Article published online: 2022-07-13

Study of Three Potential Diagnostic Biomarkers in Nasopharyngeal Carcinoma Samples from Guilan, North of Iran

Saghi Jani Kargar Moghaddam[1] Amaneh Mohammadi Roushandeh[2] Mehryar Habibi Roudkenar[2] Shadman Nemati[3] Nima Najafi-Ghalehlou[4] Toofan Pakzad[1] Masoud Hamidi[1]

[1] Department of Medical Biotechnology, Faculty of Paramedicine, Guilan University of Medical Sciences, Rasht, Iran
[2] Burn and Regenerative Medicine Research Center, Velayat Hospital, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[3] Otorhinolaryngology Research Center, Faculty of Medicine, Amiralmomenin Hospital, Guilan University of Medical Sciences, Rasht, Iran
[4] Department of Laboratory Sciences, Faculty of Paramedicine, Tabriz University of Medical Sciences, Tabriz, Iran

Address for correspondence Masoud Hamidi, BSc, MSc, PhD, Department of Medical Biotechnology, Faculty of Paramedicine, Guilan University of Medical Sciences, Rasht, Iran (e-mail: masoud.hamidi@ulb.be).

Int Arch Otorhinolaryngol 2023;27(3):e461–e470.

Abstract

Introduction Finding biomarkers for highly lethal cancers is a priority.

Objective The current study was designed to understand the clinical significance of vascular endothelial growth factor (VEGF), latent membrane protein 1 (LMP1), and tumor necrosis factor-α (TNF-α) expression as the biomarkers, and evaluate their correlation with each other, in nasopharyngeal carcinoma (NPC) in the province of Guilan, North of Iran.

Methods Gene expression was evaluated in 25 formalin-fixed paraffin-embedded (FFPE) blocks from cases of confirmed NPC and 20 FFPE samples of non-NPC by quantifying messenger ribonucleic acid (mRNA) and protein levels, using real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC) methods, respectively. Furthermore, the correlations among the protein levels of different genes, along with the patients' demographic characteristics were assessed.

Results Our findings on mRNA and protein levels demonstrated that the expression of the LMP1 gene in the NPC group was significantly elevated compared with that of the non-NPC group. In addition, the protein levels in the NPC group indicated a positive and significant correlation between LMP1 and VEGF expression. It was noted that both protein and mRNA levels showed no significant differences in the expression of TNF-α and VEGF genes between the NPC and control groups. Furthermore, there was no significant relationship between the expression of these proteins and the demographic characteristics of NPC patients.

Keywords
- nasopharyngeal carcinoma
- tumor necrosis factor-α
- vascular endothelial growth factor-A
- biomarkers

received
October 13, 2021
accepted after revision
April 17, 2022
article published online
July 13, 2022

DOI https://doi.org/10.1055/s-0042-1749371.
ISSN 1809-9777.

Thieme Revinter Publicações Ltda., Rua do Matoso 170, Rio de Janeiro, RJ, CEP 20270-135, Brazil

Conclusion Overall, a significant increase in LMP1 expression was observed in NPC patients, which may serve as a diagnostic biomarker for NPC. Also, LMP1 might be involved in NPC progression by inducing VEGF gene expression.

Introduction

One of the malignant epithelial tumors of the head and neck region is nasopharyngeal carcinoma (NPC), which prevalently arises from the fossa of Rosenmüller.[1] The global incidence of NPC has been reported to be roughly 2% and 0.2% among head and neck carcinomas and all cancer types, respectively. Every year, the highest prevalence of this disease is observed in Asia.[2,3] The most common epidemiological features attributed to NPC have considerable ethnic and geographical distributions.[4] It has been reported that higher morbidity and mortality rates of NPC are observed among males in comparison with females.[5] Also, the prognosis of NPC in females is better than in males due to physiological factors.[6] In addition to the genetic susceptibility and environmental stresses, another prominent etiological factor related to NPC is the Epstein-Barr virus (EBV), which is involved in the molecular abnormalities resulting in NPC.[7,8]

In 2005, the World Health Organization (WHO) categorized 3 main histopathological subtypes of NPC as follows: keratinizing squamous cell carcinoma (SCC) (WHO type I), non-keratinizing SCC, including differentiated (WHO type II) and undifferentiated variants (WHO type III).[9] It is remarkable that the incidence of the non-keratinizing SCC subtype is outnumbered in endemic areas and is closely interrelated with Epstein-Barr virus infections.[10] Nasopharyngeal carcinoma is a radiosensitive tumor, the anatomical location of which renders radiotherapy an optional treatment for patients with early-stage non-metastatic NPC.[3] However, it has been reported that metastasis in advanced-stage NPC leads to undesirable effects after treatment.[11,12] Therefore, using suitable detection methods to diagnose early-stage NPC might help prevent disease development and is counted as an effective treatment.[13] Nowadays, biomarkers are suggested in cancer diagnosis, especially NPC, for numerous advantages.[14–16]

