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Original Article

Evaluation of Some Prognostic Biomarkers in Human Papillomavirus-Related Multiphenotypic Sinonasal Carcinoma

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What's Known

- Human papillomavirus (HPV)-related multiphenotypic sinonasal carcinoma (HMSC) is a novel form of sinonasal carcinoma associated with high-risk HPV.
- HMSC is a rare distinct tumor with high-risk local recurrence, unknown clinicopathologic spectrum, and prognosis, and is often misdiagnosed as adenoid cystic carcinoma of the salivary gland or sinonasal squamous cell carcinoma.

What's New

- Forty patients with morphological characteristics of HMSC were evaluated for high-risk HPV, the absence of adenoid cystic carcinoma-related proteins, and the presence of squamous and myoepithelial proliferation.
- The expression of some biomarkers was associated with aggressive malignant behavior, poor survival, and poor prognosis.

Abstract

Background: Human papillomavirus (HPV)-related multi phenotypic sinonasal carcinoma (HMSC) is a recently described tumor subtype with an unknown prognosis, often misdiagnosed with other sinonasal carcinomas, and associated with high-risk HPV (HR-HPV). The present study aimed to evaluate the expression of vascular endothelial growth factor (VEGF), Bcl-2-associated X protein (BAX), epidermal growth factor receptors (EGFR), ProEx™C, and human telomerase reverse transcriptase (hTERT) and assess their association with survival and clinicopathological characteristics.

Methods: Between 2017 and 2022, 40 HMSC patients underwent surgical resection at the School of Medicine, Zagazig University Hospitals (Zagazig, Egypt). Tissue samples were examined for the presence of HR-HPV; absence of myeloblastosis (MYB), MYB proto-oncogene like 1 (MYBL1), and nuclear factor I/B (NFIB) fusions and the presence of myoepithelial proteins (calponin, S100, SMA), squamous differentiation markers (p63, p40, calponin), VEGF, BAX, ProEx™C, and hTERT by immunohistochemistry. All patients were followed up for about 54 months until death or the last known survival data. Data were analyzed using the Chi square test and Kaplan-Meier method.

Results: The expression of VEGF, hTERT, and ProEx™C was significantly associated with age, advanced tumor stages, lymph node metastasis, tumor size, mortality, relapse, poor disease-free survival (DFS), and overall survival (OS) ($P<0.001$). BAX expression was significantly associated with tumor size, age, poor DFS, and relapse ($P=0.01$, $P<0.001$, $P=0.035$, and $P=0.002$, respectively).

Conclusion: HMSC is strongly associated with HR-HPV. The expression of VEGF, EGFR, BAX, hTERT, and ProEx™C is associated with aggressive malignant behavior, poor survival, and poor prognosis, making them novel prognostic biomarkers for targeted therapeutics in HMSC.

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Introduction

Sinonasal cancer (SNC) accounts for approximately 3.6% of all head and neck malignancies and less than 0.2% of all cancers.

The annual incidence of SNC is 0.556 cases per 100,000 people.[1] Between 2016 and 2021, orofacial malignancies accounted for 3.54% of all head and neck cancers in Egypt.[2] Histological subtypes of SNC include adenoid cystic carcinoma (ACC), squamous cell carcinoma (SCC), and some other less common subtypes. These malignancies originate from seromucous glands and surface epithelium.[3] The exact cause of SNC remains unclear. However, smoking is considered a significant risk factor in most head and neck cancers. It was reported that intestinal-type sinonasal adenocarcinoma is related to occupational exposure to wood dust.[4]

Human papillomavirus (HPV) is widely accepted as the cause of 20-25% of all head and neck cancers. Most HPV-related head and neck tumors occur in the oropharynx. However, SNC accounts for 20-25% of these tumors. High-risk HPV (HR-HPV) infection is strongly associated with HPV-related multiphenotypic sinonasal carcinoma (HMSC), especially the HPV-33 strain.[5] HMSC is usually found as tissue fragments focally lined by respiratory epithelium with occasional squamous metaplasia. Histologically, HMSC is divided by fibrous hyalinizing bands into compartments with two distinct patterns, namely cribriform and solid. The cribriform pattern consists of cylindromatous microcystic spaces with basophilic mucoid material surrounded by basaloid tumor cells. In contrast, the solid pattern shows compact tumor cells with a minimum amount of eosinophilic cytoplasm and multiple atypical cells with nuclear pleomorphism and vesicular nuclei, with different areas showing high mitotic activity (50-55 mitoses per 10 high-power fields) and confluent necrosis.[5] HMSC cribriform pattern is morphologically similar to ACC but lacks translocation between myeloblastosis (MYB), MYB proto-oncogene like 1 (MYBL1), and nuclear factor I/B (NFIB). On the other hand, the solid pattern mimics SCC but differs by the presence of myoepithelial differentiation.[6] The prevalence of HMSC is still unclear. However, it appears to be less aggressive than sinonasal SCC but has a higher risk of local recurrence in up to 36% of all cases.[5] HMSC cribriform patterns are often misdiagnosed as ACC, and solid patterns as SCC. HPV encodes two late genes (L1 and L2) and six earlier genes (E1-E7). E5, E6, and E7 are the main oncogenes involved in cell proliferation and aid viral replication. These viral oncogenes can promote tumorigenesis by activating different molecular signaling pathways.[7]

