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POM121 is a novel marker for predicting the prognosis of laryngeal cancer

Ruihua Zhao[1]*, Genxiong Tang[2]*, Tengqi Wang[1], Lingli Zhang[3], Wei Wang[4], Qiangfang Zhao[4] and Kun Zhao[4]

[1]Emergency Center, Bayannur Hospital, Bayannur, [2]Department of Stomatology, Children's Hospital of Nanjing Medical University, Nanjing, [3]Department of Ophthalmology, Inner Mongolia Autonomous Region People's Hospital, Hohhot and [4]Department of Oncology, Huaian Hospital, Huaian, China

*Ruihua Zhao and Genxiong Tang contributed equally to this study

Summary. The nuclear pore membrane protein 121 (POM121) is an important member of the nuclear pore complex which regulates nucleocytoplasmic transport, but little is known about the role of POM121 in laryngeal cancer. In this study, quantitative real-time polymerase chain reaction and immunohistochemistry were performed to detect POM121 expression in laryngeal tissues. The associations between POM121 and clinicopathological characteristics and overall survival in laryngocarcinoma patients were also analyzed. The mechanism of POM121 was preliminarily explored through gene set enrichment analysis (GSEA). mRNA and protein expression of POM121 in laryngocarcinoma tissues were higher than those in nontumor tissues. High POM121 expression was positively correlated with poor differentiation (χ^2=42.391, P<0.001), advanced distant metastases (χ^2=20.346, P<0.001) and TNM stage (χ^2=23.436, P<0.001). Laryngocarcinoma patients with high POM121 level tended to have poor overall survival. GSEA confirmed that the mechanism of POM121 in laryngeal cancer may relate to sphingolipid metabolism, lysosome, fatty acid metabolism, ribosome, nucleotide excision repair and the PPAR signaling pathway. Overall, POM121 expression might be a prognostic biomarker in laryngeal cancer, and POM121 has the potential to present as a therapeutic target for laryngocarcinoma patients.

Offprint requests to: Kun Zhao, Department of Oncology, Huaian Hospital, Huaian 223200, PR China. e-mail: zhaokuntll@163.com

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Introduction

Laryngeal cancer is the third most common carcinoma in otorhinolaryngology, ranking 23rd in all types of cancer worldwide (Marioni, 2012; Lin et al., 2018). Due to atmospheric pollution and lifestyle habits, there is an upward trend in morbidity of laryngeal cancer (Wang et al., 2016). Surgery, chemotherapy and radiotherapy alone or in combination are common therapies for patients with laryngeal cancer, but the 5-year survival rate shows little improvement, and has even been reported to have decreased during last 40 years, which is controversial (Siegel et al., 2016). Unfortunately, about 60% of patients are neglected until entering advanced stage (stage III or IV) at first diagnosis (Steuer et al., 2017). Compared with total laryngectomy which can result in a severe loss of quality of life, chemotherapy presents advantages in improving organ preservation and survival rate (Li et al., 2015). Considering hysteretic diagnosis and poor survival rate, it is necessary to identify novel sensitive biomarkers for early screening, prognosis and targeted therapy.

The nuclear pore membrane protein 121 (POM121) is a constitutive part of the nuclear pore complex (NPC) which regulates the communication between cytoplasm and nucleus. POM121, NDC1 transmembrane nucleoporin, and Glycoprotein 210 are the only known nucleoporins distributed in vertebrates and POM121 is the least conserved (Guo et al., 2018). POM121 plays crucial roles in NPC assembly, NPC anchoring, and nuclear

envelope formation (Antonin et al., 2005; Funakoshi et al., 2007; Bui et al., 2014). Previous researchon POM121 paid most attention to NPC organization, but recent studies have confirmed the connections between POM121 and many diseases. Full-length POM121 enables efficient HIV-1 nuclear import, and N-terminally truncated POM121 clocks human immunodeficiency virus 1 (HIV-1) infection (Saito et al., 2017; Guo et al., 2018), indicating that POM121 participates in HIV replication. POM121 has been confirmed to modulate macrophage inflammation via NF-κB P65 nuclear accumulation (Ge et al., 2019). Furthermore, aberrant expression of POM121 in acute lymphoblastic leukemia and prostate cancer suggested that POM121 is also involved in tumorigenesis and progression (Fortschegger et al., 2014; Rodriguez-Bravo et al., 2018). The role and mechanism of POM121 in laryngeal cancer remain unclear and have great research value.