Latent membrane protein 1 is a 66kDa integral membrane protein that plays a prominent role in EBV-associated malignancies, such as NPC.[17] There are functional similarities between LMP1 and tumor necrosis factor receptor (TNFR) family members, including TNFR1 and CD40. Thus, LMP1 and TNFR family members can interact with the same proteins, such as TNFR-associated factors (TRAFs), which leads to activating the nuclear factor-κB (NF-κB) signaling pathways. This transcription factor can induce the overexpression of several genes, including vascular endothelial growth factor (VEGF).[18,19] Also, concerning the role of LMP1 in the progression of cancers, it has been reported that in hypoxic conditions, it upregulates transcription factor hypoxia-inducible factor-1 (HIF-1) and immortalizes tumors by inducing the expression of key genes involved in the invasion of cancers, which results in tumor growth and metastasis.[20] One of the key genes is VEGF, with a molecular weight of nearly 45kDa, known as an angiogenesis factor in many cancers.[21] The angiogenic properties of VEGF can culminate with cancer progression metastasis. Additionally, VEGF plays a vital role in tumor growth by inducing anti-apoptotic factors.[22] Therefore, VEGF has been employed in different cancers as a biomarker for diagnostic and prognostic purposes.[23,24]

Tumor necrosis factor-α (TNF-α) is the most important proinflammatory cytokine, mainly produced by activated macrophages.[25] It has dual functions, including tumor promotion and suppression in several cancers, and is involved in cancer development by inducing the expression of angiogenic factors, such as VEGF.[22,26] In addition, since TNF could be a cancer killer, TNF-α can be used in cancer therapeutic approaches.[26] Also, several studies have considered serum TNF-α as a novel prognostic biomarker to predict bone invasion and posttreatment distant metastasis in NPC patients.[27,28]

Objectives

Regarding the items mentioned above of LMP1, VEGF, TNF-α, and their association in cancer progression, the current study was performed to consider these three genes as potential biomarkers for the first time in NPC samples, collected from the archives of clinical laboratories in Guilan province, North of Iran. Also, differences in the expression of LMP1, VEGF, and TNF-α between cancerous and non-cancerous nasopharyngeal tissues were assessed by real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC) methods.

Materials and Methods

Patients' Tissue Samples

Between 2011 and 2018, 25 formalin-fixed paraffin-embedded (FFPE) blocks of NPC patients (9 females and 16 males; 15–82 years old) and 20 non-cancerous FFPE specimens (control group) (12 females and 8 males; 5–87 years old) were provided from the nasopharynx of individuals who had been referred to the pathology centers for reasons other than NPC, such as polyp or rhinoplasty from pathology departments in Rasht city. All specimens were selected according to the inclusion and exclusion criteria, including validation of the normal area or the adjacent normal area of nasopharyngeal tissue as inclusion criteria in the control group, confirmation of NPC tissues by two pathologists and their classification according to the WHO guideline, and, finally, absence of another disease other than NPC.

Real-time PCR

Four serial sections of each FFPE block were cut into 5-µm thick samples for real-time PCR. Then, the total ribonucleic acid (RNA) was purified from the tissue specimens using RNeasy FFPE Kit (QIAGEN GmbH, Hilden, Germany). After that, complementary deoxyribonucleic acid (cDNA) synthesis was performed from extracted RNA according to the manufacturer's instructions (Yekta Tajhiz Azma Co, Iran). To determine the expression of VEGF, LMP1, and TNF-α genes, real-time PCR was performed to evaluate mRNA levels using the Applied Biosystems StepOnePlus Real-Time PCR System (USA) by SYBR Green method. Also, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was designated as an endogenous control gene. Following that, PCR was performed in a 15-µL reaction mixture, containing 7.5 µL ready-to-use Ampliqon high ROX master mix 2x (Ampliqon A/S, Odense, Denmark), 3 µL double distilled water, 10 pmol forward primer (0.75 µL), 10 pmol reverse primer (0.75 µL), and 3 µL diluted cDNA for each sample as the template. After 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C, PCRs were done twice. Also, the sequence of primers used for the real-time PCR is shown in ►**Table 1**. The relative mRNA expression of the genes was calculated by the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry (IHC)