To date, due to its rarity, the molecular biology of HMSC has not been well-studied. It is known that the expression of vascular endothelial growth factor (VEGF), ProEx™C, Bcl-2-associated X protein (BAX), epidermal growth factor receptor (EGFR), and human telomerase reverse transcriptase (hTERT) is associated with poor prognosis of ACC of the salivary gland. However, their expressions in HMSC have not been previously evaluated. Therefore, the present study aimed to evaluate the expression of VEGF, BAX, EGFR, hTERT, and ProEx™C in patients with HMSC and assess their association with survival and clinicopathological characteristics.

Patients and Methods

A total of 40 patients with HMSC who underwent surgical resection with adjuvant radiation therapy (if needed) or definitive concurrent chemoradiation were enrolled in the study. Between 2017 and 2022, these patients were treated in various departments of the Faculty of Medicine, Zagazig University Hospital (Zagazig, Egypt). Follow-up was scheduled every three months in the first two years and every six months in subsequent years. All patients were followed up for about 54 months (range: 20-60) until death or last known survival data.

The patients were classified according to the TNM staging system (tumor size, extent of spread to the lymph nodes, and presence of metastasis) by the American Joint Committee on Cancer (AJCC) for sinonasal neoplasms.[8] Only patients who tested positive for HR-HPV; negative for MYBL1, MYB, and NFIB fusions; and positive for myoepithelial (calponin, SMA, S100) and squamous (p40, p63) differentiation markers were included in the study. The tissue samples were evaluated for the following:

- The presence of HR-HPV (a high-risk cocktail) using polymerase chain reaction (PCR).
- The absence of MYBL1, MYB, and NFIB fusions using fluorescence *in situ* hybridization (FISH) to rule out ACC.
- The presence of myoepithelial (calponin, SMA, S100) and squamous (p40, p63) differentiation markers for immunohistochemical identification of cancer.
- The expression of VEGF, ProEx™C, BAX, EGFR, and hTERT using immunohistochemistry technique and their association with clinicopathological and prognostic parameters of all patients.

The study was carried out in accordance with the ethical principles proposed by the World Medical Association for Human Studies,[8] and was approved by the Ethics Committee of Zagazig University (number: ZU-IRB#9902). Written informed consent was obtained from all patients.

HPV Genotyping Assay

Quantitative HPV-specific PCR was performed for HPV genotyping of HMSC tissue samples. DNA was extracted from 5 µm thick slides containing paraffin-embedded tumor tissues. The tissues were macrodissected from the slides, deparaffinized with xylene, and digested with 50 g/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) in a solution containing 1% sodium dodecyl sulfate at 48 °C for two days. In accordance with the manufacturer's recommendations, the DNA was extracted using ultrapure chloroform:phenol: isoamyl alcohol reagent (Invitrogen, Carlsbad, CA, USA).[9]

The L1 area of the HPV genome was amplified by consensus primers Gp5+-Gp6+ and Gp5-Gp6 using 30 µl PCR solution consisting of ammonium sulfate (16.6 mmol), tris Trizma™ crystals (67.0 mmol at pH 8.8), magnesium chloride (6.70 mmol), ethyl mercaptan (10.0 mmol), dimethyl sulfoxide (0.1 %), DMSO (3.3%), each primer (20 pmol), and platinum Taq (0.5 U).[10] The procedure for the rapid PCR in a Veriti thermal cycler was 40 cycles at 95 °C for 30 seconds, 44 °C for 60 seconds, and 72 °C for 90 seconds. Type-specific primers were used for the E6 and E7 regions of HPV types 11, 16, 18, 31, 33, 35, and 56.[11] The amplification cycle was reduced to 35 cycles, and the annealing temperature for HPV-33 and HPV-35 primers was set to 57 °C for 30 seconds.

Fluorescence in Situ Hybridization

Break-apart FISH assay for MYB, NFIB, and MYBL1 (all from Empire Genomics, Buffalo, NY, USA) was performed.[10] Tumour cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) II (ZytoVision GmbH, Bremerhaven, Germany) after hybridization.[12]

Immunohistochemistry

The tissue samples were deparaffinized for 15 min in a 56 °C oven, sectioned at a 3-5 µm thickness, and fixed on positively charged slides, and then placed in xylene for 30 min. The slides were hydrated in descending alcohol series (concentrations 95%, 85%, and 75% alcohol) for five min. The samples were then washed with phosphate-buffered saline (PBS) for 5 min.[9, 13] Antigen retrieval was performed by microwaving the samples for 20 min in a ready-to-use Dako target recovery solution (PH 6.0). Using a lint-free tissue (gauze pad), the residual liquid around the sample was carefully removed to keep the reagent within the defined area.[14-18] To inhibit endogenous peroxidase, tissue sections were treated with 3% hydrogen peroxide, incubated for 5 min, and then carefully rinsed with distilled water.

