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Original Article

Lipocalin-2 Upregulation in Nasopharyngeal Carcinoma: A Novel Potential Diagnostic Biomarker

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Abstract

Background: Lipocalin-2 (LCN2) deregulation has been reported in several types of cancer and is implicated in the proliferation, migration, angiogenesis, and progression of tumors. However, its aberrant expression has been rarely studied in nasopharyngeal carcinoma (NPC). In the present study, we investigated the expression of LCN2 in NPC patients.

Methods: In this descriptive cross-sectional study, 29 NPC and 20 non-cancerous control paraffin pathology blocks were obtained from the seven-year (2011 to 2018) archive of Razi Laboratory in Rasht, Iran. LCN2 mRNA expression was evaluated through quantitative real-time PCR. In addition, immunohistochemistry was performed to evaluate LCN2 expression at the protein level. The fold change value and total immunostaining score (TIS) were applied for quantitative evaluation. The nonparametric Mann-Whitney U test and Fisher's exact test were used through GraphPad Prism 8.3.0 software. $P<0.05$ was considered statistically significant.

Results: Our results revealed that LCN2 mRNA and protein levels in NPC tissues were significantly higher than control tissues ($P=0.028$ and $P=0.002$, respectively). At the protein level, 65.51% (19/29) of NPC patients were categorized as having high LCN2 expression (TIS>3) and 34.47% (10/29) as low expression (TIS≤3). While in the control group, 25% (5/20) of subjects represented a high expression of LCN2 (TIS>3), and 75% (15/20) showed no or weak expression (TIS≤3). No significant correlation was found between the overexpression of LCN2 at the protein level and the demographic features of the patients.

Conclusion: Our findings suggest that LCN2 might be considered a potential new diagnostic marker for NPC. However, this warrants further studies.

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Keywords ● Lipocalin-2 ● Nasopharyngeal carcinoma ● Immunohistochemistry ● Biomarkers ● Head and neck neoplasms

What's Known

- Nasopharyngeal carcinoma (NPC) is generally diagnosed in advanced stages, which primarily results in unfavorable outcomes.
- There is still no acceptable screening test despite the remarkable geographical and ethnic distribution of nasopharyngeal carcinoma.

What's New

- mRNA and protein levels in nasopharyngeal carcinoma tissues were significantly higher than control tissues.
- Lipocalin-2 (LCN2) might be considered a potential new diagnostic marker for NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is designated as being the most prevalent type of head and neck cancers and the sixth globally recognized predominant malignancy[1, 2] with a remarkable geographical and ethnic distribution.[3] Totally, 86,500 new cases of NPC and 50,000 NPC-related deaths

are reported per annum all over the world.[4] In 2018, 34,681 cases of NPC and 22,231 resultant deaths were estimated in Southeast Asian countries.[5] The interaction of genetic and environmental factors alongside Epstein-Barr virus (EBV) infection are etiological factors of NPC.[6, 7] In addition, consumption of alcohol,[8] salty and preserved foods, such as salted fish particularly during childhood, limited fresh fruit or leafy vegetables,[9] and traditional southern Chinese foods containing nitrosamine,[4] as well as smoking, even passively,[10] personal history of the ears, nose, and throat (ENT) problems such as sinusitis and otitis media,[11] polymorphisms in the histocompatibility locus antigens (HLAs),[12] and family background of cancer, especially NPC[13] have all proven to be predisposing factors of NPC. According to the demographic information, men are about twice susceptible to the disease, and NPC development reaches its peak between 50-60 years of age.[14] Based on the international guidelines, the standard therapeutic approach for NPC is radiotherapy with/without concurrent chemotherapy.[15, 16] However, locoregional recurrence is seen in 10-15% of patients following treatment.[1, 17] Due to the lack of symptoms in the early stages of the disease and having no acceptable screening test, NPC is generally diagnosed in an advanced stage, mostly resulting in unfavorable outcomes. Therefore, finding a suitable early-stage biomarker for NPC is essential.[18, 19] Over the past few decades, a number of studies reported several genes undergoing altered expression profiles in NPC cells compared to their healthy fellows. Therefore, these genes have been proposed to be considered for diagnostic and prognostic purposes.[20, 21] However, there is yet no candidate biomarker for NPC.[22, 23] One of the potential candidate biomarkers for NPC could be lipocalin-2 (LCN2)/neutrophil gelatinase-associated lipocalin (NGAL). LCN2 is a secretory protein of the lipocalin superfamily.[24] The precise function of LCN2 is not fully understood yet. However, a variety of physiological functions, including anti-oxidant,[25] anti-bacterial, and iron transporting activities[21] were reported for LCN2. Furthermore, LCN2 is subjected to conspicuous overexpression in acute and chronic inflammatory diseases, ischemic diseases,[24] and various malignancies, such as breast, colorectal, ovarian, prostate, lung, liver, and pancreatic cancers.[21] In addition, it has been revealed that LCN2 is overexpressed and thus involved in the formation of nasal polyps as a non-cancerous tissue overgrowth in the head and neck region.[26] Although upregulation of LCN2 in most cancers favors cancer promotion, there are also some reports indicating LCN2 inhibits cancer progression. This shows the complicated role of LCN2 in cancer biology.[27, 28] The present study aimed to determine the impact of LCN2 expression in NPC compared to normal non-cancerous tissues and highlighted the role of lipocalin-2 in the behavior of this malignancy.

