Original paper

High-risk human papillomavirus enhances the expression of COX-2 VEGF, EGFR, ProEx-C, and TERT proteins in human papillomavirus-related multiphenotypic sinonasal carcinoma through activation of PI3K/Akt, pRb, and TERT signalling pathways

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Human papillomavirus (HPV)-related multiphenotypic sinonasal carcinoma (HMSC) is a new type of sinonasal tumour that frequently drops out of accurate diagnosis. Human papillomavirus related multiphenotypic sinonasal carcinoma was previously known as HPV-related sinonasal carcinoma with adenoid cystic characteristics, and it is connected to high-risk HPV (HR-HPV) strains whose prognosis is unknown. We aim to evaluate PI3K/Akt, pRb, and h telomerase reverse transcriptase (TERT) signalling pathway activation through the expression of proteins cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), ProEx-C, and TERT and their prognostic and clinicopathological value in HMSC patients.

Sections of the 40 paraffin blocks of HMSC were recovered, and all samples were evaluated for the presence of a cocktail of HR-HPV, and the absence of MYB, NFIB, and MYBL1 fusions using fluorescence in situ hybridization; the presence of myoepithelial markers; S100, actin; the presence of squamous differentiation markers; calponin, p40, and p63 using PCR-based assays; and COX-2,VEGF, ProEx-C, and TERT using immunohistochemical staining. All patients were monitored for around 54 months, until death, or the last known surviving data (range 20–60 months).

A statistically significant relationship exists between COX-2 expression was significantly related to the old age group, tumour extent, relapse, mortality, and poor DFS; ($p = 0.001$), ($p = 0.01$), ($p = 0.002$), and ($p = 0.035$), respectively. While VEGF, ProEx-C, and TERT expression with the old age group, tumour extent, lymph node metastasis, advancedstaging, relapse, mortality, poor disease free survival (DFS), and overall survival ($p = 0.001$).

Human papillomavirus-related multiphenotypic sinonasal carcinoma is a unique sinonasal neoplasm with a strong link to HR-HPV strains. Expression of COX-2,

VEGF, EGFR, ProEx-C, TERT was linked to poor prognosis, survival, and aggressive malignant behaviours such as proliferation, local recurrence, and lymph node metastasis, making them novel beneficial biomarkers and targeted therapies for HMSC patients.

Key words: COX-2,VEGF, EGFR, ProEx-C, TERT, HPV, multiphenotypic sinonasal carcinoma.

Introduction

Sinonasal cancers (SNCs) account for about 3.6% of all head and neck cancers, and less than 0.2% of all malignant tumours are detected worldwide [1]. Despite the low occurrence, it includes many histological types such as adenocarcinomas, squamous cell carcinoma, cylindrical cell carcinoma, melanoma, undifferentiated carcinoma, olfactory neuroblastoma, and lymphoma [2]. This malignancy arises from the surface epithelium and the seromucous glands [3].

The factors that lead to sinonasal carcinoma development are unclear. However, some related risk factors have been noticed. Intestinal-type adenocarcinoma is a rare kind associated with occupational exposures such as wood dust, and cigarette smoking is another substantial risk factor for most head and neck malignancies [4].

Human papillomavirus (HPV) is now widely acknowledged as the cause of 20–25% of head and neck cancers [5]. Even though most HPV-related head and neck malignancies occur in the oropharynx [6], the sinonasal tract carcinoma accounts for another 20–25% of anatomical carcinomas [7]. Human papillomavirus positivity is closely associated with the sinonasal tract's non-keratinizing squamous cell carcinoma phenotype. The high-risk HPV (HR-HPV) infection is connected to HPV-related multiphenotypic sinonasal carcinoma (HMSC), formerly known as HPV-related carcinoma with adenoid cystic features. Human papillomavirus type 33 is the most common HR-HPV found in HMSC [8, 9].

Human papillomavirus-related multiphenotypic sinonasal carcinoma is a novel neoplasm that affects the sinonasal tract, characterized by a salivary-like appearance, and presented as tissue fragments focally lined by respiratory epithelium with squamous metaplasia. The tumour is divided by fibrosis and hyalinization bands into compartments. The 2 distinct patterns shown in HMSC at low magnification are cribriform and solid. The cribriform pattern is formed of cylindromatous microcystic spaces with basophilic mucoid material surrounded by basaloid tumour cells, while the solid pattern displays tumour cells with nuclear pleomorphism, vesicular nuclei, minimal eosinophilic cytoplasm, focal areas with confluent necrosis, and atypical cells with multiple nuclei and prominent nucleoli. In these areas, mitotic activity is considerable, with 50–55 mitoses per 10 HPF [8].

Although HMSC cribriform pattern morphologically resembles the most common sinonasal tumour (adenoid cystic carcinoma), it lacks MYB, NFIB, and MYBL1 translocation, and its solid type mimics squamous cell carcinoma but with myoepithelial proliferation [10].

Human papillomavirus-related multiphenotypic sinonasal carcinoma is a new distinct type with uncertain prevalence. Although it appears to be less aggressive than sinonasal squamous cell carcinoma, it has been associated with a high-risk of local recurrence (36%). Late local recurrence after initial distant metastases occurs in 5% of patients [9]. Human papillomavirus-related multiphenotypic sinonasal carcinoma cribriform pattern is usually misinterpreted as adenoid cystic carcinoma, while its solid type is misdiagnosed as squamous cell carcinoma.

Two late genes, L1 and L2, as well as 6 early genes, E1 through E7, are encoded by HPVs. The main oncogenes that encourage host cell growth and assist viral multiplication are E5, E6, and E7. They can promote tumourigenicity by activating different survival signalling pathways.

These pathways are complementary and overlapping, but E5, E6, and E7 share in the PI3K/Akt pathway activation and express different proteins, enhancing proliferation and survival [11].

A crucial mechanism for cancer survival is the PI3K/Akt system. By expressing a protein that regulates cell proliferation, growth, mobilisation, angiogenesis, and cell survival, this pathway has boosted cancer initiation, progression, metastasis, and medication resistance. As a result, blocking the route has been suggested as a cancer treatment. Numerous cancers, including breast cancer, melanoma, colon cancer, and prostate cancer, have been the focus of intensive research on targeted PI3K/Akt therapy.

E5 activates PI3K/Akt pathway

The ability of E6 and E7 to cause cancer can beenhanced by E5. Through its use of downstream target proteins like COX-2 and VEGF it can boost angiogenesis and cell proliferation while reducing cell

death. EGFR can become more responsive to EGFR stimulation when E5 is present, increasing the activation of EGFR downstream pathways (PI3K/Akt), which upregu- late to reduce apoptosis [11].

E6 activates PI3K/Akt pathway

E6 has several strategies by which it can open this pathway. Through PDZ proteins, E6 inactivates PTEN, increasing pAkt. The mTOR complex or persistent activation of receptor protein tyrosine kinases, such as the EGFR, insulin, and insulin-like growth factor receptor-β, are other ways in which E6 might activate the PI3K/Akt pathway.

E6 activates telomerase reverse transcriptase pathway

E6 can increase telomerase activity by upregulation of telomerase reverse transcriptase (TERT), through direct interaction with the (TERT) gene, or through activation of c-myc, leading to activation of telomerase activity [11].

E7/PI3K/Akt pathway

Several studies have shown that E7 can activate the PI3K/Akt pathway. The mechanism of increased Akt activity has been regarded as inhibition of pRb by E7. Increased Akt activity and loss of Rb may be mediated by protein phosphatase 2A (PP2A) [11]. Rb inactivation is associated with over-expression of S-phase activation proteins (aberrant S-phase induction), MCM2, and topoisomerase II α (TOP2A), which accumulate in HPV-transformed cells, and it is limited in the normal epithelium [12–14]. ProEx C is a newly developed immunocytochemical cocktail intended to detect cells with aberrant S-phase induction, which consists of an antibody against both (TOP2a) and MCM2 [13 –15].

Due to its rarity, the molecular biology of HMSC has not been well described. In immunohistochemistry, expression of COX-2, VEGF, EGFR, ProEx-C, and TERT are known poor prognostic factors of ACC; however, their expression was not evaluated for HMSC before, and the relationship between these markers and survival of patients is not well-known.

We evaluated the expression of the resultant proteins of these pathways for each viral protein individually and in combination. COX-2, VEGF, EGFR, ProEx-C, TERT, as a result of proteins of E5-PI3K/Akt pathway, EGFR for E5 and E6-PI3K/Akt pathway, TERT for E6-TERT pathway, and ProEx-C for E7-PI3K/Akt pathway.