Primary antibodies were VEGF monoclonal antibody (H11), catalog number MA5-13182 in a 1:20 dilution; BAX monoclonal antibody, catalog number MA5-14003 in a 1:50 dilution; and EGFR monoclonal antibody (JH121), catalog number MA5-13070 in 2 µg/mL dilution (all from Invitrogen, Thermo Fisher Scientific, USA). Furthermore, we used ProEx™C (prediluted, clone MCM2 26H6.19, MCM2 27C5.6, TOP2A SWT3D1; 3D Imaging Inc, Burlington, NC, USA); anti-hTERT, anti-telomerase catalytic subunit (RABBIT) antibody 600-401-252S (Rockland Immunochemicals, Inc., Limerick, PA, USA), SMA and S100 (clone HHF35 and 4C4.9, respectively; Ventana Medical Systems, Inc. Tucson, AZ, USA), and calponin (clone CALP; DAKO GmbH, Jena, Germany).

The tissue specimens were washed in PBS and incubated for 15 min at room temperature with biotinylated anti-mouse immunoglobulin.[19-22] Streptavidin-HRP was added to the tissue slides and washed after 15 min. Next, they were treated with diaminobenzidine (DAB) substrate, incubated for five to 10 min, and then gently washed with distilled water. The slides were submerged in Mayer's hematoxylin solution and incubated for 2-5 min, depending on the hematoxylin strength. DPX was used as mounting medium, and the tissue slides were carefully mounted with a coverslip after clearing in three changes of xylene.

Scoring System for Immunohistochemical Staining

VEGF and BAX positivity was detected as cytoplasmic staining, ProEx™C as nuclear, hTERT as cytoplasmic, nuclear, or both; and EGFR as cytoplasmic and membrane staining. The immune response in tissue samples was identified in 10 randomly selected fields by counting the percentage of stained cells in each field. These were then scored as negative staining (score 0), 1-25% stained cells (score 1), 26-50% stained cells (score 2), 51-75% stained cells (score 3), and 76-100% stained cells (score 4). The intensity of staining was scored as negative (score 0), mild (score 1), moderate (score 2), and high (score 3) intensity. The final result was deduced by multiplying the intensity score by the percentage of positive cell fraction.[19] ProEx™C nuclear staining was scored as negative (<5% of the nuclei are stained), weak (5-25% of the nuclei are positive), moderate (25-50% of the nuclei are positive), and strong (>50% of the nuclei are stained). Two pathologists blinded to the clinical data of the patients independently evaluated all slides.

Statistical Analysis

Data were analyzed using GraphPad Prism statistical software, version 7 (GraphPad Prism Software Inc., San Diego, CA, USA). The Chi square test was used to analyze the expression levels and their association with prognostic and clinicopathological parameters. Kaplan-Meier method was used to estimate the overall survival (OS) and disease-free survival (DFS) and the log-rank test to analyze the difference. Univariable and multivariate Cox regression was used to assess the effect of all variables. A two-sided P≤0.05 was considered statistically significant.

Table 1: Demographic and Clinicopathological parameters of the patients

Parameters		Patients (n, %) (N=40)
Age (years)	55.4±14.4* (range: 29-83)	
Sex	Male	16 (40%)
	Female	24 (60%)
Age group (years)	<45	8 (20%)
	≥45	32 (80%)
Primary site	Paranasal sinus	12 (30%)
	Nasal cavity	26 (65%)
	Orbit	2 (5%)
HPV type	HPV-33	34 (85%)
	HPV-35	4 (10%)
	HPV-16	2 (5%)
Tumor size	T1/T2	24 (60%)
	T3/T4	16 (40%)
Lymph node metastasis	N0	26 (65%)
	N1	6 (15%)
	N2	4 (10%)
	N3	4 (10%)
Stage	Early stage (I, II)	26 (65%)
	Advanced stage (III, IV)	14 (35%)
Distant metastasis	M0	39 (97.5%)
	M1	1 (2.5%)
VEGF expression	Low	10 (25%)
	High	30 (75%)
EGFR expression	Negative	11 (27.5%)
	Positive	29 (72.5%)
BAX expression	Negative	8 (20%)
	Positive	32 (80%)
ProEx™C expression	Negative	16 (40%)
	Positive	24 (60%)
hTERT expression	Low	18 (45%)
	High	22 (55%)
Treatment modality	Surgery	16 (40%)
	Sur+rt±cth	20 (50%)
	CCRT	4 (10%)
Relapse	Absent	16 (40%)
	Local recurrence	20 (50%)
	Distant metastasis	2 (5%)
	Died	2 (5%)
Treatment after recurrence	Surgery	8 (20%)
	Sur+rt±cth	4 (10.53%)
	Sur+reirrad±cth	6 (15.79%)
	CCRT	2 (5.26%)
	CTH	2 (5.26%)
	No TTT	16 (42.11%)
Mortality	Alive	26 (68.42%)
	Dead	12 (31.58%)

