular damage. Collectively, our preliminary results pave the way for future studies of larger sample size to validate the use of VIM as a reliable biomarker for RTR.

#### **Ethics Statement**

All procedures performed in the current study were performed in accordance with the 1964 Helsinki declaration and its later amendments. Formal written informed consent and approval by the Institutional Review Board (IRB) of Mount Sinai Medical Center of Florida were not required since the included patients were deceased at the time of the study. Chart review was carried out by CITI (Collaborative Institutional Training Initiative)certified physicians.

#### Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

#### **Code Availability**

Not applicable.

#### ORCID

Juan Carlos Alvarez Moreno Hisham F. Bahmad Christopher A. Febres-Aldana Andrés Pirela Andres Azuero Ali Salami Robert Poppiti https://orcid.org/0000-0001-7517-7044 https://orcid.org/0000-0003-3799-2595 https://orcid.org/0000-0002-4754-1344 https://orcid.org/0000-0001-5884-4909 https://orcid.org/0000-0002-4601-8383 https://orcid.org/0000-0003-3343-4035 https://orcid.org/0000-0003-2561-2583

#### **Author Contributions**

Conceptualization: RP. Data curation: JCAM, HFB, AP, AA. Formal analysis: AS. Investigation: JCAM, HFB, CAFA, AP, AA. Methodology: JCAM, HFB, AS. Project administration: RP, JCAM. Resources: JCAM. Software: AS. Supervision: RP. Validation: CAFA, RP. Visualization: JCAM, HFB, CAFA, RP. Writing—original draft: JCAM, HFB. Writing—review & editing: CAFA, AP, AA. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

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# A multicenter study of interobserver variability in pathologic diagnosis of papillary breast lesions on core needle biopsy with WHO classification

Hye Ju Kang<sup>1</sup>, Sun Young Kwon<sup>2</sup>, Ahrong Kim<sup>3</sup>, Woo Gyeong Kim<sup>4</sup>, Eun Kyung Kim<sup>5</sup>, Ae Ree Kim<sup>6</sup>, Chungyeul Kim<sup>6</sup>, Soo Kee Min<sup>7</sup>, So Young Park<sup>8</sup>, Sun Hee Sung<sup>9</sup>, Hye Kyoung Yoon<sup>10</sup>, Ahwon Lee<sup>11</sup>, Ji Shin Lee<sup>12</sup>, Hyang Im Lee<sup>13</sup>, Ho Chang Lee<sup>14</sup>, Sung Chul Lim<sup>15</sup>, Sun Young Jun<sup>16</sup>, Min Jung Jung<sup>17</sup>, Chang Won Jung<sup>18</sup>, Soo Youn Cho<sup>19</sup>, Eun Yoon Cho<sup>19</sup>, Hye Jeong Choi<sup>20</sup>, So Yeon Park<sup>21</sup>, Jee Yeon Kim<sup>22</sup>, In Ae Park<sup>23</sup>, Youngmee Kwon<sup>1</sup>

<sup>1</sup>Department of Pathology, National Cancer Center, Goyang; <sup>2</sup>Department of Pathology, Keimyung University Dongsan Medical Center, Daegu; <sup>3</sup>Department of Pathology, Pusan National University Hospital, Busan; <sup>4</sup>Department of Pathology, Inje University Haeundae Paik Hospital, Busan; <sup>5</sup>Department of Pathology, Eulji University Hospital, Seoul; <sup>6</sup>Department of Pathology, Korea University Guro Hospital, Seoul; <sup>7</sup>Department of Pathology, Hallym University Sacred Heart Hospital. Anvana: <sup>8</sup>Department of Pathology, MizMedi Hospital, Seoul; <sup>9</sup>Department of Pathology, Ewha Womans University Mokdong Hospital, Seoul; <sup>10</sup>Department of Pathology, Inje University Busan Paik Hospital, Busan; <sup>11</sup>Department of Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul: <sup>12</sup>Department of Pathology, Chonnam National University Hwasun Hospital, Hwasun; <sup>13</sup>Department of Pathology, Gyeongsang National University Changwon Hospital, Changwon; <sup>14</sup>Department of Pathology. Chungbuk National University Hospital. Cheongiu: <sup>15</sup>Department of Pathology, Chosun University Hospital, Gwangju; <sup>16</sup>Department of Pathology, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon; <sup>17</sup>Department of Pathology, Kosin University Gospel Hospital, Busan; <sup>18</sup>Department of Pathology, Green Cross Laboratories, Yongin; <sup>19</sup>Department of Pathology, Sungkyunkwan University Samsung Medical Center, Seoul; <sup>20</sup>Department of Pathology, Ulsan University Hospital, Ulsan; <sup>21</sup>Department of Pathology, Seoul National University Bundang Hospital, Seongnam; <sup>22</sup>Department of Pathology, Pusan National University Yangsan Hospital, Yangsan; <sup>23</sup>Department of Pathology, Seoul National University Hospital, Seoul, Korea

**Background:** Papillary breast lesions (PBLs) comprise diverse entities from benign and atypical lesions to malignant tumors. Although PBLs are characterized by a papillary growth pattern, it is challenging to achieve high diagnostic accuracy and reproducibility. Thus, we investigated the diagnostic reproducibility of PBLs in core needle biopsy (CNB) specimens with World Health Organization (WHO) classification. **Methods:** Diagnostic reproducibility was assessed using interobserver variability (kappa value,  $\kappa$ ) and agreement rate in the pathologic diagnosis of 60 PBL cases on CNB among 20 breast pathologists affiliated with 20 medical institutions in Korea. This analysis was performed using hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining for cytokeratin 5 (CK5) and p63. The pathologic diagnosis of PBLs was based on WHO classification, which was used to establish simple classifications (4-tier, 3-tier, and 2-tier). **Results:** On WHO classification, H&E staining exhibited 'fair agreement' ( $\kappa$ =0.21) with a 47.0% agreement rate. Simple classifications presented improvement in interobserver variability and agreement rate. IHC staining increased the kappa value and agreement rate in all the classifications. Despite IHC staining, the encapsulated/solid papillary carcinoma (EPC/SPC) subgroup ( $\kappa$ =0.16) exhibited lower agreement compared to the non-EPC/SPC subgroup ( $\kappa$ =0.35) with WHO classification, which was similar to the results of any other classification systems. **Conclusions:** Although the use of IHC staining for CK5 and p63 increased the diagnostic agreement of PBLs in CNB specimens. **Conclusions:** Although the use of IHC staining for CK5 and p63 increased the diagnostic agreement of PBLs in CNB specimens, WHO classification exhibited a higher discordance rate compared to any other classifications. Therefore, this result warrants further intensive consensus studies to improve the diagnostic reproducibility of PBLs with WHO classification.

Key Words: Papillary breast lesion; Core needle biopsy; Interobserver variability; Agreement rate

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Corresponding Author: Youngmee Kwon, MD, PhD, Department of Pathology, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang 10408, Korea Tel: +82-31-920-1742, Fax: +82-31-920-1369, E-mail: ymk@ncc.re.kr

Papillary breast lesions (PBLs) encompass a broad spectrum of proliferative diseases that account for less than 3% of breast tumors [1-3]. The histologic features of PBLs include mass-like projections attached to the wall of the dilated ducts and have a fibrovascular stalk lined by epithelial cells. PBLs can be both benign and malignant lesions, representing less than 10% of benign breast lesions and less than 2% of all breast cancers [4,5], respectively.

Ultrasound-guided core needle biopsy (CNB) is universally used in the initial pathologic approach for suspicious radiologic findings in breast lesions. PBLs constitute approximately 4.5% to 10.7% of breast lesions diagnosed on CNB [6,7]. The 5th edition of the WHO classification of tumors of the breast is the most recently updated version for pathologically diagnosing PBLs [8]. Compared with the 4th edition of the WHO classification of tumors of the breast there have been little or no changes since 2012 in terms of the diagnostic criteria and classification of PBLs [8,9]. However, differential diagnosis of PBLs remains challenging due to the limited samples obtained from CNB. The difficulty in pathologic diagnosis of PBLs increases due to the broad spectrum of histological findings and subtle differences exemplifying each category [1-3]. Moreover, the lack of reliable and reproducible criteria of its diagnosis and classification may limit diagnostic accuracy [10].

Several studies have sought to promote the interpretation reproducibility of PBLs among pathologists as an endeavor to improve diagnostic accuracy. Immunohistochemical (IHC) staining has significantly increased diagnostic agreement rates among pathologists who have exhibited unsatisfactory findings on hematoxylin and eosin (H&E) staining [11-13]. These studies suggest that additional histopathologic modalities are potentially useful in increasing the diagnostic agreement rate. Nonetheless, these studies have limitations in generalization because their results were derived from analysis among very few pathologists from a single institution [11-13]. Moreover, there is little data about the agreement rate of PBLs based on the WHO classification.

To evaluate the diagnostic reproducibility of PBLs on CNB based on the WHO classification, we investigated the interobserver variability among 20 breast pathologists working in 20 medical institutions. We intended to compare the interobserver variability between H&E and IHC stains and specify the diagnostic pitfalls in the differential diagnosis of challenging cases.

### MATERIALS AND METHODS

#### Study design and case selection

We evaluated the interobserver variability and agreement rates in 60 PBL cases on CNB among 20 breast pathologists affiliated with 20 medical institutions in Korea. Sixty PBL cases were recruited from 20 medical institutions that participated in this study. The consensus meeting of the Korean Breast Pathology Study Group (KBPSG) verified and determined the pathologic diagnosis of 60 PBL cases on CNB. Fig. 1 displays the composition of pathologic diagnoses in all 60 PBL cases. Each case constitutes one H&E and two IHC stained slides for both cytokeratin 5 (CK5) and p63. Initially, 60 H&E-stained slides were circulated to 20 breast pathologists for review. Subsequently, IHC stained slides for CK5 and p63 in the same 60 cases were circulated to the same 20 breast pathologists and re-reviewed. Interobserver variability and agreement rates were analyzed for the pathologic diagnosis of PBLs in H&E and IHC stains. Additionally, we conducted a detailed review of the challenging cases of differential diagnoses observed among our 60 PBL cases.

#### Diagnostic classification of PBLs

Pathologic classification of PBLs was conducted based on the 4th edition of the *WHO classification of tumors of the breast* [9]. In this classification [9], the PBLs were classified into 10 categories



Fig. 1. Composition of the pathologic diagnosis in all 60 papillary breast lesions. SPC, solid papillary carcinoma; IDP, intraductal papilloma; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; PCIS, papillary carcinomas in situ; EPC, encapsulated papillary carcinoma; SPC, solid papillary carcinoma.

comprising intraductal papillomas (IDP), IDPs with atypical ductal hyperplasia (ADH), IDPs with ductal carcinomas in situ (DCIS), IDPs with lobular carcinomas in situ (LCIS), papillary carcinomas in situ (PCIS), encapsulated papillary carcinomas (EPC), solid papillary carcinomas (SPC), EPCs with invasion, SPCs with invasion, and invasive papillary carcinomas (IPC). Of the WHO classification, intraductal papillary neoplasms (IDPN) were defined as a category including IDP, IDP with ADH, IDP with DCIS, IDP with LCIS, and PCIS. EPC and SPC were categorized into EPC/SPC.

In addition, using the WHO classification, we created simple classifications of PBL using 4-tier, 3-tier, and 2-tier systems as follows: 4-tier consisted of benign, atypical, in situ, and invasive; 3-tier consisted of benign, in situ, and invasive; 2-tier consisted of benign and malignant (Table 1). For instance, if EPC was diagnosed, it was categorized into in situ in the 4-tier system and malignant in the 2-tier system.

#### Immunohistochemistry

For each CNB specimen of cases, IHC staining for CK5 and p63 was performed. IHC staining for CK5 was conducted using antibodies against CK5 (XM26, Leica Biosystems, Newcastle upon Tyne, UK) with 1:200 antibody dilution and a detection kit (Ivew DAB kit, Ventana, Tucson, AZ, USA). IHC staining for p63 was performed using antibodies against p63 (BC4A4, Biocare Medical, Pacheco, CA, USA) with 1:100 antibody dilution and a detection kit (Ultraview DAB kit, Ventana). According to the manufacturer's protocol, all the procedures of IHC staining were processed by a Ventana BenchMark XT system (Ventana).

 Table 1. Diagnostic categories for PBL in the WHO, 4-tier, 3-tier, and 2-tier classifications

WHO	4-tier	3-tier	2-tier
Intraductal papilloma	Benign	Benign	Benign
IDP with ADH	Atypical		
IDP with DCIS	In situ	In situ	Malignant
IDP with LCIS			
Papillary carcinoma in situ			
Encapsulated papillary carci-			
noma			
Solid papillary carcinoma			
EPC with invasion	Invasive	Invasive	
SPC with invasion			
Invasive papillary carcinoma			

PBL, papillary breast lesion; WHO, World Health Organization; IDP, intraductal papilloma; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ; EPC, encapsulated papillary carcinoma; SPC, solid papillary carcinoma.

#### Statistical analysis

Fleiss's kappa values for interobserver variability were used in analyzing diagnostic reproducibility in H&E and IHC staining among 20 breast pathologists. Interobserver variability was classified into five categories (0.00–0.20, poor; 0.21–0.40, fair; 0.41– 0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, excellent agreement) to identify the level of reproducibility. Additionally, the average of the agreement rates in H&E and IHC staining of 60 cases was calculated in four diagnostic classifications. The agreement rate was determined by the proportion of pathologic diagnosis from 20 pathologists that was consistent with that from the consensus meeting of KBPSG. Statistical analyses were performed using STATA ver. 16.0 (StataCorp LP, College Station, TX, USA).

#### RESULTS

Interobserver variability and agreement rates in H&E and IHC staining in each classification are presented in Table 2. In the WHO classification, H&E staining exhibited 'fair agreement' ( $\kappa$  = 0.21). Kappa values increased inversely with the number of categories in the diagnostic classification (4-tier:  $\kappa$  =0.31, 3-tier:  $\kappa$  =0.42, and 2-tier:  $\kappa$  =0.44). IHC staining improved the interobserver variability in all classifications. In IHC staining, overt improvement in reproducibility was observed in 4-tier ('fair agreement' to 'moderate agreement') and 2-tier ('moderate agreement' to 'substantial agreement'). The agreement rate also exhibited similar findings with kappa values for interobserver variability. The agreement rate was generally higher in IHC staining compared to H&E staining in all classifications. Within the same staining methods, simpler diagnostic classification tended to have a higher agreement rate.

Fig. 2 shows the interobserver variability in H&E staining for all 60 PBL cases, IDPN, and EPC/SPC in each classification. There were 48 cases of IDPN and 12 cases of EPC/SPC in this

Table 2. The interobserver variability and agreement rates in H&E and IHC staining of 60 PBL cases in each diagnostic classification

	H&	E staining	IHC staining		
Diagnostic classification	к	Agreement rate (%)	к	Agreement rate (%)	
WHO	0.21	47.0	0.37	60.5	
4-tier	0.31	63.3	0.51	76.4	
3-tier	0.42	76.7	0.56	84.3	
2-tier	0.44	80.0	0.62	87.5	

H&E, hematoxylin and eosin; IHC, immunohistochemistry; PBL, papillary breast lesion; WHO, World Health Organization classification; 4-tier, 4-tier classification; 3-tier, 3-tier classification; 2-tier, 2-tier classification.



Fig. 2. Interobserver variability in the pathologic diagnoses of all 60 papillary breast lesions cases, intraductal papillary neoplasms (IDPN), and encapsulated/solid papillary carcinoma (EPC/SPC) with hematoxylin and eosin staining in all classifications. WHO, World Health Organization classification; 4-tier, 4-tier classification; 3-tier, 3-tier classification; 2-tier, 2-tier classification.



Fig. 3. Interobserver variability in the pathologic diagnoses of all 60 papillary breast lesions cases, intraductal papillary neoplasms (IDPN), and encapsulated/solid papillary carcinoma (EPC/SPC) with immunohistochemical staining in all classifications. WHO, World Health Organization classification; 4-tier, 4-tier classification; 3-tier, 3-tier classification; 2-tier, 2-tier classification.

study. Kappa values for IDPN and EPC/SPC were lower than that for all 60 PBL cases in all classifications. IDPN had lower reproducibility than all the 60 PBL cases, despite the same reproducibility ('fair agreement') in 4-tier. EPC/SPC exhibited the lowest kappa value with 'poor agreement' (WHO:  $\kappa = 0.13$ , 4-tier:  $\kappa = 0.03$ , 3-tier:  $\kappa = 0.05$ , and 2-tier:  $\kappa = 0.05$ ).