Patients and Methods

In this descriptive cross-sectional study, all specimens were collected from the archived blocks gathered in seven years from 2011 to 2018 in the Pathology Departments of Razi Laboratory (Rasht, Iran). All NPC samples were confirmed by two pathologists and classified according to the World Health Organization (WHO) classification for each type of cancer. The control samples were obtained from the nasopharynx of individuals and referred to the pathology centers for reasons other than NPC, such as polyp or rhinoplasty.

All procedures performed in this study were in accordance with the ethical standards of the Ethics Committee of Guilan University of Medical Sciences (ethics code: IR.GUMS.REC.1397.339) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

RNA Extraction and cDNA Synthesis

RNA Extraction was performed using the RNeasy FFPE Kit (Qiagen, Germany). Briefly, five micrometer (μm) sections were cut from the formalin-fixed paraffin-embedded (FFPE) tissues by microtome (MicroTech, Germany), deparaffinized using xylene (Merck, Germany), and rinsed by ethanol 96% (Dr. Mojallali Co., Iran). Then, tissue samples were incubated at room temperature for 10 min. RNAs were isolated from tissue samples according to the RNeasy FFPE Kit instruction. Briefly, buffer PKD was added to the samples and centrifuged for one min at 11,000 ×g (10,000 rpm). Then, proteinase K (Qiagen, Germany) was added and incubated according to the kit guidelines. DNase booster buffer (Qiagen, Germany) was added to the samples and incubated at room temperature for 15 min. Then, followed by the addition of ethanol, buffer RBC (Qiagen, Germany) was added and mixed thoroughly. Afterward, the samples were transferred to a RNeasy MinElute spin column and placed in a 2 mL collection tube. Samples were centrifuged for 15 sec at ≥8000 ×g (≥10,000 rpm). Buffer RPE (Qiagen, Germany) was added to the RNeasy MinElute spin column and centrifuged for 15 sec at ≥8000 ×g (≥10,000 rpm). Then, Buffer RPE was

added to the RNeasy MinElute spin column and centrifuged for two min at ≥8000 ×g (≥10,000 rpm) to wash the spin column membrane. The RNeasy MinElute spin column was placed in a new 2 mL collection tube and centrifuged at full speed for five min. Then, the RNeasy MinElute spin column was placed in a new 1.5 mL collection tube. Finally, RNase-free water (Qiagen, Germany) was added directly to the spin column membrane and centrifuged for one min at full speed to elute the RNA. The quality and quantity of the extracted RNA were evaluated by NanoDrop 2000 (Thermo Fisher Scientific, USA). Afterward, the cDNA was synthesized using a cDNA synthesis kit (Yektatajhiz Azma Co., Iran).

Quantitative Real-Time PCR

Expression of the LCN2 gene and the housekeeping gene GAPDH were assessed by SYBR Green real-time PCR dye using Applied Biosystems™ StepOnePlus™ Real-Time PCR System (USA). The corresponding primer sequences were as follows: LCN2 forward primer: 5'-CTGTCCCAATCGACCAGTGT-3', LCN2 reverse primer: 5'-CCAGCTCCCTCA ATGGTGTT-3'; GAPDH forward primer: 5'-GACAGTCAGCCGCATCTTCT-3', GAPDH reverse primer: 5'-GCGCCCAAT ACGACCAAATC-3'. PCR condition was a pre-activation stage of 15 min at 95 °C (initial denaturation), followed by 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. The reactions were run in duplicate for each sample. Finally, the difference in the expression of LCN2 mRNA in the two samples was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry Assay