*Mean±SD (standard deviation); HPV: Human papillomavirus; VEGF: Vascular endothelial growth factor; EGFR: Epidermal growth factor receptor; BAX: Bcl-2-associated X protein; hTERT: Human telomerase reverse transcriptase; Sur+rt±cth: Surgery followed by postoperative irradiation with/without chemotherapy; Sur+reirrad±cth: Surgery followed by postoperative re-irradiation with/without chemotherapy; CCRT: Chemoradiotherapy; CTH: Chemotherapy; TTT: Transpupillary thermotherapy

Table 2: Association of clinicopathological and outcome parameters with the expression of VEGF, BAX, EGFR, ProEx™C, and hTERT in 40 HMSC patients

Parameters			VEGF		P value	BAX		P value	EGFR		P value	ProEx™C		P value	hTERT		P value
			Low (n=10)	High (n=30)		Low (n=8)	High (n=32)		Negative (n=11)	Positive (n=29)		Negative (n=14)	Positive (n=26)		Low (n=18)	High (n=22)	
		Total	n (%)			n (%)			n (%)			n (%)			n (%)		
Sex	Male	16	4 (25%)	12 (75%)	0.649	5 (31.2%)	11 (68.8%)	0.292	4 (25%)	12 (75%)	0.533	10 (62.5%)	6 (37.5%)	0.582	7 (43.8%)	9 (56.2%)	0.578
	Female	24	6 (25%)	18 (75%)		9 (37.5%)	15 (62.5%)		7 (29.2%)	17 (70.8%)		12 (50%)	12 (50%)		11 (45.8%)	13 (54.2%)	
Age group (years)	<45	8	7 (87.5%)	1 (12.5%)	<0.001*	6 (75%)	2 (25%)	<0.001*	8 (100%)	0 (0%)	<0.001*	8 (100%)	0 (0%)	<0.001*	8 (100%)	0 (0%)	<0.001*
	≥45	32	3 (9.4%)	29 (90.6%)		2 (6.2%)	30 (93.8%)		3 (9.4%)	29 (90.6%)		6 (18.8%)	26 (81.2%)		10 (31.2%)	22 (68.8%)	
Primary site	Paranasal sinus	12	6 (50%)	6 (50%)	<0.001*	5 (41.7%)	7 (58.3%)	<0.001*	7 (58.3%)	5 (41.7%)	0.008	9 (75%)	3 (25%)	<0.001*	9 (75%)	3 (25%)	0.039
	Nasal cavity	26	3 (11.5%)	23 (88.5%)		1 (3.8%)	25 (96.2%)		3 (11.5%)	23 (88.5%)		4 (15.4%)	22 (84.6%)		8 (30.8%)	18 (69.2%)	
	Orbit	2	1 (50%)	1 (50%)		2 (100%)	0 (0%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)		1 (50%)	1 (50%)	
Tumor size	T1/T2	24	10 (41.7%)	14 (58.3%)	0.002	8 (33.3%)	16 (66.7%)	0.01	11 (45.8%)	13 (54.2%)	0.001	14 (58.3%)	10 (41.7%)	<0.001*	18 (75%)	6 (25%)	<0.001*
	T3/T4	16	0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)		0 (0%)	16 (100%)	
Lymph node metastasis	Absent	26	10 (38.5%)	16 (61.5%)	0.006	7 (26.9%)	19 (73.1%)	0.140	11 (42.3%)	15 (57.7%)	0.004	14 (53.8%)	12 (46.2%)	<0.001*	18 (69.2%)	8 (30.8%)	<0.001*
	Present	14	0 (0%)	14 (100%)		1 (20%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
Distant metastasis	M0	39	10 (25.6%)	29 (74.4%)	0.750	8 (20.5%)	31 (79.5%)	0.800	11 (28.2%)	28 (71.8%)	0.725	14 (35.9%)	25 (64.1%)	0.650	18 (46.2%)	21 (53.8%)	0.55
	M1	1	0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)	
Stage	Early (I/II)	26	10 (38.5%)	16 (61.5%)	0.006	7 (26.9%)	19 (73.1%)	0.140	11 (42.3%)	15 (57.7%)	0.004	14 (53.8%)	12 (46.2%)	<0.001*	18 (69.2%)	8 (30.8%)	<0.001*
	Advance (III/IV)	14	0 (0%)	14 (100%)		1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
Relapse	Absent	16	9 (56.2%)	7 (43.8%)	<0.001*	5 (31.2%)	11 (68.8%)	0.002	11 (100%)	6 (37.5%)	<0.001*	14 (87.5%)	2 (12.5%)	<0.001*	13 (81.2%)	3 (18.8%)	0.001
	Present	22	0 (0%)	22 (100%)		1 (4.5%)	21 (95.5%)		0%)	22 (100%)		0 (0%)	22 (100%)		4 (18.2%)	18 (81.8%)	