Material and methods

This prospective cohort study included 40 patients with HMSC, who underwent surgical resection with adjuvant radiotherapy if needed or definitive concurrent chemoradiation, at Zagazig University Hospital Otorhinolaryngology, Ophthalmology, and General Surgery, Clinical Oncology, and Medical Oncology Faculty of Medicine, Zagazig, Egypt. Follow-up was every 3 months for the first 2 years, then every 6 months for the next 5 years, from 2017 to 2022. All patients were monitored for around 54 months (range 20–60 months), until death or the last known surviving data. The study (no. ZU-IRB#9902) was approved by the Institutional Review Board (IRB) – Faculty of Medicine, Zagazig University, Egypt (no. ZU-IRB#/3/2543), Ethical Committee in the Faculty of Medicine, Beni-Suef University, Egypt (no. FMBSRUEC/070617), and the Institute Ethics Review Board (Faculty of Medicine, Benghazi University, Benghazi, Libya). The study was carried out following the Ethics of the World Medical Association (Helsinki Declaration of 1975, as revised in 2000) for human studies [16]. Each patient completed a written informed consent form before being included in the study. All cases are classified using the American Joint Committee on Cancer TNM categories (tumour – nodus – metastases) for malignancies of the nasal cavity and paranasal sinuses, 8th edition 2017 [9].

Sections of 40 paraffin blocks were generated, and all samples were assessed for the following: 1 – the presence of HR-HPV (a high-risk cocktail) using PCR-based assays, 2 – the absence of MYB, NFIB, and MYBL1 fusions by fluorescence in situ hybridization (FISH) to exclude adenoid cystic carcinoma, 3 – the expression of myoepithelial markers (S100, SMA, calponin) using immunohistochemistry to exclude sinonasal squamous cell carcinoma, 4 – the expression of squamous differentiation markers (p40, p63), and expression of COX-2, VEGF, EGFR, ProEx-C, and TERT by IHC. The correlation between clinicopathological and prognostic parameters and expression levels of COX-2, VEGF, EGFR, ProEx-C, and TERT, individually and in combination, was determined.

The combined expression was evaluated by combining the positive expression of positive and negative evaluated markers with the high expression of high and low evaluated markers.

All patients were followed-up until death or until the last known alive date for approximately 54 months (range 20–60 months).

Quantitative polymerase chain reaction for human papillomavirus detection

Quantitative HPV-specific PCR was used to genotype HPV in HMSC tissue samples. On 5-mm-thick slides, DNA was extracted from formalin-fixed, paraffin-embedded tumour tissues. The tissues were deparaffinized with xylene, macrodissected from the slides, and digested with 50 g/ml proteinase K (Boehringer

Mannheim) in the presence of 1% sodium dodecyl sulphate for 2 days at 48°C. The DNA was subsequently extracted using UltraPure Phenol: chloroform: isoamyl alcohol reagents according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) [17].

As stated earlier, the procedure was as follows: the L1 region of the HPV genome was used to amplify the consensus primers Gp5–Gp6 and Gp5+–Gp6+ using the following 30l PCR solution, as previously described [18]. 16.6 mM ammonium sulphate, 67.0 mM Tris-Trizma prepared crystals (pH 8.8), 6.7 mM magnesium chloride, 10.0 mM 2-MercaptoEthanol, 0.1 percent dimethyl sulphoxide, 3.3% DMSO, 20 pmol primers, and 0.5 U platinum taq. The rapid PCR protocol in a Veriti thermal cycler was 95°C for 30 seconds, 44°C for 60 seconds, and 72°C for 90 seconds for 40 cycles (Applied Biosciences). For the E6 and E7 regions of HPV types 11, 16, 18, 31, 33, 35, and 56, type-specific primers were used, as previously mentioned [19], except for the amplification cycle being lowered to 35 seconds and the annealing temperature for the HPV 33 and 35 primers being adjusted to 57°C for 30 seconds.

Fluorescence in situ hybridization

MYB break-apart FISH assay (Empire Genomics, Buffalo, NY, USA) and MYBL1 (Empire Genomics) and NFIB (Empire Genomics) break-apart FISH assays (Empire Genomics) break-apart FISH assays (Empire Genomics) break-apart FISH assays (Empire Genomics) break-apart FISH assays [20]. Tumour nuclei were counterstained with 4×, 6-diamidino-2-phenylindole (DAPI) II after hybridization (ZytoVision GmbH, Bremerhaven, Germany). A total of 100 nuclei were counted, and only nuclei with the entire nuclear membrane visible were scored. Breakaway signals in 10% of cells were thought to indicate rearrangement [7, 17].

Immunohistochemistry

Immunohistochemistry was performed by using anti VEGF, COX-2, EGFR, ProEx-C and TERT antibodies [21-25]. Vascular endothelial growth factor in a 1/20 dilution (a mouse monoclonal antibody (H11) Catalogue # MA5-13182, Invitrogen, Thermo Fisher Scientific, USA), COX-2 a rabbit monoclonal anti-COX-2 antibody (diluted 1: 50; BIOCARE MEDICAL, USA), EGFR in 2 μg/ml dilution (a mouse monoclonal antibody (JH121) Catalogue # MA5-13070, Invitrogen, Thermo Fisher Scientific, USA) ProExC (prediluted, clone MCM2 26H6.19, MCM2 27C5.6, TOP2A SWT3D1; TriPath Imaging Inc, Burlington, NC), TERT, anti-telomerase catalytic subunit (RABBIT) antibody-600-401-252S (Rockland Immunochemicals, Inc., Limerick, PA, USA), muscle-specific actin (clone HHF35; Ventana), calponin (clone CALP; Dako), and S100 (clone 4C4.9; Ventana [26-30].

Evaluation of immunohistochemical staining

ProEx-C scoring

ProExC nuclear staining of less than 5% of cells was considered negative; staining of 5–25% of cells was considered weak; 25–50% was considered the moderate expression; and staining of more than 50% was considered strong.

COX-2, VEGF, EGFR, and TERT scoring

The COX-2 and VEGF positivity were detected as cytoplasmic staining, TERT as nuclear, cytoplasmic, or both, and EGFR as membrane and cytoplasm staining. The immunoreactions in tumour cells were determined in 10 randomly selected fields by counting the percentage of positive cells (cytoplasmic staining) in each field and scoring them as follows: 0 (negative), 1–25% (score 1), 26–50% (score 2), 51–75% (score 3), and 76–100% (score 4). The staining intensity was measured as follows: 0 – indicates negative, 1 – indicates mild, 2 – indicates moderate, and 3 – indicates strong intensity. The final scoring was calculated by multiplying the percentage of positive cells by the intensity score [31]. All slides were independently evaluated by 2 investigators who had no prior knowledge of the clinical data of the patients.

Statistical analysis

We performed statistical analyses using Graph Pad software (Graph Pad version 7.0). COX-2, VEGFR, EGFR, ProEx-C, and TERT expression levels and association with clinicopathological and prognostic parameters were analysed by the χ^2 test.

We measured disease free survival (DFS) pattern and overall survival (OS) rates by using the Kaplan-Meier method, and we analysed the differences in survival using the log-rank test.

The prognostic values of these 10 variables were tested by the univariate and multivariate COX proportional hazard models. All statistical tests were 2 sided. $P < 0.05$ was considered statistically significant.

Results

Forty cases were diagnosed as HMSCs. The clinicopathologic features and staging data are summarized in Table I. Thirty-four (85%) of the recorded cases were HPV 33, 4 (10%) cases were HPV 35, and 2 cases (5%) were HPV 16. The distribution of the cases within the sinonasal tract was as follows: 26 (65%) were confined to the nasal cavity, 12 (30%) of the cases were in the paranasal sinuses, with 2 cases arising in orbit (10%). The average age was 56.6 years ±15.3 years. Sixteen patients (40%) were male, while 24 (60%) were female. Patients were divided into 8 (20%) patients under 45 years of age and 32 (80%)

Table I. Clinicopathological parameters of the studied human papillomavirus related multiphenotypic sinonasal carcinoma patients