IHC staining generally improved the interobserver reliability in all 60 PBL cases and IDPN in all classifications (Fig. 3). The reproducibility of IDPN improved to the same level of all 60 PBL cases except that in 2-tier (all 60 PBL cases: 'substantial agreement' and IDPN: 'moderate agreement'). However, the kappa values were lowest in EPC/SPC with 'poor agreement' (WHO:  $\kappa = 0.16$ , 4-tier:  $\kappa = 0.04$ , 3-tier:  $\kappa = 0.05$ , and 2-tier:  $\kappa = 0.06$ ) similar to that in H&E staining, which demonstrated that IHC staining did not improve the diagnostic agreement of EPC/SPC in contrast with all 60 PBL cases and IDPN.

In our 60 cases, five cases were particularly challenging for differential diagnosis with a relatively high discordance rate (Table 3). The presence of apocrine metaplasia (Supplementary Fig. S1) and flat epithelial atypia-like features (Supplementary Fig. S2) made it difficult to distinguish benign from malignant intraductal lesions. Regarding the differential diagnosis between in situ and invasive lesions, we found three challenging cases including one large cystic mass with no myoepithelial cells along the papillae (Supplementary Fig. S3), one with a solid multinodular pattern and smooth contours (Supplementary Fig. S4), and one with a predominant solid multinodular and jigsaw pattern (Supplementary Fig. S5).

#### DISCUSSION

For 60 PBL cases obtained from CNB, we assessed the interobserver variability and agreement rates in pathologic diagnoses among 20 breast pathologists. In an analysis with the WHO classification, pathologic diagnosis in H&E staining showed 'fair agreement' ( $\kappa = 0.21$ ) with an agreement rate of 47.0%. This result is comparable to those of previous studies in line with ours. In H&E staining for 57 cases of PBLs, three pathologists demonstrated a substantial agreement ( $\kappa = 0.79$ ) in reproducibility and an 86% agreement rate with seven diagnostic categories [11]. Additionally, an analysis with five diagnostic categories indicated moderate agreement ( $\kappa = 0.54$ ) and a 44% agreement rate in 129 PBL cases by H&E staining among four pathologists [12].

Compared with the previous results, it seems that our kappa values and agreement rates are relatively low. The plausible explanations for this finding may be the number of pathologists and the complexity of the diagnostic categories. Our study was performed to assess interobserver variability within 10 diagnostic categories among 20 pathologists. The number of pathologists and diagnostic categories is greater than those of other studies conducted with three pathologists with seven categories [11] and four pathologists with five categories [12].

It seems that the greater number of pathologists makes it harder to obtain a consistent diagnosis for any lesion compared to fewer pathologists. Moreover, a more complicated diagnostic category contributes to lower reproducibility as found in our study. We observed improved reproducibility in simple diagnostic categories, showing the highest kappa value (0.44) and agreement rate (80.0%) in 2-tier. Additionally, the characteristics of diagnostic classification may contribute to the change of reproducibility in our study. The WHO classification features

Table 3. Examples of challenging cases and their challenging points with diagnostic agreement rates in IHC staining among 20 breast pathologists

Challenging case	Challenging point (%)	Diagnosis (agreement rate, n/20)
Apocrine metaplasia	Benign (55)	IDP (30%, 6/20)
		IDP with ADH (25%, 5/20)
	Malignant (45)	IDP with DCIS (35%, 7/20)
		PCIS (10%, 2/20)
Flat epithelial atypia-like features	Benign (30)	IDP with ADH (30%, 6/20)
	Malignant (70)	IDP with DCIS (35%, 7/20)
		PCIS (20%, 4/20)
		EPC (15%, 3/20)
Large cystic pattern with fibrous capsule but no or rare myoepithelial cells	In situ (95)	IDP with DCIS (5%, 1/20)
		PCIS (70%, 14/20)
		EPC (20%, 4/20)
	Invasive (5)	IPC (5%, 1/20)
Solid multinodular pattern with smooth contours but no or rare myoepithelial cells	In situ (65)	EPC (5%, 1/20)
		SPC in situ (60%, 12/20)
	Invasive (35)	SPC invasive (30%, 6/20)
		IPC (5%, 1/20)
Solid multinodular and jigsaw pattern with ragged contours but no myoepithelial cells	In situ (75)	PCIS (20%, 4/20)
		EPC (40%, 8/20)
		SPC in situ (15%, 3/20)
	Invasive (25)	SPC invasive (10%, 2/20)
		IPC (15%, 3/20)

IHC staining, immunohistochemical staining for CK5 and p63; IDP, intraductal papilloma; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; PCIS, papillary carcinoma in situ; EPC, encapsulated papillary carcinoma; IPC, invasive papillary carcinoma; SPC, solid papillary carcinoma.

determinacy in diagnosis. However, the pathology category classification (B1-B5) published by the UK National Health Service Breast Screening Programme (NHSBSP) allows for probability in differential diagnosis [14]. In practice, the use of these reporting systems exhibited higher reproducibility ( $\kappa = 0.54$ ) compared to our study ( $\kappa = 0.21$ ) [12]. Therefore, it is assumed that the adoption of this diagnostic classification would lead to the higher reproducibility in our cases.

Elmore et al. [15] investigated the concordance rate of the pathologic diagnosis of non-PBLs on CNB among 115 pathologists recruited from eight U.S. states with consensus-derived reference diagnoses. Their study showed that the overall concordance rate was 75.3% (95% confidence interval, 73.4% to 77.0%; 5,194 of 6,900 interpretations). The diagnostic concordance rate on CNB is lower in PBLs (63.3% in our study and 44% in the previous study [12]) compared to non-PBLs (75.3%) with similar diagnostic categories despite fewer pathologists, indicating more complicated diagnostic difficulty in PBLs.

Multicenter studies are thought to be superior to single-center studies in presenting generalized results in breast pathology. However, to my knowledge, there has been no multicenter study examining the reproducibility of PBLs [15]. In contrast, our study was conducted for 20 pathologists from 20 multiple medical institutions, conferring more generalizability on our findings in PBLs. Additionally, it is noted that our results were derived from breast pathologists. One study indicated that breast pathologists are more accurate in diagnosing CNB guided PBLs compared to non-breast pathologists [13]. In that study, interobserver variability was 'fair agreement' ( $\kappa = 0.38$ ) between the breast pathologists and non-breast pathologists. Therefore, our results suggest the difficulty in diagnosing PBLs even in breast pathologists, warranting improving diagnostic accuracy for PBLs.

IHC staining increases diagnostic accuracy and improves interobserver variability [16]. CK5 is used in distinguishing between hyperplastic and neoplastic epithelial proliferation in PBLs [17,18]. p63 is a nuclear protein that is specific for myoepithelial cells without manifestation in blood vessels and myofibroblasts [19,20]. IHC staining helps to make an accurate diagnosis on PBLs through the ability of CK5 and p63 in identifying monoclonal epithelial proliferation and the presence of myoepithelial cells [17,21], respectively. Our study also showed that the application of IHC staining generally improved interobserver variability and agreement rates in diagnosing PBLs. Nonetheless, it is noted that the utilization of IHC staining is limited in EPC/SPC with very low kappa values.

EPC and SPC are distinctive variants of PCIS, each accounting for <1% of breast carcinomas [22]. Morphological differentiation in H&E staining has a decisive role in diagnosing EPC/ SPC because IHC staining is less helpful. Despite differential points including cystic versus solid and single versus multiple in morphology [23], some cases on CNB practically exhibit overlaps like a transition from single to multiple ductal lesions and cystic to solid appearance with a gradual cystic filling of proliferation [24]. Additionally, definitive cut-off criteria have yet to be determined, which may decrease the diagnostic agreement rates among pathologists. Supplementary Table S1 presents the distribution of histologic patterns and pathologic diagnoses of 12 EPC/SPC cases based on 2-tier or 4-tier classifications, revealing that most pathologists diagnosed most EPC/SPC cases as malignant in 2-tier classification. Nonetheless, it is interesting that kappa values remained low in 2-tier classification for EPC and SPC. This was attributed to the technical limitations of the formula used to calculate kappa values. In cases where the observed agreement was asymmetrically lopsided, kappa values can be drastically lowered due to increased chance agreement rates [25]. Therefore, the tipping effect of lopsided pathologic diagnoses by 20 pathologists induced low kappa values even in 2-tier classification.

We intended to describe five challenging histologic patterns of PBL with diagnostic pitfalls even in IHC staining, specifically apocrine metaplasia, flat epithelial atypia-like features, large cystic masses with no myoepithelial cells along the papillae, and predominant solid multinodular masses with smooth contours or jigsaw patterns. Benign PBLs are often exaggerated by the presence of apocrine metaplasia [10]. Apocrine metaplasia is characterized by abundant eosinophilic cytoplasm with CK5 (-) and a lack of myoepithelial cells with p63 (-) [26,27]. Therefore, the first case with apocrine metaplasia confounded the distinction between benign and malignant intraductal lesions, leading to a diagnostic disagreement even in IHC staining. The WHO classification defined flat epithelial atypia as columnar cell lesions with nuclear atypia [9]. In contrast with non-PBLs, no definite concept of flat epithelial atypia associated with PBLs has been suggested or proposed until now. In the second case with flat epithelial atypia-like features, we observed a high proportion of diagnosis in IDP with ADH (30%) and IDP with DCIS (35%). This heterogeneous diagnosis may be attributable to the difficulty in determining the size ( $\geq$ 3 mm in DCIS or < 3 mm in ADH) of histologically identical epithelial proliferation [10]. The third case with a large cystic pattern revealed that PCIS was the most common diagnosis (70%), followed by EPC (20%). EPC is histologically similar to PCIS in some ways, but EPC is characterized by a single cystic or nodular pattern without myoepithelial cells along the papillae and at the periphery, occasionally forming a thick fibrous capsule [10]. The helpful point of distinguishing PCIS from EPC is the presence of myoepithelial cells at the periphery of the PCIS [28]. However, our case presented completely CK5 (–) and sparsely and focally p63 (+) at the periphery, potentially leading to diagnostic disagreement. It is important to differentiate between in situ and invasive lesions for the management and prognostication of PBLs [24]. In the fourth case with a solid multinodular pattern, there were smooth contours and focal suspected microinvasions without immunoreactivity of both CK5 and p63. Although SPC occupied 90% of diagnoses, it was classified as both in situ (60%) and invasive (30%). The fifth case with a solid multinodular and jigsaw pattern was challenging to distinguish between in situ and invasive lesions. Moreover, a solid multinodular pattern suggested SPC, but a fragmented lesion may be misinterpreted as one that fell out of a cystic lesion reminiscent of EPC. Limited materials and fragmented samples in CNB specimens may be the main factors contributing to these disagreements of diagnoses. Interestingly, all 12 cases of EPC/SPC were categorized to the latter three histologic patterns for not only challenging but also helping differential diagnoses as shown in Supplementary Table S1. In papillary carcinomas that were difficult to differentiate between in situ and invasive lesions, such as the above fourth and fifth cases, it is recommended to diagnose them as PCIS or of uncertain invasiveness on CNB to avoid overtreatment, especially in the current era of preoperative (neoadjuvant) chemotherapy.

Our study has some limitations. First, we could not include very rare cases such as IDP with LCIS, EPC with invasion, and IPC because of their extreme rarity especially on CNB. Therefore, PBL cases included in this study actually belong to seven categories as shown in Fig. 1 in contrast to 10 categories of the WHO classification. Second, our cases did not have information on the final diagnosis from excisional biopsy or surgical resection. Although the consensus meeting of KBPSG proposed the pathologic diagnosis of 60 PBL cases, their diagnoses were not likely to guarantee the correctness of the diagnosis. Third, there was no clinical and radiologic information about the 60 PBL cases in our raw data. The absence of this information may be an obstacle in determining an accurate diagnosis and prognosis. Fourth, we did not use the most recently updated version of the WHO classification published in 2019. Nonetheless, because there was no difference between the 4th and 5th editions in diagnosing and classifying PBLs, the concern for the discrepancy between the two versions is minimal [8,9].

In conclusion, our study demonstrated that interobserver variability in the pathologic diagnosis of PBLs was unsatisfactory among 20 breast pathologists from 20 multiple medical institutions. Although IHC staining improved interobserver variability and agreement rates in diagnosing PBLs, diagnostic reproducibility was still limited in specific cases including EPC/SPC. Therefore, more intensive consensus studies are necessary to improve the diagnostic agreement and categorization of PBLs with the WHO classification. Further studies should continue to develop effective modalities in distinguishing PBLs especially on CNB.

#### **Supplementary Information**

The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2021.07.29.

#### **Ethics Statement**

This study was approved by the National Cancer Center Institutional Review Board with a waiver of informed consent (NCC2018-0214).

#### Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

#### **Code Availability**

Not applicable.

#### ORCID

Hye Ju Kang Sun Young Kwon Ahrong Kim Woo Gyeong Kim Eun Kyung Kim Ae Ree Kim Chungyeul Kim Soo Kee Min So Young Park Sun Hee Sung Hye Kyoung Yoon Ahwon Lee Ji Shin Lee Hyang Im Lee Ho Chang Lee Sung Chul Lim Sun Young Jun Min Jung Jung Chang Won Jung Soo Youn Cho Eun Yoon Cho Hye Jeong Choi So Yeon Park Iee Yeon Kim In Ae Park Youngmee Kwon

https://orcid.org/0000-0002-7427-6275 https://orcid.org/0000-0002-8410-0185 https://orcid.org/0000-0003-2317-8920 https://orcid.org/0000-0003-1694-6602 https://orcid.org/0000-0001-6701-6988 https://orcid.org/0000-0001-9645-2156 https://orcid.org/0000-0002-9636-5228 https://orcid.org/0000-0002-7099-9433 https://orcid.org/0000-0003-1159-1486 https://orcid.org/0000-0001-9345-1131 https://orcid.org/0000-0003-0714-8537 https://orcid.org/0000-0002-2523-9531 https://orcid.org/0000-0003-4634-2228 https://orcid.org/0000-0002-5932-8217 https://orcid.org/0000-0003-4687-8348 https://orcid.org/0000-0001-6179-691X https://orcid.org/0000-0002-3007-2094 https://orcid.org/0000-0002-2831-9430 https://orcid.org/0000-0003-4809-9612 https://orcid.org/0000-0001-9714-7575 https://orcid.org/0000-0003-4675-4492 https://orcid.org/0000-0002-5124-8589 https://orcid.org/0000-0002-0299-7268 https://orcid.org/0000-0002-0503-984X https://orcid.org/0000-0001-6667-3484 https://orcid.org/0000-0002-1370-2496

#### **Author Contributions**

Conceptualization: IAP, ARK, YK. Data curation: SYK, ARK, WGK, EKK, CK, SKM, SYP, SHS, HKY, AL, JSL, HIL, HCL, SCL, SYJ, MJJ, CWJ, SYC, HJC, IAP. Formal analysis: HJK, YK. Funding acquisition: AK, JYK, YK. Investigation: HJK, YK. Methodology: AK, JYK, YK. Project administration: SYP, EYC, YK. Resources: SYK, AK, WGK, EKK, CK, SKM, SHS, HKY, AL, JSL, HIL, HCL, SYJ, MJJ, HJC, EYC, SYP, JYK, IAP, YK. Supervision: IAP, AK, YK. Validation: HJK, YK. Visualization: HJK, YK. Writing—original draft: HJK. Writing—review & editing: HJK, YK. Approval of final manuscripts: all authors.

