Received: 24 June 2021 | Revised: 17 November 2021 | Accepted: 26 January 2022

DOI: 10.1111/iep.12430

INTERNATIONAL JOURNAL OF Experimental Pathology WILEY

ORIGINAL ARTICLE

Orchestrated expression of vasculogenic mimicry and laminin-5γ2 is an independent prognostic marker in oral squamous cell carcinoma

Depanwita Saha[1] | **Debarpan Mitra**[1] | **Neyaz Alam**[2] | **Sagar Sen**[2] | **Saunak Mitra Mustafi**[3] | **Syamsundar Mandal**[4] | **Biswanath Majumder**[5] | **Nabendu Murmu**[1]

[1]Department of Signal Transduction and Biogenic Amines, Chittaranjan National Cancer Institute, Kolkata, India

[2]Department of Surgical Oncology, Chittaranjan National Cancer Institute, Kolkata, India

[3]Department of Pathology, Chittaranjan National Cancer Institute, Kolkata, India

[4]Department of Epidemiology and Biostatistics, Chittaranjan National Cancer Institute, Kolkata, India

[5]Departments of Molecular Profiling, Cancer Biology and Molecular Pathology, Mitra Biotech, Bangalore, India

Correspondence
Nabendu Murmu, Department of Signal Transduction and Biogenic Amines, Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Kolkata 700026, India.
Email: nabendu.murmu@cnci.ac.in

Present address
Biswanath Majumder, Oncology Division, Bugworks Research, C-CAMP, Bangalore, India

Funding information
This work was financially supported by the Science and Engineering Research Board (SERB), Government of India, under Grant Project No: EEQ/2016/000345.

Abstract

Vasculogenic mimicry (VM), an endothelial cell–independent alternative mechanism of blood supply to the malignant tumour, has long been considered as an adverse prognostic factor in many cancers. The correlation of VM with laminin-5γ2 and the assessment of their harmonized expression as an independent risk factor have not been elucidated yet in oral squamous cell carcinoma (OSCC). CD31/PAS staining stratified 116 clinically diagnosed OSCC specimens into VM+ and VM− cohorts. The expression pattern of laminin-5γ2 and its upstream modulator MMP2 was evaluated by immunohistochemistry and Western blot. The Kaplan–Meier and Cox regression analyses were performed to assess the survival and prognostic implications. The presence of VM demonstrated a significant correlation with the expression of laminin-5γ2 ($p < .001$) and MMP2 ($p < .001$). This pattern was mirrored by the significant upregulation of laminin-5γ2 and MMP2 in VM+ cohorts compared with the VM− ones. Furthermore, co-expression of VM and laminin-5γ2 was significantly associated with tumour grade ($p = .010$), primary tumour size ($p < .001$), lymph node metastasis ($p = .001$) and TNM stages ($p < .001$) but not with patients' age, gender, tobacco and alcohol consumption habit. Vasculogenic mimicry and laminin-5γ2 double-positive cohort displayed a significantly poorer disease-free survival (DFS) and overall survival (OS). Vasculogenic mimicry, laminin-5γ2 and their subsequent dual expression underlie a significant prognostic value for DFS [hazard ratio (HR) = 9.896, $p = .028$] and OS [HR = 21.401, $p = .033$] in OSCC patients. Together, our findings imply that VM along with laminin-5γ2 is strongly linked to the malignant progression in OSCC and VM and laminin-5γ2 coordination emerges as a critical prognostic biomarker for OSCC.

KEYWORDS
co-expression, laminin-5γ2, OSCC, prognosis, survival, vasculogenic mimicry

1 | INTRODUCTION

Oral cancer is the 6th most common malignancy in the world.[1] Oral squamous cell carcinoma (OSCC) accounting for over 90% of oral cancers[2] is one of the most common causes of cancer-related deaths in the developing countries including India and the South-East Asia. The estimated incidence and mortality due to lip, oral cavity cancer in the world are 2.0% and 1.8% respectively.[3] Annually 75,000–80,000 new oral cancer cases are being reported in India.[4] The use of tobacco in various forms including cigarette, bidi, hookah, betel nut and betel quid is the major risk factor for OSCC.[5] Severe alcoholism, HPV infection, dietary deficiencies and poor oral hygiene are the other common identified risk factors.[6] Metastasis and postoperative recurrence are the most common reasons for poor 5-year survival[7] that further increases failure of treatment. There are multiple clinicopathological factors responsible for this poor outcome. Growing tumours survive in the nutrient- and oxygen-deficit state using diverse strategies. In 1999, Maniotis et al.[8] demonstrated that when blood supply cannot meet the need of rapid tumour growth, some aggressive, metastatic and genetically dysregulated cancer cells mimic the endothelial cells and form pseudovascular channel–like structures called vasculogenic mimicry (VM). It was first described in human uveal melanoma as periodic acid–Schiff (PAS)–positive patterned vascular network and enables the tumours to form matrix-embedded vascular structures carrying plasma and blood cells to fulfil the increasing nutrient and metabolic demands in tumour microenvironment.[9] Core matrix proteins such as laminin, heparan sulphate proteoglycan and collagens IV and VI have been identified in these patterns.[10] Though vasculogenic mimicry is considered as an established prognostic marker in melanoma,[11,12] breast cancer,[13,14] glioblastoma,[15] ovarian cancer,[16] lung cancer,[17] prostate cancer[18] and digestive cancers,[19] the underlying molecular phenotypes inducing it in OSCC and their prognostic significance are poorly understood.

Laminin-5 (Ln-5), a component of extracellular matrix (ECM) adhesion molecules, is expressed predominantly in the epithelial basal membrane structure that promotes static adhesion and hemidesmosome formation.[20] However, the cleavage of short arm of γ2 subunit of laminin-5 by matrix metalloproteinases (MMPs) such as MMP2 and MT1-MMP leads to its switching from static to migratory form resulting in cell migration and/or invasion.[21,22] In the context of the molecular mechanism influencing VM, the downstream signalling of VE-cadherin, EphA2 and VEGF choreographs the cleavage of laminin-5 into the pro-migratory γ2x and γ2' fragments through activated MMP2, implicating the roles of the extracellular matrix remodelling in positively regulating the formation of VM network.[23]

Although the differential expression of laminin-5γ2 has been associated with tumour invasion and lymph node metastasis in OSCC[24,25] and with the poor survival outcome in TCGA database–derived head and neck cancer (HNC) cohorts ($n = 502$),[26] its correlation with VM phenotype and the prognostic significance of their coordinated expression have not been elucidated yet. Here, we aim to investigate the phenotypic characteristics of VM structures in OSCC tumour tissues and evaluate the expression of laminin-5γ2 and its upstream modulator MMP2, as well as their correlation in the process of the acquisition of VM structure in OSCC microenvironment. Finally, we have undertaken the survival and risk factor assessment of VM-laminin-5γ2 coordination in a defined patient cohort to enlighten a novel and promising therapeutic target of OSCC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