Briefly, FFPE samples were cut into 3-µm thick sections and placed on glass slides, coated with Silane (Azma Cell Aria, Iran). After deparaffinization and dehydration, the FFPE sections were exposed to heat-induced antigen retrieval (HIAR) in an autoclave for 20 minutes at 121°C, using ethylenediaminetetraacetic acid (EDTA) buffer for VEGF and TNF-α (pH 8) and LMP1 (pH 9). In the next step, the slides were transferred into three different series of phosphate-buffered saline (PBS) (5 minute each), respectively. Then, endogenous peroxidase activity was inhibited by H_2O_2 blocking for 10 minutes. The tissues were treated with Mouse Monoclonal anti-VEGF antibody (Diagnostic Biosystems, Pleasanton, CA, USA), Mouse Monoclonal anti-TNF-α Antibody (Diagnostic BioSystems), and Mouse Monoclonal anti-human Epstein-Barr Virus/LMP1 Antibody (Master Diagnóstica, Granada, Spain) for 1.5 hour at room temperature. To detect VEGF and TNF-α antibodies, the slides were incubated with peroxidase-labeled Mouse/Rabbit PolyVue Plus HRP/DAB Detection System (Diagnostic BioSystems). According to the manufacturer's protocol, LMP1 expression was detected by Master Polymer Plus Detection System (Master Diagnóstica). Afterward, the slides were stained with 3,3'-diaminobenzidine (DAB) substrate solution, incubated for 7 minutes, and counterstained with Harris' hematoxylin. Human normal colon, breast cancer, and Hodgkin lymphoma tissue samples were considered positive controls for TNF-α, VEGF, and LMP1, respectively.

Quantification of IHC

To quantify the expression of the proteins by IHC findings, the total immunostaining score (TIS) was defined. In this method, the proportion score (PS) represents the percentage of positive staining tumor cells in four scales (1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, >75%) and the intensity score (IS) indicates the average staining intensity compared with control cells (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). The TIS was calculated by (PS x IS) that equals nine values as follows: 0, 1, 2, 3, 4, 6, 8, 9, and 12. Each protein expression level (VEGF, LMP1, and TNF-α) was measured as 1, no expression (TIS 0); 2, weak expression (TIS 1–3); 3, moderate expression (TIS 4, 6, and 8); and 4, intense expression (TIS 9 and 12). Total immunostaining score 0–3 has been expounded/interpreted as negative and TIS > 3 as positive.[29]

Statistical Analysis

All statistical calculations were performed using the GraphPad Prism 8.3.0 software (GraphPad Software Inc., La Jolla, CA, USA). The median of each group was computed and the non-parametric Mann-Whitney U test was used to evaluate the differences between the two groups. Also, correlations between the protein levels of LMP1, TNF-α, and VEGF in the NPC and non-cancerous groups were separately assessed using the Spearman rank correlation coefficient. Moreover, the Fisher exact test was used to determine the significant correlation between the demographic characteristics of the patients and the overexpression of the proteins mentioned above. A p-value lower than 0.05 ($p < 0.05$) was considered as statistically significant.

Results

LMP1 Expression in NPC and Normal Tissue Samples

The results of real-time PCR illustrated that the messenger RNA (mRNA) levels of LMP1 were significantly higher in NPC tissues than in normal samples ($p = 0.0064$) (►**Table 2** and ►**Fig. 1**), as well as the protein levels of LMP1

Table 1 Primer sequences

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession No.	Amplicon
GAPDH	GACAGTCAGCCGCATCTTCT	GCGCCCAATACGACCAAATC	NM_001357943	104
LMP1	AACAAAGGAGGTGACCAGGG	AAGCAGCGTAGGAAGGTGTG	MN244677	105
VEGF	TGTGAATGCAGACCAAAGAAAGA	GCTTTCTCCGCTCTGAGCAA	NM_001025366	196
TNF-α	CTGGGGCCTACAGCTTTGAT	GGCCTAAGGTCCACTTGTGT	NM_000594	126

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMP1, latent membrane protein1; TNF-α, tumor growth factor-α; VEGF, vascular endothelial growth factor.

Table 2 Differential protein and mRNA expression of LMP1, VEGF, and TNF-α genes in the NPC and normal groups

	NPC median	Q1	Q3	Mean ± SD	Normal median	Q1	Q3	Mean ± SD	*P*-value
mRNA level									
LMP1	2.563	2.297	4.949	4.317 ± 5.296	1	0.707	1.414	1.344 ± 1.472	0.0064**
VEGF	1.071	0.615	4.309	2.487 ± 2.979	1	0.615	2.462	1.483 ± 1.367	0.645
TNFα	1.148	0.411	4.618	3.651 ± 6.210	1	0.329	2.639	1.750 ± 1.869	0.519
Protein level									
LMP1	2	1.5	4	2.480 ± 1.610	0	0	1	0.6000 ± 0.9403	< 0.0001****
VEGF	4	3.5	6	4.560 ± 1.781	4	3.25	5.5	4.200 ± 1.196	0.558
TNFα	6	2	8	5.200 ± 3.253	6	6	8	6.400 ± 1.729	0.249

Abbreviations: LMP1, latent membrane protein 1; mRNA, messenger ribonucleic acid; NPC, nasopharyngeal carcinoma; SD, standard deviation; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
** $p < 0.01$
**** $p < 0.0001$.