Parameters		Total	VEGF		P value	BAX		P value	EGFR		P value	ProEx™C		P value	hTERT		P value
			Low (n=10)	High (n=30)		Low (n=8)	High (n=32)		Negative (n=11)	Positive (n=29)		Negative (n=14)	Positive (n=26)		Low (n=18)	High (n=22)	
			n (%)			n (%)			n (%)			n (%)			n (%)		
Mortality	Dead	12	0 (0%)	12 (100%)	0.015	2 (16.7%)	10 (83.3%)	0.548	1 (8.3%)	11 (91.7%)	0.185	1 (8.3%)	11 (91.7%)	0.021	1 (8.3%)	11 (91.7%)	0.002
	Alive	28	10 (35.7%)	18 (64.3%)		6 (21.4%)	22 (78.6%)		10 (357%)	18 (643%)		13 (46.4%)	15 (53.6%)		17 (60.7%)	11 (39.3%)	
Disease-free survival (DFS)																	
Mean (months)			58.9	47.364	0.001	56.25	49.2	0.035	59.00	47.016	<0.001*	59.214	45.474	<0.001*	56.667	45.627	<0.001*
Median DFS			NR	48.0		NR	52.0		NR	48.00		NR	44.0		NR	40.0	
5-year DFS			90%	23%		87.5%	32.9%		90.9%	22.1%		92%	13%		77%	15%	
Overall survival (OS)																	
Mean (months)			60.0	56.22	0.113	58.857	56.723	0.262	60.00	56.08	0.08	60.0	55.61	0.021	59.882	54.786	0.002
Median OS			NR	NR		NR	NR		NR	NR		NR	NR		NR	58.0	
5-year OS			88.9%	62.1%		85.7%	64.6%		88.9%	60.8%		91.7%	56.1%		94.1%	47.7%	

NR: Not reached

Results

A total of 40 patients were diagnosed with HMSC and their demographic and clinicopathological characteristics were obtained (table 1). The patients were divided into two age groups, namely <45 years old (n=8) and ≥45 years old (n=32). Based on the TNM staging system, the size of the tumor in 24 (60%) and 16 (40%) patients were in stages T1/T2 and T3/T4, respectively. The nasal cavity was the primary site of the tumor with no lymph node involvement in 26 (65%) patients. Lymph node metastasis N1, N2, and N3 was observed in 6 (15%), 4 (10%), and 4 (10%) patients, respectively. During the initial evaluation, there was no distant metastasis in 39 (97.5%) patients. Of all patients, 26 (65%) had tumor stage I/II, and 14 (35%) had advanced tumor stage III/IV. Treatment modality in 16 (40%) patients was only surgery, 20 (50%) received postoperative irradiation with/without chemotherapy, and 4 (10%) received chemoradiotherapy. There was no relapse in 16 (40%) patients, 20 (50%) had local recurrence, 2 (5%) had distant metastases after treatment, and 2 (5%) patients died. Of the patients with relapse, 8 (20%) underwent surgery, 4 (10.53%) underwent surgery plus irradiation with/without chemotherapy, 6 (15.79%) underwent surgery plus re-irradiation with/without chemotherapy, 2 (5.26%) received chemoradiotherapy, and 2 (5.26%) received chemotherapy.

Immunohistochemical Evaluations

The results of immunohistochemistry tests for the expression of VEGF, BAX, EGFR, ProEx™C, and hTERT in relation to clinicopathological parameters of all patients are presented in table 2.

VEGF: Of the 40 patients, 30 (75%) showed high expression, and 10 (25%) had low expression of VEGF (figures 1A and 1B). A strong association was found between high VEGF expression and tumor size (P=0.002), ≥45 age group (P<0.001), advanced tumor stages III and IV (P=0.006), and lymph node metastasis (P=0.006). Relapse was associated with high positive expression of VEGF compared to patients with negative expression (P<0.001). The five-year DFS in patients with low VEGF expression was 90% compared to those with high VEGF expression (23%) (P=0.001). However, the five-year OS was significantly higher in patients with low VEGF expression (88.9%) than those with high expression (62.1%). Furthermore, there was a significant association between high VEGF expression and mortality (P=0.015).