The expression of the LCN2 at the protein level was evaluated by IHC. First, 3 µm of the paraffin-embedded tissue sections were prepared on silane-coated slides. Then, the sections were incubated at 65 °C for 60 min. For deparaffinization, slides were floated in two different series of xylene (five min each), rehydrated in decreasing concentrations of ethanol (100%, 96%, 80%, and 70%), and washed in distilled water. Antigen retrieval was carried out by heating the slides for 20 min at 121 °C in ethylenediaminetetraacetic acid (EDTA) buffer (pH=8). Then, after slow cooling, the slides were washed three times with phosphate-buffered saline (PBS) for 15 min. After endogenous peroxidase blocking with 3% H_2O_2 for 10 min, blocking the nonspecific protein binding was performed by a solution of 0.1% bovine serum albumin (BSA) in PBS. Then, the sections were incubated with rabbit monoclonal anti-lipocalin-2 antibody (Abcam, USA, dilution 1:100) overnight at 4 °C. Then, after washing with PBS, the slides were treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Abcam, USA, dilution 1:300) for primary antibody detection. Next, the slides were incubated with 3,3'-diaminobenzidine (DAB) substrate solution according to the manufacturer's protocol (Kiazist Co., Iran). Finally, the sections were counterstained with the Harris Hematoxylin and dehydrated in ascending series of ethanol concentrations. Human normal tonsil tissue was considered the LCN2 positive control.

Total Immunostaining Scoring

The total immunostaining score (TIS) was used to quantify LCN2 expression. The proportion score (PS) represents the approximate fragment of positive-staining tumor cells (1: 0-25%, 2: 26-50%, 3: 51-75%, 4: >75%). In addition, the intensity score (IS) indicates the approximate staining severity compared with the control cells (0: no staining, 1: weak staining, 2: moderate staining, 3: strong staining). Finally, TIS was calculated by multiplying PS and IS together that equals nine values 0, 1, 2, 3, 4, 6, 8, 9, and 12. The LCN2 expression level was measured as 1: no expression (TIS=0), 2: weak expression (TIS=1-3), 3: moderate expression (TIS=4,6,8), 4: intense expression (TIS=9,12). TIS>3 was considered LCN2 overexpression.[2]

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.3.0 software (GraphPad Software, USA). The variables were reported in numbers and percentages. The differences between the two groups were evaluated by the nonparametric Mann-Whitney U test, and the comparison of LCN2 overexpression according to the demographic characteristics of the NPC patients was performed by Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

LCN2 Upregulation in Human NPC Tissues

In order to evaluate the expression of LCN2 at the mRNA level, RT-qPCR was performed. The results revealed that LCN2 mRNA was significantly upregulated in the NPC group compared to the normal group (figure 1 and table 1) (P=0.028). Next, IHC was performed to evaluate the expression of LCN2 at the protein level. Consistent with the RT-qPCR results, intense LCN2 immunoreactivity was detected

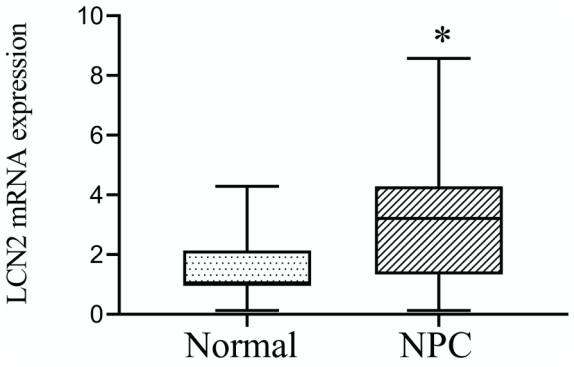

Figure 1: Quantitative RT-PCR was used to assess Lipocalin-2 expression in nasopharyngeal carcinoma and normal specimens. NPC: Nasopharyngeal carcinoma; LCN2: Lipocalin-2. (Nonparametric Mann-Whitney U test, *P=0.028)

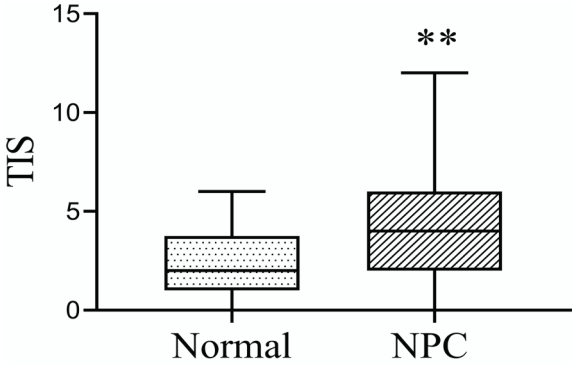

Figure 2: The figure indicated the quantitative analysis of lipocalin-2protein expression by immunohistochemistry, followed by total immunostaining score calculations in the nasopharyngeal carcinoma and normal samples. TIS: Total immunostaining score; NPC: Nasopharyngeal carcinoma; IHC: Immunohistochemistry; LCN2: Lipocalin-2. (Nonparametric Mann-Whitney U test, **P=0.002)