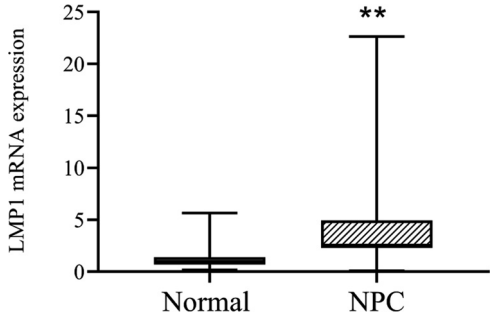

Fig. 1 LMP1 expression in NPC and normal tissue samples evaluated by quantitative reverse transcription polymerase chain reaction. LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma; ** $p < 0.01$.

($p < 0.0001$) (►**Table 2** and ►**Figs. 2** and **3**). As shown in ►**Table 3**, 9/25 (36%) of NPC samples revealed moderate expression of LMP1 (TIS > 3), and there was no intense expression. Also, 16/25 (64%) of NPC samples demonstrated low expression of LMP1 (TIS ≤ 3), among which 12/16 (48%) and 4/16 (16%) exhibited weak and negative expression, respectively. It is noteworthy that overexpression of LMP1 was not observed in the normal group (TIS > 3). Also, 7/20 and 13/20 of normal samples showed weak and negative expression, respectively (►**Table 3**).

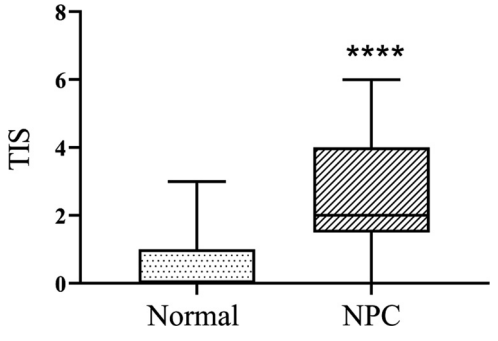

Fig. 2 Quantitative analysis of LMP1 protein expression by IHC, followed by TIS calculations in NPC and normal samples. LMP1, latent membrane protein 1; TIS, total immunostaining score; NPC, nasopharyngeal carcinoma; IHC, immunohistochemistry; **** $p < 0.0001$.

TNF-α and VEGF expression in NPC and Normal Samples

Contrary to the above-mentioned results of LMP1 overexpression in NPC patients compared with normal individuals, the results of both mRNA and protein levels of VEGF and TNF-α did not show a statistically significant difference between NPC and normal subjects (►**Table 2** and ►**Figs. 4** and **5**).

Correlation between the Protein Levels of LMP1, TNF-α, and VEGF in NPC and Non-Cancerous Tissue Samples

There was a significant and linear relation between the protein levels of VEGF and LMP1 in NPC tissues ($r = 0.410$, $p = 0.042$). However, no significant association between TNF-α and two other markers (LMP1 and VEGF) were found (►**Table 4**). Also, no significant relationship among the protein levels of the three markers in non-cancerous tissue specimens was observed (►**Table 5**).

Association of LMP1, TNFα, and VEGF Expression and Demographic Characteristics

As shown in ►**Table 6**, the protein levels of LMP1, TNF-α, and VEGF demonstrated no significant correlation with demographic features (age, sex, and NPC subtype).

Discussion

Nasopharyngeal carcinoma is a severe disease with 50,000 deaths annually.[30,31] There are several approaches for the diagnosis of NPC, but none of them is efficient. Therefore, late diagnosis in advanced-stage NPC leads to ineffective treatment approaches in NPC patients.[32] In recent years, biomarkers with diagnostic and prognostic capabilities in many cancers have drawn the interest of researchers.[15] Therefore, identifying NPC-specific biomarkers may also lead to better prognosis and treatment strategies.[33] In the present study, the expression of three genes, including LMP1, VEGF, and TNF-α was investigated for the first time in NPC FFPE