#### **Conflicts of Interest**

S.Y.P., editor-in-chief of the *Journal of Pathology and Translational Medicine*, were not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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# Immunohistochemical expression of programmed death-ligand 1 and CD8 in glioblastomas

Dina Mohamed El Samman<sup>1</sup>, Manal Mohamed El Mahdy<sup>1</sup>, Hala Sobhy Cousha<sup>1</sup>, Zeinab Abd El Rahman Kamar<sup>1</sup>, Khaled Abdel Karim Mohamed<sup>2</sup>, Hoda Hassan Abou Gabal<sup>1</sup>

Departments of <sup>1</sup>Pathology and <sup>2</sup>Clinical Oncology, Ain Shams University, Cairo, Egypt

**Background:** Glioblastoma is the most aggressive primary malignant brain tumor in adults and is characterized by poor prognosis. Immune evasion occurs via programmed death-ligand 1 (PD-L1)/programmed death receptor 1 (PD-1) interaction. Some malignant tumors have responded to PD-L1/PD-1 blockade treatment strategies, and PD-L1 has been described as a potential predictive biomarker. This study discussed the expression of PD-L1 and CD8 in glioblastomas. **Methods:** Thirty cases of glioblastoma were stained immunohistochemically for PD-L1 and CD8, where PD-L1 expression in glioblastoma tumor tissue above 1% is considered positive and CD-8 is expressed in tumor infiltrating lymphocytes. The expression of each marker was correlated with clinicopathologic parameters. Survival analysis was conducted to correlate progression-free survival (PFS) and overall survival (OS) with PD-L1 and CD8 expressed in six of 30 cases. CD8-positive tumor-infiltrating lymphocytes (CD8<sup>+</sup> TILs) had a median expression of 10%. PD-L1 and CD8 were positively correlated (p=.001). High PD-L1 expression was associated with worse PFS and OS (p=.026 and p=.001, respectively). Correlation of CD8<sup>+</sup> TILs percentage with age, sex, tumor site, laterality, and outcomes were statistically insignificant. Multivariate analysis revealed that PD-L1 was the only independent factor that affected prognosis. **Conclusions:** PD-L1 expression in patients with glioblastoma is robust; higher PD-L1 expression is associated with lower CD8+ TIL expression and worse prognosis.

Key Words: Glioblastoma; Programmed death ligand 1; Tumor-infiltrating lymphocytes; CD8; Survival analysis

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Corresponding Author: Dina Mohamed El Samman, MSc, Department of Pathology, Faculty of Medicine, Ain Shams University, Abbassyia Square, Ramsis Street, Cairo 11566, Egypt Tel: +20-1123342337, +20-1147509744, Fax: +20-224346753, +20-224346041, E-mail: dina\_elsamman@med.asu.edu.eg, dina.elsamman@yahoo.com

Glioblastoma is a neuroepithelial tumor of the central nervous system (CNS); it is characterized by extremely aggressive behavior and poor prognosis. It constitutes 12%–15% of all intracranial tumors, 50% of all astrocytic tumors [1], and 40%–50% of all primary brain tumors [2]. Glioblastoma is the most common primary malignant brain tumor in adults [3], and the World Health Organization (WHO) classifies it as a grade IV astrocytoma [4,5]. It constitutes 33.7% of all astrocytic tumors and 16.3% of primary malignant CNS tumors [6].

It is the third most common CNS tumor after fibrillary astrocytoma and medulloblastoma [7].

The current standard management for newly diagnosed glioblastoma consists of maximal safe resection, followed by radiotherapy and then adjuvant chemotherapy with temozolomide [1,8]. Despite the advances in therapeutic approaches, such as surgery, radiotherapy, and chemotherapy over the past decade, the overall survival (OS) of patients with glioma remains unsatisfactory [9].

Glioblastoma long has been recognized as an immunosuppressive tumor characterized by activation of several immune escape mechanisms, including upregulation of programmed deathligand 1 (PD-L1), also known as B7-H1 [10].

During infection or inflammation in normal tissue, PD-L1/ programmed death receptor 1 (PD-1) interaction plays an important role in preventing autoimmunity during the immune response. On the other hand, in the tumor microenvironment, PD-L1/PD-1 interactions evade tumor immunity by inactivating cytotoxic T lymphocytes/CD8-positive tumor-infiltrating lymphocytes (CD8<sup>+</sup> TILs) [11].

Immunohistochemical assessment of the PD-L1 protein in

several studies on melanoma, non-small cell lung cancer, and other tumors showed that it positively correlates with response to PD-1 targeting therapy. This makes it a potential predictive biomarker for treatment response of these tumors to targeted PD-1/PD-L1 blocking therapy and prognosis [12-15].

Accordingly, immunotherapy in the form of immune checkpoint inhibitors might offer a new opportunity for management of glioblastoma since these have succeeded in the treatment of other tumors. Given this context, the present study aimed to determine the roles of PD-L1 expression and its effects on the immune microenvironment represented in CD8<sup>+</sup> TILs in patients with glioblastoma.

There have been efforts to develop more accurate predictive biomarkers of patient response to checkpoint blockade, particularly anti–PD-1/PD-L1 [16]. However, systematic studies on the expression of PD-L1 in human tissue samples are limited. Thus, we aimed to characterize PD-L1 expression and its association with clinicopathologic parameters in human glioblastoma cases to provide a basis for clinical trials and translational biomarker research. We also investigated PD-L1 expression and its correlation with patient prognosis.

### MATERIALS AND METHODS

This is a retrospective cohort study that included formalinfixed paraffin-embedded (FFPE) tissue blocks from 30 patients with glioblastoma. Medical records were collected from the archives of the Clinical Oncology Department and the Pathology Laboratory of Ain Shams University Hospital from January 2017 to January 2020.

The following inclusion criteria were used to select tumor tissue blocks for final analysis: (1) histopathological diagnosis of glioblastoma, (2) available paraffin-embedded tissue diagnostic biopsy data, and (3) available clinical follow-up data after initial diagnosis. Clinicopathologic variables of age, gender, tumor location, and tumor laterality were recorded. Survival indicators (OS and progression-free survival [PFS]) were identified using the follow-up data.

The paraffin-embedded tissue blocks of 30 pretreatment glioblastoma cases were cut into sections with a thickness of 4 µm and subjected to the following.

(1) Routine hematoxylin and eosin (H&E) staining: The H&Estained slides of the tissue biopsies were prepared to confirm glioblastoma diagnosis based on the 2016 CNS WHO classification.

(2) Assessment of isocitrate dehydrogenase 1 (*IDH1*) mutation: *IDH1* mutation was evaluated by immunohistochemistry [5]. Anti IDH-1 (R-132 H) mouse monoclonal antibody (clone H09) from Dianova was used to identify *IDH1* wild- and mutant-types in FFPE sections using a BenchMark Ventana (GX) automated immunostainer (Roche, Rotkreuz, Switzerland). Twentyseven cases (90%) were negative for *IDH1* mutation (IDH-1 wild), and three (10%) cases were positive (*IDH1* mutant).

(3) Immunohistochemical staining for PD-L1 and CD8: Regarding immunohistochemical staining, 4-µm paraffin-embedded tissue sections were prepared and stained using the Bench-Mark Ventana (GX) automated immunostainer. We first loaded the slides, antibodies, and ultra-view detection kit dispensers into the BenchMark instrument, we selected the standard pretreatment protocol CC1 in the automatic immunostainer device, and set the antibody incubation for 32 minutes at 37°C. When staining was complete, the slides were removed from the instrument, rinsed well with soap and water 10 times, and then washed with a buffer (reaction buffer concentrate  $[\times 10]$  from Ventana [Ref 950-300, LOT G 24035]). The following primary antibodies were used: (1) PD-L1 NBP2-15791 rabbit/human polyclonal antibody from Novus Biological (Littleton, CO, USA) diluted to 1:200 and (2) CD8 C8/144B mouse monoclonal antibody from Cell Marque (Ref. 108-98, key code CMC 10829030, Darmstadt, Germany) diluted to 1:100.

Non-neoplastic lymph node tissue sections were used as positive controls for PD-L1, and non-neoplastic spleen sections were used as positive controls for CD8. Control slides were obtained from the histopathology paraffin embedded tissue blocks archived in the Pathology Lab, Faculty of Medicine, Ain Shams University Hospitals, and the study was conducted. Slides were prepared and used as control slides. Both positive and negative control slides were included in each run. Neagtive control slides used are gliobalstoma tissue sections, primary antibodies were not applied on them, instead the buffer is used on it. Three runs were done, in each run a negative control slide was used. All specimens were reviewed independently using light microscopy in at least five areas ( $400 \times$  magnification) by investigators who were blinded to immunohistology and clinical data.

PD-L1 expression was recorded according to cellular localization and distribution of the immunohistochemical signal, whether diffuse/fibrillary or membranous, with a positivity cutoff value > 1%, according to Berghoff et al. [8]. Any PD-L1 expression in the slide > 1% is considered positive whereas any expression of PD-L1 on the slide  $\leq 1\%$  is considered negative. The extent of diffuse/fibrillary PD-L1 expression throughout the tumor tissue was scored semiquantitatively according to percentage of cells expressing PD-L1 in the non-necrotic tumor area: (1) no PD-L1–positive tumor areas,  $(2) \le 25\%$ , (3) 26% - 50%, (4) 51% - 75%, and (5) > 75%. The patients were categorized further into high (PD-L1 > 50%) and low (PD-L1  $\le 50\%$ ) PD-L1 expression groups using the median value as a cutoff point (50%) [8].

CD8+ TIL expression was evaluated quantitatively by examining each section using at least five high-power fields (×40 objective and ×10 eyepiece) and the most abundant TILs to calculate the percentage of expression [17]: (1) Each slide was examined to detect the 5 hpfs (×40) with the most abundant TIIs, (2) In those 5 hpfs with most abundant TIIs, TILs were calculated as a percentage against the tumor tissue background using the automated image analyzer mentioned in the methods, and (3) The median percentage out of those 5 hpfs was referred to as the percentage of CD8<sup>+</sup> TILs for this case. Counting was conducted using the Leica Q Win V.3 program (Wetzlar, Germany), an image analyzer, installed on a computer in the Histology Department, Faculty of Medicine, Ain Shams University (CPU: Ryzen 4600H, Graphics: AMD Radeon Pro 5300 XT, RAM: 32 GB 2666MHz DDR4, Screen: 27-inch [diagonal] 5120×2880 Retina 5K display, Storage: 4TB SSD). The computer was connected to a Leica DM2500 microscope. Furthermore, the densities were scored at  $200 \times 400 \times$  magnification in predefined regions of interest within the glioblastoma (intra-tumoral) and the perivascular region. The percentage of TILs in each case was recorded from the median field. The patients were categorized further into high (CD8<sup>+</sup> TILs > 10%) and low (CD8<sup>+</sup> TILs  $\leq$  10%) CD8 infiltration groups using the median value as a cutoff point (10%) [17]. Semiquantitative evaluation criteria were used to describe TIL infiltration density as either sparse, moderate, or dense [18].

#### Statistical analysis

The collected data were revised, coded, tabulated, and input into a PC using the SPSS ver. 20 for Windows (IBM Corp., Armonk, NY, USA). Suitable analyses were conducted according to the type of data obtained for each parameter. Parametric numerical data were expressed as mean ± standard deviation (SD) and range, whereas nonparametric numerical data were expressed as median and interquartile range (IQR). Nonnumerical data were expressed as frequency and percentage. Mann-Whitney U test was used to assess the statistical significance of differences in nonparametric variables between the two study groups. Fisher's exact test was used to examine the relationship between two qualitative variables when the expected count was less than 5 in more than 20% of cells. Correlation analysis using Pearson's method was used to assess the strength of the association between two quantitative variables. The correlation coefficient (r) defines the strength (magnitude) and direction (positive or negative) of the linear relationship between two variables. Spearman's method was used as a nonparametric measure of rank correlation where the correlation coefficient (rs) defined the strength (magnitude) and direction (positive or negative) of the relationship between two variables. Very weak, weak, moderate, strong, and very strong correlations were defined as r or rs values of 0-0.19, 0.2-0.39, 0.40-0.59, 0.6-0.79, and 0.8-1, respectively. The endpoints of this study were disease-free survival and OS. PFS was calculated as the time between initial biopsy and disease progression or last follow-up. OS was calculated as the time between initial biopsy and death or last follow-up. Patients who were deceased at the time of data cutoff were censored at the last date the patient was known to be alive. Survival distributions were estimated using the Kaplan-Meier method. Correlations of OS and PFS to clinical parameters, PD-L1 and CD-8 expression were done using log-rank test and Kaplan-Meier curves. The Cox regression was used for modeling the time to a specified event, considering the values of other given variables. Univariate and multivariable analyses were conducted using Cox's proportional hazards model. Statistical significance was set at p < .05, and p = .01 was defined as highly significant.

### RESULTS

#### Clinical characteristics of patients

We studied 30 patients with histopathologically diagnosed grade IV glioblastoma according to the 2016 WHO criteria. Their ages ranged from 29 to 75 years (mean, 50.9); 22 were male (73.3%), and eight (26.7%) were female. Regarding tumor location and laterality as determined via magnetic resonance imaging (MRI), 13/30 cases (44.8%) were located in the right hemisphere, 15/30 (51.7%) in the left hemisphere, and 1/30 (3.4%) crossed the midline, appearing bilateral. On MRI, the sites of glioblastoma included all lobes (frontal, parietal, occipital, and temporal), most frequently in the frontal and parietal lobes (20% and 16.7%, respectively). Thalamic and parasagittal sites were seen. Some cases were bridging into more than one lobe, including the frontoparietal, parieto-occipital, temporofrontal, temporo-occipital, and temporoparietal lobes.

#### PD-L1 expression

All 30 cases (100%) demonstrated positive PD-L1 staining in tumor cells in a diffuse/fibrillary pattern, with a mean value of 57.6% and a median of 50%. Among the PD-L1–positive cases, five (16.7%) had  $\leq$ 25% positive staining of the tumor cells, 10 (33.3%) had 26%–50% positive staining, five (16.7%) had



Fig. 1. Glioblastoma sections stained for CD8 and programmed death-ligand 1 (PD-L1). CD8<sup>+</sup> tumor-infiltrating lymphocytes (TIL) membranous/cytoplasmic staining in the high infiltration group (A) and low infiltration group (B). CD8<sup>+</sup> TIL membranous/cytoplasmic staining showing a perivascular distribution (C) and an intra-tumoral distribution (D). PD-L1<sup>+</sup> tumor cells showing a diffuse/fibrillary pattern (E) and diffuse/fibrillary and membranous patterns (F).

51%-75% positive staining, and the remaining 10 (33.3%) had > 75% positive staining. The median was used to categorize further the cases into high (PD-L1 > 50%) and low (PD-L1  $\leq$  50%) PD-L1 expression groups. Lastly, six cases showed positive membranous expression alongside the diffuse/fibrillary expression, as illustrated in Fig. 1.

#### CD8 expression

The percentage of CD8<sup>+</sup> TILs ranged from 1% to 40% (mean, 14.33%  $\pm$  12.5%; median [IQR], 10% [5%–10%]). The low infiltration group (CD8<sup>+</sup> TIL  $\leq$  10%) represented 56.66% of all cases (n = 17), whereas the high infiltration group (CD8<sup>+</sup> TIL > 10%) represented 43.33% of all cases (n = 13), as illustrated in Fig. 1. The CD8<sup>+</sup> TILs were classified semiquantitatively as either sparsely (12 cases, 40%), moderately (11 cases, 36.7%), or densely (7 cases, 23.33%) stained. Regarding the distribution of CD8<sup>+</sup> TILs, the majority of cases (20 cases, 66.7%) was perivascular, eight cases (26.7%) were intra-tumoral, and the remaining two cases (23.3%) were both perivascular and intra-tumoral.

Table 1.	Correlations	between	clinical	characteristics	and	PD-L1
expressio	n					

	PD-L1, n (%)		Test of s	ignifica	ince
	Low expression group ≤50%ª	High expression group >50%ª	$\chi^2$	p- value	Significance
Age (yr)			1.2	.273	NS
<53	9 (60.0)	6 (40.0)			
≥53	6 (40.0)	9 (60.0)			
Sex			Fisher exact test	.682	NS
Male	12 (54.5)	10 (45.5)			
Female	3 (37.5)	5 (62.5)			
Laterality			0.54	.464	NS
Left	6 (46.2)	7 (53.8)			
Right	9 (60.0)	6 (40.0)			

PD-L1, programmed death-ligand 1; NS, not significant.

 $^{\rm a}{\rm 50\%}$  is the cutoff point used to categorize PD-L1 expression into low and high expression groups.

# Correlations between PD-L1, CD8 expression, and clinical characteristics

No statistical significance was detected regarding the effect of

 Table 2. Correlations between clinical characteristics and CD8 expression

	С	D8	Test of s	significa	nce
	Low infiltration group ≤10%ª	High infiltration group >10% <sup>a</sup>	χ²	p- value	Significance
Age (yr)			0.14	.713	NS
<53	9 (60.0)	6 (40.0)			
≥53	8 (53.3)	7 (46.7)			
Sex			Fisher exact test	>.99	NS
Male	12 (54.5)	10 (45.5)			
Female	5 (62.5)	3 (37.5)			
Laterality			2.39	.122	NS
Left	9 (69.2)	4 (30.8)			
Right	6 (40.0)	9 (60.0)			

NS, not significant.