Table 1: Differential protein and mRNA expression of lipocalin-2 gene in nasopharyngeal carcinoma and normal groups

Variables	NPC Median	Q1	Q3	Mean±SD	Normal Median	Q1	Q3	Mean±SD	P value
LCN2 (mRNA)	3.215	1.339	4.287	3.644±2.932	1	1	2.140	1.557±1.450	0.0288*
LCN2 (Protein)	4	2	6	4.655±3.154	2	1	3.75	2.200±1.576	0.0022**

LCN2: Lipocalin-2; NPC: Nasopharyngeal carcinoma; SD: Standard deviation; Q: Quartile; *P<0.05, **P<0.01; Nonparametric Mann–Whitney U-test was used. Total sample size was 49.

Table 2: Lipocalin-2 expression in nasopharynx tissues determined by immunohistochemistry

	No LCN2 overexpression		LCN2 Overexpression	
Variables	No expression (TIS=0) n (%)	Weak expression (TIS=1-3) n (%)	Moderate expression (TIS=4,6,8) n (%)	Intense expression (TIS=9,12) n (%)
NPC tissues (29)	4 (13.79)	6 (20.68)	16 (55.17)	3 (10.34)
Normal tissues (20)	3 (15)	12 (60)	5 (25)	0

LCN2: Lipocalin-2; NPC: Nasopharyngeal carcinoma; TIS: Total immunostaining score; Total sample size was 49.

in the NPC patients compared to the controls (figures 2 and 3). As shown in table 2, 13.79% (4/29) of the NPC specimens showed negative expression (TIS=0), and 20.68% (6/29) showed weak expression (TIS=1-3) of LCN2. However, 55.17% (16/29) of the cases represented moderate expression (TIS=4,6,8), and 10.34% (3/29) represented intense expression (TIS=9,12) of LCN2. Percentages of samples indicating a TIS of 2, 3, 4, 6, 8, 9, and 12 were 13.79% (n=4), 6.89% (n=2), 17.24% (n=5), 34.48 % (n=10), 3.44 % (n=1), 3.44% (n=1), and 6.89% (n=2), respectively. In the normal samples, scores 0, 1, 2, 3, 4, and 6 were detected in 15% (n=3), 15% (n=3), 40% (n=8), 5 % (n=1), 20% (n=4), and 5% (n=1), respectively. However, intense expression (TIS=9,12) was not detected in the normal group. Moreover, there was a significant difference in TIS between the control and the patient groups (P=0.002) (figure 2 and table 1). There was no difference in LCN2 overexpression between the two NPC groups.

Based on the IHC results, 19 out of 29 patients exhibited an increase in LCN2 expression (TIS>3), while 10 out of 29 patients showed no increase in the expression of LCN2 (TIS≤3) (table 2). The present study was performed on 29 NPC specimens (20 men and 9 women; 15-82 years old) based on the availability of samples and 20 control (non-cancerous) specimens (8 men and 12 women; 5-87 years old). Most NPC patients aged between 50 and 70 years. It is noteworthy that, based on the demographic parameters, there was no difference in LCN2 upregulation between the two groups of NPC patients (table 3).

Discussion

Our findings revealed that the expression of LCN2 both at the protein and the mRNA levels in NPC tissues was markedly upregulated compared to the normal tissues. In fact, 19 out of the 29 NPC patients showed higher expression of LCN2. Of note, in the current study, NPC patients were divided into two groups according to their demographic features. No difference between