The surgical and clinically confirmed OSCC tissue specimens from a sum total of 116 patients (median age: 54 years, range: 28–80 years) were obtained from Chittaranjan National Cancer Institute, Kolkata, during May 2014–April, 2015. Patients with history of recurrence or preoperative chemotherapy and radiotherapy were excluded. Informed written consent was obtained from all the patients prior to specimen collection. The study was approved by the Institutional Ethics committee (IEC Ref: A-4.311/53/2014) in accordance with the ethical guidelines of Declaration of Helsinki (1964) and its later amendments. Tumour-node-metastasis stages were evaluated according to 8th edition of the American Joint Committee on Cancer (AJCC), and tumour grade was classified according to World Health Organization (WHO) standards. For OSCC patients with complete clinicopathological information and follow-up data, the overall survival (OS) time was calculated as the time interval from the date of surgery of the patients to their oral cancer–related death, and disease-free survival (DFS) was noted as the time interval from the date of surgery to the first documentation of local recurrences or distant metastasis. Parameters that are associated with diagnosis, prognosis and treatment of OSCC such as age, anatomic location of primary tumour, histological grade, habit of tobacco and alcohol consumption, tumour size, lymph node metastasis, and TNM stage group have been recorded (Table 1).

2.2 | Immunohistochemistry/PAS dual staining

Immunohistochemistry (IHC) coupled with PAS staining was performed with the primary antibodies against CD31 or PECAM-1 (Santa Cruz Biotechnology, Inc; rabbit monoclonal; clone: M-20, dilution: 1:100), laminin-5 (ϒ2 chain) (Merck; mouse monoclonal; clone: D4B5, dilution: 1:100) and MMP2 (Novus Biologicals; mouse monoclonal; clone: 8B4, dilution: 1:100) as per previously described methods.[14,27]

2.3 | Evaluation of vasculogenic mimicry and IHC markers

Vasculogenic mimicry (VM) was identified through the detection of CD31–negative and PAS-positive lumen–like structures surrounded by tumour cells (but not with endothelial cells) with or without red blood cells inside the lumen[28–32] (Figure 1A). The vascular structures were observed for structural integrity with no incidence of haemorrhage, necrosis or inflammatory cell infiltration in close proximity.[33–36] The VM density with respect to the overall vascular density has been assessed according to the modified method described by Weinder et al,[37] 1991, Shao et al, 2008,[38] and Zhou et al 2019.[39] The total number of CD31+ and CD31- lumen–like vascular structures, surrounded by tumour cells or endothelial cells, was considered as the overall vascular density. The areas of highest vascular density were found by observing the slides at 200× magnification. VM vessels were individually counted in 5 randomly selected 200× magnification field. The average percentage of VM has been evaluated relative to the overall vascular density and graded on the basis of following score: 0, negative; 1, <20%; 2, 20-<40%; 3, 40-<60%; and 4, ≥60%. The immunohistochemical score of our studied markers (laminin-5γ2 and MMP2) was determined by considering intensity of staining and proportion (%) of stained cells.[40–45] All the staining results were blindly evaluated by two experienced pathologists in a semi-quantitative manner. To account the intra-tumoral heterogeneity of antibody expression, ten randomly selected represented fields (under 400× magnification) from different areas of each slide were evaluated by two qualified pathologists (manual method). The staining intensity was determined on the basis of the following score: 0, negative; 1, mild; 2, moderate; and 3, strong staining, and the percentage (proportion) of positively stained cells per field was scored as follows: 0, <10%; 1, <25% of positively stained cells; 2, <50% of positively stained cells; and 3, >50% of positive cells.[46–48] The final immunohistochemical staining score of each sample was determined by summation of staining intensity and percentage (proportion) of positively stained cells, which ranged from 0 to 6. The final staining score 0–3 was considered as negative staining and that of 4–6 was considered as positive staining.[38,49] The semi-quantitative evaluation of pathologists was further validated by IHC profiler plugin[50] compatible with ImageJ (Figure S1). To normalize the digital image analysis with the

TABLE 1 Demographic and clinicopathological profile of OSCC patients

Patients' characteristics	*n* (%)
Age (years)	
<55	62 (53.45)
≥55	54 (46.55)
Gender	
Male	85 (73.28)
Female	31 (26.72)
Tobacco consumption	
Yes	50 (43.10)
No	66 (56.90)
Alcohol consumption	
Yes	10 (8.62)
No	106 (91.38)
Tumour location	
Lip	8 (6.89)
Tongue	17 (14.65)
Buccal mucosa	39 (33.62)
Gingiva	24 (20.69)
Floor of mouth	4 (3.45)
Retromolar trigone (RMT)	5 (4.31)
Others[a]	19 (16.38)
Grade	
Well	73 (62.93)
Moderate	41 (35.34)
Poor	2 (1.72)
Primary tumour status	
T1	64 (55.17)
T2	8 (6.89)
T3	18 (15.52)
T4	26 (22.41)
Lymph node metastasis	
N0	66 (56.89)
N1	27 (23.27)
N2	21 (18.10)
N3	2 (1.72)
TNM stage group	
I (T1N0M0)	50 (43.10)
II (T2N0M0)	5 (4.31)
III (T3N0M0, T1-3N1M0)	23 (19.83)
IV (T4N0M0-T1-4N1-3M0)	38 (32.76)

[a]Others include alveolar mucosa, hard palate and soft palate.