 $^{\rm a}10\%$  is the cutoff point used to categorize CD8 expression into low and high infiltration groups.

age, sex, or laterality on PD-L1 (p = .273, p = .682, and p = .464, respectively) (Table 1) or CD8 (p = .713, p > .99, and p = .122, respectively) (Table 2) expression. PD-L1 expression was significantly associated with the proportion of CD8<sup>+</sup> TILs (p = .001), with a moderate inverse correlation according to Spearman's method (rs = -0.573).

#### Survival analyses

From the date of diagnosis to January 2020, 26 of 30 (86.7%) patients did not survive; only 4 (13.3%) patients were alive by the end of the study. The PFS had a mean of 10 months and a median of 7 months (95% confidence interval [CI], 7.5 to 12.5; SD, ± 7.03), whereas the mean OS of patients with glioblastoma was 18 months, and the median OS was 13 months (95% CI, 14 to 21).

#### Correlations between clinical characteristics and survival

Patients younger than 53 years (the median age in our study) had a mean PFS of 9.21 months (95% CI, 5.89 to 12.51) and a mean OS of 18.47 months (95% CI, 13.50 to 23.40), whereas patients 53 years and older had a mean PFS of 10.80 months (95% CI, 6.94 to 14.66) and a mean OS of 16.87 months (95% CI, 11.56 to 22.17).

Males had a mean PFS of 11.90 months (95% CI, 8.89 to 14.90) and a mean OS of 18.97 months (95% CI, 14.80 to 23.12), whereas females had a mean PFS of 4.75 months (95% CI, 3.15 to 6.35) and a mean OS of 13.6 months (95% CI, 7.34 to 19.90).

Patients with tumors arising in the left cerebral hemisphere had a mean PFS of 10.69 months (95% CI, 6.49 to 14.89) and a

mean OS of 19 months (95% CI, 13.60 to 24.50), whereas those with tumors arising in the right cerebral hemisphere had a mean PFS of 9.87 months (95% CI, 6.33 to 13.40) and a mean OS of 17.50 months (95% CI, 12.50 to 22.60).

Sex was significantly correlated with PFS (p = .004), with worse prognosis in female patients. PFS was not correlated with age (p = .430) or laterality (p = .760). OS had no correlation with age (p = .690), sex (p = .317), or laterality (p = .647).

#### PD-L1 expression and survival

Individuals with high tumor PD-L1 expression (PD-L1 > 50%) had shorter mean PFS (7.47 months; 95% CI, 5.40 to 9.50) and OS (10.8 months, 95% CI, 7.90 to 13.70), indicating worse prognosis. Those with low PD-L1 expression (PD-L1  $\leq$  50%), on the other hand, had longer mean PFS (12.50 months; 95% CI, 8.24 to 16.80) and OS (24.60 months; 95% CI, 19.90 to 29.20). Thus, PD-L1 expression had a significant correlation with PFS and OS (p = .026 and p=.001, respectively), denoting worse prognosis in the high expression group, as demonstrated in Fig. 2.

#### CD8 immunohistochemical expression and survival

The low infiltration group (CD8<sup>+</sup> TIL  $\leq$  10%) had shorter mean PFS (8.24 months; 95% CI, 5.40 to 11) and OS (14.59 months; 95% CI, 10.67 to 18.50) compared to the high infiltration group (CD8<sup>+</sup> TIL > 10%), which had longer mean PFS (12 months; 95% CI, 8 to 16.59) and OS (21 months; 95% CI, 15.57 to 27). However, the proportion of CD8<sup>+</sup> TILs was not significantly correlated with either PFS or OS (p = .093 and p = .066, respectively), as demonstrated in Table 3 and Fig. 2.

# Multivariate analysis of clinical characteristics and PD-L1 and CD8 expression

Multivariate analyses were performed using Cox regression to determine the variables that were independently predictive of PFS and OS. PD-L1 expression was the only factor with independent prognostic significance (hazard ratio, 6.30; p = .001) for OS only; age, sex, and CD8<sup>+</sup> TIL proportion were not statistically significant in terms of PFS and OS. This indicates that positive PD-L1 expression greater than 50% increased the risk of fatality by 6.3 times, as demonstrated in Table 4.

## DISCUSSION

Glioblastoma is the most common and aggressive primary malignant brain tumor in adults [19]. PD-L1 is expressed robustly



Fig. 2. Kaplan-Meier curves showing survival functions. (A) Percentage of CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) in relation to progression-free survival (PFS). (B) Percentage of CD8<sup>+</sup> TILs in relation to overall survival (OS). (C) Programmed death-ligand 1 (PD-L1) expression in relation to PFS. (D) PD-L1 expression in relation to OS.

in most glioblastoma cases, and clinical trials using PD-1/PD-L1targeted immunotherapy have shown improved median OS in patients with glioblastoma [20]. Tumor immunology and immunotherapy are emerging trends in cancer therapy. Thus, studies on PD-L1 are relevant considering its role in glioblastoma, its effect on survival analysis, and its potential role in disease management. Moreover, analysis of immunological parameters, such as TILs, and their correlation with PD-L1 expression (representing the microenvironmental immune response to PD-L1 expression) is crucial for development of successful immunotherapies [21].

We studied the expression of PD-L1 and CD8 within the context of survival analysis in glioblastoma cases. In our study, PD-L1 expression was detected in all glioblastoma cases, ranging from 20% to 90%. This was in accordance with Wintterle et al. [22], Wilmotte et al. [23], Berghoff et al. [8], and Nduom et al. [3], who also reported high PD-L1 expression in glioblas-

toma cases (100%, 85.2%, 88%, and 95%, respectively). On the other hand, Yao et al. [24], Avril et al. [25], Liu et al. [4], and Zeng et al. [26] reported weaker PD-L1 expression in glioblastoma (75%, 76.5%, 45%, and 50% respectively). Xue et al. [2] emphasized that the positive rate of PD-L1 expression varied by study and ranged from 6.1% to 100%. Pooled analysis demonstrated an overall positive percentage of PD-L1 protein expression (44.72%) [2].

In our study, positive PD-L1 membranous staining was apparent in 36.7% of all cases (n = 11). This was in agreement with Berghoff et al. [8] and Nduom et al. [3]; the former found strong membranous PD-L1 immunostaining in 44 of 117 cases (37.6%), whereas the latter reported a higher percentage of PD-L1 membranous expression (60.6%), which might be attributed to their larger sample size (n = 345). On the contrary, Garber et al. (2017) [27] reported a higher percentage of PD-L1 membranous expres-

		PFS (mo)			OS (mo)	
	Mean±SE	95% CI	p-value	Mean±SE	95% CI	p-value
Age (yr)			.432			.690
<53	$9.20 \pm 1.69$	5.89-12.51		$18.47 \pm 2.51$	13.54-23.39	
≥53	$10.80 \pm 1.97$	6.94-14.66		$16.87 \pm 2.71$	11.56-22.17	
Sex			.004			.317
Male	$11.90 \pm 1.54$	8.89-14.92		$18.97 \pm 2.12$	14.82-23.12	
Female	4.70±0.82	3.15-6.35		$13.63 \pm 3.21$	7.34–19.91	
Laterality			.760			.647
Left	$10.69 \pm 2.14$	6.49-14.89		19.08±2.78	13.63-24.52	
Right	$9.87 \pm 1.80$	6.33-13.40		$17.57 \pm 2.58$	12.51-22.62	
PD-L1			.026			<.001
≤50% <sup>a</sup>	$12.53 \pm 2.19$	8.24-16.83		$24.57 \pm 2.37$	19.93-29.20	
>50%ª	$7.47 \pm 1.05$	5.41-9.53		$10.80 \pm 1.48$	7.90-13.70	
CD8+ TILs			.093			.066
$\leq 10\%^{b}$	$8.24 \pm 1.44$	5.41-11.06		$14.59 \pm 2$	10.67-18.50	
>10% <sup>b</sup>	$12.30 \pm 2.18$	8.03-16.59		$21.41 \pm 2.98$	15.57-27.26	

Table 3. Univariate survival analysis of glioblastoma according to immunohistochemistry scores of PD-L1 and CD8<sup>+</sup> TILs and other clinicopathologic factors

PD-L1, programmed death-ligand 1; TILs, tumor-infiltrating lymphocytes; PFS, progression-free survival; OS, overall survival; SE, standard error; CI, confidence interval.

<sup>a</sup>50% is the cutoff point used to categorize PD-L1 expression into low and high expression groups; <sup>b</sup>10% is the cutoff point used to categorize CD8 expression into low and high infiltration groups.

 
 Table 4. Multivariate analysis of the effects of independent factors on progression-free survival and overall survival using Cox survival analysis

	Progression-free survival			(	Overall survival		
	HR	95% CI	p-value	HR	95% Cl	p-value	
Age ≥53 yr	0.60	0.26-1.41	.241	0.63	0.25-1.6	.334	
Female sex	2.56	0.93–7.07	.069	1.25	0.47–3.28	.653	
PD-L1 >50% <sup>a</sup>	1.82	0.68–4.83	.232	6.30	2.22-17.91	.001 <sup>b</sup>	
CD8 > 10%°	0.63	0.27-1.49	.295	0.53	0.2-1.4	.203	

HR, hazard ratio; Cl, confidence interval; PD-L1, programmed death-ligand 1. <sup>a</sup>50% is the cutoff point used to categorize PD-L1 expression into low and high expression groups; <sup>b</sup>Significant; <sup>c</sup>10% is the cutoff point used to categorize CD8 expression into low and high infiltration groups.

sion (60.6%). Although the results of our study and some of the aforementioned studies seem numerically different, they are not necessarily conflicting as they all demonstrate that glioblastomas have high PD-L1 expression.

The differences in results could be explained by the techniques used. For example, tissue microarray uses smaller tumor tissue samples (1 mm), which are more prone to sampling bias and falsenegative cases than are studies using full slides, wherein nonsampled tumor parts in tissue microarray are interpreted as false negatives regarding PD-L1 expression. Generally, the difference in PD-L1 expression across studies can be attributed to variability in the methodology of PD-L1 assessment, utilized antibodies, immunohistochemical staining protocols, sample size, methods of preparing the tumor tissues, and diagnostic standards (e.g., expression patterns and positivity cutoffs). Therefore, further research to establish uniform standards is necessary.

All glioblastoma cases investigated herein showed CD8<sup>+</sup> TIL expression (mean, 14.3%; median, 10%; range, 1%–40%), and we used the median as a cutoff value to categorize the cases into low and high infiltration groups, similar to Han et al. [17], who reported a median of 3.4% and used it for categorization. Both studies used the same quantitative evaluation protocol, examining each section using at least five high-power fields with the most abundant TILs to record the median expression and, thus, categorize cases into two groups. However, in our study, we used an automated image analyzer that recorded the percentage of TILs, whereas Han et al. [17] manually counted the number of TILs. Our sample size was smaller (30 cases) than that of Han et al. [17] (90 cases).

The low infiltration group represented 56.66% of all cases, while the high infiltration group represented 43.3% of all cases. TILs were detected in all cases, and the majority was of sparse density and perivascular in distribution. This finding is in line with the findings of Yang et al. [28], Rutledge et al. [29], Han et al. [17], and Berghoff et al. [8]. This observation regarding the distribution of TILs in glioblastoma suggests that TILs do not migrate easily into the immunosuppressive tumor microenvironment but instead arrest mainly in the perivascular space. The lower total number of TILs in our study and in the study of Han et al. [17] compared to that of other tumors, such as ovarian cancer [21] and

breast cancer [30], could be related to the blood-brain barrier or the specific local microenvironment.

The expression of PD-L1 and CD8<sup>+</sup> TIL showed a significant, moderate, inverse correlation (rs = -0.573, p = .001). This can be interpreted according to the findings of Avril et al. [25], who explained that PD-L1 produced by glioma cell lines affects the activation and recruitment of T-cells and decreases the production of lymphocytic interferon- $\gamma$  and interleukins 2 and 10. According to Wintterle et al. [22], the expression of PD-L1 in malignant tumors inhibits CD4<sup>+</sup> and CD8<sup>+</sup> T-cells via PD-1.

In our study, age, sex, and laterality were not correlated with either PD-L1 or the proportion of CD8<sup>+</sup> TILs. This observation was in line with the findings of Berghoff et al. [8].

The median OS in our study was 13 months, which is in line with the findings of Zeng et al. [26] and Xue et al. [2], who reported an OS of 12 and 14.6 months, respectively. The median PFS in our study was 7 months (95% CI, 7.5 to 12.5), whereas Ballmann et al. [31] reported a median PFS of 5.3 months (95% CI, 5.0 to 5.6).

Cox regression analysis found no significant difference in OS in terms of age, sex, or laterality and no difference in PFS in terms of age or laterality. This is in concordance with Han et al. [17] but not with Berghoff et al. [8], who reported older age to be a negative prognostic indicator. On the other hand, we found female patients to have a significantly shorter PFS, but Han et al. [17] reported that sex was not correlated with either OS or PFS

In our study, patients with high PD-L1 expression (> 50%) had shorter OS than those with low expression. This is in concordance with Liu et al. [4] and Nduom et al. [3].

Multivariate analysis revealed that PD-L1 is an independent factor in relation to OS, which agrees with the results of Berghoff et al. [8] and Nduom et al. [3]. However, this contrasted with the findings of Preusser et al. [32], Zeng et al. [26], and Xue et al. [2]. The conflict in results with Zeng et al. [26] can be explained by their inclusion of glioma cases of all grades. This conflict needs to be studied further to confirm and determine the prognostic value of PD-L1 expression in glioblastoma prognosis. It should also be noted that PD-L1 was significantly correlated with PFS. However, the current literature lacks an explanation for this, and further studies are warranted.

The effect of PD-L1 expression on survival can be explained by the need got PD-1 and its ligand, PD-L1, for immunosuppression of T-cells, tumor cells, and antigen-presenting cells (APCs). Their interaction leads to inhibition of early T-cell activation, abolishing their cytotoxic activity and hindering the production of inflammatory cytokines [33-35].

Although the high CD8<sup>+</sup> TIL infiltration group had longer OS and PFS than the low infiltration group, this was not statistically significant in our study. This is in accordance with Kim et al. [36] and Han et al. [17], who reported that CD8<sup>+</sup> TILs alone could not predict effectively patient outcome in glioblastoma. They also concluded that CD8<sup>+</sup> TILs might not be effective prognostic markers in gliomas because high-grade gliomas have multiple mechanisms of mediating immunosuppression, such as anergy stimulation by tumor APCs, antigen loss, cytokine immunosuppression, and T-cell apoptosis [37]. However, according to Han et al. (2014), the combination of high CD4<sup>+</sup> TILs and low CD8<sup>+</sup> TILs was significantly associated with shorter PFS and OS [17]. These results suggest that effective antitumor immunity requires an appropriate ratio of CD8/CD4<sup>+</sup> TIL in gliomas. On the other hand, CD8<sup>+</sup> TILs have a known favorable effect on the survival of patients with breast cancer [30], ovarian cancer [21], or colorectal cancer [33,38,39].

Glioblastoma is an aggressive brain tumor with a poor prognosis. PD-L1 is expressed extensively in glioblastomas and can be considered a negative prognostic indicator and a predictive biomarker for the selection of patients for immunotherapy. Cases with high PD-L1<sup>+</sup> expression (>50%) have a worse prognosis. The proportion of CD8<sup>+</sup> TILs is lower in cases of high PD-L1 expression, indicating possible immune evasion of glioblastomas exerted by PD-L1. More research is needed in this field to provide more opportunities for patients with glioblastoma to benefit from immunotherapy using PD-L1 blockers.

#### **Ethics Statement**

All procedures performed in the current study were approved by the National Research Ethics Committee (FMASU MD 428/2017) in accordance with the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all participants included in this study.

#### Availability of Data and Material

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

#### **Code Availability**

Not applicable.