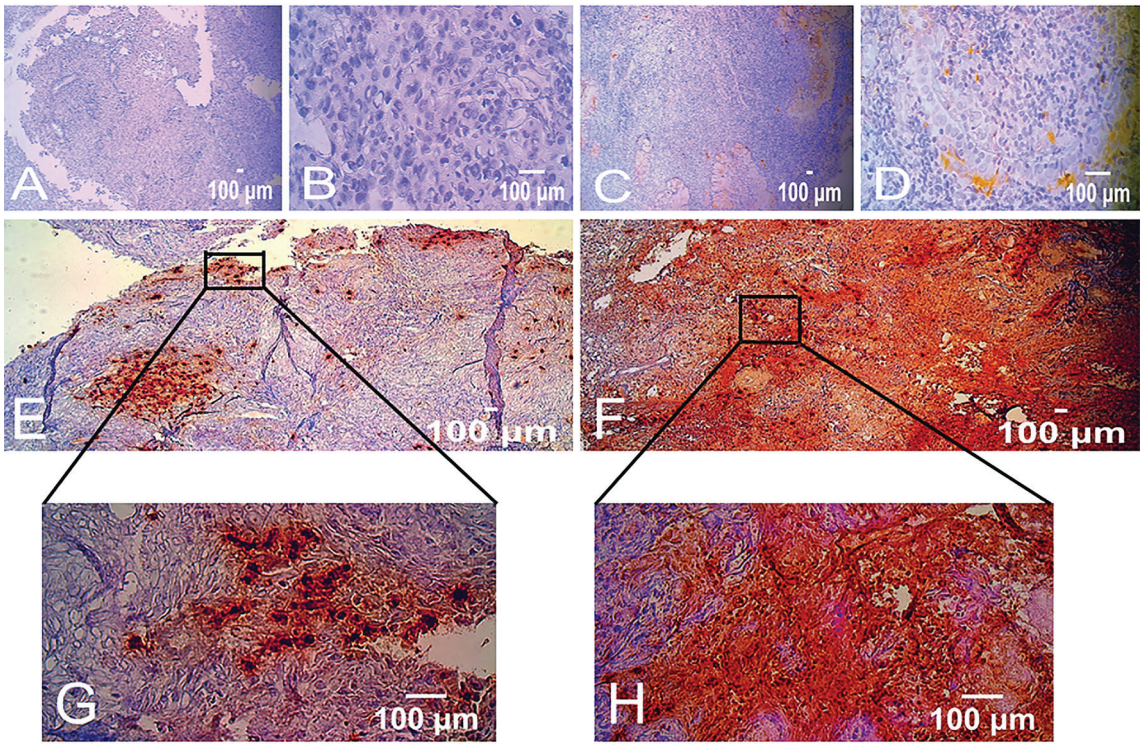

Figure 3: Photomicrographs of immunohistochemistry staining for the nasopharyngeal carcinoma and control samples were used to assess lipocalin-2 protein. The staining intensity was calculated using the total immunostaining score. A) Negative staining (TIS=0) in normal tissues of the nasopharynx (×100), B) Negative staining (TIS=0) in normal tissues (×400), C) Weak staining (TIS=2) in normal tissues (×100), D) Weak staining (TIS 2) in normal tissues (×400), E) Moderate staining (TIS=6) in NPC tissues (×100), F) Intense staining (TIS=12) in NPC tissues (×100), G) Moderate staining (TIS=6) in NPC tissues (×400), H) Intense staining (TIS=12) in NPC tissues (×400).

Table 3: Comparison between the demographic characteristics and lipocalin-2 overexpression in nasopharyngeal carcinoma patients

Demographic features		NPC Patients (n=29) n (%)	LCN2 overexpressed patients (n=19) n (%)	P value
Age (year)	>30	26 (89.65)	17 (58.62)	>0.999
	≤30	3 (10.35)	2 (6.90)	
Sex	Women	9 (31.03)	4 (13.79)	0.204
	Men	20 (68.97)	15 (51.72)	
Type of tumor	Keratinizing squamous cell carcinoma (WHO type I)	2 (6.89)	1 (3.45)	>0.999
	Undifferentiated non-keratinizing squamous cell carcinoma (WHO type III)	27 (93.10)	18 (62.07)	

LCN2: Lipocalin-2; WHO: World health organization; NPC: Nasopharyngeal carcinoma; Fisher's exact test was used.

the two NPC groups was detected. Hence, the current study was conducted to investigate the expression of LCN2 in NPC as a potentially novel candidate biomarker. Unraveling the molecular mechanisms underlying cancer initiation and progression has been intriguing for both clinical and basic scientists. In this regard, pinpointing some key molecules involved in cancer might provide an opportunity not only to better understand the disease but also to introduce potentially novel therapeutic strategies.