Factor	Staging date
Age (years) range, mean ± SD	29–83, 55.4 ±14.4
Age group, *n* (%)	
< 45 years	8 (20.00)
≥ 45 years	32 (80.00)
Sex, *n* (%)	
Male	16 (40.00)
Female	24 (60.00)
HPV type, *n* (%)	
HPV-33	34 (85.00)
HPV-35	4 (10.00)
HPV-16	2 (5.00)
Primary site, *n* (%)	
Paranasal sinus	12 (30.00)
Nasal cavity	26 (65.00)
Orbit	2 (5.00)
Tumor extent, *n* (%)	
T1/T2	24 (60.00)
T3/T4	16 (40.00)
Lymph node involvement, *n* (%)	
N0	26 (65.00)
N1	6 (15.00)
N2	4 (10.00)
N3	4 (10.00)
Metastasis, *n* (%)	
M0	39 (97.50)
M1	1 (2.50)
Stage, *n* (%)	
Early (I/II),	26 (65.00)
Advanced (III/IV),	14 (35.00)
VEGF, *n* (%)	
Weak	10 (25.00)
Strong	30 (75.00)
COX-2, *n* (%)	
Negative	8 (20.00)
Positive	32 (80.00)
EGFR, *n* (%)	
Negative	11 (27.50)
Positive	29 (72.50)
ProEx-C, *n* (%)	
Negative	16 (40.00)
Positive	24 (60.00)
TERT, *n* (%)	
Low	18 (45.00)
High	22 (55.00)
Combined positive and high expression, *n* (%)	
Positive	20 (50.00)
Negative	20 (50.00)
Treatment modality, *n* (%)	
Surgery	16 (40.00)
Sur + rt+/–cth	20 (50.00)
CCRT	4 (10.00)
Relapse, *n* (%)	
Absent	16 (40.00)
Local rec	20 (50.00)
Dist. Mets	2 (5.00)
Lost	2 (5.00)
Treatment of recurrence, *n* (%)	
Surgery	8 (20.0)
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, *n* (%)	
Dead	12 (31.58)
Alive	26 (68.42)

patients over 45 years of age. The patients were in stages T1/T2 (n = 24, 60%) and T3/T4 (n = 16, 40%) at the time of presentation. Twenty-six (65%) cases were restricted to the primary site at the time of diagnosis, without evidence of lymph node metastases, while lymph node involvement was observed in the remaining cases, as follows: N1 had 6 cases (15%), N2 had 4 cases (10%), and N3 had 4 cases (10%). None of the 40 cases had distant metastases at the time of diagnosis. According to stage, 26 (65%) cases of HMSC were classified as early-stage (I/II), while 14 (35%) cases were classified as advanced stage

(III/IV). For 40 patients, treatment and follow-up data were available. For the first treatment, 16 (40%) patients had only surgical resection, 20 (50%) cases had postoperative therapeutic radiation, and 4 (10%) patients had radiation with concurrent chemotherapy. Sixteen patients (40%) did not relapse, whereas 20 of the 40 patients (50%) acquired local recurrences, 2 developed distant metastases (5%) following therapy, and 2 patients (5%) were lost. Of the relapsed cases, 8 (21%) underwent surgery alone, 4 (10.53%) underwent surgery plus radiation with/without chemotherapy, 6 (15.97%) underwent surgery and re-irradiation and chemotherapy, 2 (5.26%) received concurrent chemoradiotherapy, and 2 (5.26%) received chemotherapy (Table I).

Histological type confirmation

Areas resemble adenoid cystic carcinoma showing tubular architecture with biphasic inner ductal and outer myoepithelial lining separated by hyalinized fibrous bands, other areas showing solid squamous differentiation with nuclear pleomorphism and hyperchromasia. Focally, basaloid cell nests with eosinophilic ductal cells in between were seen. The MYB, NFIB, and MYBL1 fusions were negative, while myoepithelial markers S100, SMA, and calponin and squamous differentiation markers p40, and p63 were all positive.

VEGF expression

The expression of VEGF and clinicopathological features are summarized in Table II. Thirty of the 40 cases (75%) showed high expression for VEGF, whereas 10 of the 40 cases (25%) showed low expression (Fig. 1). The sex of the patients had no statistically significant link with high VEGF expression ($p = 0.649$). High VEGF expression was shown to have a statistically significant association with the old age group ($p < 0.001$), lymph node metastasis ($p = 0.006$), marked tumour extent ($p = 0.002$), and advanced clinical stages III and IV ($p = 0.006$) (Table II).

The relationship between the expression of VEGF and outcomes in 40 HMSC patients

Relapse was significantly associated with high positive expression of VEGF than in those with negative expression ($p < 0.001$), the 5-year DFS in patients with low expression of VEGF was 90%, which was high significantly better than the 5-year DFS in high positive cases 23% ($p = 0.001$). Also, there was a significant association between mortality and high expression of VEGF, $p = 0.015$, whereas 5-year overall survival was considerably higher in patients with low expression of VEGF (88.9%) compared to patients who expressed high positive VEGF (62.1%, at 95% CI) (Fig. 2).

COX-2 expression

The expression of COX-2 and clinicopathological features are summarized in Table II. Thirty-two of the 40 cases (80%) showed high expression for COX-2, whereas 8 of the 40 cases (20%) were low expression (Fig. 3). The sex of the patients had no statistically significant link with high COX-2 expression ($p = 0.292$). High COX-2 expression was shown to have a statistically significant association with the old age group ($p < 0.001$) and marked tumour extent ($p = 0.01$) (Table II).

The relationship between the expression of COX-2 and outcomes in 40 HMSC patients

Relapse was significantly associated with higher positive expression of COX-2 than in those with negative expression ($p = 0.002$); the 5-year DFS in patients with low expression of COX-2 was 87.5%, which was high significantly better than the 5-year DFS in high positive cases, i.e. 32.9% ($p = 0.035$) at 95% CI (Fig. 4).

Epidermal growth factor receptor expression

The expression of EGFR and clinicopathological features are summarized in Table II. Twenty-nine of the 40 cases (72.5%) were positive for EGFR, whereas 11 of the 40 cases (27.5%) were negative (Fig. 5). The sex of the patients had no statistically significant link with EGFR expression ($p = 0.533$). Positive EGFR expression was shown to have a statistically significant association with the old age group > 45 y, $p < 0.001$, lymph node metastasis ($p = 0.004$), marked tumour extent ($p = 0.001$), and advanced clinical stages III and IV ($p = 0.004$) (Table II).

The relationship between the expression of epidermal growth factor receptor and outcomes in 40 HMSC patients

Relapse was significantly more strongly associated with positive expression of EGFR than in those with negative expression ($p < 0.001$); the 5-year DFS in patients with negative expression of EGFR was 90.9%, which was significantly better than the 5-year DFS in positive cases: 22.1% ($p = 0.001$) at 95% CI (Fig. 6).

ProEx-C expression

The expression of ProEx-C and clinicopathological features are summarized in Table II. Twenty six of the 40 cases (65%) were positive for ProEx-C, whereas 14 of the 40 cases (35%) tested negative (Fig. 7). The sex of the patients had no statistically significant link with ProEx-C expression ($p = 0.582$). ProEx-C expression was shown to have a statistically significant association with the old age group

Table II. Relation between clinicopathological, outcome parameters, and VEGF, BAX, ProEx-C and TERT expression in the studied patients

		VEGF					*P*-value	COX-2				*P*-value	EGFR				*P*-value	ProEx-C				*P*-value	TERT				*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		
	Subdivisions	N	N	%	N	%		N	%	N	%		N	%	N	%		N	%	N	%		N	%	N	%	
Age group	< 45 Years	8	7	87.5	1	12.5	< 0.001*	6	75.0	2	25.0	< 0.001*	8	100.0	0	.0	< 0.001	8	100.00	0	0.00	< 0.001*	8	100	0	0	< 0.001*
	≥ 45 years	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	
Sex	Male	16	4	25.0	12	75.0	0.649	5	31.2	11	68.8	0.292	4	25.0	12	75.0	0.533	10	62.50	6	37.50	0.582	7	43.8	9	56.2	0.578
	Female	24	6	25.0	18	75.0		9	37.5	15	62.5		7	29.2	17	70.8		12	50.00	12	50.00		11	45.8	13	54.2	
Primary site	Paranasal sinus	12	6	50.0	6	50.0	< 0.001*	5	41.7	7	58.3	< 0.001	7	58.3	5	41.7	0.008	9	75.0	3	25.0	< 0.001	9	75.0	3	25.0	0.039
	Nasal cavity	26	3	11.5	23	88.5		1	3.8	25	96.2		3	11.5	2	88.5		4	15.4	22	84.6		8	30.8	18	69.2	
	Orbit	2	1	50.0	1	50.0		2	100.0	0	.0		1	50.0	1	72.5		14	50.0	1	50.0		1	50.0	1	50.0	
Tumor extent	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	75.0	6	25.0	< 0.001*
	T3/T4	16	0	0.0	16	100.0		0	.0	16	100.0		0	.0	16	100.0		0	.0	16	100.0		0	.0	16	100.0	
Lymph node involvement	Absence	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*
	Presence	14	0	0.0	14	100.0		1	20.0	13	92.9		0	.0	14	100.0		0	.0	14	100.0		0	.0	14	100.0	
Metastasis	M0	39	10	25.6	29	74.4	0.750	8	20.5	31	20.5	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	.0	1	.0		0	.0	1	100.0		0	.0	1	100.0		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*
	Advanced (III/IV)	14	0	.0	14	100.0		1	7.1	1	92.9		0	.0	14	100.0		0	.0	14	100.0		0	.0	14	100.0	
Relapse	Absent	16	9	56.2	7	43.8	< 0.001*	5	31.2	11	68.8	0.002	1	4.3	22	95.7	< 0.001*	13	81.2	22	100	< 0.001*	13	81.2	3	18.8	0.001
	Presence	22	0	.0	22	100.0		1	4.5	21	95.5		10	62.5	6	37.5		0	0	3	18.8		4	18.2	18	81.8	
Mortality	Dead	12	0	.0	12	100.0	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		9	34.6	17	65.4		13	46.4%	15	53.6		17	60.7	11	39.3	
DFS (months)																											
Mean (months) (95% CI)			58.900		47.364		0.001	56.25		49.20		0.035	59.00		47.016		0.001	59.214		45.474		< 0.001*	56.667		45.627		< 0.001*
Median			NR		48.000			NR		52.000			NR		48.00			NR		44.000			NR		40.000		
5-year (%)			90		23			87.5		32.9			90.9		22.1%			92		13			77		15		
OS (months)																											
Mean (months) (95% CI)			60.00		56.22		0.113	58.857		56.723		0.262	60.00		56.08		0.08	60.00		55.61		0.021	59.882		54.786		0.002
Median			NR		NR			NR		NR			NR		NR			NR		NR			NR		58.0		
5-year (%)			88.9		62.1			85.7		64.6			88.9		60.8%			91.7		56.1			94.1		47.7		