#### ORCID

Dina Mohamed El Samman Manal Mohamed El Mahdy Hala Sobhy Cousha Khaled Abdel Karim Mohamed Hoda Hassan Abou Gabal https://orcid.org/0000-0002-1447-4982 https://orcid.org/0000-0003-3108-0528 https://orcid.org/0000-0003-4867-2984 https://orcid.org/0000-0003-3629-0217 https://orcid.org/0000-0003-2552-7645

#### **Author Contributions**

CConceptualization: DMES, MMEM, HSC, KAKM, HHAG. Formal analysis: DMES, MMEM, HHAG. Investigation: DME, MME, HHAG. Methodology: DMES, MMEM, HSC, KAKM, HHAG. Supervision: HSC, MMEM, KAKM, HHAG. Writing—original draft preparation: DMES, HHAG. Writing—review & editing: DMES, MMEM, HSC, KAKM, HHAG. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

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# Programmed death-ligand 1 expression and tumor-infiltrating lymphocytes in non-small cell lung cancer: association with clinicopathologic parameters

Gaurav Garg<sup>1</sup>, Kuruswamy Thurai Prasad<sup>2</sup>, Navneet Singh<sup>2</sup>, Parul Gupta<sup>1</sup>, Valliappan Muthu<sup>2</sup>, Ashim Das<sup>1</sup>, Amanjit Bal<sup>1</sup>

Departments of <sup>1</sup>Histopathology and <sup>2</sup>Pulmonary and Critical Care Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India

**Background:** Data on the prevalence of programmed death-ligand 1 (PD-L1) expression and tumor-infiltrating lymphocytes (TILs) in non-small cell lung cancer (NSCLC) and their clinical significance in Indian patients are limited. **Methods:** Newly diagnosed NSCLC cases (adenocarcinoma or squamous cell carcinoma [SqCC] histology) were included in the present study. The TILs were evaluated based on morphology on hematoxylin and eosin-stained slides. PD-L1 expression in tumors was assessed using immunohistochemistry with rabbit monoclonal antibody (SP263) on the Ventana automated immunostainer. Tumors with PD-L1 expression >50% on tumor cells were considered PD-L1-positive. Tumors in which TILs occupy >25% of stroma were considered to have high TILs. The present study included 128 cases of NSCLC (67 adenocarcinoma, 61 SqCC). PD-L1 positivity was observed in 17.2% of the patients with NSCLC. Baseline characteristics of PD-L1-positive subjects were similar to PD-L1-negative subjects except for a higher prevalence of liver metastasis (18.2% vs. 2.8%; p=.018) and a higher probability of diagnosis from extrapulmonary biopsies. High TILs were observed in 20% and 25% of the patients with NSCLC, respectively, however, neither were predictors of survival in SqCC.

Key Words: Lung neoplasms; Non-small cell lung carcinoma; PD-L1; Immunotherapy; Tumor-infiltrating lymphocytes

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Corresponding Author: Amanjit Bal, MD, DNB, Department of Histopathology, Postgraduate Institute of Medical Education and Research, 5th Floor, Research Block-A, Chandigarh 160012, India

Tel: +91-172-2755104, Fax: +91-172-2744401, E-mail: docaman5@hotmail.com

Interaction of tumor cells with their microenvironment may result in infiltration of the tumor by immune cells of the host. Although such immune cell infiltration does not universally occur in all tumors, when present, these tumor-infiltrating lymphocytes (TILs) may provide tumor-specific immune response to prevent further tumorigenesis. However, tumor cells may evade host immunity through the expression of several transmembrane proteins including programmed death-ligand 1 (PD-L1). PD-L1–targeted therapy is a form of immunotherapy which targets the components of the PD-L1 pathway to prevent downregulation of anti-tumoral immunity. Unfortunately, PD-L1–targeted therapy is effective in < 50% of non-small cell lung cancers (NSCLCs) despite the presence of PD-L1 expression. However, in general, better results are observed in patients with higher PD-L1 expression [1–4]. Therefore, testing for PD-L1 expression using

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immunohistochemistry is often performed before initiating PD-L1–targeted therapy. The prevalence of PD-L1 positivity in NSCLC varies widely across studies performed in different parts of the world [2–5]. A large part of this difference could be attributed to methodological differences which is supported by the results of a recent multinational study (EXPRESS) which showed similar PD-L1 expression in advanced NSCLCs across various geographical regions when a uniform testing strategy was used [6]. However, the reported prevalence of PD-L1 expression in Indian patients with NSCLC, albeit with different methods, is much lower [7,8]. Furthermore, the PD-L1 expression in NSCLC has been associated with certain clinicopathological characteristics and poor clinical outcomes in some but not all studies [5]. The intensity of tumor infiltration by TILs has been associated with survival [9,10]. In the present study, the prevalence and prog-

nostic significance of PD-L1 expression in a cohort of NSCLC patients from North India were reported.

### MATERIALS AND METHODS

#### Patients

This was a single institute, prospective, observational study conducted over 2 years. Consecutive patients with newly diagnosed NSCLC with adenocarcinoma or squamous cell carcinoma (SqCC) histology were considered for inclusion in this study. Subjects with a biopsy sample insufficient for the necessary histopathological analyses were excluded from the study.

#### Histopathological examination

All specimens were processed for paraffin sections for routine hematoxylin and eosin (H&E) staining and immunohistochemistry. The specimens included lung biopsies (bronchoscopic biopsies and computed tomography [CT]/positron emission tomography (PET)–guided biopsies) and biopsies from metastatic sites (lymph nodes, pleura, bone, and brain). Cytology samples were not included in the study. Based on H&E, the tumors were categorized as adenocarcinoma or SqCC as defined by the 2015 World Health Organization classification of lung tumors [11]. Tumors in which histological subtyping on the basis of routine H&E staining was difficult, immunohistochemistry with p63, thyroid transcription factor 1, cytokeratin (CK) 5/6, CK7, neuron-specific enolase, and CD56 was used.

#### Tumor-infiltrating lymphocytes

The TILs were evaluated based on morphology on H&Estained slides. Only lymphocytes and plasma cells were included in the scoring. Necrotic areas within the tumor and alveolar macrophages were not included in the scoring [12]. TILs were assessed in five areas of the tumor to determine the mean TIL score. The TIL score was assigned based on the proportion of tumor stroma occupied by TILs (TIL0, 0 to  $\leq 5\%$ ; TIL1, >5% to  $\leq 25\%$ ; TIL2, > 25 to  $\leq 50\%$ ; TIL3, >50%). Subjects with a score of TIL0 were considered to have low TILs and subjects with a score of TIL2 or TIL3 were considered to have high TILs.

#### PD-L1 expression

PD-L1 expression in tumors was assessed using immunohistochemistry with rabbit monoclonal antibody (SP263) on the Ventana automated immunostainer. Detection was optimized with the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). For positive controls, sections of the human placenta, as recommended in the data sheet of SP263 antibody (Fig. 1A) as well as human tonsil, were included in each batch. A negative control was run for each case. At least 100 viable tumor cells were scored. Tumor cells were considered to express PD-L1 when they showed complete or partial membranous staining with or without cytoplasmic staining of any intensity. PD-L1 expression on tumor cells was assigned scores based on the proportion of tumor cells (TC0, 0 to <1%; TC1, ≥1 to ≤50%; TC2, >50%) expressing PD-L1. Tumors with a PD-L1 expression on tumor cells >50% (TC2) were considered PD-L1–positive.

#### Mutation analysis

Epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (ALK) rearrangements were evaluated in subjects with adenocarcinoma histology. *EGFR* mutation analysis was performed using real-time PCR (EnteroGen, Agility Biotech, Los Angeles, CA, USA). ALK rearrangements were identified on immunohistochemistry performed on a Ventana BenchMark XT automated slide-processing system using the D5F3 clone [13].

#### **Clinical details**

The following clinical parameters were recorded at baseline: age, sex, smoking status, body mass index, performance status, and TNM. Performance status was evaluated using the Eastern Cooperative Oncology Group scale [14]. Contrast-enhanced CT scan of the thorax and upper abdomen (including the liver and adrenals) or whole-body PET was obtained for baseline staging evaluation in all patients. Tumor staging was performed using the seventh edition of the American Joint Committee on Cancer (AJCC) TNM classification [15]. Tumor staging was also performed using the eighth edition of the AJCC TNM classification in 106 patients (Supplementary Table S1). Because the proportion of patients between the seventh and eighth edition of the AJCC TNM classification was not significantly different, and to ensure completeness of data, the staging in the seventh edition of the AJCC TNM classification was used in the present study.

Subjects were treated with chemotherapy, targeted therapy, immunotherapy, radiotherapy, or surgery as indicated by tumor histopathology, mutation status, and clinical status, as previously described [16]. Briefly, subjects with adenocarcinoma without any driver mutation were treated with pemetrexedbased platinum doublet followed by maintenance pemetrexed therapy until disease progression [17]. Subjects with squamous histology were treated with docetaxel or gemcitabine-based platinum doublet. All patients receiving chemotherapy were administered at least four cycles of chemotherapy before response assessment using the Response Evaluation Criteria in Solid Tumors ver. 1.0 [18]. Subjects with sensitizing *EGFR* mutation or *ALK* rearrangement were treated with appropriate EGFR tyrosine kinase or ALK inhibitors, respectively [19,20].

#### Statistical analysis

Continuous variables were expressed as the mean and standard deviation and categorical values were expressed as the numbers and percentages. The differences between continuous and categorical variables were analyzed using the Mann-Whitney U test and the chi-square test (or Fisher exact test), respectively. Overall survival (OS) was defined as the time between initial diagnosis and date of death or last follow-up. The cutoff date for survival analysis was November 30, 2018. Survival curves were generated using the Kaplan-Meir method and were compared using the log-rank test. Multivariate analysis to identify predictors of survival was performed using the Cox proportional hazard model. Statistical analyses were performed using the commercial statistical package SPSS (IBM SPSS Statistics, ver. 22, IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

#### RESULTS

A total of 128 cases of NSCLC were included in the present study (Table 1); 103 (80.5%) were males and the median age of the study population was  $61 \pm 15$  years. The majority of patients had advanced disease (84.1% were stage IIIB/IV) and 44.8% of the patients had extrathoracic disease at diagnosis. Tissue samples (small biopsies: endobronchial biopsies, transbronchial biopsies, and imaging-guided core biopsies) were predominantly obtained from the lung (82.4%) and metastatic lymph nodes (8%), and 9.6% were resection samples. Adenocarcinoma and SqCC were observed in 52.3% and 47.7% of the subjects, respectively. A larger proportion of patients who were younger, female, non-smokers, and presented with advanced disease had adenocarcinoma. EGFR and ALK alterations were observed in 6.2% and 5.4% of the subjects, respectively. Chemotherapy (74.2%) was the most commonly used first-line therapy. Due to economic constraints, PD-L1-targeted therapy despite PD-L1 expression could not be provided to any patient in this study.

Based on immunohistochemistry, the majority of patients had PD-L1 expression on < 1% of tumor cells (TC0, 61.7%) (Table 2, Fig. 1). PD-L1 expression on  $\ge 1\%$  of tumor cells (TC1

#### Table 1. Baseline clinical characteristics

Characteristic	Total (n = 128)	Adenocarcinoma (n=67)	SqCC (n=61)	p-value
Age (yr)	$59.5 \pm 11.1$	57.5±11.4	$61.8 \pm 10.5$	.047
Male sex	103 (80.5)	45 (67.2)	58 (95.1)	<.001
Smokers <sup>a</sup>	95 (74.2)	39 (58.2)	56 (94.9)	<.001
Body mass index (kg/m²)	$20.3 \pm 4.0$	$20.9 \pm 3.9$	19.7 (4.0)	.093
ECOG PS score $\geq 2$	19 (14.8)	9 (13.4)	10 (16.4)	.638
TNM stage at diagnosis <sup>a</sup>				.024 <sup>b</sup>
I	2 (1.6)	2 (3.0)	0	
II	5 (4.0)	2 (3.0)	3 (5.1)	
IIIA	13 (10.3)	2 (3.0)	11 (18.6)	
IIIB	27 (21.4)	9 (13.4)	18 (30.5)	
IV	79 (62.7)	52 (77.6)	27 (45.8)	
Extrathoracic disease <sup>c</sup>	56 (44.8)	41 (61.2)	15 (25.9)	<.001
Biopsy site <sup>c</sup>				.087 <sup>d</sup>
Lung	103 (82.4)	50 (75.8)	53 (89.8)	
Lymph node	10 (8.0)	6 (9.1)	4 (6.8)	
Pleura	5 (4.0)	5 (7.6)	0	
Other	7 (5.6)	5 (7.6)	2 (3.4)	
EGFR-positive	8 (6.2)	8 (11.9)	0	.005
ALK-positive	7 (5.4)	7 (10.4)	0	.009
First-line treatment				.011
Chemotherapy	95 (74.2)	49 (73.1)	46 (75.4)	
Targeted therapy <sup>e</sup>	16 (12.5)	13 (19.4)	3 (4.9)	
Other	17 (13.3)	5 (7.5)	12 (19.7)	

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

SqCC, squamous cell carcinoma; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; SD, standard deviation.

<sup>®</sup>Data were not available in two patients; <sup>®</sup>For comparison of stage IIIB or IV between adenocarcinomas and SqCCs; <sup>®</sup>Data were not available in three patients; <sup>@</sup>For comparison of lung biopsy between adenocarcinomas and SqCCs; <sup>®</sup>Patients who had a high probability of underlying *EGFR* mutation (e.g., non-smoking females) in whom molecular testing could not be performed (inadequate tissue for molecular analysis in the initial sample with the patient unwilling or unfit for a repeat invasive procedure) and whose performance status did not permit the use of chemotherapy were provided targeted therapy on compassionate grounds. Therefore, the number of patients with driver mutations and the number of patients who received targeted therapy was not equal.

or TC2) was observed in 38.3% of the subjects. However, this did not differ between subjects with adenocarcinoma or SqCC (p = .335). TIL1 (31.3%) and TIL0 (23.4%) were the most commonly observed TIL scores (Table 2, Fig. 2). High TILs (TIL occupying > 25% of tumor stroma, TIL2 or TIL3) were observed in 26.6% of patients. Infiltration > 50% of tumor stroma by TILs (TIL3) occurred in the least number of cases (7.8%). Difference in the TIL scores between adenocarcinoma and SqCC was not observed (p = .126). The baseline characteristics were not different between subjects with high and low TILs (data not shown). A higher proportion of patients with PD-L1 expression

Table 2.	. Histopathological	characteristics	of the ov	erall population

Variable	Total (n = 128)	Adenocarcinoma (n=67)	SqCC (n=61)	p-value
PD-L1 TC score				.335ª
TC0	79 (61.7)	44 (65.7)	35 (57.4)	
TC1	27 (21.1)	8 (11.9)	19 (31.1)	
TC2	22 (17.2)	15 (22.4)	7 (11.5)	
TIL score <sup>b</sup>				.126°
TILO	30 (23.4)	18 (26.9)	12 (19.7)	
TIL1	40 (31.3)	22 (32.8)	18 (29.5)	
TIL2	24 (18.8)	10 (14.9)	14 (23.0)	
TIL3	10 (7.8)	4 (6.0)	6 (9.8)	

Values are presented as number (%).

The TC score was assigned based on the proportion of tumor cells expressing PD-L1 (TC0: <1%, TC1: ≥1 but ≤50%, TC2: >50%). The TIL score was assigned based on the proportion of tumor stroma occupied by TILs (TIL0: <5%, TIL1: <25%, TIL2: >25 but <50%, TIL3: >50%).

SqCC, squamous cell carcinoma; PD-L1, programmed death-ligand 1; TC, tumor cell; TILs, tumor-infiltrating lymphocytes.

<sup>a</sup>For comparison of the proportion of subjects with PD-L1 expression on  $\geq 1\%$  of tumor cells (TC1 or TC2) between adenocarcinomas and SqCCs; <sup>b</sup>TIL score was not available for 26 patients; <sup>c</sup>For comparison of the proportion of subjects with  $\geq 25\%$  of tumor stroma infiltrated by TILs (TIL2 or TIL3) between adenocarcinomas and SqCCs.

 $\geq$  1% on tumor cells (TC1 or TC2: 58.8% vs. 35.7%, p = .026) had high TILs than low TILs.