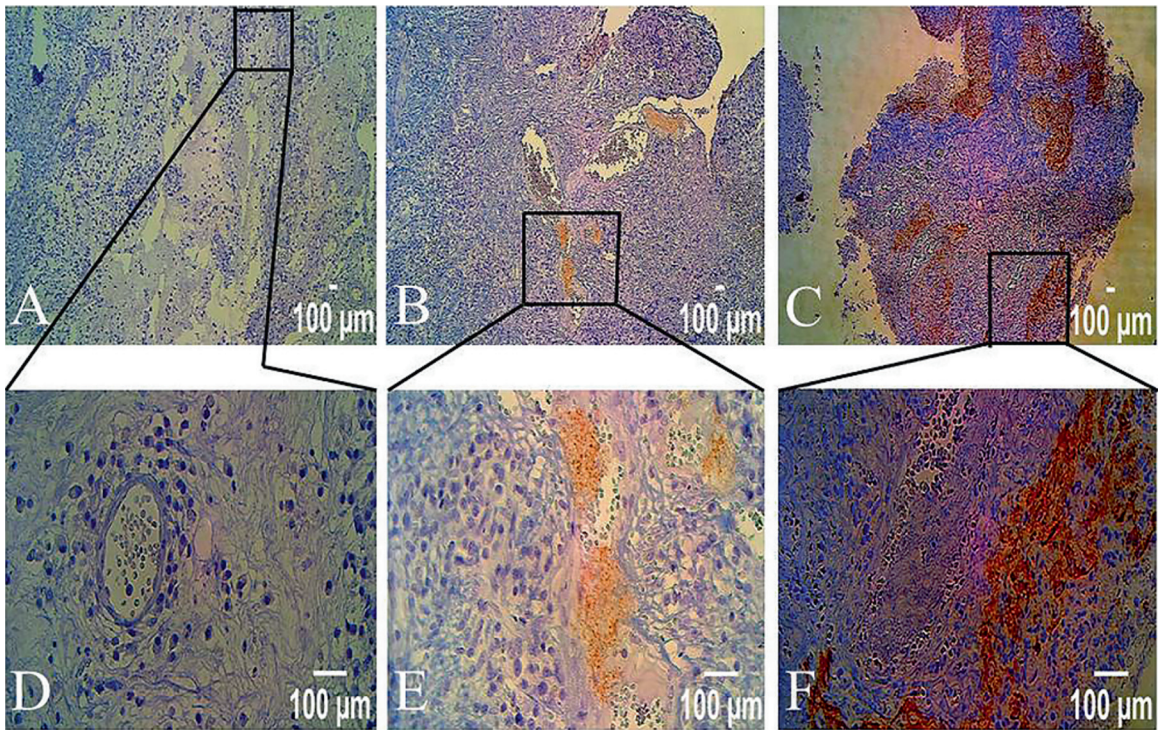

Fig. 3 Photomicrographs of IHC stained NPC and normal tissue samples to assess LMP1 protein. The intensity of staining was calculated using TIS. (**A**) Negative staining (TIS 0) in normal tissues of nasopharynx (100x). (**B**) Weak staining (TIS 2) in normal tissues (100x). (**C**) Moderate staining (TIS 6) in NPC tissues (100x). (**D**) Negative staining (TIS 0) in normal tissues (400x). (**E**) Weak staining (TIS 2) in normal tissues (400x). (**F**) Moderate staining (TIS 6) in NPC tissues (400x). Scale bar, 100µm. NPC, nasopharyngeal carcinoma; IHC, immunohistochemistry; LMP1, latent membrane protein 1; TIS, total immunostaining.

specimens of the inhabitants of Guilan province to consider them as a set of potential biomarkers in the diagnosis of NPC. The correlation between the expression of each of the three markers with each other and with the demographic characteristics (age and gender) and subtype of the tumor was evaluated in NPC patients. The results showed that mRNA and protein levels of LMP1 were significantly increased in NPC patients compared with normal individuals. However, no significant difference was detected in the expression of the other two markers (VEGF and TNF-α) between the NPC and control groups.

In this regard, Xu et al. reported that both the protein and mRNA levels of VEGF increased dramatically in patients with endometrial cancer compared with control subjects. In

Table 3 LMP1, VEGF, and TNF-α expression in nasopharynx tissue specimens evaluated by immunohistochemistry

	No proteins (LMP1, VEGF, TNFα) overexpression				Proteins (LMP1, VEGF, TNFα) overexpression			
	No expression		Weak expression		Moderate expression		Intense expression	
	(TIS 0)		(TIS 1–3)		(TIS 4, 6, 8)		(TIS 9, 12)	
	n	%	n	%	n	%	n	%
NPC tissues (25)								
(LMP1)	4	16%	12	48%	9	36%	0	0%
(VEGF)	0	0%	6	24%	18	72%	1	4%
(TNFα)	4	16%	3	12%	15	60%	3	12%
Normal tissues (20)								
(LMP1)	13	65%	7	35%	0	0%	0	0%
(VEGF)	0	0%	5	25%	15	75%	0	0%
(TNFα)	0	0%	0	0%	16	80%	4	20%

Abbreviations: LMP1, latent membrane protein1; NPC, nasopharyngeal carcinoma; TIS, total immunostaining score; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