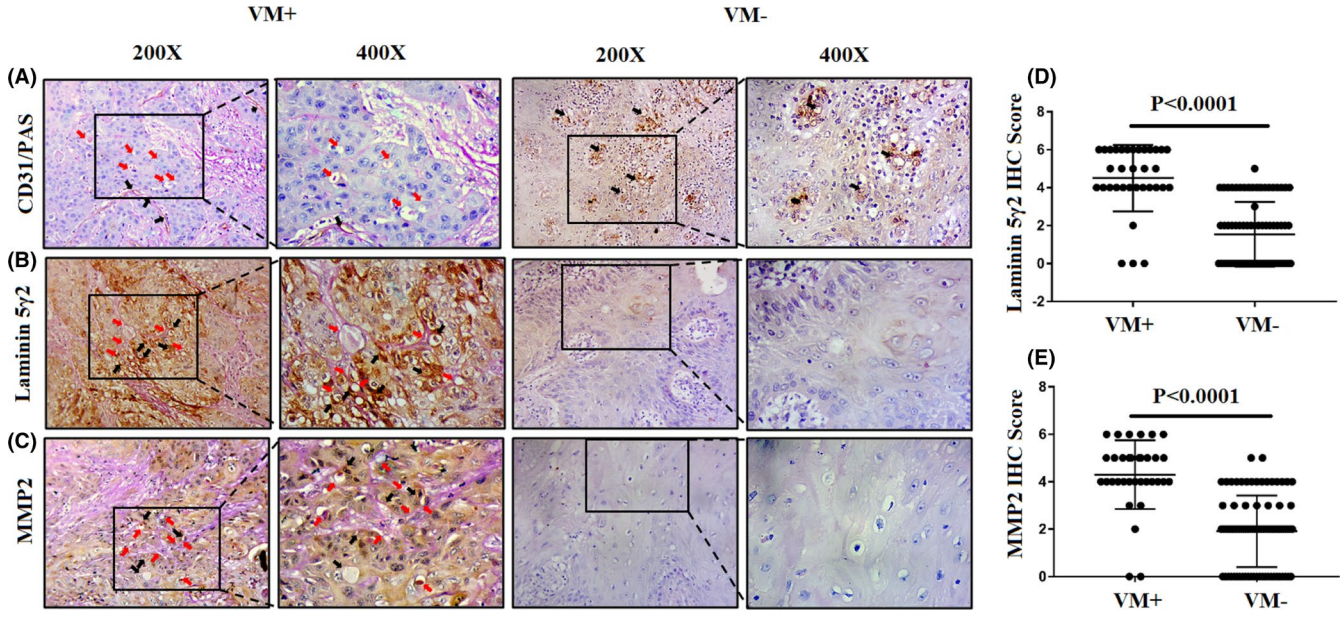

FIGURE 1 (A) CD31-PAS staining showing VM and endothelial structure in OSCC. Red arrows indicate PAS-positive and CD31-negative VM architecture, and black arrows represent endothelial structure showing CD31-positive staining with or without PAS staining (200× and 400× magnification). Representative images for immunohistochemical status (coupled with PAS staining) of (B) laminin-5γ2 and (C) MMP2 in VM-positive and VM-negative OSCC cohorts (200× and 400× magnification). Red arrows indicate PAS-positive networks, and black arrows indicate specific expression of proteins (B,C). Quantitative analysis of (D) laminin-5γ2 and (E) MMP2 expressions was revealed by immunohistochemical scores. The differences between VM-positive and VM-negative groups were calculated by the *t* test ($p < .0001$)

pathologists' manual analysis, the IHC profiler–generated four-tier staining pattern was scored from 0, negative; 1, low positive; 2, moderately positive; and 3, high positive similar to the manual assessment. Further, the total percentage of positive staining (1, low positive; 2, moderately positive; and 3, high positive) was determined from their individual percentage contribution and also scored from 0 to 3 scale in accordance with the pathologists' consideration of score for proportion (%) of stained cells.[46–48] The final immunohistochemical score (0–6) of each sample was determined by summation of the percentage score of total positive staining and the total intensity score contributed by different degrees of positive staining pattern to harmonize the digital assessment with the manual findings inferring the final score ≤3 as negative staining and that of >3 as positive staining.

2.4 | Western blot

Western blot analysis was performed using 3–5 mg of tissue specimens from few representative samples of both VM+ ($n = 15$) and VM− ($n = 15$) groups as per our previously described methods.[51] Fifty micrograms of total protein extract and appropriate primary antibodies against laminin-5 (ϒ2 chain) (Merck; mouse monoclonal; Clone: D4B5, Dilution: 1:100) and MMP2 (Novus Biologicals; mouse monoclonal; Clone: 8B4, Dilution: 1:100) was used. β-Actin was used as a loading control.

2.5 | Statistical analysis

All the statistical analyses were performed using SPSS 17 software (SPSS Inc) and GraphPad Prism version 7.00 software. The chi-squared (χ^2) test was performed to find the associations between clinical–pathological parameters and VM and laminin-5γ2, and correlation among laminin-5γ2, MMP2 expression and VM was determined by the Spearman correlation test. The t test was used to compare two means. Kappa value was calculated to assess the agreement between two pathologists and two methods. The Kaplan–Meier survival analysis followed by the log rank test was used to compare the survival patterns. The multivariate Cox proportional hazard regression model was also used where overall survival (OS) and disease-free survival (DFS) were calculated. $p < .05$ was taken to be statistically significant.

3 | RESULTS

3.1 | Evaluation of VM in OSCC tissue specimens

Vasculogenic mimicry (VM) was identified in 29.31% of OSCC tissue specimens. Based on the CD31/PAS staining, the total patient population was stratified into VM-positive (VM+) and VM-negative (VM−) cohorts (Figure 1A). 34

of 116 (29.31%) cases were VM-positive, and 82 (70.69%) cases were VM-negative.

3.2 | Correlation of VM with the differential expression of laminin-5γ2 and MMP2

The immunohistochemical expression of laminin-5γ2 and MMP2 was observed in OSCC tumour cell cytoplasm and in tumour stroma. The expression of laminin-5γ2 and MMP2 was positively correlated to VM at their individual level ($r = .6642$, $p \leq .001$ and $r = .6201$, $p \leq .001$ respectively), and the expression of laminin-5γ2 and MMP2 was also significantly correlated ($r = .5046$ and $p \leq .001$) with each other (Figure S2A–C). The expression pattern of laminin-5γ2 and MMP2 in VM-positive and VM-negative cohorts has been represented in Figure 1B,C. The quantitative data indicated the significantly elevated expression of laminin-5γ2 and MMP2 in VM-positive cohorts ($p < .0001$; Figure 1D,E). The mean immunohistochemical score of laminin-5γ2 and MMP2 in VM-positive cohorts was 4.50 ± 1.75 and 4.29 ± 1.45 (mean ± SD), respectively, whereas the same for the VM-negative cohorts was 1.74 ± 1.54 and 1.91 ± 1.51 respectively. Figure S1 shows the significantly positive correlation ($R^2 > .9$, $p < .001$) between the IHC scoring of two pathologists and between digital and manual methods. Kappa statistics was used to assess the interobserver and intermethod agreement, which revealed almost perfect strength of agreement ($\kappa = 0.81–1.00$) between the scoring of two pathologists and substantial strength of agreement ($\kappa = 0.61–0.80$)[52] between digital and manual methods confirming the reliability of our IHC scoring method. The differential expressional status of the studied markers was also validated through quantitative Western blot analysis (Figure S2D,E), which indicated significantly higher intensity of immunoreactive bands in VM-positive groups compared with the VM-negative ones ($p < .05$).