BAX: Of the 40 patients, 32 (80%) showed high expression, and 8 (20%) had low expression of BAX (figures 1C and 1D). High BAX expression

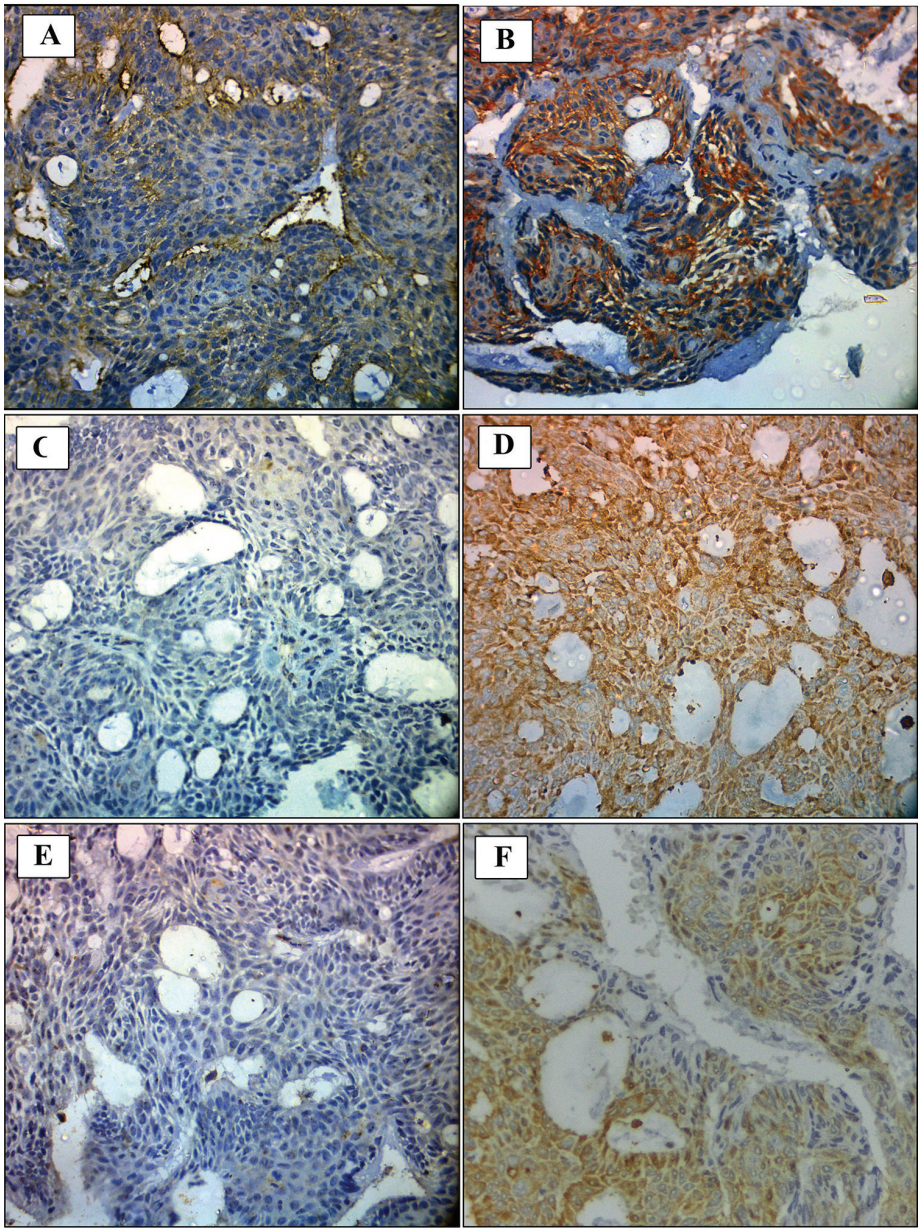

Figure 1: Immunohistochemistry of vascular endothelial growth factor (VEGF) shows (A) negative expression and (B) positive cytoplasmic expression in human papillomavirus-related multiphenotypic sinonasal carcinoma (HMSC) (×400). Immunohistochemistry of Bcl-2-associated X protein (BAX) shows (C) negative expression and (D) positive cytoplasmic expression in HMSC (×400). Immunohistochemistry of epidermal growth factor receptor (EGFR) shows (E) negative expression and (F) positive cytoplasmic expression in HMSC (×400).

was significantly associated with marked tumor size (P=0.010) and ≥45 age group (P<0.001). Relapse was significantly associated with high expression of BAX compared to patients with low expression (P=0.002). The five-year DFS was significantly higher in patients with low expression of BAX (87.5%) than those with high expression (32.9%) (P=0.035).

***EGFR*:** Of the 40 patients, 29 (72.5%) were EGFR-positive, and only 11 (27.5%) were EGFR-negative (figures 1E and 1F). The expression of EGFR-positive was significantly associated with advanced tumor stages III and IV (P=0.004), lymph node metastasis (P=0.004), tumor size (P=0.001), and ≥45 age group (P<0.001). Relapse was significantly associated with EGFR-positive compared to patients with EGFR-negative (P<0.001). The five-year DFS in patients with EGFR-negative was significantly higher than those with EGFR-positive (90.9% vs. 22.1%).