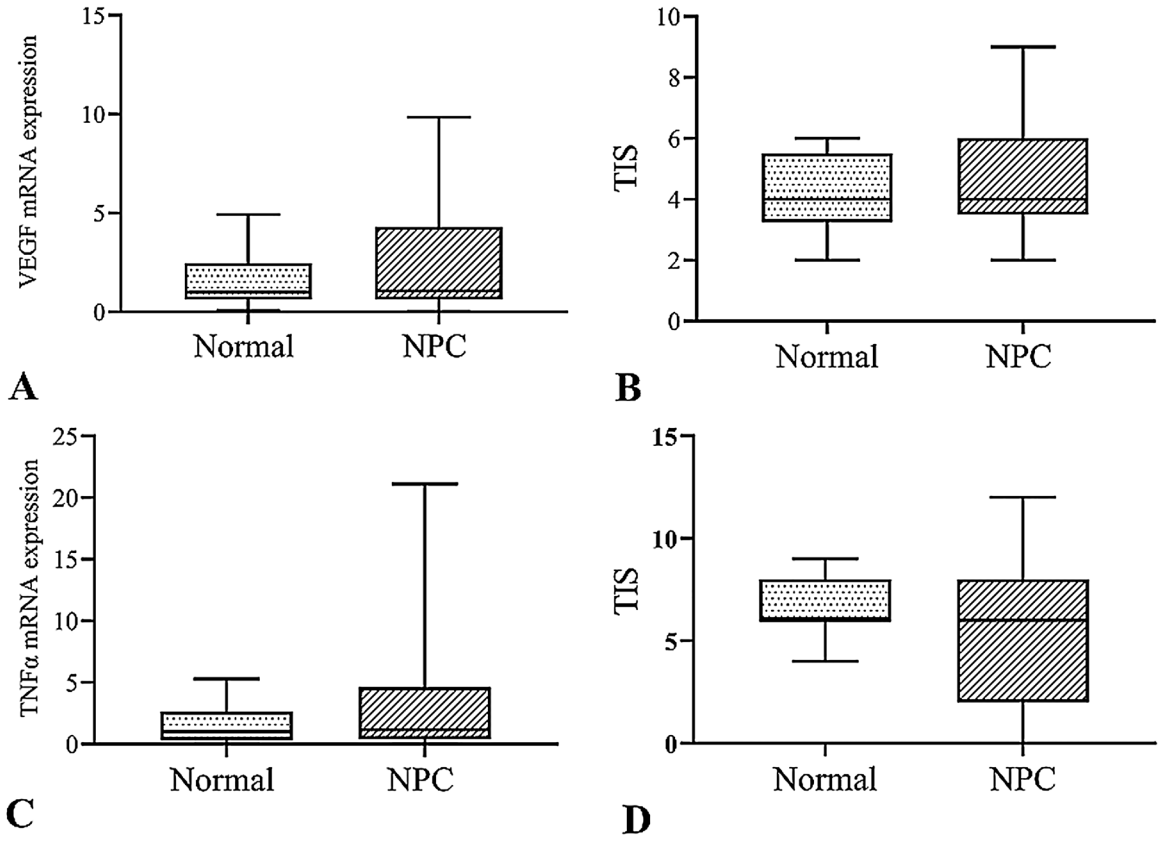

Fig. 4 (**A**) VEGF expression in NPC and normal tissue samples evaluated by quantitative RT-PCR. (**B**) Quantitative analysis of VEGF protein expression by IHC, followed by TIS calculations in the NPC and normal samples. (**C**) TNF-α expression in NPC and normal tissue samples evaluated by quantitative RT-PCR. (**D**) Quantitative analysis of TNFα protein expression by IHC, followed by TIS calculations in the NPC and normal samples. VEGF, vascular endothelial growth factor; TNF-α, tumor growth factor-α; TIS, total immunostaining score; IHC, immunohistochemistry; NPC, nasopharyngeal carcinoma.

addition, they suggested that VEGF might be a diagnostic biomarker in early-stage endometrial cancer and even a target in treatment interventions. Also, elevated VEGF expression was significantly associated with higher levels of differentiation and stage of the disease.[34] However, no significant difference was found in the levels of VEGF expression between the gastric cancer patients and the normal group.[35]

In another study, it was found that 66.7% of all NPC patients showed a significantly elevated level of VEGF expression compared with the controls.[36] It is noted that, in a study, 76% of all NPC patients presented a high level of VEGF expression with no significant difference compared with controls. It is noteworthy that many studies have demonstrated the role of VEGF angiogenesis in cancer progression and proliferation.[22] In this regard, Pan et al. showed that increasing VEGF levels leads to the progression of the NPC stage. It was revealed that VEGF could be considered a prognostic factor in patients with lower survival.[37] According to another study, VEGF expression had significant relationship with metastasis and tumor recurrence in NPC patients.[38]

Numerous studies have confirmed the role of LMP1 in the development and progression of NPC. For instance, Hariwiyanto et al. showed a significant and inverse correlation between the expression of LMP1 and response to treatment.[39] However, it is disputed in some clinical studies whether LMP1 has no communication with metastasis and tumor progression.[40] In the present study, there was a significant relationship between the expression of LMP1 and VEGF markers in NPC patients, which may indicate the role of LMP1 in NPC development through VEGF. Inconsistent with our findings, Paydas et al. reported a positive and significant relationship between the protein levels of VEGF and LMP1 genes in non-Hodgkin lymphoma patients, most probably showing an association between angiogenesis and viral carcinogenesis.[41] Also, the demographics (age and sex) of non-Hodgkin lymphoma patients did not show significant association with VEGF and LMP1 protein expression, which is consistent with our results[41]. However, in a report conducted by Chrestella et al., there was no significant relationship between these two proteins (VEGF and LMP1) in NPC, which is in discrepancy with previous studies and our findings.[42]