> 45 y, $p < 0.001$. A significant statistical association was seen between ProEx-C expression with lymph node metastasis ($p < 0.001$), marked tumour extent ($p < 0.001$), and advanced clinical stages III and IV ($p < 0.001$) (Table II).

The relationship between the expression of ProEx-C and outcomes in 40 HMSC patients

Relapse was more significantly associated with positive expression of ProEx-C than in those with negative expression ($p < 0.001$); the 5-year DFS in patients with negative expression of ProEx-C was 92%, which was significantly better than the 5-year DFS in positive cases, at 13% ($p < 0.001$). Also, there was a significant association between mortality and positive ProEx-C, $p = 0.021$, whereas 5-year overall survival was significantly higher in patients with negative expression of ProEx-C, at 91.7%, compared to patients who expressed positive ProEx-C: 56.1% ($p = 0.02$) at 95% CI (Fig. 8).

Telomerase reverse transcriptase expression

The expression of TERT and clinicopathological features are summarized in Table II. Twenty-two of the 40 cases (55%) showed high expression for TERT, whereas 18 of the 40 cases (45%) showed low expression (Fig. 9). The sex of the patients had no statistically significant link with high TERT expression ($p = 0.578$). High TERT expression was shown to have a statistically significant association with the old age group ($p < 0.001$), lymph node metastasis ($p < 0.001$), marked tumour extent ($p < 0.001$), and advanced clinical stages III and IV ($p < 0.001$) (Table II).

The relationship between the expression of telomerase reverse transcriptase and outcomes in 40 HMSC patients

Relapse was more significantly associated with high positive expression of TERT than in those with negative expression ($p = 0.001$); the 5-year DFS in patients with low expression of VEGF was 77%, which was significantly better than the 5-year DFS in high positive cases, at 15% ($p < 0.001$). Also, there was a significant association between mortality and high expression of TERT $p = 0.02$, whereas 5-year overall survival was significantly higher in patients with low expression of TERT, i.e. 94.1%, compared to patients who expressed high positive TERT, i.e. 47.7%, at 95% CI (Fig. 10).

Combined VEGF/COX-2/EGFR/ProEx-C/TERT expression

The combined expression of COX-2/VEGF/EGFR/ProEx-C/TERT and clinicopathological features are summarized in Table III. Twenty of the 40 cases (50%) showed combined expression of COX-2/VEGF/EGFR/ProEx-C/TERT, whereas the other 20 (50%) showed the absence of the combination of the 5 markers. The sex of the patients had no statistically significant link with the combined expression ($p = 0.374$). The combined expression was shown to have a statistically significant association with the old age group ($p = 0.002$), lymph node metastasis ($p < 0.001$), marked tumour extent ($p < 0.001$), and advanced clinical stages III and IV ($p < 0.001$) (Table III).

The relationship between the combined expression of COX-2/VEGF/EGFR/ProEx-C/TERT and outcomes in 40 HMSC patients

Relapse was more significantly associated with patients who showed combined expression than in those with the absence of combined expression ($p = 0.001$); the 5-year DFS in patients with combined expression was 70%, which was high significantly better than the 5-year DFS in patients with the absence of combined expression, i.e. 11.1% ($p < 0.001$). Also, there was a significant association between mortality and patients who showed combined expression, $p = 0.02$, whereas 5-year overall survival was significantly higher in patients with absent combined expression, i.e. 89.2%, compared to patients who expressed combined expression, i.e. 47.5%, ($p = 0.003$), at 95% CI (Fig. 11).

Discussion

Human papillomavirus-related multiphenotypic sinonasal carcinoma is a unique HPV-related tumour with many of the histologic characteristics of sinonasal adenoid cystic carcinomas but lacks MYB/MYBL1/NFIB translocation and has features of squamous differentiation but exhibits myoepithelial proliferation. Despite its aggressive appearance and delayed clinical course marked by high local recurrence rates, little is known about HMSC's clinical behaviour [32].

We diagnosed 40 cases of the newly described HMSC in our study, which presented clinically as a polypoid tumour in the nasal cavity, paranasal sinuses, and orbit, which leads to nasal obstruction, discharge, epistaxis, ocular symptoms, and pain [20].

There are few advanced molecular investigations on adenoid cystic carcinoma and its various subtypes, and patients continue to have a poor prognosis. A deeper comprehension of the biological processes underlying this tumour's development may assist in clarifying some of its clinical characteristics and provide further knowledge that may lead to novel therapeutic approaches.

Both genders in our study were affected, with women being the majority, which was in agreement with Bishop *et al.* [20]; however, Rupp *et al.* [33] showed that only women were affected in their study.

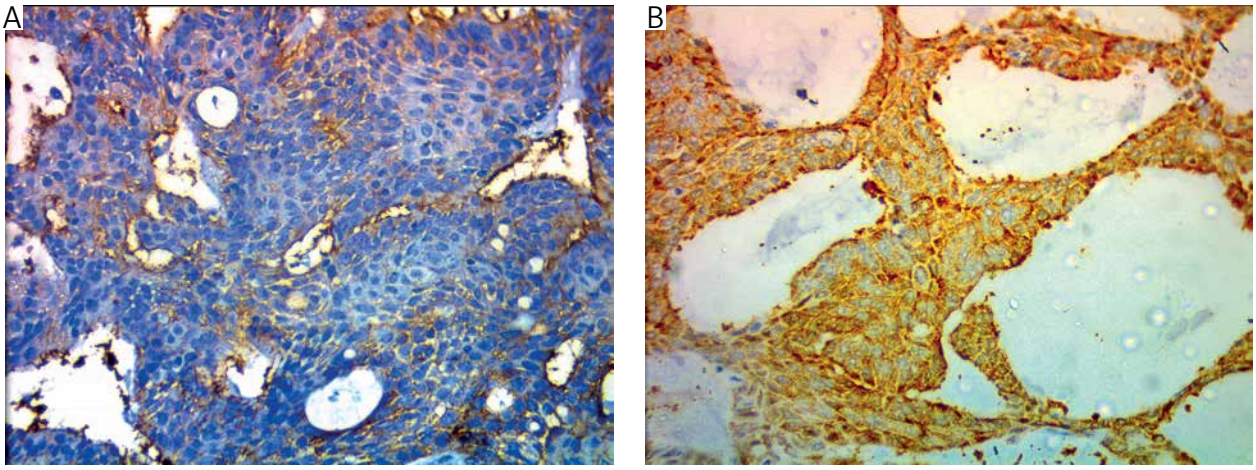

Fig. 1. Immunohistochemistry of vascular endothelial growth factor (VEGF): low VEGF expression (A) and high VEGF expression (B) in human papillomavirus-related multiphenotypic sinonasal carcinoma (400×)