In this study, we detected POM121 expression in laryngeal cancer tissues by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC), and explored its association with clinicopathological parameters and prognosis. Gene set enrichment analysis (GSEA) was performed to preliminarily speculate on the action mechanism of POM121 in laryngeal cancer.

Materials and methods

Tissue specimens and clinicopathological data

A total of 559 patients treated at Bayannur Hospital from September 2008 to March 2013 were recruited for the study. All the patients signed consent forms, and none of them underwent immunotherapy, chemotherapy or radiation therapy before biopsy or surgery. There were 657 formalin-fixed, paraffin-embedded samples collected in this research, including 398 laryngeal cancers, 43 vocal leukoplakia, 56 laryngeal papilloma, 38 chronic hypertrophic laryngitis, 24 laryngeal keratosis and 98 pericarcinomatous tissues that were set as control. Clinicopathologic information was acquired from medical records, such as age, gender, tobacco consumption, alcohol consumption, location, growth pattern, histological type, differentiation, depth of invasion, lymph node metastasis, distant metastasis, Tumor Node Metastasis (TNM) stage, and clinical data about treatment. The treatment methods included surgery, radiotherapy and chemotherapy. Clinical/radiological follow-up methods were as follows: the first follow-up time was 1 month after operation, then every 2 months, and an imaging examination after 6 months in the first year. After that, an annual imaging examination was required, with chest imaging when necessary. The follow-up time was every 3 months in the second year, every four to six months at 3-5 years and every 6 to 12 months at more than 5 years. Patients who had undergone neck radiation required a thyroid stimulating hormone test every 6-12 months. This research obtained the approval from the Human Ethics Committees of Bayannur Hospital (Approval No: BSYY2018019).

qTR-PCR

Total RNA was extracted from 17 pairs of cancerous tissues and matched normal laryngeal tissues using Trizol reagent (#R0016, Beyotime, Shanghai, China). cDNA was reversely transcribed from extracted RNA using BeyoRT™ II First Strand cDNA Synthesis Kit (#D7168L, Beyotime, China) following the manual, and amplified using BeyoFast™ SYBR Green qPCR Mix (#D7265, Beyotime, China) on a Quant Studio™ 6 Flex Real-Time PCR System. Human β-actin was regarded as the internal control to detect POM121 mRNA levels. The following primers were constructed and used in cDNA amplification: human β-actin forward, 5'-ACAGAGCCTCGCCTT TGC-3', and reverse, 5'-CCACCATCACGCCCTGG-3'; and POM121 forward, 5′-CGTTTGCCTTCAACGTGAGC-3', and reverse, 5'-AAAAGTGTTGCCGAAAG GTGC-3'. All experiments were repeated in triplicate, and comparative quantification of POM121 was calculated as $2^{-\Delta\Delta Ct}$.

IHC analysis

After comparison with hematoxylin-eosin staining images, three typical cores were selected from each tissue sample. Tissue microarrays (TMAs) were constructed by removing 1 mm diameter cores and transferring cores to a recipient block using a TMA Grand Master (3DHISTECH, Budapest, Hungary). Paraffin-embedded TMAs were cut into 4-μm thick sections and then baked at 67°C for 2 h. After deparaffinization in xylene and rehydration in alcohol gradient, sections were boiled in citrate buffer (pH=6) with a microwave oven, inducing antigen retrieval. We blocked sections with 3% hydrogen peroxide for 10 min to block endogenous peroxidase. Subsequently, tissue samples were incubated with a polyclonal rabbit anti-POM121 antibody (1:100, ab190015, Abcam, Cambridge, UK) overnight and then with a horseradish peroxidase-labeled goat anti-rabbit IgG H&L (1:2000, ab205718, Abcam, UK) at 37°C for 1 h. Finally, the sections were stained by diaminobenzidine tetrahydrochloride and counterstained by haematoxylin.

Evaluation of the staining

The IHC sections stained for POM121 were scored independently by two pathologists who were blinded to patients' information. For POM121 assessment, staining intensity was scored into four levels: 0, negative; 1, weak; 2, medium; and 3, strong. The score of cells with different staining intensity was 100 × staining intensity score × percentage of cells in the same level. The final score of POM121 in a tissue section was the sum of four intensity percentage scores with the range from 0 (no staining) to 300 (100% of cells with strong staining).

GSEA

We downloaded the RNAseq data of laryngocarcinoma patients from NCBI-Gene Expression Omnibus database (Available online: https://www.ncbi.nlm.nih.gov/geo/ query/acc.cgi?acc=GSE27020) and conducted the analysis on GSEA 3.0 software. All tissues were categorized into high POM121 group and low POM121 group according to the median POM121 mRNA level. Functional gene sets were defined based on KEGG gene set, and a total of 1000 permutations were calculated. P value <0.05 and FDR <0.25 for a gene set were recognized as significantly different.