It has been found that the LMP1 expression was significantly increased in patients with oral cancer compared with the control groups.[43] In the research by Rosales-Perez et al., 10 out of 25 NPC paraffin blocks showed positive and higher protein levels of LMP1. It is noteworthy to mention that 9 out of 25 NPC blocks were positive for LMP1, as represented in the present study. In addition, parallel with our results, the prevalence of NPC was higher among men than among

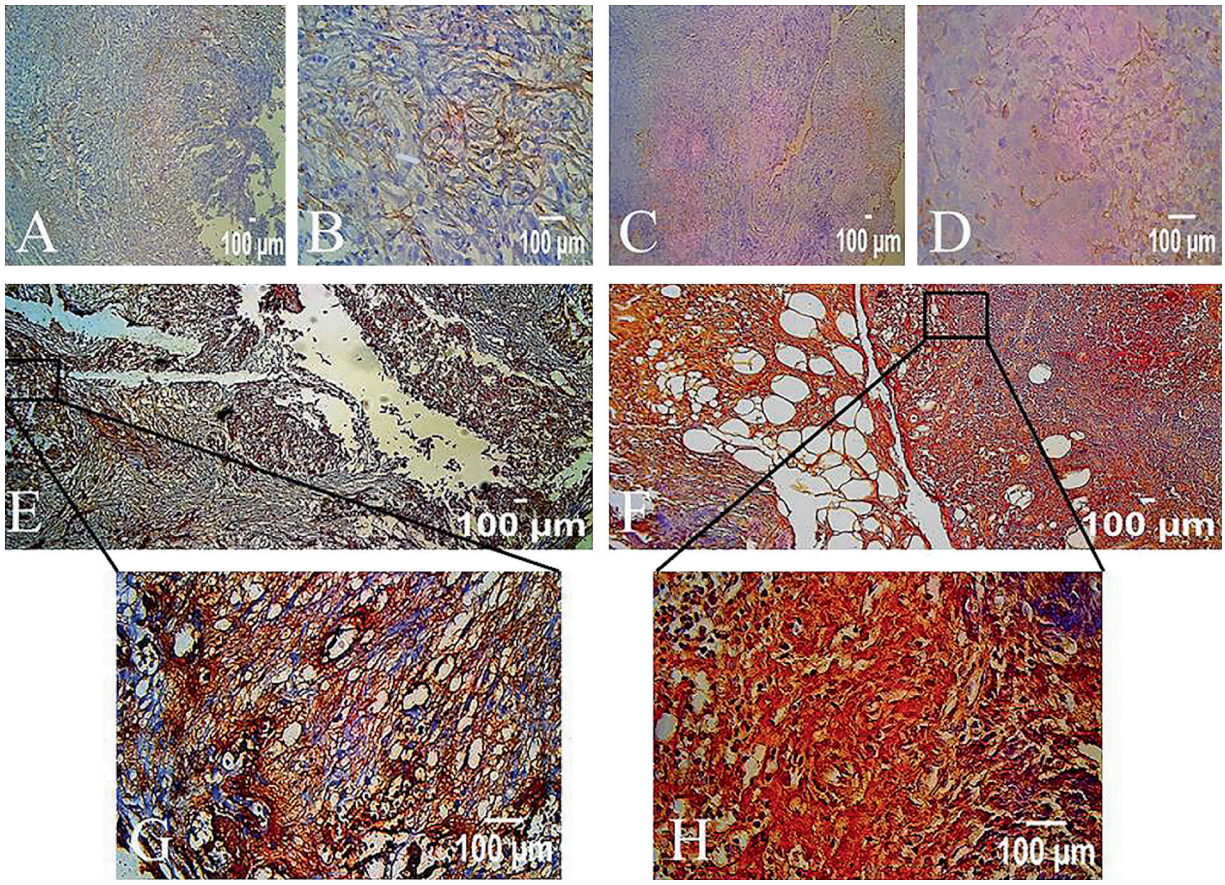

Fig. 5 Photomicrographs of IHC stained NPC and control samples to assess VEGF and TNF-α proteins. The intensity of staining was calculated using TIS. (**A**) Weak staining of VEGF (TIS 2) in normal tissues of nasopharynx (100x). (**B**) Weak staining of VEGF (TIS 2) in normal tissues (400x). (**C**) Weak staining of TNF-α (TIS 2) in NPC tissues (100x). (**D**) Weak staining of TNF-α (TIS 2) in NPC tissues (400x). (**E**) Intense staining of VEGF (TIS 12) in NPC tissues (100x). (**F**) Intense staining of TNFα (TIS 12) in normal tissues (100x). (**G**) Intense staining of VEGF (TIS 12) in NPC tissues (400x). (**H**) Intense staining of TNFα (TIS 12) in normal tissues (400x). VEGF, Vascular endothelial growth factor; TNF-α, tumor growth factor-α; TIS, Total immunostaining score; IHC, Immunohistochemistry; NPC, Nasopharyngeal carcinoma.