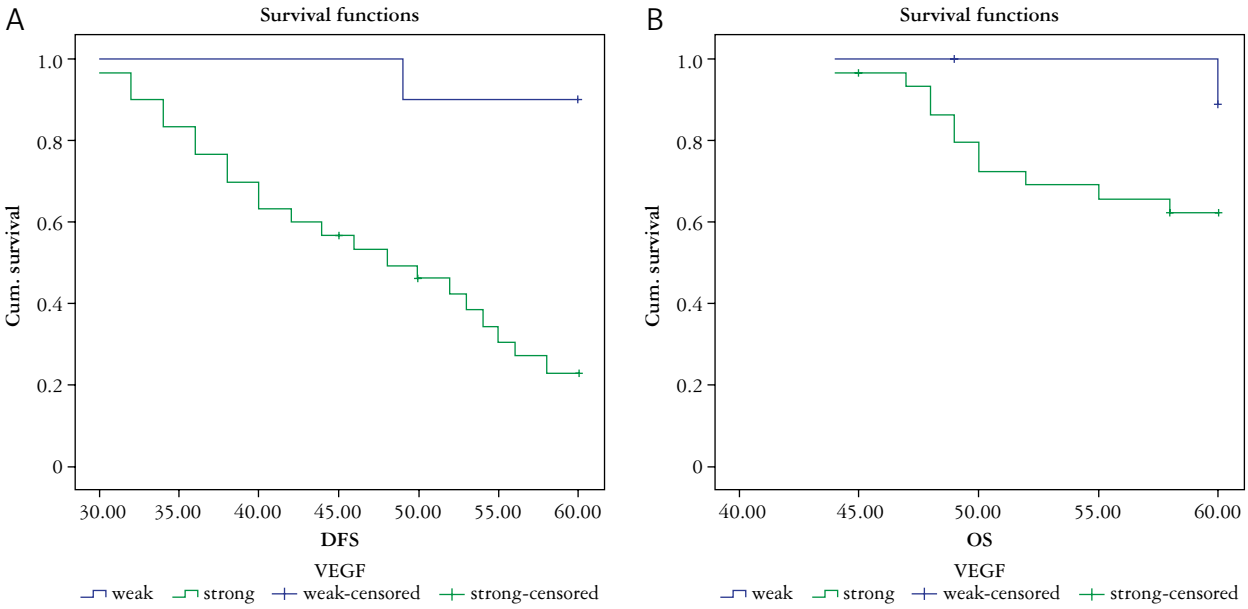

Fig. 2. Kaplan-Meier plot curve: dense fine speckled pattern stratified by vascular endothelial growth factor (VEGF) expression (A) and overall survival stratified by VEGF expression (B)

DFS – dense fine speckled, OS – overall survival

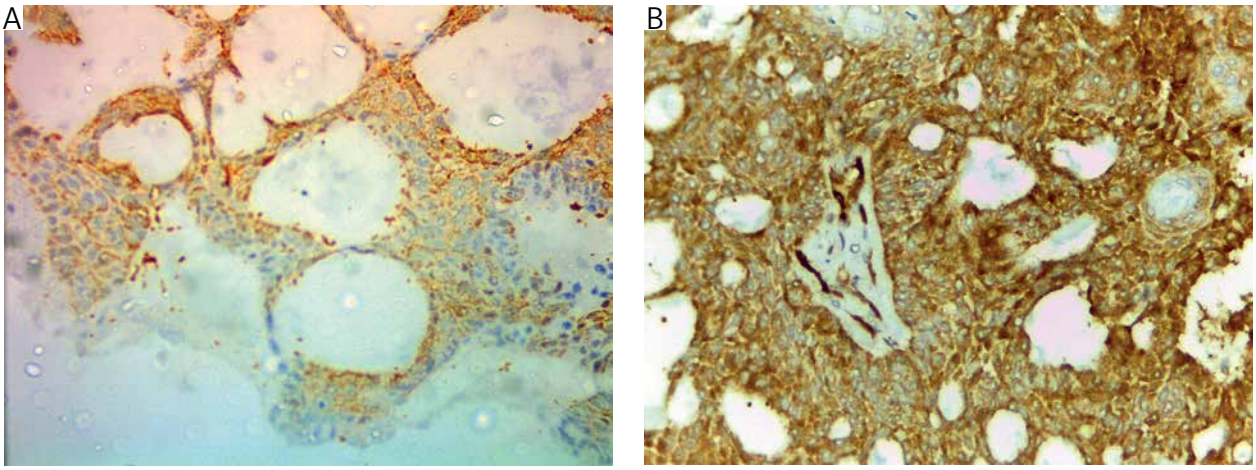

Fig. 3. Immunohistochemistry of COX-2: low COX-2 (A) and high COX-2 (B) in human papillomavirus related multiphenotypic sinonasal carcinoma (400×)

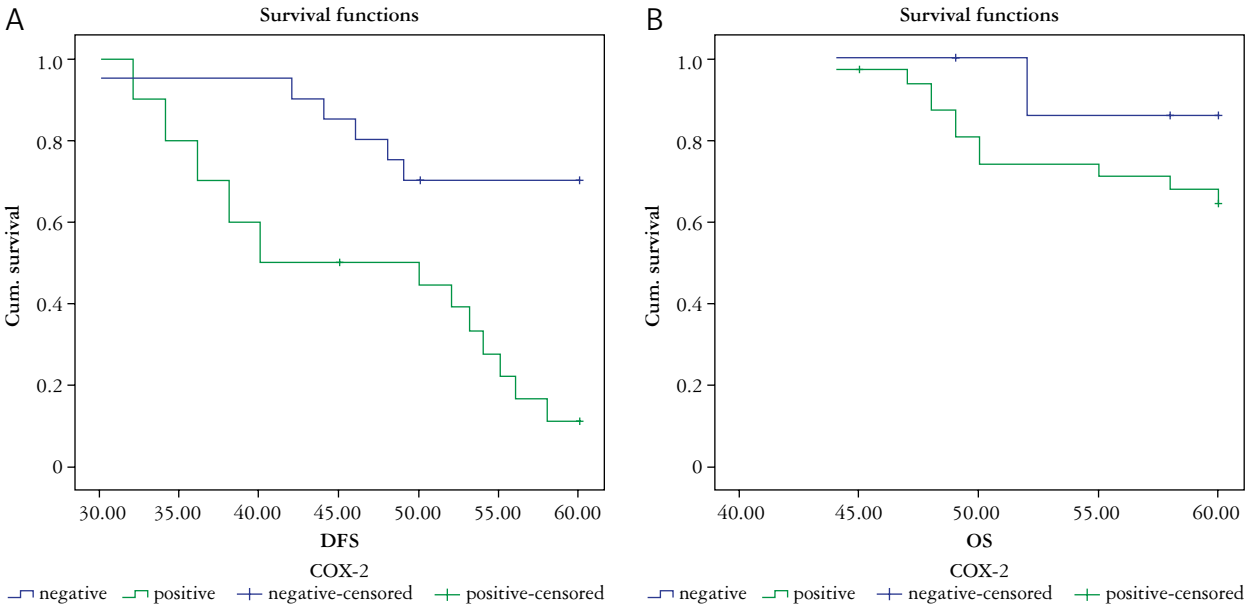

Fig. 4. Kaplan-Meier plot curve: dense fine speckled stratified by coexpression (COX-2) (A) and overall survival stratified by COX-2 (B)

COX-2 – coexpression, DFS – dense fine speckled, OS – overall survival

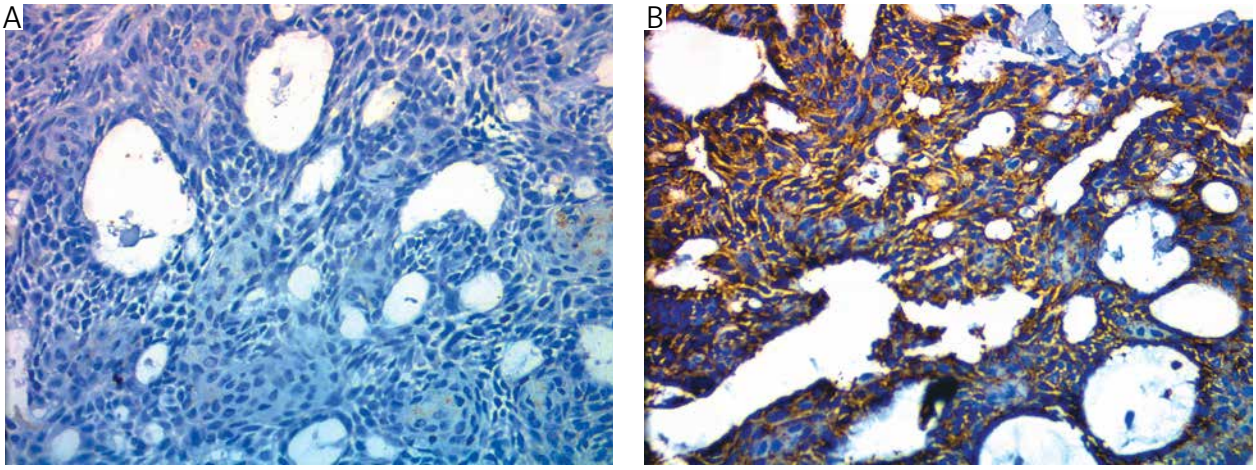

Fig. 5. Immunohistochemistry of epidermal growth factor receptor (EGFR): negative EGFR expression (A) and positive EGFR expression (B) in human papillomavirus related multiphenotypic sinonasal carcinoma (400×)