A total of 22 subjects (17.2%) were classified as PD-L1–positive (PD-L1 expression on tumor cells > 50%, TC2). Most of the clinical characteristics of PD-L1–positive subjects were not different from PD-L1–negative subjects (Table 3). However, subjects who were PD-L1–positive had a higher prevalence of liver metastasis at baseline (18.2% vs. 2.8%; p = .018). PD-L1–positive subjects were also more often diagnosed based on extrapulmonary biopsies (Table 3). The median OS (95% confidence interval [CI]) was 7.6 (6.5–8.8) months in subjects who were PD-L1–negative compared with 8.5 (1.1–16.0) months in subjects who were PD-L1–positive (log-rank p = .584) (Fig. 3). On multivariate analysis, sex was the only factor associated with survival (hazard ratio, 0.39; 95% CI, 0.16 to 0.98; p = .046) (Table 4). The survival of patients with tumors showing high TILs was not different from subjects with low TILs (data not shown).



Fig. 1. Photomicrographs (programmed death-ligand 1 [PD-L1] staining using SP263 clone) showing positive control (PD-L1 staining in placenta) (A), TC2 (PD-L1 expression on >50% of tumor cells [TCs]) (B), TC1 (PD-L1 expression on 1%–50% TCs) (C), and TC0 (PD-L1 expression on <1% of TCs) (D).



Fig. 2. Photomicrographs (x 200) of hematoxylin and eosin staining showing TILO (0%–5% of tumor stroma occupied by tumor-infiltrating lymphocytes [TILs]) (A), TIL3 (>50% of tumor stroma occupied by TILs) (B).

### DISCUSSION

In this study, 17.2% of the patients with NSCLC could be labelled as PD-L1–positive (PD-L1 expression on tumor cells > 50%). PD-L1 expression  $\geq$ 1% on tumor cells was observed in 38.3% of the subjects. PD-L1–positive subjects had a higher prevalence of liver metastasis and were more often diagnosed using biopsy samples obtained from extrapulmonary sites. The PD-L1 expression did not affect survival in the study population. High TILs (TILs occupying > 25% of tumor stroma) were observed in 26.6% of the subjects, however, it did not affect survival. A larger proportion of patients with higher PD-L1 expression on tumor cells was more associated with subjects with high TILs than low TILs.

The assessment of PD-L1 expression based on immunohistochemistry is complicated by several factors including intra-tumor heterogeneity and inter-assay variation [21]. Therefore, the estimated prevalence of PD-L1–positive NSCLCs varies widely (7%– 75%) [5,22,23]. In the multinational EXPRESS study, PD-L1 expression on  $\geq$  50% and  $\geq$  1% of tumor cells was observed in 22% and 52% of patients with advanced NSCLC, respectively, using the 22C3 pharmDx kit [6]. In the present study, PD-L1 positivity was observed in 17.2% of NSCLCs (38.3% had PD-L1 expression  $\geq$  1% on tumor cells). The SP263 antibody clone was used because its performance is comparable or better than the other available antibody clones [24-26]. In prior studies from India, a PD-L1 positivity rate of 27% (>5% PD-L1 expression on tumor cells) and 34% ( $\geq$ 1% PD-L1 expression on tumor cells) was reported using the SP142 and SP263 clones, respectively [7,8]. Overall, the reported rates of PD-L1 expression in lung cancer in India appears to be less than international estimates. Whether this difference is due to methodological differences or true geographic differences is unclear.

PD-L1 expression has been associated with male sex, smoking, advanced tumor stage, SqCC histology, and *EGFR* mutation [5,22,27,28]. In the present study, association between PD-L1 expression and sex, smoking, or TNM stage, was not found. Liver metastasis was more common in subjects who were PD-L1–positive. The PD-L1 positivity rate was not affected by histology.

The association between PD-L1 expression and survival is controversial [5,7,22]. In a meta-analysis of 41 studies, PD-L1 expression was associated with poor survival in NSCLCs, specifically in subgroups of patients with adenocarcinoma or early disease [5]. In contrast, another study showed that PD-L1 expression resulted in worse prognosis in SqCC but not adenocarcinoma [22]. Furthermore, in another study, PD-L1 positivity in immune cells was found associated with better prognosis in resected NSCLCs [7]. In the present study, PD-L1 expression was not as-

Variable	PD-L1 negative (n=106)	PD-L1 positive (n=22)	p-value
Age (yr)	$59.6 \pm 10.6$	$59.5 \pm 13.6$	.934
Male sex	88 (83.0)	15 (68.2)	.240
Smokersª	82 (77.3)	13 (59.1)	.051
Body mass index (kg/m²)	$20.5 \pm 4.0$	$19.3 \pm 3.7$	.308
ECOG score ≥2	17 (16.0)	2 (9.1)	.525
TNM stage at diagnosis <sup>a</sup>			.649
I	2 (1.9)	0	
1	4 (3.8)	1 (4.5)	
IIIA	11 (10.4)	2 (9.1)	
IIIB	25 (23.6)	2 (9.1)	
IV	62 (58.5)	17 (77.3)	
Extrathoracic disease at baseline <sup>b</sup>	44 (41.5)	12 (54.5)	.311
Liver metastasis	3 (2.8)	4 (18.2)	.018
Biopsy site <sup>b</sup>			.022
Lung	91 (85.8)	12 (54.5)	
Lymph node	6 (5.7)	4 (18.2)	
Pleura	3 (2.8)	2 (9.1)	
Other	4 (3.8)	3 (13.6)	
Histology			.102
Adenocarcinoma	52 (49.1)	15 (68.2)	
Squamous cell carcinoma	54 (50.9)	7 (31.8)	
EGFR-positive	5 (4.7)	3 (13.6)	.138
ALK-positive	5 (4.7)	2 (9.1)	.345
First-line treatment			.664
Chemotherapy	80 (75.5)	15 (68.2)	
Targeted therapy	12 (11.3)	4 (18.2)	
None	14 (13.2)	3 (13.6)	
TIL score (%)°			.890
0–5	23 (28.0)	7 (31.8)	
6–25	33 (40.2)	7 (31.8)	
26–50	18 (22.0)	6 (27.3)	
>50	8 (9.8)	2 (9.1)	

 Table 3. Difference in clinical characteristics between PD-L1–positive and PD-L1–negative subjects

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

Tumors with PD-L1 expression > 50% in tumor cells (TC2) were considered PD-L1-positive.

PD-L1, programmed death-ligand 1; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; TIL, tumor-infiltrating lymphocyte; SD, standard deviation.

<sup>a</sup>Data were not available in two patients; <sup>b</sup>Data were not available in three patients; <sup>c</sup>TIL score was not available for 24 patients.

#### sociated with survival.

Infiltration  $\geq 20\%$  of tumor stroma with TILs has been observed in 50% of subjects with NSCLCs [26]. Higher TILs have been associated with higher PD-L1 expression and better survival in NSCLCs [9,10,29]. In the present study, approximately onefourth of the tumors showed high TILs (infiltration > 25% of tumor stroma by lymphocytes TIL2 or TIL3). Although high TILs were associated with higher PD-L1 expression in both TILs and tumor cells, high TILs did not affect survival in our study cohort.



Fig. 3. Kaplan-Meir plots for overall survival (OS). OS of programmed death-ligand 1 (PD-L1)–positive subjects was not different from PD-L1–negative subjects. The median (95% confidence interval) OS was 7.6 months (6.5–8.8) in subjects who were PD-L1–negative compared with 8.5 months (1.1–16.0) in subjects who were PD-L1-positive (log-rank p=.584).

Table 4. Multivariate analysis for predictors of OS

Variable	HR (95% CI)	p-value
Age	1.00 (0.98–1.03)	.708
Female sex	0.39 (0.16–0.98)	.046
Smoking	0.84 (0.36-2.00)	.682
ECOG PS ≥2	0.66 (0.30-1.45)	.297
TNM stage IIIB or IV	1.68 (0.82-3.44)	.154
Adenocarcinoma histology	0.78 (0.46-1.33)	.358
PD-L1-positive	1.47 (0.78-2.77)	.231

Tumors with PD-L1 expression >50% on tumor cells (TC2) or PD-L1 expression of 1%-50\% on tumor cells (TC1) with PD-L1 expression >10% in TILs (IC2) were considered PD-L1-positive.

OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; PD-L1, programmed death-ligand 1; TIL, tumor-infiltrating lymphocytes.

This was a single-center study with a relatively small sample size, thus, the results, especially subgroup analyses, should be interpreted cautiously. The TNM stage was not a prognostic factor in the present study for several reasons. The study had a relatively small sample size (n = 128). A disproportionately larger proportion of patients had stage IV disease (62.7% with stage IV disease in this study compared with 51.1% with stage IV disease in a previous analysis from our center with a much larger sample size (n = 1,501) [14]. The EGFR and ALK positivity rate observed in this study was considerably lower than what is generally observed at our center possibly due to selection bias [30]. Subjects who had undergone mutation testing could have been excluded from this study because they might have been left with inadequate tissue specimen for additional histopathological analyses.

Furthermore, a considerable proportion of lung cancer patients diagnosed based on cytology samples were not included in this study.

In conclusion, the present study results showed PD-L1 positivity and high TILs can be observed in approximately one-fifth and one-fourth of the patients with NSCLC, respectively. However, PD-L1 expression or high TILs did not affect the OS in our study cohort.

#### Supplementary Information

The Data Supplement is available with this article at https://doi.org/10.4132/ jptm.2021.08.08.

#### **Ethics Statement**

Written informed consent was obtained from all the study participants. The study was approved by the Institutional Ethics Review Committee (Reference number: NK/4398/MD/381).

#### Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

#### **Code Availability**

Not applicable.

#### ORCID

Gaurav Garg	https://orcid.org/0000-0002-1658-9441
Kuruswamy Thurai Prasad	https://orcid.org/0000-0001-7690-6595
Navneet Singh	https://orcid.org/0000-0002-8389-0701
Parul Gupta	https://orcid.org/0000-0001-7035-1752
Valliappan Muthu	https://orcid.org/0000-0003-0410-8468
Ashim Das	https://orcid.org/0000-0002-2031-7000
Amanjit Bal	https://orcid.org/0000-0002-1457-8589

#### **Author Contributions**

Conceptualization of study: AB. Data curation: GG, KTP, NS, PG. Formal analysis: AB, AD, VM. Investigation: GG. Methodology: AB, AD, VM, GG, PG. Resources: AB, NS, KTP, GG, PG. Supervision: AB. Validation: AB, AD, KTP, NS. Writing-original draft: AB, GG. Writing-review & editing: AB, KTP, PG, AD, NS. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

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# Rosette-forming epithelioid osteosarcoma in the rib: a rare case of location and morphology

#### Sun-Ju Oh

Department of Pathology, Kosin University College of Medicine, Busan, Korea

The rib is an unusual location for osteosarcoma and is reported in only 2% of all cases. The major histological variants of osteosarcoma are osteoblastic, chondroblastic, and fibroblastic, with a few rare variants including one epithelioid type. This report describes a 44-year-old male with an osteolytic mass in the right seventh rib. Histological examination revealed osteosarcoma with unique features of epithelioid appearance and rosette structures. To the best of our knowledge, this is the first reported case of a rosette-forming osteosarcoma of the rib that showed epithelioid morphology. Despite successful surgery, the patient's prognosis was poor because this malignancy had an unusual location within the axial skeleton and was a rare histological variant.

Key Words: Osteosarcoma; Ribs; Epithelioid; Rosette

Received: May 7, 2021 Revised: May 25, 2021 Accepted: June 22, 2021 Corresponding Author: Sun-Ju Oh, Department of Pathology, Kosin University College of Medicine, 262 Gamcheon-ro, Seo-gu, Busan 49267, Korea Tel: +82-51-990-6333, Fax: +82-51-990-3080, E-mail: 10highpowerfield@gmail.com

Osteosarcoma (OSA) is the most common primary malignant bone tumor, and it typically occurs in adolescents and young adults, with a slight male predominance [1]. OSA primarily affects the metaphysis of the long bones and, less frequently, the axial skeleton, including the ribs [2]. Various pathological subtypes of conventional OSA have been recognized, including osteoblastic, chondroblastic, and fibroblastic subtypes. This report describes a rare case of epithelioid OSA that involved the rib with special rosette-forming structure morphological features. Particular attention was paid to review the OSA in the rib, which is an atypical location for a lesion, and the rare histologic rosetteforming epithelioid OSA variant.

# **CASE REPORT**

A 44-year-old male presented with a mass in the right posterior thorax. He reported intermittent upper back pain for the 6 months prior to admission. An initial chest radiograph showed a nonhomogeneous mass that involved the posterolateral aspect of the right 7th rib, and contrast-enhanced chest computed tomography (CT) indicated an ill-defined osteolytic lesion with coarse calcification (Fig. 1). Overall image analysis suggested a malignant bone tumor, including chondrosarcoma. The patient underwent wide surgical excision of the rib with tumor-free margins.

Gross examination revealed resected bone with a 6-cm-sized hemorrhagic mass with cortical disruption (Fig. 2A). Microscopically, the tumor was arranged in a trabecular or rosette-like growth pattern that contained a fibrillar matrix in the center (Fig. 2B, C). Higher magnification exhibited epithelioid tumor cells with a plasmacytoid feature of eccentrically located nuclei and an abundant cytoplasm (Fig. 2C, D). The nuclei showed a clumped chromatin pattern, and each contained 1 or 2 prominent nucleoli. Mitoses were frequent, up to 12 per 10 high-power fields. An area of well-defined nests was identified, whereas blood-filled cystic spaces exhibited telangiectatic features (Fig. 2E). An irregularly interconnected coarse trabecular pattern of immature osteoid deposition was obvious throughout the tumor (Fig. 2F). For immunohistochemical panels, the neoplastic cells stained negatively for cytokeratin AE1/AE3, leukocyte common antigen, CD138, CD99, synaptophysin, desmin, and CD34, with the only exception of a positive reaction for vimentin. The final histologic diagnosis was a rosette-forming epithelioid osteosarcoma.

The postoperative systemic workup did not reveal metastasis, and the patient was treated with adjuvant radiotherapy for pro-



Fig. 1. Radiologic images. (A) Chest radiograph showing an oval mass (arrow) in the right thorax. (B) Computed tomography scan of the chest, revealing a large, heterogeneously enhanced mass (arrow) that originated from the right seventh rib, with lytic destruction of the bone and coarse calcification.



Fig. 2. Epithelioid osteosarcoma of the rib. (A) Macroscopic picture of an ill-defined lytic mass of the rib with hemorrhagic cystic degeneration. (B) Microscopic view of the trabecular and rosette-forming pattern that contained a fibrillar matrix in the center. (C) Rosette-like structures of plasmacyoid tumor cells with eccentrically located nuclei and abundant pale cytoplasm. (D) Mixed epithelioid and plasmacytoid tumor cells. (E) Blood-filled cystic spaces showing telangiectatic features. (F) Neoplastic bones produced by malignant tumor cells are found throughout the lesion.

phylactic purposes. After one year of follow-up, however, recurrence was noted on the adjacent ribs and chest wall, for which the patient underwent palliative surgery followed by six cycles of adjuvant chemotherapy that consisted of doxorubicin and cisplatin. Tumor progression continued with multiple metastases to both lungs and the liver, and the patient died four and a half years after initial diagnosis.

# DISCUSSION

Primary OSA of the ribs is uncommon, and accounting for only 40 (2%) of the total 1,952 cases of OSA reported in the largest series; to date, very few cases have been reported [1-5]. Most of the cases that affect the ribs occurred in patients during their fourth decade, which was late compared to cases of the long bone, which primarily occur in the second decade. The major histological variants of conventional OSA are subdivided as osteoblastic, chondroblastic, and fibroblastic, while a few other variants are classified as giant cell-rich, osteoblastoma-like, and clear cell [1]. Scranton et al. first reported the rare epithelioid OSA subtype [6]. Several subsequent reports have described cases of epithelioid OSA that affect long bones and the lumbar spine, maxilla, and mandible [7-10]. Only one epithelioid OSA that originated in the ribs was reported prior to this case [11]. In one of these epithelioid cases, tumor cells showed various patterns of sheet, alveolar, acinar, trabecular, organoid, hemangiopericytoma-like, and rosette-like appearances [9]. Rosette-forming OSA was illustrated in only two previous studies [8,9]. In the two studies, Okada et al. found a rosette-like structure in 16 of 280 cases of OSA, which corresponded with a rate of 5.7% [9].

Histopathological differential diagnosis in epithelioid tumors of the rib are comprised of various malignant neoplasms including metastatic carcinoma, malignant melanoma, and any kind of malignant mesenchymal tumor with an epithelioid appearance such as a fibrosarcoma, leiomyosarcoma, and an undifferentiated sarcoma. Although immunohistochemistry is helpful for differentiation, the tissue samples must be carefully examined to identify malignant osteoid formation by tumor cells, which is pathognomonic for OSA. Metaplastic bone formations and calcifications are relatively common in many benign and malignant tumors, but only lace-like osteoid formation by malignant cells is important in the differential diagnosis.