women, and the undifferentiated variant of NPC was more common than keratinizing subtype in the NPC group, as reported in the study by Rosales-Perez et al. Also, no significant correlation was found between the demographic characteristics of NPC patients (sex, age, NPC subtype) and LMP1 expression. It seems that the expression pattern of LMP1 depends on the type of cancer. So that Almedia et al. did not find any positive and significant elevation of protein levels of LMP1 in thyroid cancer patients.[44,45]

Evidence has shown the important role of proinflammatory cytokine TNF-α in many inflammatory processes related to carcinogenesis and cancer progression; however, there

Table 4 Correlation among the expressions of each of the three markers studied (LMP1, VEGF, TNFα) at the protein level in NPC tissue samples

NPC group		LMP1	VEGF	TNFα
LMP1	r	1	0.410	0.363
	P		0.042*	0.075
VEGF	r	0.410	1	0.146
	P	0.042*		0.485
TNFα	r	0.363	0.146	1
	P	0.075	0.485	

Abbreviations: LMP1, latent membrane protein1; NPC, nasopharyngeal carcinoma; r, relation; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; *p*, *p*-value
*$p < 0.05$.

Table 5 Correlation among the protein levels of each of the three markers (LMP1, VEGF, and TNF-α) in normal tissue samples

Normal group		LMP1	VEGF	TNFα
LMP1	r	1	0.410	0.363
	P		0.042	0.075
VEGF	r	0.410	1	0.146
	P	0.042		0.485
TNFα	r	0.363	0.146	1
	P	0.075	0.485	

Abbreviations: LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma; r, relation; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; *p*, *p*-value.

Table 6 Association between demographic features and LMP1, VEGF, and TNFα overexpression

Demographic features	Number of patients		LMP1 overexpression		VEGF overexpression		TNFα overexpression	
			n (%)	*p* value	n (%)	*p* value	n (%)	*p* value
Age (years)								
> 30	23 (92%)		8 (32%)		17 (68%)		16 (64%)	
				> 0.9999		> 0.9999		> 0.9999
≤ 30	2 (8%)		1 (4%)		2 (8%)		2 (8%)	
Sex								
Female	9 (36%)		3 (12%)		7 (28%)		4 (16%)	
				> 0.9999		> 0.9999		0.0581
Male	16 (74%)		6 (24%)		12 (48%)		14 (56%)	
Type of tumor								
Keratinizing squamous cell carcinoma (WHO type I)	2 (8%)		1 (4%)		2 (8%)		1 (4%)	
				> 0.9999		> 0.9999		0.4900
Undifferentiated non-keratinizing squamous cell carcinoma (WHO type III)	23 (92%)		8 (32%)		17 (68%)		17 (68%)	

Abbreviations: LMP1, latent membrane protein 1; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

was no statistically significant relationship between TNF-α protein expression and any of the demographic characteristics in the NPC group. In this regard, Yu et al. also showed that TNF-α expression was not associated with age, sex, and NPC subtype. However, they reported a direct link between increased TNF-α expression and bone metastasis in NPC patients.[46] Susilo et al. also reported a significant association between TNF-α protein expression and advanced-stage NPC.[28] In addition, as reported by Akinmoladun et al., no significant difference was found in the serum levels of TNF-α in patients with head and neck cancers, such as NPC, compared with the normal group, which is consistent with our findings.[47]

It is noted that mRNA was extracted from FFPE specimens in the present study. Due to the high sensitivity of RNA to temperature, rapid degradability, and instability, mRNA isolation from archived FFPE blocks was the main challenge in our study.[48] However, the samples with desirable quality and quantity have been considered for detecting the gene expression. It is noted that several samples were excluded because of unsuitable RNA quality. In addition, in a study conducted by Tabola et al., it was mentioned that long-term storage of FFPE blocks resulted in destructive effects on the quality of the extracted RNA. Therefore, it is necessary to optimize the storage time of paraffin blocks. In this investigation, optimizing the storage time of FFPE blocks was considered 3 years.[49] However, in another study, it was reported that there was no significant difference in the quality of RNAs isolated from 10-year-old FFPE specimens compared with recently obtained FFPE specimens for several months. However, the quantity of RNA was higher in recently archived FFPE blocks than in 10-year-old blocks.[50] Furthermore, using FFPE tissue samples in isotope labeling and microdissection technologies provides an appropriate alternative to frozen/fresh tissues to find suitable biomarkers.[51]

Conclusion

It can be concluded that high expression of LMP1 in patients with NPC was observed, which may serve as a diagnostic biomarker in NPC patients. In addition, LMP1 might be involved in NPC progression by inducing VEGF gene expression. However, there was no significant difference in both the protein levels and gene expression of VEGF and TNF-α between NPC patients and the normal group. It is noteworthy that the number of samples in our study was limited; therefore, future studies should focus on including more samples to clarify the association between different genes involved in NPC progression.

Conflict of interests

The authors have no conflict of interests to declare.

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