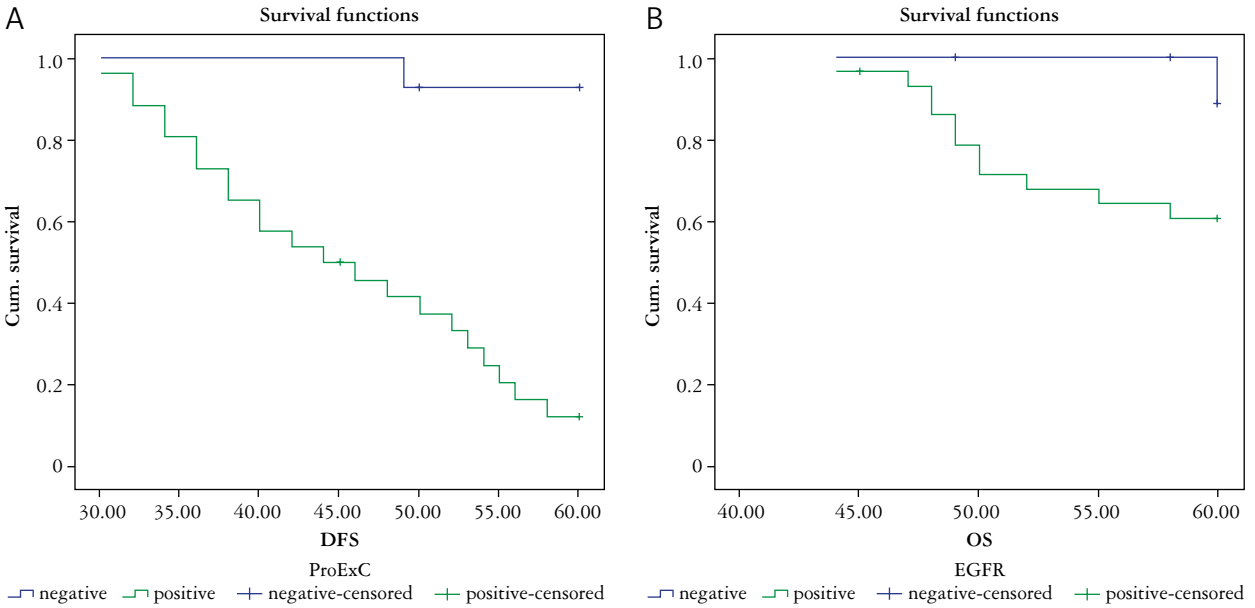

Fig. 6. Kaplan-Meier plot curve: dense fine speckled pattern stratified by epidermal growth factor receptor (EGFR) expression (A) and showed overall survival stratified by EGFR expression (B)

DFS – dense fine speckled, EGFR – epidermal growth factor receptor, OS – overall survival

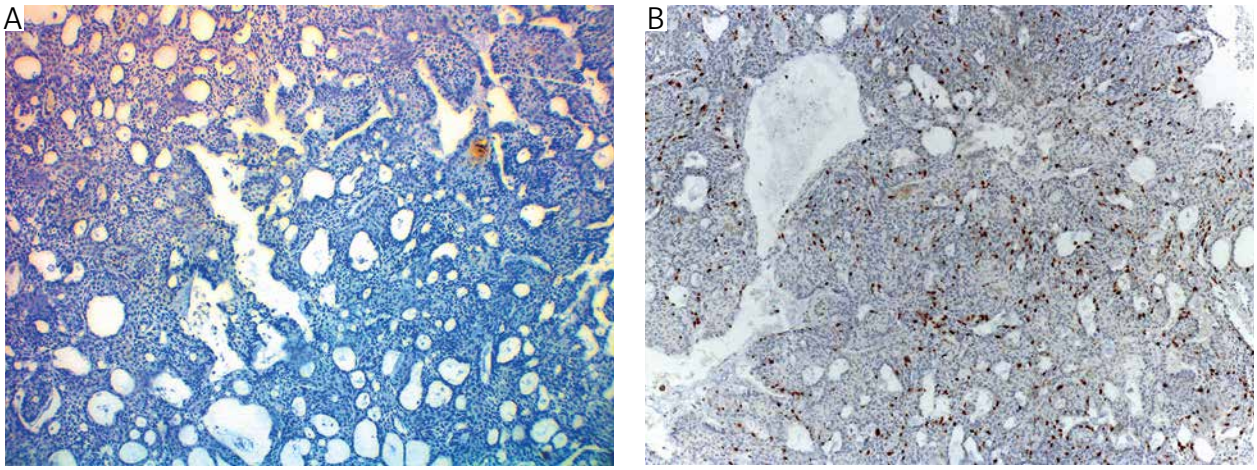

Fig. 7. Immunohistochemistry of proEx-C: negative proEx-C expression (A) and positive proEx-C expression (B) in human papillomavirus related multiphenotypic sinonasal carcinoma (100×)

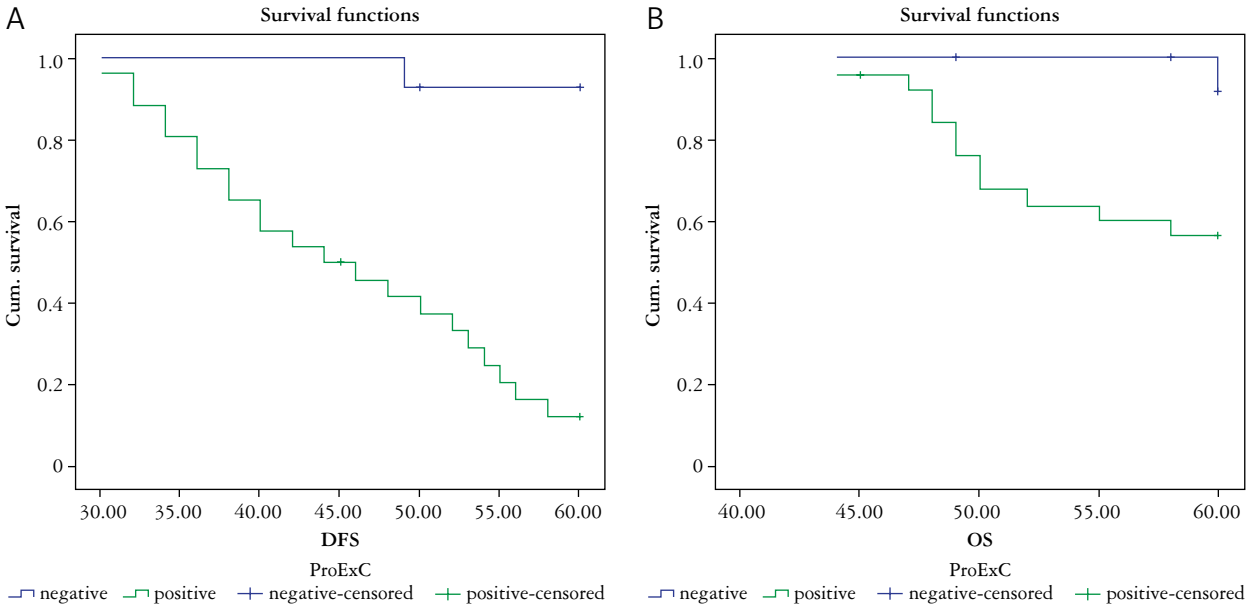

Fig. 8. Kaplan-Meier plot curve: dense fine speckled pattern stratified by proEx-C expression (A) and overall survival stratified by proEx-C expression (B)

DFS – dense fine speckled, EGFR – epidermal growth factor receptor, OS – overall survival