Reported outcomes of patients with OSA indicated traumatic improvements after wide excision and multi-agent chemotherapy [1]. This strategy of extensive surgical resection and adjuvant chemotherapy are also effective in OSA of the ribs and increased the likelihood of relapse-free survival [2]. Another study, however, indicated that there was no apparent difference in survival rates between patients who underwent surgery alone and those who underwent both surgery and chemotherapy [4]. Surgical resection should include the entire thickness of the chest wall with a wide margin, including the adjacent ribs, intercostal muscles, pleura, and vertebrae. After resection, large chest wall defects need to be covered with tissue flaps or mesh material [4].

The prognosis of OSA in the axial skeleton, including the rib, is generally poorer than results reported for cases in the extremity [1]. The 5-year survival rate for patients with rib OSA was much lower, at 27%, despite multimodal chemotherapy and aggressive surgery, compared to the higher 65%–75% survival rate in patients with OSA in an extremity [4]. Okada et al. [9] reported that rosette-forming OSA is more aggressive than other histological variants; the 5-year survival rate was 15% compared to 45% for OSA without rosettes. In addition, 75% of patients with rosette characteristics died of multiple lung metastases despite extensive surgery with wide surgical margins and systemic chemotherapy [9].

In conclusion, this is the first report of a rosette-forming epithelioid OSA that involved the rib. This case illustrates the unusual morphological features. Because this OSA is a rare subtype and associated with a poor prognosis, additional information regarding proper treatment is needed.

#### **Ethics Statement**

This study was approved by the institutional review board (IRB) of Kosin University Gospel Hospital with a waiver of informed consent (IRB No. 2018-01-020).

#### Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

#### **Code Availability**

Not applicable.

#### ORCID

Sun-Ju Oh https://orcid.org/0000-0001-6013-8579

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

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# Primary testicular carcinoid tumor with marked lymphovascular invasion

Hyun Jung Lee<sup>1,2</sup>, Joon Young Park<sup>1</sup>, So Young Kim<sup>1</sup>, Chung Su Hwang<sup>1</sup>, Jung Hee Lee<sup>1</sup>, Dong Hoon Shin<sup>1</sup>, Jee Yeon Kim<sup>1,2</sup>

<sup>1</sup>Department of Pathology, School of Medicine, Pusan National University, Yangsan; <sup>2</sup>The Research Institute for Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, Yangsan, Korea

Testicular carcinoid tumors are very rare, accounting for less than 1% of all testicular tumors. We report a rare case of a testicular carcinoid tumor with extensive lymphatic invasion. A 42-year-old man presented with a painless, enlarged right testicular mass. There was no history of injury or discomfort in this region. Right radical orchiectomy was performed, which showed a well-defined, non-encapsulated solid white mass with calcification (7.0 × 4.5 × 3.5 cm) and absence of cystic components. Microscopic examination using hematoxylin and eosin staining of the tumor sections identified organoid, trabecular, and solid patterns with rosette formation. Extensive multifocal lymphatic invasion was observed. Immunohistochemistry was positive for synaptophysin, chromogranin, and CD56. Testicular carcinoid tumors usually show good prognoses; however, there was extensive lymphovascular invasion in this case. Thus, in the case of unusual presentation of the disease, close follow-up is necessary.

Key Words: Testicular carcinoid tumor; Testicular neoplasm; Lymphovascular invasion; Orchiectomy

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Corresponding Author: Jee Yeon Kim, MD, Department of Pathology, Pusan National University School of Medicine, 49 Busandaehak-ro, Mulgeum-eup, Yangsan 50612, Korea Tel: +82-55-360-1861, Fax: +82-55-360-1865, E-mail: jeykim@pusan.ac.kr

Testicular carcinoid tumors are very rare, with a prevalence of less than 1% among all testicular tumors [1]. The first case of testicular carcinoid tumor was reported as an element of a benign cystic teratoma by Simon et al. in 1954 [2]. Approximately 10% of testicular carcinoid tumors are complicated by the carcinoid syndrome [3,4]. Testicular carcinoid tumors can be divided into three subgroups: primary testicular carcinoid tumors, carcinoid tumors associated with teratoma, and carcinoid metastasis to the testis [5].

Carcinoid tumors were initially considered benign [6]; however, they are now regarded as potentially malignant neoplasms [7]. Furthermore, benign and malignant carcinoid tumors are difficult to differentiate histologically, making it important to identify metastases. The preferred therapy for carcinoid tumors is surgical excision. Other therapies include somatostatin analogs, interferon- $\alpha$ , chemotherapeutics, and radiation. The treatment with somatostatin analogs is limited to symptom control in patients with carcinoid syndrome and stabilization of disease progression.

There are only a few studies on testicular carcinoid tumors, and the clinical implications of its diagnosis remain unclear. We report a very rare case of a carcinoid tumor of the testis with extensive lymphatic invasion but no manifestation of carcinoid syndrome.

# CASE REPORT

A 42-year-old man presented with a painless, enlarged right testicular mass confirmed by ultrasonography ( $7.0 \times 4.5 \times 3.5$  cm in size). The patient did not have a history of injury or discomfort in this region. A computed tomography scan revealed a 7.0 cm homogenous enhancing mass with calcification in the right testicle. Results of laboratory analysis were as follows:  $\alpha$ -fetoprotein (AFP), 5.03 ng/mL (normal range, 0.1 to 930 ng/mL), beta-human chorionic gonadotropin, < 0.5 mIU/mL (normal range, 0 to 5 mIU/mL); and testosterone, 1.83 ng/mL (normal range, 1.2 to 10.19 ng/mL).

Right radical orchiectomy was performed, and it revealed a well-defined, non-encapsulated solid white mass with calcification  $(7.0 \times 4.5 \times 3.5 \text{ cm})$  and no cystic components. The cut surface was clear and evenly white, with no hemorrhage or necrosis (Fig. 1). The spermatic cord did not show any abnormalities. Following the surgery, the patient has been alive and well for 13



Fig. 1. Gross appearance of the right testicular mass. (A) A well-defined, non-encapsulated solid white mass is observed. (B) The cut surface is clear and evenly white without hemorrhage or necrosis.



Fig. 2. Microscopic findings of the testicular carcinoid tumor. (A) Organoid, trabecular, and solid patterns are visible. (B) The cells have a uniform polygonal appearance with fine granular cytoplasm and round nuclei containing fine chromatin and small nucleoli. (C, D) Extensive multifocal lymphatic invasion is detected.

months without lymph node metastasis or recurrence.

On microscopy, a hematoxylin and eosin stained tumor section showed organoid, trabecular, and solid patterns with rosette formation. The cells had a uniform polygonal shape with fine granular cytoplasm and round nuclei containing fine chromatin and small nucleoli (Fig. 2A, B). The mitotic figure was 0 per highpower field (HPF). Ki67 proliferation index was less than 1%, and tumor necrosis was absent. Extensive multifocal lymphovascular invasion was identified (Fig. 2C, D). There were no germ cell neoplasia in situ or other germ cell tumor components and no teratomatous component. The tumor had invaded the rete testis (pT2). Hemorrhage and necrosis were not observed. Further evaluation by immunohistochemistry showed the tumor was positive for synaptophysin, chromogranin, and CD56 (Fig. 3A, B) and negative for GATA3, OCT3/4, CD30, AFP, and c-kit. CD31 showed positivity in the lymphovascular endothelium (Fig. 3C), and D2-40 showed positivity in the lymphatic endothelium (Fig.

3D). Based on the morphological and immunohistochemical findings, this case was diagnosed as primary testicular carcinoid tumor. The tumor was staged as pT2N0M0.

## DISCUSSION

The incidence of testicular tumors is low, and testicular carcinoid tumors account for only 1% of all testicular tumors [8]. Primary testicular carcinoid tumors are exceedingly rare, and only a few cases have been reported in the literature. These tumors present in patients typically between their second and ninth decades of life, with an average diagnosis age of 46 years [9,10]. Testicular carcinoid tumors often do not elicit symptoms, or they cause symptoms similar to those of other testicular tumors. The symptoms are not specific, and the carcinoid syndromes are often not recognized until the histological diagnosis is confirmed.

In contrast to most ovarian carcinoid tumors that present as



Fig. 3. Immunohistochemical staining is positive for chromogranin and (A) synaptophysin (B). (C) CD31 shows positivity for lymphovascular endothelium. (D) D2-40 shows positivity for lymphatic endothelium.

teratomatous components, most testicular carcinoid tumors are histologically pure [11,12]. Carcinoid tumors are 15 times more common in the ovaries than in the testicles. Ovarian carcinoid tumors are most often associated with mature cystic teratomas, which constitute the most common type of ovarian germ cell tumors in females up to 30 years of age [13].

Histogenesis of testicular carcinoid tumors has not yet been defined. There is a strong rationale that testicular carcinoid tumors are of germ cell origin. Most tumor cases in the testes, especially solitary, large tumors, are likely to be primary. Testicular tumors are considered primary when metastasis from another primary tumor has been reasonably ruled out. Malignant outcomes have been reported in approximately 16% of cases. Features correlated with adverse outcomes include coagulative necrosis, 2 to 10 mitoses per 10 HPFs, atypical mitoses, and atypical carcinoid tumor-like features [14]. The present case was primary testicular carcinoid tumor, and no metastasis was identified. Coagulative necrosis and mitosis, which are usually seen in atypical carcinoid tumor, were not identified. Compared to previously reported cases, this tumor showed extensive lymphovascular invasion without atypical carcinoid tumor pattern.

The key features of testicular carcinoid tumors are organoid and trabecular patterns, with tumor cells arranged in solid nests or cords. The tumor cells have uniform polygonal shapes with fine granular cytoplasm and round nuclei containing fine chromatin and small nucleoli.

Testicular carcinoid tumors usually have a good prognosis; however, when there is an unusual presentation of the disease, such as extensive lymphatic invasion as in this case, a close follow-up is necessitated. The presence of multifocal lymphovascular invasion in this case was striking and warranted close clinical follow-up and further work-up.

This is the first report of a primary testicular neuroendocrine tumor with extensive lymphatic invasion. Additionally, patients with malignant tumors exhibit a prolonged clinical course before death, which usually results from distant metastases. Therefore, a long-term follow-up evaluation is necessary for patients with this neoplasm.

#### **Ethics Statement**

This study was approved by the Institutional Review Board of the Pusan National University Yangsan Hospital (05-2021-173), and the need for informed consent was waived.

#### Availability of Data and Material

All data generated or analyzed during the study are included in this published article.

#### **Code Availability**

Not applicable.

#### ORCID

Hyung Jung Lee	https://orcid.org/0000-0002-2995-6060
Joon Young Park	https://orcid.org/0000-0002-4193-2520
So Young Kim	https://orcid.org/0000-0003-3076-9147
Chung Su Hwang	https://orcid.org/0000-0001-9520-0301
Jung Hee Lee	https://orcid.org/0000-0003-3003-2217
Dong Hoon Shin	https://orcid.org/0000-0002-4980-9295
Jee Yeon Kim	https://orcid.org/0000-0002-0503-984X

#### **Author Contributions**

Conceptualization: HJL. Data curation: JYP, HJL. Formal analysis: SYK, HJL. Investigation: CSH, HJL. Validation: JHL, HJL. Visualization: HJL. Writing—original draft: HJL. Writing—review & editing: HJL, DHS, JYK. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

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# *Fusobacterium nucleatum*: caution with interpreting historical patient sample cohort

Kate L. F. Johnstone<sup>1</sup>, Sinead Toomey<sup>1</sup>, Stephen Madden<sup>2</sup>, Brian D. P. O'Neill<sup>3</sup>, Bryan T Hennessy<sup>1</sup>

<sup>1</sup>Medical Oncology Laboratory, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin; <sup>2</sup>Data Science Centre, Royal College of Surgeons in Ireland, Dublin; <sup>3</sup>St Luke's Radiation Oncology Network, Dublin, Ireland

Fusobacterium nucleatum (Fn) was first noted to be associated with colorectal cancer (CRC) in 2012 [1]. Since then there have been several publications in retrospective cohorts analyzing the relationship between colorectal tumor fusobacterial abundance, clinical and molecular characteristics and clinical outcomes [1]. The majority of studies, including 'Prognostic impact of Fusobacterium nucleatum depends on combined tumor location and microsatellite instability status in stage II/III colorectal cancers treated with adjuvant chemotherapy' by Oh et al. [2] published in Journal of Pathology and Translational Medicine, utilize quantative polymerase chain reaction for the NusG gene in Fn relative to a control prostaglandin transporter gene (SLCO2A1). Many of these studies utilize formalin-fixed paraffin-embedded (FFPE) tissue that has been stored for up to two decades. Although this enables a long follow-up period for clinical outcomes, recent data from our lab shows that advanced age of sample significantly impairs the ability to detect Fn.

Oh et al. [2] analyzed 747 surgical samples of stage III and high-risk stage II CRCs that had been preserved in FFPE format. These participants had surgery between 2005 and 2012 making their specimens 6–13 years old at time of analysis. Five hundred and ninety-three samples were of quality to be included in the analysis. Oh et al. [2] noted a detection rate for Fn of 68.8%. They used a median cutoff to divide Fn-positive results into Fnhigh and -low then grouped Fn-low with Fn-negative for analysis. Oh et al. [2] found participants with Fn-high CRCs to have

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Corresponding Author: Kate L. F. Johnstone, MBChB

Medical Oncology Laboratory, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Smurfit Building, Beaumont Hospital, Dublin 9, Ireland Tel: +353-1-809-3823, Fax: +353-1-809-3778, E-mail: katejohnstone@rcsi.com

significantly higher T score and were more likely to be proximal cancers. Fn-high non-sigmoid colon cancers that subsequently received adjuvant chemotherapy had significantly improved survival compared to Fn-low/negative. This association was intensified among the MSI-low sub-group.

In their discussion, Oh et al. [2] make note of their unpublished data showing more recently collected and embedded specimens had higher rates of Fn positivity. They infer Fn detection rate could be affected by storage time.

Based on our data, we would like to support this unpublished finding of Oh et al. [2] and demonstrate a significant negative relationship between storage time of FFPE tissue and detection of Fn.

We analyzed fusobacterial abundance in diagnostic biopsies from 202 locally advanced rectal cancers (LARCs) from patients diagnosed and treated in Irish hospitals between 2000 and 2020, using the same qPCR method as Oh et al. [2].

Ten point four percent of LARC diagnostic biopsies were positive for Fn. The age of sample at time of analysis was measured in months between diagnostic biopsy and analysis by qPCR. Positive samples were more likely to be younger. The median sample age among Fn-positive samples was 57 months, compared to 134 months for Fn-negative samples. The age of samples was significantly different between negative and positive groups on Wilcoxon Rank-sum (z = 3.568, p < .001) (Fig. 1).

The samples in our study, and that by Oh et al. [2], were preserved in FFPE format. FFPE processing can induce DNA damage itself and over time DNA slowly degrades in FFPE format and length of amplifiable DNA fragments become shorter [3,4]. Most research on DNA degradation focuses on mammalian, eukaryotic DNA but there is emerging evidence that FFPE





preservation may disproportionately affect bacterial DNA [4,5].

A review of published studies detecting Fn with the same qPCR method with change in cycle thresholds (qPCR  $\Delta$ CT) shows some tendency for older sample cohorts to have lower rates of Fn detection. Table 1 compares rates of Fn detection from lowest to highest between publications that used similar methods to evaluate Fn in CRC tumor samples, format samples were stored in and the age of the samples at time of analysis. Sample age was calculated from the difference between years of collection and date of publication, but it is likely DNA may have been extracted years before analysis and that analysis may have occurred sometime before publication in some cases [6-27]. Only Oh et al. [2] and Mima et al. [8-10] commented that year of diagnosis (and therefore age of sample) was associated with Fn detection rates. There appears to be a pattern between age of cohorts and rates of detection of Fn, but frozen samples and fluorescent in situ hybridization or ddPCR method appear to increase sensitivity too. Unfortunately, as studies did not include supplementary data or reports of age of samples meta-analysis was not possible, but we acknowledge this would be ideal.