***ProEx™C*:** We observed positive staining for ProEx™C in the samples of 26 (65%) of the 40 patients, and 14 (35%) stained negative (figures 2A to 2D). The expression of ProEx™C was significantly associated with ≥45 age group (P<0.001), tumor size (P<0.001), advanced tumor stages III and IV (P<0.001), and lymph node metastasis (P<0.001). Relapse was

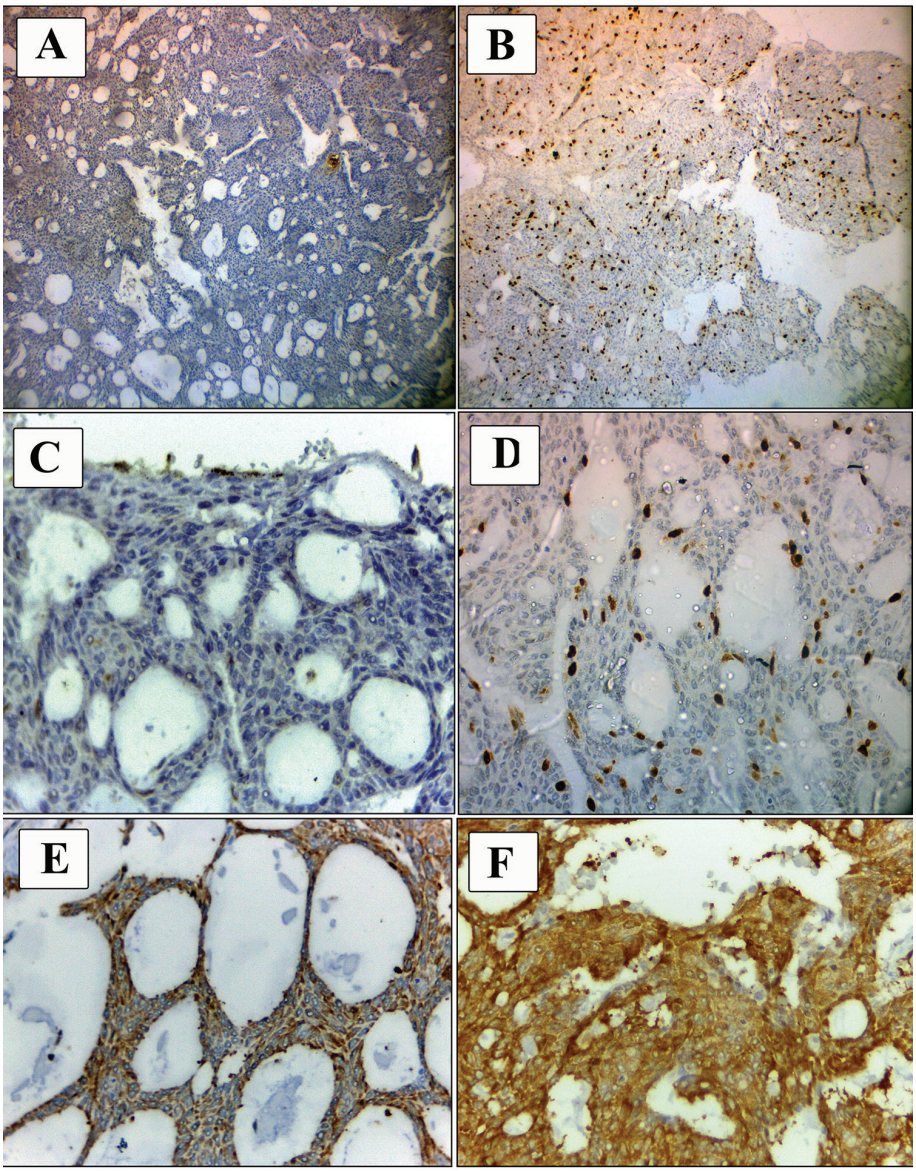

Figure 2: Immunohistochemistry of proEx™C shows (A) negative expression and (B) positive nuclear expression in HMSC (×100). High magnification (×400) images of (C) negative expression and (D) positive nuclear expression of proEx™C in HMSC. Immunohistochemistry of human telomerase reverse transcriptase (hTERT) shows (E) negative expression and (F) positive cytoplasmic expression in HMSC (×400).

significantly associated with positive expression of ProEx™C compared to patients with negative expression (P<0.001). The five-year DFS in patients with negative expression of ProEx™C (92%) was significantly higher than those with positive expression (13%) (P<0.001). The five-year OS was higher in patients with negative expression of ProEx™C (91.7%) than those with positive expression (56.1%) (P=0.02). Positive expression of ProEx™C was significantly associated with mortality (P=0.021).

hTERT: Of the 40 patients, 22 (55%) showed high expression, and 18 (45%) had low expression of hTERT (figures 2E and 2F). High expression of hTERT was significantly associated with lymph node metastasis (P<0.001), ≥45 age group (P<0.001), tumor size (P<0.001), and advanced tumor stages III and IV (P<0.001). Relapse was significantly associated with high hTERT expression compared to patients with low expression (P=0.001). The five -year DFS in patients with low expression of hTERT (75%) was significantly higher than those with high expression (15%) (P<0.001). Besides, the five -year OS was higher in patients with low expression of hTERT (94.1%) than those with high expression (47.7%). Furthermore, there was a significant association between high expression of hTERT and mortality (P=0.02).