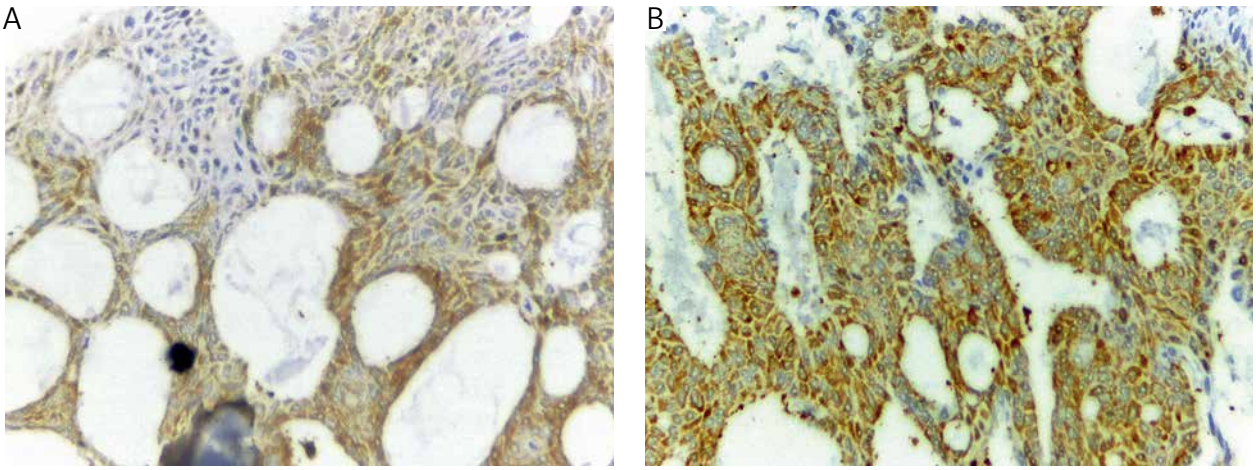

Fig. 9. Immunohistochemistry of h telomerase reverse transcriptase (TERT): low TERT expression (A) and high TERT expression (B) in human papillomavirus-related multiphenotypic sinonasal carcinoma (400×)

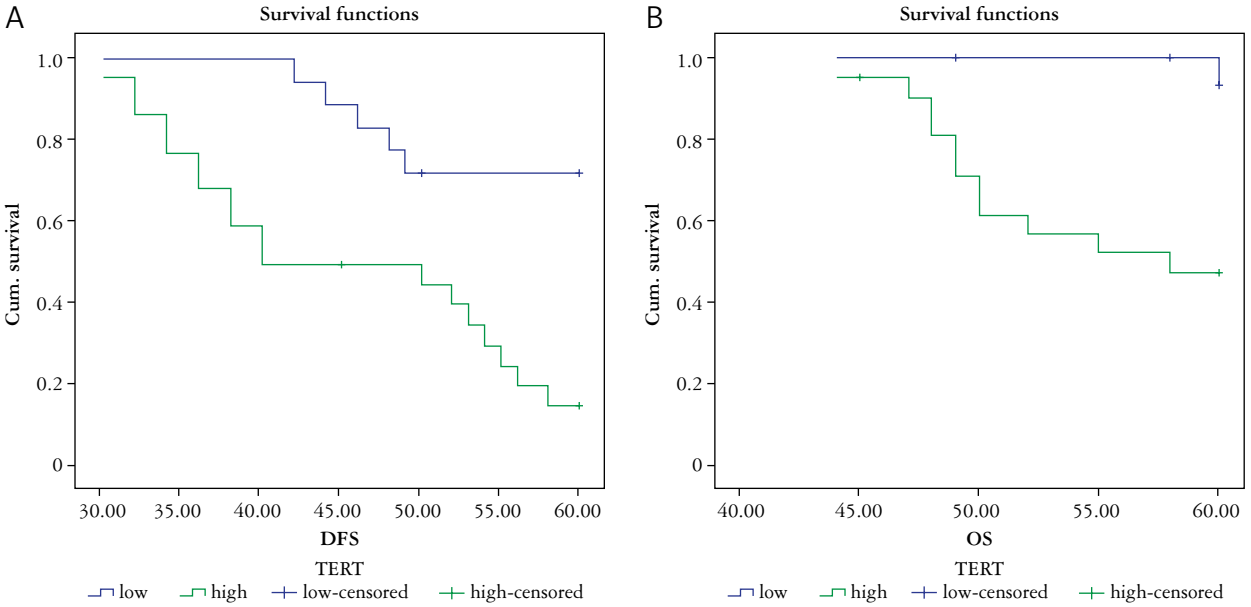

Fig. 10. Kaplan-Meier plot curve: stratified by h telomerase reverse transcriptase (TERT) expression (A) and overall survival stratified by TERT expression (B)

DFS – dense fine speckled, OS – overall survival, TERT – telomerase reverse transcriptase

Table III. Relation between clinicopathological, outcome parameters, and VEGF, BAX, ProEx-C and TERT co-expression in the studied patients

Combined expression of the five markers			COX-2/VEGF/EGFR/ProEx-C/TERT				*p*-value
			Negative *n* = 20		Positive *n* = 20		
	Subdivisions	*n*	*n*	%	*n*	%	
Age group	< 45 years	8	8	100.0%	0	.0%	0.002
	≥ 45 years	32	12	37.5%	20	62.5%	
Sex	Male	16	7	43.8	9	56.2	0.374
	Female	24	13	54.2	11	45.8	
Tumor extent	T1/T2	24	20	83.3%	4	16.7%	< 0.001*
	T3/T4	16	0	.0%	16	100.0%	
Lymph node involvement	Absence	26	19	73.1%	7	26.9%	< 0.001*
	Presence	14	1	7.1%	13	92.9%	
Metastasis	M0	39	20	51.3%	19	48.7%	0.500
	M1	1	0	.0%	1	100.0%	
Stage	Early (I/II)	26	19	73.1%	7	26.9%	< 0.001*
	Advanced (III/IV)	14	1	7.1%	13	92.9%	
Relapse	Absent	16	6	26.1%	17	73.9%	< 0.001*
	Presence	22	14	87.5%	2	12.5%	
Mortality	Dead	12	2	16.7%	10	83.3%	0..020
	Alive	28	17	65.4%	9	34.6%	
DFS (%)			70		11.1		< 0.001*
OS (%)			89.2		47.5		0.003

In our study, we observed 20 patients (50%) with local recurrences and 2 patients (5%) with a metastatic spread that was near to what Ward *et al.* [32] found in their literature evaluation of 57 cases, reporting that 36.4% of patients had local recurrence and 4.5% of cases acquired distant metastases. Ahn *et al.* and Shah *et al.* [9, 34] discovered that late recurrences had been reported with a follow-up of roughly

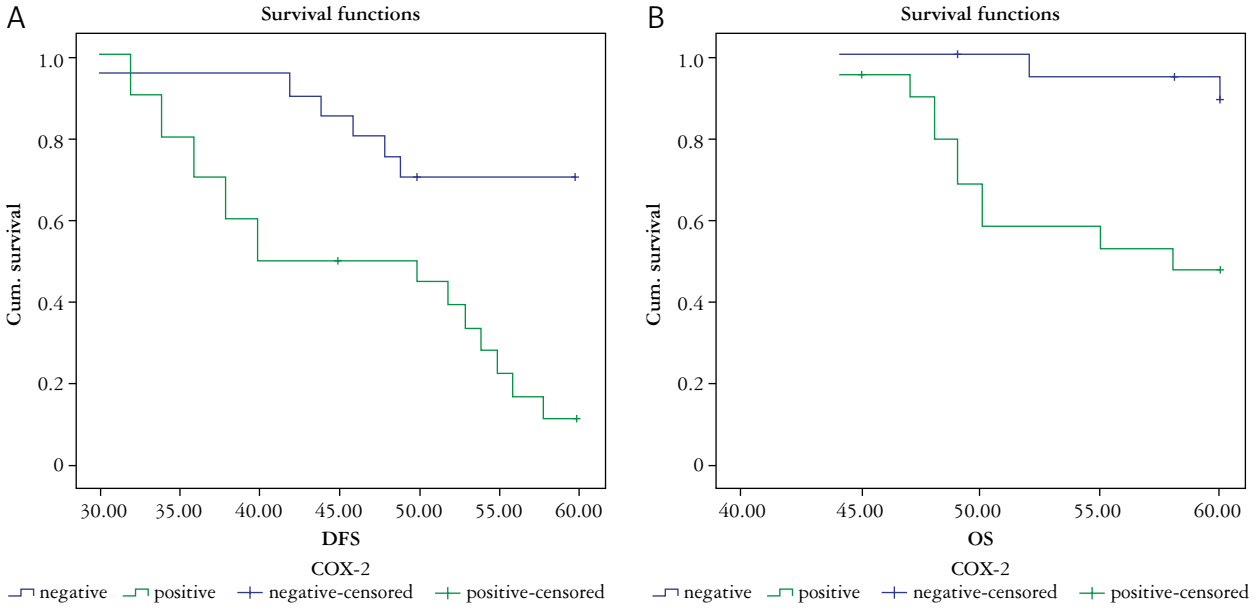

Fig. 11. Kaplan-Meier plot curve: dense fine speckled pattern stratified by co-expression of (vascular endothelial growth factor – VEGF, coexpression – COX-2, epidermal growth factor receptor – EGFR, ProEx-C and telomerase reverse transcriptase – TERT) (A) and overall survival stratified by (VEGF, COX-2, EGFR, ProEx-C, and TERT) co-expression (B)

50 months, while Rupp *et al.* [33] found no recurrence or metastases in their study.