Statistical analysis

All statistical analyses were carried out with SPSS 19.0 statistical software package (SPSS, Inc., USA). The comparison of POM121 mRNA between laryngocarcinoma tissues and matched normal tissues was conducted by the Wilcoxon Signed Rank test. The scores of POM121 in IHC were converted into dichotic data (low or no vs high) using the cutoff value 125, which was selected by the X-tile software program (The Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab) (Zhao et al., 2017). The association between POM121 expression and clinicopathological parameters was analyzed using the χ^2 test. The Kaplan-Meier method and the log-rank test were utilized to evaluate survival curves of patients. A cox proportional hazards model was constructed to analyze the univariate and multivariate hazard ratios. A two-tailed p-value <0.05 was defined as statistically significant.

Results

POM121 mRNA and protein were overexpressed in laryngocarcinoma tissues

To investigate the mRNA expression of POM121 in laryngeal cancer, 17 pairs of laryngocarcinoma tissues and matched normal laryngeal mucosa were detected through qRT-PCR. As shown in Fig. 1, POM121 mRNA level in laryngocarcinoma tissues was significantly higher than that in adjacent noncancerous tissues (p<0.001).

We also detected POM121 protein expression in 398 laryngeal cancers, 43 vocal leukoplakia, 56 laryngeal papilloma, 38 chronic hypertrophic laryngitis, 24 laryngeal keratosis and 98 pericarcinomatous tissues. POM121 protein, which was localized in nuclear envelope, was overexpressed in laryngocarcinoma tissues (Fig. 2). High POM121 expression was presented in 255 of 398 cases of laryngocarcinoma tissues (64.07%), whereas only 15.31% non-tumortissue samples (15/98) showed high POM121 expression (p<0.001, Table 1). Furthermore, high POM121 incidences in vocal leukoplakia (34.88%), laryngeal papilloma (41.07%), chronic hypertrophic laryngitis (31.58%) and laryngeal keratosis tissues (29.17%) was higher than that in pericarcinomatous tissues, but lower than that in laryngeal carcinoma (Table 1).

High POM121 expression was correlated with advanced clinicopathological features in laryngeal cancer

This study further explored the relationship between POM121 expression and clinicopathologic

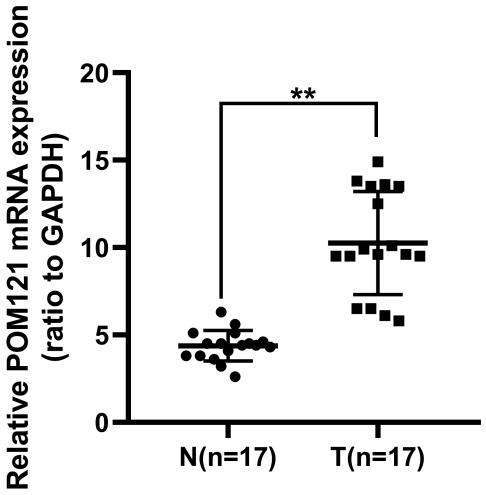

Fig. 1. POM121 mRNA expression in 17 pairs of laryngeal tissues. POM121 mRNA levels in the 17 laryngocarcinoma tissues (T) were higher than those in matched normal tissues (N) (P< 0.05).

Table 1. POM121 protein expression in laryngocarcinoma tissues.

Characteristics	n	POM121 expression (%)		χ^2	P
		Low or no	High		
Laryngeal carcinoma	398	143 (35.93)	255 (64.07)	212.766	<0.001*
Vocal leukoplakia	43	28 (65.12)	15 (34.88)		
Laryngeal papilloma	56	33 (58.93)	23 (41.07)		
Chronic hypertrophic laryngitis	38	26 (68.42)	12 (31.58)		
Laryngeal keratosis	24	17 (70.83)	7 (29.17)		
Pericarcinomatous tissue	98	83 (84.69)	15 (15.31)		

Table 2. POM121 protein expression level and laryngocarcinoma patient clinicopathological characteristics.