3.3 | Association of VM and laminin-5γ2 with the clinicopathological features

The association of individual and coordinated expression of VM and laminin-5γ2 with the clinicopathological characteristics of patients has been summarized in Table 2. The result showed 30 (25.86%) VM–laminin-5γ2 double-positive cases, 4 (3.44%) VM-positive laminin-5γ2 negative cases, 21 (18.10%) VM-negative laminin-5γ2–positive cases and 61 (52.58%) VM–laminin-5γ2 double-negative cases. The presence of vasculogenic mimicry in OSCC was significantly associated with tumour grade ($p = .002$), primary tumour status ($p < .001$), lymph node metastasis ($p = .005$) and TNM stage group ($p < .001$) but not with patient's age, sex and tobacco or alcohol consumption habit. It has been significantly found that 50% (1/2) cohort of the poorly differentiated tumour grade developed VM, whereas 48.78% (20/41) of the moderately differentiated group and 17.80% (13/73) of the well-differentiated group were found to be VM-positive. It is also noteworthy that 59.09% (26/44) patients of T3 and T4 primary tumour status significantly developed VM compared with the T1 and T2 group [11.11% (8/72)]. Similarly, the occurrence of VM was also significantly prevalent in the patients with positive nodal status compared with the negative ones [46% (23/50) vs 16.66% (11/66)], as well as in the patients with TNM stage groups III and IV [47.54% (29/61)] compared with TNM stage groups I and II [9.09% (5/55)]. The positive rate of VM was also significantly associated with the expression of laminin-5γ2 in OSCC. 43.9% of the total patients (51/116) showed positive expression of laminin-5γ2. Among them, the VM-positive group was found to have significantly increased level of expression of laminin-5γ2 [88.23% (30/34)] compared with the VM-negative [25.61% (21/82)] counterparts. Similar to the findings for the association of VM with the clinicopathological features, the positive expression of laminin-5γ2 was mostly observed in the patients with T3 and T4 primary tumour status (68.18%, $p < .001$) and TNM stage groups III and IV (57.37%, $p = .002$). Interestingly, the double-positive expressional status of VM–laminin-5γ2 was also significantly associated with tumour grade ($p = .010$), primary tumour status ($p < .001$), lymph node metastasis ($p = .00133$) and TNM stage group ($p < .001$) indicating the strong correlation between VM and laminin-5γ2 in the pathogenesis of OSCC. In addition to laminin-5γ2, we have also found a significant positive association of its upstream modulator MMP2 with VM ($p < .001$; Table S1). In association with the significant positive correlation of MMP2 with VM and laminin-5γ2, we have also demonstrated the significant association of MMP2 and VM-MMP2 dual positivity with the other established prognostic features of OSCC (Table S1). These data indicated the deterministic role of laminin-5γ2 and its activator molecule MMP2 in the occurrence of VM and progression of OSCC.

3.4 | Correlation of the positive expression of laminin-5γ2 and VM with disease-free and overall survival

To understand the collaborative prognostic significance of VM–laminin-5γ2, the 5-year survival rate was calculated for total of 116 patients with respect to DFS and OS. After completion of the follow-up (median follow-up

TABLE 2 Association between VM, laminin-5γ2 and their dual expression with clinicopathological characteristics of oral squamous cell carcinoma (OSCC)

Patients' characteristics	VM				Laminin-5γ2				VM and laminin-5γ2 dual expression			
	Positive	Negative			Positive	Negative			Positive	Negative		
	n (%)	*n* (%)	χ^2	*p* value	*n* (%)	*n* (%)	χ^2	*p* value	*n* (%)	*n* (%)	χ^2	*p* value
Age (years)												
<55	17 (14.65)	45 (38.79)	0.230	0.632	23 (19.83)	39 (33.62)	2.55	0.110	14 (12.07)	48 (41.38)	0.748	0.387
≥55	17 (14.65)	37 (31.89)			28 (24.14)	26 (22.41)			16 (13.79)	38 (32.76)		
Gender												
Male	23 (19.83)	62 (53.45)	0.778	0.378	37 (31.89)	48 (41.38)	0.02	0.875	20 (17.24)	65 (56.03)	0.903	0.342
Female	11 (9.48)	20 (17.24)			14 (12.07)	17 (14.65)			10 (8.62)	21 (18.10)		
Tobacco consumption												
Yes	12 (10.34)	38 (32.76)	1.20	0.274	19 (16.37)	31 (26.72)	1.27	0.260	12 (10.34)	38 (32.76)	0.159	0.690
No	22 (18.96)	44 (37.93)			32 (27.59)	34 (29.31)			18 (15.52)	48 (41.38)		
Alcohol consumption												
Yes	2 (1.72)	8 (6.89)	0.458	0.499	2 (1.72)	8 (6.89)	2.55	0.110	2 (1.72)	8 (6.89)	0.196	0.658
No	32 (27.59)	74 (63.79)			49 (42.24)	57 (49.14)			28 (24.14)	78 (67.24)		
Grade												
Well	13 (11.21)	60 (51.72)	12.6	**0.002***	28 (24.14)	45 (38.79)	2.53	0.283	12 (10.34)	61 (52.59)	9.19	**0.010***
Moderate	20 (17.24)	21 (18.10)			22 (18.96)	19 (16.38)			17 (14.65)	24 (20.69)		
Poor	1 (0.86)	1 (0.86)			1 (0.86)	1 (0.86)			1 (0.86)	1 (0.86)		
Primary tumour status												
T1	5 (4.31)	59 (50.86)	33.6	**<0.001***	17 (14.65)	47 (40.52)	18.5	**<0.001***	4 (3.45)	60 (51.72)	30.4	**<0.001***
T2	3 (2.59)	5 (4.31)			4 (3.45)	4 (3.45)			3 (2.59)	5 (4.31)		
T3	10 (8.62)	8 (6.90)			12 (10.34)	6 (5.17)			8 (6.89)	10 (8.62)		
T4	16 (13.79)	10 (8.62)			18 (15.52)	8 (6.89)			15 (12.93)	11 (9.48)		
Lymph node metastasis												
N0	11 (9.48)	55 (47.41)	12.9	**0.005***	20 (17.24)	46 (39.65)	6.91	0.075	8 (6.89)	58 (50)	15.7	**0.001***
N1	14 (12.07)	13 (11.21)			16 (13.79)	11 (9.48)			13 (11.21)	14 (12.07)		
N2	8 (6.89)	13 (11.21)			14 (12.07)	7 (6.03)			8 (6.89)	13 (11.21)		
N3	1 (0.86)	1 (0.86)			1 (0.86)	1 (0.86)			1 (0.86)	1 (0.86)		
TNM stage group												
I+II	5 (4.31)	50 (43.10)	20.6	**<0.001***	16 (13.79)	39 (33.62)	9.39	**0.002***	4 (3.45)	51 (43.96)	18.9	**<0.001***
III+IV	29 (25)	32 (27.59)			35 (30.17)	26 (22.41)			26 (22.41)	35 (30.17)		

TABLE 2 (Continued)