Human papillomavirus has been linked to the development of numerous head and neck cancers, with oropharyngeal carcinomas being the most frequent. The prevalence of HPV-driven head and neck cancers has increased dramatically over the previous 3 decades [32].

The processes of HPV infection in the sinonasal tract are still unclear; surprisingly, 3 of 4 patients were hospital staff interacting with HPV-infected lesions [32]. Bergbrant *et al.* [35] showed that HPV may be discovered on the body surface (e.g. nostrils) of health care providers. However, Kofoed *et al.* [36] proved that the overall HPV detection rate of health care employees was not significantly higher than that of other employees.

One of the diagnostic features of HMSC is the association of HPV that can be detected with in situ hybridizations of HPV RNA, as in our study, where we demonstrated HPV-33 in 85%, HPV-35 in 10%, and HPV-16 in 5% of cases. Another diagnostic feature of HMSC is that the lack of translocation of MYB/MYBL1 in HMSC clearly distinguishes it from the solid variant of adenoid cystic carcinoma, and myoepithelial differentiation sets it apart from sinonasal squamous cell carcinoma [20]. In our 40 cases of HMSC, the morphology of most patients showed areas exhibiting adenoid cystic characteristics with myoepithelial differentiation and other parts showing tumour involvement resembling nonkeratinized squamous cell carcinoma. The morphology and HPV types found (33, 35, 16) were comparable to those reported in earlier studies by [20, 33, 37]. However, a study by Rupp *et al.* [33] showed that one of the cases (HMSC) had a pronounced glomeruloid pattern with more pleomorphic cells and an associated HPV type 82. Furthermore, HPV (33, 35) types are phylogenetically identical to the other HPV types but are rarely discovered in cervical cancer, accounting for only 2% of HPV-related cervical cancer [38].

Although the exact cell of origin in HMSC is unknown, the multiphasic expression of pancytokeratin combined with biphasic staining of basal/myoepithelialcells with p40, p63, calponin, and smooth muscle actin, as well as its morphology, points to a salivary gland origin [20, 39].

It is the first time to examine VEGF, COX-2, EGFR, ProEx-C, TERT immunoexpression in HMSC and evaluated their association with prognostic and clinicopathological outcomes, so our results were discuses with the most relevant tumours.

Vascular endothelial growth factor is the most potent and specific agent among many molecules capable of initiating angiogenesis, and it has been widely assessed in various types of cancer. Vascular endothelial growth factor was proven to be overexpressed through the activation of the PI3K/Akt pathway by the E5 oncoprotein of HPV. In our study, 30 patients (75%) were positive for VEGF, which is significantly associated with the old age group, tumour extent, lymph node metastases, advanced tumour stages, relapse, and mortality. That was in agreement with Lee *et al.* [40], who showed that 71% of patients had high VEGF expression in adenoid cystic carcinoma of the salivary gland. Association of VEGF expression with poor prognosis of adenoid cystic carcinoma has been reported in previous studies [41–44] that reported a significant as-

sociation of high expression of VEGF with advanced tumour stage, local recurrence, and poor OS rat; on the other hand, Lee *et al.* [40] reported that VEGF expression was not related to the recurrence or the survival rate of salivary gland ACC.

Coexpression is a different protein that the PI3K/Akt pathway can impact. Most cell types do not express COX-2, an enzyme that transforms arachidonic acid into prostaglandins when they are not inflamed. However, inflammatory conditions can lead to higher COX-2 levels, which have a direct impact on cell growth and death. Increased COX-2 expression, which in turn contributes to carcinogenesis by preventing some cancers from undergoing apoptosis and boosting angiogenesis, has been linked to NF-B activation [45].

COX-2 was expressed strongly in 32 patients (80%) of HMSC in the current study, which followed the results of Akrish *et al*., 2009, while Haymerle *et al.*, 2016 [46, 47] found COX-2 highly expressed in 83% and 87% of patients, respectively, In our study, high COX-2 expression was significantly associated with the old age group, tumour extent, relapse, and mortality that was in accordance with Haymerle *et al.* [47], who found that overexpression of COX-2 was associated to poor survival, and cyclooxygenase inhibitors are a potential therapeutic option in an adjuvant setting or for patients with unresectable tumours of the minor salivary glands.

Epidermal growth factor receptor, a transmembrane tyrosine kinase receptor, is upstream of PI3K/Akt pathway activation that activates downstream signalling, which stimulates mitosis, proliferation and inhibition of apoptosis [48, 49]. It has been well documented that activation of the EGFR pathway plays an important role in the pathogenesis and progression of various tumours. Recently, anti-EGFR agents have been effectively used for tumour treatment [50].

In the current study, 29 patients (72%) were positive for EGFR, which was in accordance with Ettl *et al.* [51], who found EGFR expressed in 77% of patients with salivary gland adenoid cystic carcinoma. In our study, positive expression of EGFR was significantly related to the old age group, tumour extent, and lymph node metastases, and relapse was in line with Ettl *et al.* [51], who stated that overexpression of EGFR correlates with poor prognosis in salivary gland carcinomas, and that EGFR is a new therapeutic target with a good impact on survival as recorded by preliminary data from a clinical trial that had shown tumour response to gefitinib, an EGFR inhibitor. On the other hand Saida *et al.* [50] reported that no correlation was found between EGFR expression and clinicopathological factors.

Pro-Ex-C is a marker for higher-risk HPV, causing overexpression of cell cycle proteins such as mini-chromosome maintenance protein 2 (MCM2) and topoisomerase II-a (TOP2A), which are overexpressed when viral DNA integrates into the host genome, leading to aberrant S-phase induction [52]. In many epithelial tumours, such as the cervix, endometrium, bladder, thyroid, and oesophagus, the Pro-Ex-C marker is an immunohistochemical cocktail including antibodies against both TOP2A and MCM2 proteins that detect early dysplastic changes, premalignant lesions, and prognostic outcomes [52–57].

In the current investigation, 24 patients (65%) tested positive for ProEx-C, significantly linked to the old age group, tumour extent, lymph node metastases, advanced tumour stages, relapse, and mortality. It was clarified that Pro-Ex-C expression had predictive and prognostic values, which was in agreement with Kungoane [58], who stated that MCM2 overexpression was linked to poor prognosis in salivary gland neoplasms, and Vargas *et al.*, Jaehne *et al.*, Savera *et al.*, and Ghazy *et al*. [59–62] reported that high grades of different salivary gland carcinoma were significantly associated with high MCM2 expression. While Vivatvakin *et al.* [63] found that downregulation of MCM2 and Ki67 is associated with advanced laryngeal squamous cell carcinoma stages.

In recent investigations, the MCM2 protein was shown to be a sensitive proliferation indicator with higher sensitivity than standard proliferation markers like Ki-67 because they identify both cycling and noncycling cells with proliferative potential [62, 64].

Telomerase's ability to catalyse is improved by HPV E6. Telomerase reverse transcriptase collaborates with an NF-B subunit to control the expression of TNF, IL-6, IL-8, and MMP-9, which trigger the epithelial-mesenchymal transition and promote metastasis [49]. We found that in 22 patients, 55% showed high expression of TERT, followed Shigeishi *et al.* [65], who found that TERT was highly expressed in 66% of cases with salivary gland adenoid cystic carcinoma. Also, high TERT expression was significantly linked to the old age group, tumour extent, lymph node metastases, advanced tumour stages, relapse, and mortality.

Combined expression of VEGF, COX-2, EGFR, ProEx-C, and TERT in HMSC patients was found in 20 (50%) patients and was significantly associated with the old age group, tumour extent, lymph node metastases, advanced tumour stages, relapse, and mortality.

Vascular endothelial growth factor, COX-2, EGFR, and ProEx-C expression are linked to the oncogenic pathways of higher-risk HPV in various epithelial malignancies, as well as the prognosis of these tumours, at the molecular level

Conclusions

Human papillomavirus-related multiphenotypic sinonasal carcinoma is a unique sinonasal neoplasm that has a strong link to HR-HPV strains. Expression

of VEGF, COX-2, EGFR, ProEx-C was linked to poor survival and aggressive malignant behaviours such as proliferation, local recurrence, and lymph node metastasis, making them novel prognostic biomarkers and targeted therapies for HMSC patients.

The authors declare no conflict of interest.

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