Characteristics	n	POM121 expression		Pearson χ^2	P-value
		Low or no	High		
Total	398	143 (35.93)	255 (64.07)		
Gender				0.004	0.951
Male	194	70 (36.08)	124 (63.92)		
Female	204	73 (35.78)	131 (64.22)		
Age				0.015	0.904
<60	196	71 (36.22)	125 (63.78)		
≥60	202	72 (35.64)	130 (64.36)		
Tobacco consumption				0.000	0.990
YES	195	70 (35.90)	125 (64.10)		
NO	203	73 (35.96)	130 (64.04)		
Alcohol consumption				0.000	0.984
YES	209	75 (35.89)	134 (64.11)		
NO	189	68 (35.98)	121 (64.02)		
Location				3.216	0.200
Supraglottic	123	52 (42.28)	71 (57.72)		
Glottic	250	82 (32.80)	168 (67.20)		
Subglottic	25	9 (36.00)	16 (64.00)		
Growth pattern				1.360	0.507
Exophytic	196	76 (38.78)	120 (61.22)		
Expansive	60	20 (33.33)	40 (66.67)		
Ulcerative	142	47 (33.10)	95 (66.90)		
Histological type				0.099	0.999
Squamous cell carcinoma	353	127 (35.98)	226 (64.02)		
Adenocarcinoma	11	4 (36.36)	7 (63.64)		
Undifferentiated carcinoma	12	4 (33.33)	8 (66.67)		
Lymphatic sarcoma	9	3 (33.33)	6 (66.67)		
Fibrosarcoma	13	5 (38.46)	8 (61.54)		
Differentiation				42.391	<0.001*
Well	193	99 (51.30)	94 (48.70)		
Moderate	160	40 (25.00)	120 (75.00)		
Poor	45	4 (8.89)	41 (91.11)		
Depth of invasion				3.335	0.503
Tis	22	10 (45.45)	12 (54.55)		
T1	15	7 (46.67)	8 (53.33)		
T2	79	32 (40.51)	47 (59.49)		
T3	152	49 (32.24)	103 (67.76)		
T4	130	45 (34.62)	85 (65.38)		
Lymph node metastasis				3.395	0.335
N0	43	18 (41.86)	25 (58.14)		
N1	37	17 (45.95)	20 (54.05)		
N2	167	60 (35.93)	107 (64.07)		
N3	151	48 (31.79)	103 (68.21)		
Distant metastasis				20.346	<0.001*
M0	316	131 (41.46)	185 (58.54)		
M1	82	12 (14.63)	70 (85.37)		
TNM stage				23.436	<0.001*
0	28	13 (46.43)	15 (53.57)		
I	46	23 (50.00)	23 (50.00)		
II	72	35 (48.61)	37 (51.39)		
III	62	22 (35.48)	40 (64.52)		
IV A + IV B	124	40 (32.26)	84 (67.74)		
IV C	66	10 (15.15)	56 (84.85)		
Treatment methods				26.321	<0.001*
Surgery	193	102 (52.8%)	91 (47.2%)		
Surgery+radiotherapy	147	32 (21.8%)	115 (78.2%)		
Radiotherapy+ chemotherapy	58	9 (15.5%)	49 (84.5%)		

characteristics in 398 laryngocarcinoma tissues (Table 2). Laryngocarcinoma patients with high POM121 expression were significantly correlated with poor differentiation (χ^2=42.391, P<0.001), advanced distant metastases (χ^2=20.346, P<0.001) TNM stage (χ^2=23.436, P<0.001), and treatment approaches (χ^2=26.321, P<0.001). However, there was no connection between POM121 and age, gender, tobacco consumption, alcohol consumption, location, growth pattern, histological type, depth of invasion, or lymph node metastasis.

High POM121 expression was correlated with poor prognosis in laryngeal cancer

To determine the prognostic value of POM121 level, we analyzed the association between POM121 expression and overall survival of patients with laryngeal carcinoma. Kaplan-Meier survival curves confirmed that patients with high POM121 expression had poorer survival than those with low POM121 expression (P<0.001, Fig. 3), indicating that the level of POM121 protein was significantly related to OS of laryngocarcinoma patients, as well as distant metastases, TNM stage and differentiation. Univariate analysis proved the prognostic value of POM121 expression, regardless of differentiation, distant metastasis and TNM stage. Multivariate analysis showed that high POM121 expression (HR, 1.531; 95% CI, 1.259-2.064; p<0.001), differentiation (HR, 1.106; 95% CI, 1.149-1.397; p<0.001), distant metastasis (HR,1.129; 95% CI, 1.149-1.451; p<0.001), TNM stage (HR, 1.263; 95% CI, 1.088-1.652; p<0.001) were independent poor prognostic factors for laryngocarcinoma patients (Table 3).