Patients' characteristics	VM				Laminin-5γ2				VM and laminin-5γ2 dual expression			
	Positive	Negative			Positive	Negative			Positive	Negative		
	n (%)	*n* (%)	χ^2	*p* value	*n* (%)	*n* (%)	χ^2	*p* value	*n* (%)	*n* (%)	χ^2	*p* value
VM												
Positive	-	-	-	-	30 (25.86)	4 (3.45)	38.3	**<0.001***	-	-		
Negative	-	-			21 (18.10)	61 (52.59)			-	-		

* Significant values i.e. $p < 0.05$ are denoted in bold.

period: 56 months, range: 16–60 months), 36 patients (31.03%) were dead due to local recurrence or metastasis after surgery and 62 (53.45%) patients were alive with the rest being lost to follow-up or died of other diseases unrelated to OSCC and they were considered as censored for further analysis. The Kaplan–Meier plot of DFS and OS in OSCC patients with differential status of VM, laminin-5γ2 and their dual existence have been shown in Figure 2. The follow-up data demonstrated that the mean DFS of VM-positive cohort and laminin-5γ2–positive cohort was significantly inferior to that of VM-negative (log rank = 92.052, $p < .001$) and laminin-5γ2–negative (log rank = 40.575, $p < .001$) cohort; VM–laminin-5γ2 double-positive cohort also had significantly worse DFS compared with the respective double-negative cohorts (log rank = 125.283, $p < .001$). The distinguishing DFS rate among these groups was also reflected with the OS time. The mean OS of VM-positive cohort, laminin-5γ2–positive cohort and VM–laminin-5γ2 double-positive cohort was significantly poorer than that of VM-negative (log rank = 80.363, $p < .001$), laminin-5γ2 negative (log rank = 45.209, $p < .001$) and VM–laminin-5γ2 double-negative (log rank = 114.464, $p < .001$) cohorts respectively. These findings interpreted that VM and laminin-5γ2 either individually or together are important indicators of DFS and OS in OSCC patients. Additionally, we have also found the significant difference in the Kaplan–Meier plot of DFS and OS in the OSCC patients with differential expression of MMP2 and of VM-MMP2 duality (Figure S3).

3.5 | Prognostic impact of paired VM–laminin-5γ2 positivity on disease-free and overall survival

Based on the significant findings of univariate analysis, indicating the significance of clinicopathological parameters such as tumour grade, primary tumour status, lymph node metastasis, TNM stage group and occurrence of VM independently, as well as in conjunction with the expression of laminin-5γ2 in DFS and OS, the multivariate Cox proportional hazard regression model was applied to assess their role as independent survival risk factors (Table 3). The multivariate analysis revealed that in addition to primary tumour status and lymph node metastasis, the occurrence of VM [hazard ratio (HR): 1.696; 95% CI :1.030–2.791; $p = .038$], positive expression of laminin-5γ2 (HR: 1.327; CI: 1.013–1.739; $p = .040$) and VM–laminin-5γ2 double-positive status (HR: 9.896; CI: 1.286–76.173; $p = .028$) were proved to be independent risk factors for DFS. Similar to DFS, the occurrence of VM (HR: 3.081; CI: 1.428–6.651; $p = .004$), positive expression of laminin-5γ2 (HR: 1.424;

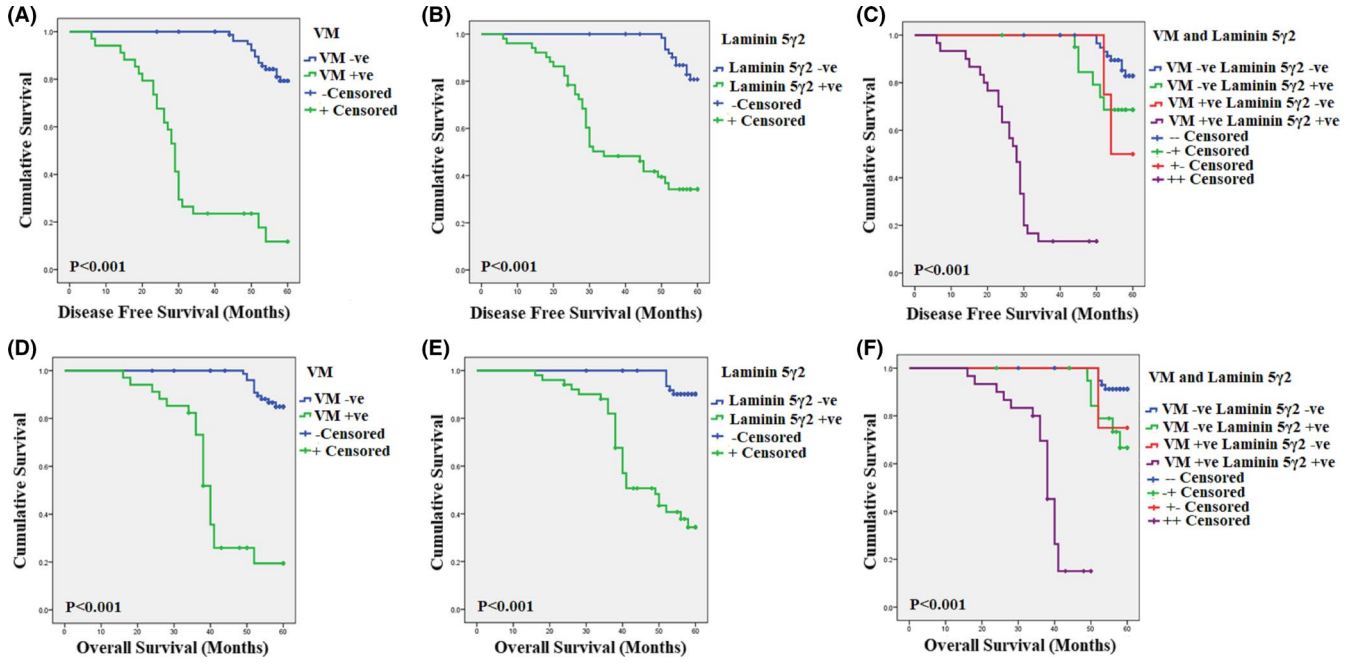

FIGURE 2 Kaplan–Meier analysis of the disease-free survival (DFS) and overall survival (OS) rate of patients with OSCC. DFS of patients in relation to (A) VM, (B) differential expressional status of laminin-5γ2, (C) VM and laminin-5γ2 dual status and OS of patients in relation to (D) VM, (E) differential expressional status of laminin-5γ2, (F) VM and laminin-5γ2 dual status

TABLE 3 Assessment of prognostic factors of disease-free survival (DFS) and overall survival (OS) by multivariate analysis of Cox proportional hazard model