Our observations suggested that LCN2 might be considered a new biomarker for NPC patients. It is worth mentioning that one of the major obstacles to NPC treatment is distant metastasis.[29] Therefore, identifying early and specific biomarkers for NPC might result in a better prognosis or the development of an efficient therapeutic strategy. Supporting this notion recently, several studies have shown that LCN2 is implicated in cancer initiation, progression, and resistance to radio- and/or chemo-therapeutics.[21, 30, 31] As far as we are aware, there are few studies dealing with the evaluation of LCN2 expression in NPC. More recently, Guo and colleagues reported that high expression levels of LCN2 mark more prolonged survival and better prognosis in NPC.[32] Besides, this

study noted no significant association between high expression of LCN2 at the protein levels and the demographic parameters, including age, sex, and NPC subtypes. Moreover, they found that exogenous overexpression of LCN2 in NPC cells suppresses their proliferation, migration, and invasion potential.[32] In contrast, Miyamoto and others reported that upregulation of LCN2 and its receptor, SLC22A17, results in poorer survival of endometrial carcinoma (EC) patients.[33] The controversial outcomes might be due to the different roles of LCN2 in cancer biology.

Some studies have indicated the critical roles of LCN2 in cancer metastasis; however, it has been shown that there is no significant association between LCN2 protein expression level and lymph node metastasis in oral squamous cell carcinoma.[2, 20, 21, 28] In another study, it was reported that LCN2 was downregulated in oral cancer tissues and was strongly associated with differentiation severity, tumor development stage, and lymph node metastasis. Therefore, LCN2 can serve as a biomarker for identifying the differentiation severity, prognosis, and disease acuteness.[20] In addition, Zhang and colleagues showed that the LCN2 expression was obviously associated with the differentiation of esophageal squamous cell carcinoma.[34] It is suggested that the LCN2 expression pattern is different even in different types of cancer. Barresi and others investigated the LCN2 expression in 30 surgically resected renal tumors by immunohistochemistry. They found that LCN2 was expressed at different levels in several histotypes of renal tumors. They reported the highest expression level of LCN2 in a couple of renal cell carcinoma (RCC) subtypes, namely the papillary RCC (PRCC) and chromophobe RCC (ChRCC), which reflects an increased need for iron uptake. This finding suggests the possible therapeutic application of iron chelators as anti-cancer agents against these tumors. In other words, iron uptake modulation by iron chelators could be exploited in anti-cancer therapies against these neoplasia.[35] Furthermore, LCN2 has been reported as a potential prognostic and/or diagnostic biomarker in various cancer types, specifically liver, prostate, and lung cancers assuming the highest prevalence, mortality, and incidence worldwide.[21, 36] For instance, Zhang and colleagues demonstrated that a high expression level of LCN2 and its receptor, neutrophil gelatinase-associated lipocalin receptor (NGALR), were both associated with an overall decreased survival rate in patients with hepatocellular carcinoma. In addition, a significant correlation was found between LCN2 and NGALR expression in advanced stages, vascular invasion, and tumor recurrence.[37] In the present study, mRNA was extracted from FFPE archived blocks to verify the expression of LCN2 in NPC patients and control subjects. It is noteworthy that the quality and the quantity of the isolated RNA from the samples were relatively low, which is one of the relevant problems with the mRNA extraction from FFPE blocks. Because of the low quality of the mRNA, some samples were discarded and not considered for further analysis. However, it has been shown that FFPE tissue samples can be used not only for retrospective and prospective biomarker discovery but also for comparing to fresh/frozen samples with the added advantage of stability at room temperature. However, prolonged storage and fixation time of FFPE specimens can question the efficiency of genomic and proteomic analyses.[38, 39] Furthermore, in most studies drawing upon samples from two to 20 years ago, the quality of RNAs, including mRNAs, was lower than those stored for one year or less.[40] Our study had some limitations, such as prolonged storage of FFPE tissue blocks and unavailability of clinicopathological information from patients.

Conclusion

Herein, the deregulation of LCN2 in NPC patients was reported. Our findings revealed the LCN2 upregulation in NPC patients in comparison to the control group at both gene and protein levels. On top of that, LCN2 might be considered a novel potential biomarker in NPC. However, further studies are required in this regard.

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Authors' Contribution

S.J.K.M: Data analysis and curation, drafting, and critical revision of the manuscript; A.M.R: Study concept and design, drafting, and critical revision of the manuscript; M.H: Acquisition and interpretation of data, drafting, and critical revision of the manuscript; Sh.N: Acquisition and interpretation of data, drafting, and critical revision of the manuscript; A.J.N: Data analysis, drafting, and critical revision of the manuscript; M.H.R: Study concept and design, drafting, and critical revision of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work, such that the 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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