As shown by us and Oh et al. [2], the ability to detect Fn from FFPE CRC tissue using qPCR declines significantly with time. Even with adjustment for age of sample, the association between Fn-status and prognosis (or other clinical outcomes) may be underestimated by using older cohorts with FFPE specimens. This is important to bear in mind for interpretation of previously published retrospective cohorts and suggests that more recently diagnosed cohorts may be more appropriate to use in the future.

#### **Ethics Statement**

Tumor samples were obtained from patients enrolled on the TRI-LARC clinical trial (NCT02151019). Historical tumor samples were obtained from tumor banks under the auspices of Institutional Review Board-approved protocols. Informed consent for tumor biobanking and research studies was obtained from all patients.

#### Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

#### **Code Availability**

Not applicable.

#### ORCID

Kate L. F. Johnstone	https://orcid.org/0000-0001-7735-2053
Sinead Toomey	https://orcid.org/0000-0002-1365-8173
Stephen Madden	https://orcid.org/0000-0002-0489-4588
Bryan T Hennessy	https://orcid.org/0000-0002-7871-6477

#### **Author Contributions**

Conceptualization: KLFJ, ST, BTH. Data curation: KLFJ, ST. Formal analysis: KLFJ, SM. Funding acquisition: ST, BTH, BDPO. Investigation: KLFJ, ST. Methodology: KLFJ, ST. Supervision: ST, BTH. Validation: KLFJ, ST. Writing—original draft: KLFJ. Writing—review & editing: KLFJ, ST, SM, BTH. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

#### **Funding Statement**

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Author (year published)	Rate of Fn detection in CRC tumors (%)	Detection method of Fn	Sample form	Sample age
Nosho et al. (2016) [6]	8.6	qPCR ∆CT	FFPE	Unclear
Author's work	10.4	qPCR ∆CT	FFPE	0–20 yr
Lee et al. (2018) [7]	12 in FFPE 98 in methacarn 100 in frozen tissues	qPCR ∆CT	FFPE, methacarn or frozen	6–13 yr
Mima et al. (2015, 2016) [8-10]	13	qPCR ∆CT	FFPE	7–20 yr
Borowsky et al. (2021) [11]	13.7	qPCR ΔCT	FFPE	8 to > 26 years NB updated same cohort used by Mima et al., may have used FN data from previous analysis in 2015 for older samples
Shariati et al. (2021) [12]	23	qPCR ∆CT	Fresh frozen	0–2 yr
Kunzmann et al. (2019) [13]	31 'high' for FN but unclear % positive	qPCR ∆CT	Fresh frozen	6–10 yr
Yan et al. (2017) [14]	38	qPCR ∆CT	Frozen	4–5 yr
Okita et al. (2020) [15]	38	qPCR ∆CT (different human genome control gene)	FFPE	Age unclear
Yamamoto et al. (2021) [16]	45	qPCR ∆CT	FFPE	3–4 yr
Tahara et al. (2014) [17]	52 for FN 73.8 for pan-fusobacterium	qPCR ∆CT	Unclear	Unclear
Lee et al. (2020) [18]	54	qPCR ∆CT	FFPE	Age unclear
Chen et al. (2019) [19]	55	qPCR ∆CT	FFPE	<2 yr
lto et al. (2015) [20]	56	qPCR ∆CT	FFPE	1–13 yr
Serna et al. (2020) [21]	57 pre-treatment (rectal cancers only)	FISH for Fn RNA	FFPE	2–13 yr
Tunsjo et al. (2019) [22]	60	qPCR ∆CT (used B-globin as control gene)	Allprotect tissue reagent, unclear time to DNA extraction or storage before analysis	2–5 yr
Yu et al. (2016) [23]	61.3 overall	FISH for Fn RNA	FFPE	1–2 yr
Kashani et al. (2020) [24]	68	PCR of Fn and electrophoresis without control gene	Fresh samples	1–3 years but may have extracted DNA immediately
Oh et al. (2019) [2]	68.8	qPCR ∆CT	FFPE	5–13 yr Excluded 20% due to poor quality
Yamaoka et al. (2018) [25]	75	ddPCR	Frozen	10–18 yr
Kim et al. (2020) [26]	82	qPCR relative to other bacterial genes	Fresh samples with immediate DNA extraction	4–5 yr
Li et al. (2016) [27]	87.1	PCR ACT	Frozen	3–4 yr

Table 1. Comparison of Fn detection rates in publications analyzing CRC tumors by detection method, sample storage format, and sample age

Comparison of Fn detection rates in publications analyzing CRC tumors by detection method, sample storage format and sample age. There is some tendency for older sample cohorts to have lower rates of detection, for frozen or fresh samples to have higher detection rates compared to FFPE, and ddPCR and FISH may be more sensitive methods than the ubiquitous quantitative polymerase chain reaction for Fn relative to control gene (qPCR  $\Delta$ CT). Fn, *Fusobacterium nucleatum*; CRC, colorectal cancer; qPCR  $\Delta$ CT, ubiquitous quantitative polymerase chain reaction for Fn relative to control gene; FFPE, formalin-fixed paraffin-embedded; ddPCR, digital droplet polymerase chain reaction; FISH, fluorescent in situ hybridization.

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# **Comment on "Breast implant-associated anaplastic large cell lymphoma:** the first South Korean case"

#### Il-Kug Kim, Tae Gon Kim

Department of Plastic and Reconstructive Surgery, Yeungnam University College of Medicine, Daegu, Korea

#### Dear Editor.

We read the paper, "Breast implant-associated anaplastic large cell lymphoma: the first South Korean case," with great interest [1]. The authors described a Korean case of breast implant-associated anaplastic large cell lymphoma (BIA-ALCL), including the clinical manifestations and pathologic features. They claimed that it was the first South Korean BIA-ALCL case. However, to our understanding, this is not true. One of the co-authors of the paper, Dr. Eun Key Kim, presented this case at the Breast Reconstruction Symposium held on June 10, 2020, and described the case as "the second BIA-ALCL case in Korea" [2]. The first case of BIA-ALCL in Korea was a 44-year-old female patient diagnosed and treated at Yeungnam University Medical Center in August 2019, and the case was presented on November 9, 2019, at the 77th PRS KOREA, the main academic conference organized by the Korean Society of Plastic and Reconstructive Surgeons [3].

The first case of BIA-ALCL in Korea led to several important changes for plastic surgeons. After the first BIA-ALCL case was reported, all hospitals in Korea stopped using textured breast implants and switched to smooth breast implants. Moreover, Korean medical insurance does not cover the treatment for any complications of uninsured cosmetic surgery. BIA-ALCL is regarded as one such complication. Without insurance, the cost of BIA-ALCL treatment is an excessive burden on most patients. Hence, the medical staff and the Korean Society of Plastic Surgeons requested the Health Insurance Review and Assessment Service to cover BIA-ALCL, and it was approved. As such, the

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Corresponding Author: Tae Gon Kim, MD, PhD Department of Plastic and Reconstructive Surgery, Yeungnam University College of Medicine, 170 Hyeonchung-ro, Nam-gu, Daegu 42415, Korea Tel: +82-53-620-3480, Fax: +82-53-626-0705, E-mail: kimtg0919@daum.net

first case of BIA-ALCL in Korea is a meaningful and memorable case for all plastic surgeons.

The case report of the first case of BIA-ALCL in Korea was published online in December 2020 and published in print in April 2021 [4]. Molecular analysis and histopathologic analysis were performed, although these were time-consuming. Meanwhile, the authors published the second case of BIA-ALCL in Korea as the first case. This may be the first published case report but it is not the first identified case [1]. Hence, in our opinion, this should be amended.

#### **Ethics Statement**

Not applicable.

#### Availability of Data and Material

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

#### **Code Availability**

Not applicable.

#### ORCID

Tae Gon Kim https://orcid.org/0000-0002-6738-4630 Il-Kug Kim https://orcid.org/0000-0002-2428-8403

#### **Author Contributions**

Conceptualization: IKK, TGK. Writing-original draft: IKK. Writing-review & editing: IKK, TGK. Approval of final manuscript: all authors.

#### **Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.

#### **Funding Statement**

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NEWSLETTER

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#### PathologyOutlines.com

# **Solid tumors and NGS panel selection** Guoli Chen and Patricia C. Tsang Geisinger Medical Laboratories, Geisinger Health, Danville and Wilkes-Barre, PA, USA

What's new in molecular genetic pathology 2021:

Received: August 26, 2021 Accepted: August 31, 2021 Corresponding Author: Patricia C. Tsang, MD Geisinger Medical Laboratories, Geisinger Health, Wilkes-Barre, PA, USA E-mail: patctsang@hotmail.com

#### ORCID

Guoli Chen https://orcid.org/0000-0002-4426-6156 Patricia C. Tsang https://orcid.org/0000-0003-0523-9793

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#### Abstract

The linchpin of precision medicine is molecular genetic and genomic testing. Molecular biomarkers are important for establishing precise diagnoses and for predicting therapeutic responses that enable cancer patients to receive personalized and targeted treatment. Below are highlights of the current considerations in next generation sequencing (NGS) panel selection, and in molecular testing of solid tumors of the lung, digestive system, thyroid and soft tissue.

# NEXT GENERATION SEQUENCING PANEL SELECTION

- The use of targeted NGS panels (comprising up to several hundred genes) is more common than whole exome sequencing (WES) in clinical practice. Pros and cons of WES or large targeted panels compared to small hotspot panels include the following:
  - Comprehensive tumor profiling maximizes the chances of uncovering clinically relevant alterations.
  - They require more tumor tissue, cost more for data analysis and storage, and entail more complex interpretation.

• FDA-approved liquid biopsy using circulating cell-free DNA is an excellent tool when tumor tissue is inaccessible or limited [1]. While liquid biopsy has a faster turnaround time, it has a higher false-negative rate than standard biopsy NGS.

# NON-SMALL CELL LUNG CARCINOMA (NSCLC)

- Molecular profiling is used to predict therapeutic efficacies in NSCLCs and typically includes EGFR, KRAS, ROS1, ALK, MET, BRAF, RET, ERBB2 (HER2), and NTRK [2]. Also useful is PD-L1 (programmed cell death ligand-1) by immunohistochemistry (IHC) to predict response to immune checkpoint inhibitors.
- EGFR mutations involve 10%-35% of lung adenocarcinoma in Western populations (and as high as 50% in Asian populations) and 3% of lung squamous cell carcinoma (SqCC). Mutations can predict therapeutic response to EGFR-specific tyrosine kinase inhibitors (TKIs).
  - Acquired resistance inevitably develops, mostly commonly due to *EGFR* T790M missense mutation, accounting for ~50% of acquired resistance mutations. FDA-approved EGFR-inhibiting drugs that specifically target T790M or exon 20 insertion mutation are available.
- *MET* amplification (by FISH or NGS) is responsible for 5%-20% of anti-EGFR resistance.
- KRAS mutations are detectable in 25%-35% of lung adenocarcinoma, and 5% of SqCC. Activating mutations in codons 12 and 13, and, less commonly, codon 61, predict unfavorable prognosis and EGFR-TKI resistance. A KRAS inhibitor drug that specifically targets the G12C missense mutation (seen in ~13% of NSCLCs) is available.
- ALK activating rearrangements occur in ~5% of lung adenocarcinoma, particularly in young non-smokers. The most common fusion gene product is *EML4-ALK*, which predicts response to ALK inhibitors. FISH is the gold standard

for testing, but IHC, RT-PCR and NGS can also be used.

- *ROS1* gene fusion (1%-2% of NSCLCs) signifies response to certain ALK inhibitors due to homology between the rearranged *ROS1* and *ALK* genes.
- *MET* exon 14 skipping mutations, *BRAF* V600E mutation, *RET* gene fusion and *NTRK* gene fusion can predict therapeutic response to their respective inhibitors.

### COLORECTAL CARCINOMA (CRC) AND CHOLANGIOCARCINOMA

- EGFR targeted treatment is effective in the absence of *KRAS* and *NRAS* mutations. Mutational analyses of *KRAS* and *NRAS* genes should include codons 12, 13, 59, 61, 117 and 146. Other drug resistance biomarkers include *BRAF* mutations and *PIK3CA/PTEN* deregulation.
- Despite its low prevalence in CRC (2%–3%), *ERBB2 (HER2)* amplification is emerging as a potential therapeutic target [3]. Furthermore, activation of *ERBB2 (HER2)* signaling causes resistance to anti-EGFR therapy in a subset of patients with metastatic CRC.
- *NTRK* fusions can be evaluated in CRC due to the availability of targeted therapies.
- FGFR gene fusion is detected in ~10% of intrahepatic cholangiocarcinoma for which two FGFR-targeted tyrosine kinase inhibitors have been approved - pemigatinib and infigratinib.

# **THYROID CARCINOMA**

- Papillary thyroid carcinoma may harbor BRAF mutations (especially V600E), TERT promoter mutations, and, less commonly, RET/PTC1/2/3 rearrangements and RAS mutations.
- PAX8-PPAR7 fusion, RAS mutations, and TERT mutations are enriched in follicular thyroid carcinoma, while BRAF V600E is unusual. RAS mutations are also seen in a minority of benign follicular adenomas.

- More than 60% of medullary thyroid carcinomas show somatic *RET* mutations.
- Anaplastic thyroid carcinomas can harbor *TERT, TP53* and/or *BRAF* mutations.
- Preoperative molecular testing is valuable in thyroid nodules with indeterminate FNA cytology. The gene panel typically includes *TERT, BRAF, PAX8/PPARY, RAS, RET/PTC* and *TP53*. Below are three commonly used commercial platforms:
- Thyroseq<sup>TM</sup> involves next-generation DNA and RNA sequencing of 112 genes to stratify thyroid nodules as likely benign or likely malignant.
- Afirma<sup>®</sup> Gene Sequencing Classifier is an RNA-based test. Similar to ThyroSeq<sup>™</sup>, it is mostly a rule-out assay with acceptable rule-in capability.

 ThyGeNEXT<sup>®</sup>/ThyraMIR<sup>®</sup> uses a combination of two tests. If no mutation is found in the first panel by DNA and RNA sequencing, another test is performed using micro-RNA expression.

# **BONE AND SOFT TISSUE TUMORS**

• Molecular testing has led to the discovery of new mesenchymal tumor entities. For example, *CIC-DUX4* sarcoma is a recently described small round blue cell tumor associated with more aggressive disease than Ewing sarcoma with characteristic *EWSR1-FL11* fusion. Immunohistochemical detection of ETV4, a transcriptional target of *CIC-DUX4*, is a useful diagnostic tool.



**Fig. 1.** Dedifferentiated liposarcoma with neoplastic spindle cells in perigastric mass. (A) H&E stain, 40 x. (B) FISH shows abnormally amplified *MDM2* gene (orange signals) relative to CEP12 control (green). *MDM2/* CEP12 ratio > 2.0:1 normal cut-off.

- Amplification of the *MDM2* gene detectable by IHC/FISH/NGS is helpful for confirming a diagnosis of atypical lipomatous tumor/ well-differentiated liposarcoma (~93%) or dedifferentiated liposarcoma (~97%). The oncogenicity of *MDM2* is related to *TP53* inactivation (Fig. 1).
- Gene fusion testing by FISH or NGS is helpful for classifying rhabdomyosarcomas [4].
  - Recurrent gene fusions are detected in ~80% of alveolar rhabdomyosarcoma (ARMS), and involve mostly the *FOXO1* gene fusing with either *PAX3* or *PAX7*. Fusion positive ARMS is associated with a worse prognosis than fusion negative ARMS.
  - Embryonal rhabdomyosarcoma (ERMS) characteristically lacks gene fusions, but shows chromosomal losses/gains and gene mutations. ERMS is similar to fusion negative ARMS in clinical behavior.
  - Most congenital/infantile spindle cell and sclerosing rhabdomyosarcomas show NCOA2 in addition to VGLL2 gene fusions. This entity is associated with a favorable prognosis. However, patients who harbor MYOD1 mutations tend to have aggressive disease.

### Reference

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# **Meet the Authors**

Dr. Chen is an Associate and Attending pathologist at Geisinger Medical Center, where he participates in clinical, academic and teaching services in surgical pathology and molecular diagnostics. He has actively published and lectured on topics related to molecular pathology of solid tumors.

Dr. Tsang joined the Pathology Outlines editorial board in 2019 and has been its Deputy Editor-in-Chief for Clinical Pathology since 2020. She is a Clinical Associate Professor and Laboratory Medical Director in Geisinger's Northeast Region and has been a practicing molecular pathologist for more than a decade.