	Disease-free survival (DFS)				Overall survival (OS)			
			95% CI				95% CI	
Variables	**HR**	***p* value**	**Lower**	**Upper**	**HR**	***p* value**	**Lower**	**Upper**
Grade	1.759	0.099	0.900	3.439	1.219	0.625	0.551	2.699
Primary tumour status	1.821	**0.003***	1.221	2.717	1.559	0.054	0.992	2.449
Lymph node metastasis	2.944	**<0.001***	1.632	5.311	2.865	**0.002***	1.478	5.554
TNM stage group	0.307	0.123	0.068	1.379	0.494	0.486	0.068	3.589
VM only	1.696	**0.038***	1.030	2.791	3.081	**0.004***	1.428	6.651
Laminin-5γ2 only	1.327	**0.040***	1.013	1.739	1.424	**0.026***	1.043	1.945
VM and Laminin-5γ2 double positive	9.896	**0.028***	1.286	76.173	21.401	**0.033***	1.276	358.980
MMP2 only	1.091	0.582	0.801	1.485	1.084	0.697	0.721	1.630
VM and MMP2 double positive	1.047	0.951	0.238	4.619	0.282	0.232	0.035	2.251

* Significant values i.e. $p < 0.05$ are denoted in bold.

CI: 1.043–1.945; $p = .026$) and the simultaneous double-positive expression of VM–laminin-5γ2 (HR: 21.401; CI: 1.276–358.980; $p = .033$) were also found to be independent risk factor for OS. In support of the significant findings on the survival endpoints, the positive expression of MMP2 and its co-existence with VM were also considered for the analysis of risk factor assessment, but neither of them proved to be the independent prognostic factor for DFS and OS in our OSCC cohorts.

4 | DISCUSSION

Alternative vascularization influences the poor prognosis of cancer patients,[53,54] evoking tumour resistance towards anti-angiogenic and anti-neoplastic therapy.[55] Vasculogenic mimicry is a leading pathological entity representing this state to which prompted us for a more comprehensive evaluation of VM and associated prognostic biomarkers underpinning in OSCC.

In this present study, we have evaluated the correlation of VM with expression of laminin-5γ2 in predicting the survival and prognosis of OSCC. We have inferred that the occurrence of VM is significantly prevalent in the poorly differentiated tumour with increased primary tumour size, higher lymph-node metastasis and TNM stage, which reflected the mechanistic link of VM to the invasion and metastasis attributing the aggressive and malignant progression of OSCC. Contextually, commonalities have been observed in other malignant tumours.[56,57] Laminin-5γ2 complements ECM remodelling and is considered to be one of the most common downstream signalling proteins in molecular cascades associated with VM, that is TGF-β,[58] VE-cadherin, EphA2, PI3-K[59] and MMP2.[60,61] Moreover, laminin receptor integrin β1-mediated FAK signalling has also been associated with VM-like network formation in human fibrosarcoma cells.[62] On the contrary, the cooperative interaction between MMP2 and laminin-5γ2 has been well established in a number of malignancies including glioblastoma[58] and aggressive melanoma when cultured on a three-dimensional ECM.[63] Our study delineated a significant interrelation of VM, laminin-5γ2 and their coordinated alignment with the histological and conventional prognostic parameters such as tumour grade, primary tumour size, lymph node metastasis and TNM stage group highlighting the impact of integrating multiple facets of these markers that may benefit while assessing the risk factors in OSCC. The double positivity of vasculogenic mimicry–laminin-5γ2, as well as their individual positive expression, also had the significantly poorer DFS and OS in our study, which may act as a tool to predict a worse prognostic indication. Although a few recent studies indicated the individual prognostic significance of some VM-associated biomarkers including LGR5,[64] ALDH1, Beclin1, p16[49] and extracellular IL17-F,[65] the combinatorial approach of VM with its associated biomarkers[14,66] is still a less explored area in OSCC. In this context, our investigation confirmed for the first time that both VM and laminin-5γ2 in combination provide better prognostic significance with higher statistical power including increased hazard ratio [(HR) = 9.896, $p = .028$ (DFS) and HR = 21.401, $p = .033$ (OS)] compared with individual expression of VM [(HR) = 1.696, $p = .038$ (DFS) and HR = 3.081, $p = .004$ (OS)] and laminin-5γ2 [HR) = 1.327, $p = .040$ (DFS) and HR = 1.424, $p = .026$ (OS)]. Collectively, these findings indicate the complementarity of VM and laminin-5γ2 as powerful risk factor for DFS and OS in OSCC. Our study also illustrated that in spite of being the upstream modulator of laminin-5γ2, MMP2 was not found to be performed as an independent risk factor in association with VM. We further validated the manual quantification data with the inputs from automatic profiler and showed a linear pattern.[67] Indeed, digital quantitative pathology is an evolving modality and needs further validation before its routine adoption as stand-alone method. Knowing the therapeutic challenges of late refractory oral malignancies and roles of novel prognostic biomarkers in informed treatment decision, these findings will provide important contextual guidance for defining appropriate clinical strategies.

In conclusion, the study revealed that the expression of the extracellular matrix protein Laminin-5γ2 coordinated with VM is significantly associated with tumour grade, primary tumour size, lymph node metastasis and TNM stage. Co-expression of vasculogenic mimicry with laminin-5γ2 underlines the independent prognostic impact and correlates with the decreased disease-free and overall survival in OSCC patients. Further validation of these findings in large independent studies would provide important predictive opportunities for better guidance towards effective treatments.

ACKNOWLEDGEMENT

The study was supported in part by the Chittaranjan National Cancer Institute (CNCI), Kolkata, India. Dr Nabendu Murmu sincerely thanks Dr Jayanta Chakrabarti, Director, CNCI, Kolkata, India, for his active support.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

DS and NM conceptualized and designed the study. DS and DM performed the experiments and analysed the data. NA and SS supplied tissue specimens. SMM validated the data and provided clinical insights. SM interpreted the data and performed statistical analysis. DS, BM and NM drafted the manuscript. BM and NM reviewed and edited the manuscript. All the authors approved the final version of the manuscript.