Discussion

The results showed that the expression of VEGF, hTERT, and ProEx™C was significantly

associated with age, advanced tumor stages III and IV, lymph node metastasis, tumor size, relapse, poor DFS, poor OS, and mortality. Moreover, the expression of BAX was significantly associated with tumor size, age, poor DFS, and relapse (0.01, <0.001, 0.035, and 0.002, respectively).

HMSC is an HPV-related tumor with several histological features of ACC without MYB, MYBL1, or NIFIP translocation. While HMSC has squamous differentiation properties, it exhibits myoepithelial proliferation. Despite its aggressive behavior and high local recurrence rate, little is known about the clinical characteristics of HMSC. Therefore, an in-depth understanding of the biological processes involved in its development is needed to clarify its clinical characteristics and develop more effective therapeutic agents.[23]

In the present study, we diagnosed 40 patients with HMSC. Clinical presentation included polypoid tumors in the nasal cavity, nasal sinuses, and orbits that led to epistaxis, nasal obstruction, nasal discharge, pain, and visual symptoms.[10] Both sexes were affected, but in line with a previous study, women were in the majority. However, another study reported that only women were affected.[20] Of the 40 patients in our study, 20 (50%) had local recurrence and 2 (5%) had metastatic spread, which was consistent with a previous study reporting 36.4% and 4.5%, respectively.[23] Two other studies reported late recurrences with a follow-up of 50 months.[5, 24] However, Rupp and colleagues observed no recurrences or metastases in four patients with HMSC.[20]

One of the diagnostic criteria of HMSC is its association with HPV. We found that HPV-33 was present in 85%, HPV-35 in 10%, and HPV-16 in 5% of our patients. Other characteristics of HMSC are the lack of MYB/MYBL1 translocation, which distinguishes it from ACC, and the presence of myoepithelial differentiation, which distinguishes it from sinonasal SCC.[10] The morphology and HPV types (16, 33, and 35) in our patients were similar to those reported in previous studies.[10, 20, 21] However, Rupp and colleagues reported that one patient with HMSC had different morphology, i.e., glomerular patterns, more pleomorphic cells, and HPV-82.[20] Although the exact origin of cell in HMSC is unknown, the expression of pan-cytokeratin combined with biphasic staining of basal and myoepithelial proliferation, as well as their morphological characteristics, are indicative of the salivary gland origin.[10]

The majority of our patients (75%) showed high expression of VEGF, which was significantly associated with tumor size, age, advanced tumor stages, lymph node metastasis, recurrence, and mortality. Our results are in line with those of a previous study reporting that 71% of their patients had high expression of VEGF in ACC of the salivary gland.[22] Another study reported that the expression of VEGF was associated with poor prognosis of ACC, and its high expression was associated with advanced tumor stages, local recurrence, and poor OS.[25] However, Lee and colleagues observed that VEGF expression was not associated with survival rate or recurrence of ACC of salivary glands.[22]

In the present study, BAX was strongly expressed in 32 (80%) patients and was significantly associated with tumor size, age, relapse, and mortality. Our results were consistent with previous studies reporting high expression of BAX in 83% of their patients and its association with poor survival.[26, 27] Our results also showed that 29 (72%) patients were EGFR-positive, and its expression was significantly associated with tumor size, age, lymph node metastasis, and recurrence. These are in line with the results of a previous study reporting that 77% of their patients with ACC of salivary glands were EGFR-positive, and the expression was associated with poor prognosis.[24] In our patients, 65% tested positive for ProEx™C, which was significantly associated with tumor size, age, advanced tumor stages, lymph node metastasis, recurrence, and mortality. This is consistent with the conclusions of previous studies reporting that high expression of minichromosome maintenance protein 2 (MCM2) was associated with poor prognosis and advanced stages of salivary gland carcinomas.[28, 29] In contrast, another study reported that the downregulation of Ki-67 and MCM2 is associated with advanced tumor stages.[30] In line with the outcome of a study by Shigeishi and colleagues,[31] our results showed that 22 (55%) patients had high expression of hTERT, and it was strongly associated with tumor size, age, advanced stages, lymph node metastasis, recurrence, and mortality. Overall, our results indicated that the expression of VEGF, BAX, EGFR, hTERT, and ProEx™C was significantly associated with poor prognosis of HMSC. Large-scale prospective studies with more in-depth molecular assessments are recommended to substantiate our findings.

Conclusion

HMSC was strongly associated with HR-HPV. The expression of VEGF, BAX, EGFR, hTERT, and ProEx™C was shown to be associated with poor survival and aggressive malignant behavior,

making them novel prognostic biomarkers for targeted therapies in HMSC.

Authors' Contribution

All authors have equally contributed to the study conception and design; data acquisition, analysis, and interpretation; and drafting and revising the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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