REFERENCES

1. Borse V, Konwar AN, Buragohain P. Oral cancer diagnosis and perspectives in India. *Sens Int*. 2020;1:100046.
2. Tandon P, Dadhich A, Saluja H, Bawane S, Sachdeva S. The prevalence of squamous cell carcinoma in different sites of oral cavity at our Rural Health Care Centre in Loni, Maharashtra – a retrospective 10-year study. *Contemp Oncol (Pozn)*. 2017;21(2):178-183.
3. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 Cancers in 185 countries. *CA Cancer J Clin*. 2021;1-121.
4. Mishra N. Oral cancer: a study in retrospection. *Natl J Maxillofac Surg*. 2019;10(1):1-2.
5. Tenore G, Nuvoli A, Mohsen A, et al. Tobacco, alcohol and family history of cancer as risk factors of oral squamous cell carcinoma: case-control retrospective study. *Applied Sci*. 2020;10(11):3896. doi:10.3390/app10113896

6. Khalesi S. A review of head and neck squamous cell carcinoma risk factors with more focus on oral cancer. *J Dent & Oral Disord*. 2016;2(6):1032.
7. Sun L, Chin RI, Gastman B, et al. Association of disease recurrence with survival outcomes in patients with cutaneous squamous cell carcinoma of the head and neck treated with multimodality therapy. *JAMA Dermatol*. 2019;155(4):442-447.
8. Maniotis AJ, Folberg R, Hess A, et al. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol*. 1999;155(3):739-752.
9. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol*. 2000;156(2):361-381.
10. Folberg R, Maniotis AJ. Vasculogenic mimicry. *APMIS*. 2004;12(7–8):508-525.
11. Zhang Z, Imani S, Shasaltaneh MD, et al. The role of vascular mimicry as a biomarker in malignant melanoma: a systematic review and meta-analysis. *BMC Cancer*. 2019;19(1):1134.
12. Bhattacharyya S, Mitra D, Ray S, et al. Reversing effect of Lupeol on vasculogenic mimicry in murine melanoma progression. *Microvasc Res*. 2019;121:52-62.
13. Andonegui-Elguera MA, Alfaro-Mora Y, Cáceres-Gutiérrez R, Caro-Sánchez C, Herrera LA, Díaz-Chávez J. An overview of vasculogenic mimicry in breast cancer. *Front Oncol*. 2020;10:220.
14. Mitra D, Bhattacharyya S, Alam N, et al. Phosphorylation of EphA2 receptor and vasculogenic mimicry is an indicator of poor prognosis in invasive carcinoma of the breast. *Breast Cancer Res Treat*. 2020;179(2):359-370.
15. Yue WY, Chen ZP. Does vasculogenic mimicry exist in astrocytoma? *J Histochem Cytochem*. 2005;53(8):997-1002.
16. Yu L, Zhu B, Wu S, et al. Evaluation of the correlation of vasculogenic mimicry, ALDH1, KiSS-1, and MACC1 in the prediction of metastasis and prognosis in ovarian carcinoma. *Diagn Pathol*. 2017;12(1):23.
17. Wang Y, Yang R, Wang X, et al. Evaluation of the correlation of vasculogenic mimicry, Notch4, DLL4, and KAI1/CD82 in the prediction of metastasis and prognosis in non-small cell lung cancer. *Medicine (Baltimore)*. 2018;97(52):e13817.
18. Sharma N, Seftor RE, Seftor EA, et al. Prostatic tumor cell plasticity involves cooperative interactions of distinct phenotypic subpopulations: role in vasculogenic mimicry. *Prostate*. 2002;50(3):189-201.
19. Ren HY, Shen JX, Mao XM, et al. Correlation between tumor vasculogenic mimicry and poor prognosis of human digestive cancer patients: a systematic review and meta-analysis. *Pathol Oncol Res*. 2019;25(3):849-858.
20. Giannelli G, Antonaci S. Biological and clinical relevance of Laminin-5 in cancer. *Clin Exp Metastasis*. 2000;18(6):439-443. doi:10.1023/a:1011879900554
21. Malina R, Motoyama S, Hamana S, Maruo T. Laminin-5 gamma2 chain and matrix metalloproteinase-2 expression in the neoplastic changes of uterine cervical squamous epithelium. *Kobe J Med Sci*. 2004;50(3–4):123-130.
22. Rousselle P, Scoazec JY. Laminin 332 in cancer: when the extracellular matrix turns signals from cell anchorage to cell movement. *Semin Cancer Biol*. 2020;62:149-165.
23. Delgado-Bellido D, Serrano-Saenz S, Fernández-Cortés M, Oliver FJ. Vasculogenic mimicry signaling revisited: focus on non-vascular VE-cadherin. *Mol Cancer*. 2017;16(1):65.
24. Yellapurkar S, Natarajan S, Boaz K. Expression of laminin in oral squamous cell carcinomas. *Asian Pac J Cancer Prev*. 2018;19(2):407-441.
25. Ono Y, Nakanishi Y, Ino Y. Clinicopathologic significance of laminin-5 γ2 chain expression in squamous cell carcinoma of the tongue immunohistochemical analysis of 67 lesions. *Cancer*. 1999;85(11):2315-2321.
26. You GR, Cheng AJ, Lee LY, et al. Prognostic signature associated with radioresistance in head and neck cancer via transcriptomic and bioinformatic analyses. *BMC Cancer*. 2019;19(1):64.
27. Sun B, Qie S, Zhang S, et al. Role and mechanism of vasculogenic mimicry in gastrointestinal stromal tumors. *Hum Pathol*. 2008;39:444-451.
28. Wang W, Lin P, Sun B, et al. Epithelial-mesenchymal transition regulated by EphA2 contributes to vasculogenic mimicry formation of head and neck squamous cell carcinoma. *Biomed Res Int*. 2014;2014:1-10.
29. Liu R, Yang K, Meng C, Zhang Z, Xu Y. Vasculogenic mimicry is a marker of poor prognosis in prostate cancer. *Cancer Biol Ther*. 2012;13(7):527-533.
30. Zhao B, Wu M, Hu Z, et al. Thrombin is a therapeutic target for non-small-cell lung cancer to inhibit vasculogenic mimicry formation. *Sig Transduct Target Ther*. 2020;5:117.
31. Meng J, Chen S, Lei Yy H, et al. Hsp90β promotes aggressive vasculogenic mimicry via epithelial–mesenchymal transition in hepatocellular carcinoma. *Oncogene*. 2019;38:228-243.
32. You B, Sun Y, Luo J, et al. Androgen receptor promotes renal cell carcinoma (RCC) vasculogenic mimicry (VM) via altering TWIST1 nonsense-mediated decay through lncRNA-TANAR. *Oncogene*. 2021;40:1674-1689.
33. Sun B, Zhang S, Zhang D, et al. Vasculogenic mimicry is associated with high tumor grade, invasion and metastasis, and short survival in patients with hepatocellular carcinoma. *Oncol Rep*. 2006;16(4):693-698.
34. Yu P, Zhu X, Zhu JL, et al. The Chk2-PKM2 axis promotes metabolic control of vasculogenic mimicry formation in p53-mutated triple-negative breast cancer. *Oncogene*. 2021;40:5262-5274.
35. Cheng L, Wang Q, Tao X, et al. FOXM 1 induces vasculogenic mimicry in esophageal cancer through β-catenin /Tcf4 signaling. *Diagn Pathol*. 2020;15:14.
36. Shao B, Zhao X, Liu T, et al. LOXL2 promotes vasculogenic mimicry and tumour aggressiveness in hepatocellular carcinoma. *J Cell Mol Med*. 2019;23(2):1363-1374.
37. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis – correlation in invasive breast carcinoma. *New Engl J Med*. 1991;324:1-8.
38. Shao Z, Zhang WF, Chen XM, Shang ZJ. Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: correlation with the angiogenesis and clinical outcome. *Oral Oncol*. 2008;44(12):1110-1117.
39. Zhou Y-T, Cai W-W, Li Y, et al. Correlations between quantitative parameters of contrast-enhanced ultrasound and vasculogenic mimicry in murine tumor model: a novel noninvasive technique for assessment? *Biol Proced Online*. 2019;21(1):11.
40. McCarty KS, Szabo E, Flowers JL, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res*. 1986;46:4244s-4248s.

41. Krajewska M, Krajewski S, Epstein JI, et al. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. *Am J Pathol*. 1996;148(5):1567-1576.
42. Gkouveris I, Nikitakis N, Avgoustidis D, Karanikou M, Rassidakis G, Sklavounou A. ERK1/2, JNK and STAT3 activation and correlation with tumor differentiation in oral SCC. *Histol Histopathol*. 2017;32(10):1065-1076.
43. Wu DH, Wang TT, Ruan DY, et al. Combination of ULK1 and LC3B improve prognosis assessment of hepatocellular carcinoma. *Biomed Pharmacother*. 2018;97:195-202.
44. Kukita A, Sone K, Oda K, et al. Histone methyltransferase SMYD2 selective inhibitor LLY-507 in combination with poly ADP ribose polymerase inhibitor has therapeutic potential against high-grade serous ovarian carcinomas. *Biochem Biophys Res Commun*. 2019;513(2):340-346.
45. Zhou W, Chen H, Ruan Y, Zeng X, Liu F. High expression of TRIM15 Is associated with tumor invasion and predicts poor prognosis in patients with gastric cancer. *J Invest Surg*. 2021;34(8):853-861.
46. Sun B, Zhang S, Zhang D, et al. Identification of metastasis-related proteins and their clinical relevance to triple-negative human breast cancer. *Clin Cancer Res*. 2008;14(21):7050-7059.
47. Sun H, Zhang D, Yao Z, et al. Anti-angiogenic treatment promotes triple-negative breast cancer invasion via vasculogenic mimicry. *Cancer Biol Ther*. 2017;18(4):205-213.
48. Dong WW, Li J, Li J, et al. Reduced expression of oestrogen receptor-β is associated with tumour invasion and metastasis in oestrogen receptor-α-negative human papillary thyroid carcinoma. *Int J Exp Pathol*. 2018;99(1):15-21.
49. Wang Y, Wang X, Zhang Y, et al. Vasculogenic mimicry and expression of ALDH1, Beclin1, and p16 correlate with metastasis and prognosis in oral squamous cell carcinoma. *Int J Clin Exp Pathol*. 2018;11(3):1599-1609.
50. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One*. 2014;5:e96801.
51. Ray S, Saha D, Alam N, et al. Exposure to chewing tobacco promotes primary oral squamous cell carcinoma and regional lymph node metastasis by alterations of SDF1α/CXCR4 axis. *Int J Exp Pathol*. 2021;102(2):80-92.
52. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977;33(1):159-174.
53. Cao Z, Bao M, Miele L, Sarkar FH, Wang Z, Zhou Q. Tumour vasculogenic mimicry is associated with poor prognosis of human cancer patients: a systemic review and meta-analysis. *Eur J Cancer*. 2013;49:3914-3923.
54. Hujanen R, Almahmoudi R, Karinen S, Nwaru BI, Salo T, Salem A. Vasculogenic mimicry: a promising prognosticator in head and neck squamous cell carcinoma and esophageal cancer? a systematic review and meta-analysis. *Cells*. 2020;9(2):507.
55. Belotti D, Pinessi D, Taraboletti G. Alternative vascularization mechanisms in tumor resistance to therapy. *Cancers (Basel)*. 2021;13(8):1912.
56. Yang JP, Liao YD, Mai DM, et al. Tumor vasculogenic mimicry predicts poor prognosis in cancer patients: a meta-analysis. *Angiogenesis*. 2016;19(2):191-200.
57. Zhang J, Qiao L, Liang N, et al. Vasculogenic mimicry and tumor metastasis. *J BUON*. 2016;21(3):533-541.
58. Ling G, Wang S, Song Z, et al. Transforming growth factor-β is required for vasculogenic mimicry formation in glioma cell line U251MG. *Cancer Biol Ther*. 2011;12(11):978-988. doi:10.4161/cbt.12.11.18155
59. Hess AR, Seftor EA, Gruman LM, Kinch MS, Seftor RE, Hendrix MJ. VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry. *Cancer Biol Ther*. 2006;5(2):228-233.
60. Lu XS, Sun W, Ge CY, Zhang WZ, Fan YZ. Contribution of the PI3K/MMPs/Ln-5γ2 and EphA2/FAK/Paxillin signaling pathways to tumor growth and vasculogenic mimicry of gallbladder carcinomas. *Int J Oncol*. 2013;42(6):2103-2115.
61. Larson AR, Lee CW, Lezcano C, et al. Melanoma spheroid formation involves laminin-associated vasculogenic mimicry. *Am J Pathol*. 2014;184(1):71-78.
62. Kawahara R, Niwa Y, Simizu S. Integrin β1 is an essential factor in vasculogenic mimicry of human cancer cells. *Cancer Sci*. 2018;109(8):2490-2496. doi:10.1111/cas.13693
63. Seftor RE, Seftor EA, Koshikawa N, et al. Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res*. 2001;61(17):6322-6327.
64. Wu Z, Song W, Cheng Z, Yang D, Yu L. Expression of LGR5 in oral squamous cell carcinoma and its correlation to vasculogenic mimicry. *Int J Clin Exp Pathol*. 2017;10(11):11267-11275.
65. Almahmoudi R, Salem A, Hadler-Olsen E, Svineng G, Salo T, Al-Samadi A. The effect of interleukin-17F on vasculogenic mimicry in oral tongue squamous cell carcinoma. *Cancer Sci*. 2021;112:2223-2232.
66. Xing P, Dong H, Liu Q, et al. ALDH1 expression and vasculogenic mimicry are positively associated with poor prognosis in patients with breast cancer. *Cell Physiol Biochem*. 2018;49:961-970.
67. Miles GJ, Powley I, Mohammed S, et al. Evaluating and comparing immunostaining and computational methods for spatial profiling of drug response in patient-derived explants. *Lab Invest*. 2021;101(3):396-407.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Saha D, Mitra D, Alam N, et al. Orchestrated expression of vasculogenic mimicry and laminin-5γ2 is an independent prognostic marker in oral squamous cell carcinoma. *Int J Exp Path*. 2022;103:54–64. doi:10.1111/iep.12430