Table 3. Univariate and multivariate analyses of the prognostic factors for overall survival in laryngocarcinoma.

		Univariate analysis			Multivariate analysis		
		HR	P-value	95%CI	HR	P-value	95%CI
POM121 expression	High vs Low or No	1.962	<0.001*	1.483 / 2.687	1.531	<0.001*	1.259 / 2.064
Age(year)	<60 vs ≥60	0.897	0.379	0.704 / 1.143			
Gender	Male vs Female	0.861	0.24	0.670 / 1.106			
Depth of invasion	Tis vs T1 + T2 vs T3 + T4	0.804	0.059	0.642 / 1.008			
Lymph node metastasis	N0 vs N1 + N2 + N3	1.425	0.095	0.941 / 2.158			
Distant metastasis	M0 vs M1	1.134	<0.001*	1.053 / 1.365	1.129	<0.001*	1.149 / 1.451
TNM stage	0 vs I + II + III+ IV A + IV B vs IV C	1.276	<0.001*	0.978 / 1.455	1.263	<0.001*	1.088 / 1.652
Differentiation	Well vs Moderate vs Poor	1.157	<0.001*	1.055 / 1.326	1.106	<0.001*	1.149 / 1.397

P<0.05; HR: Hazard ratio; CI: Confidence interval.

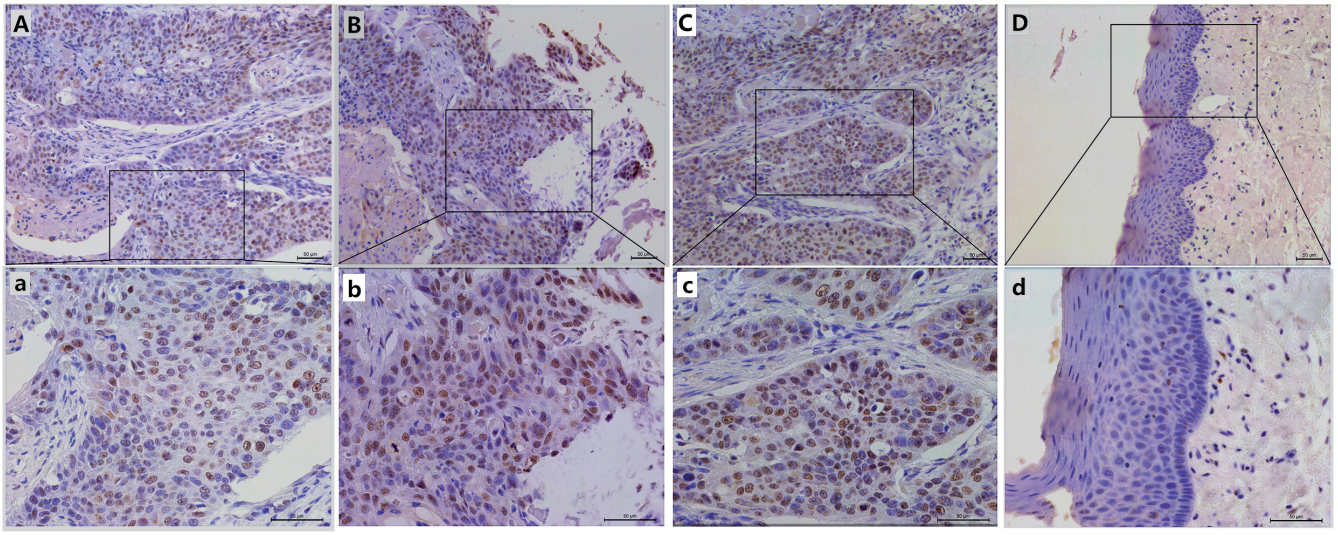

Fig. 2. Representative images of POM121 protein in laryngeal tissues. **A.** Well differentiated squamous carcinoma with high POM121 expression. **B.** Moderately differentiated squamous carcinoma with high POM121 expression. **C.** Poorly differentiated squamous carcinoma with high POM121 expression. **D.** Pericarcinomatous laryngeal tissues with no POM121 expression. Scale bars: A-D, 500 μm; a-d 50 μm.

POM121 expression was correlated with specific KEGG pathways.

Differential gene expression in tissues with high and low POM121 expression was explored through GSEA. Compared to low POM121 group, high POM121 group expressed differential genes which were enriched in sphingolipid metabolism, lysosome, fatty acid metabolism, ribosome, nucleotide excision repair and peroxisome proliferator activated receptor (PPAR)

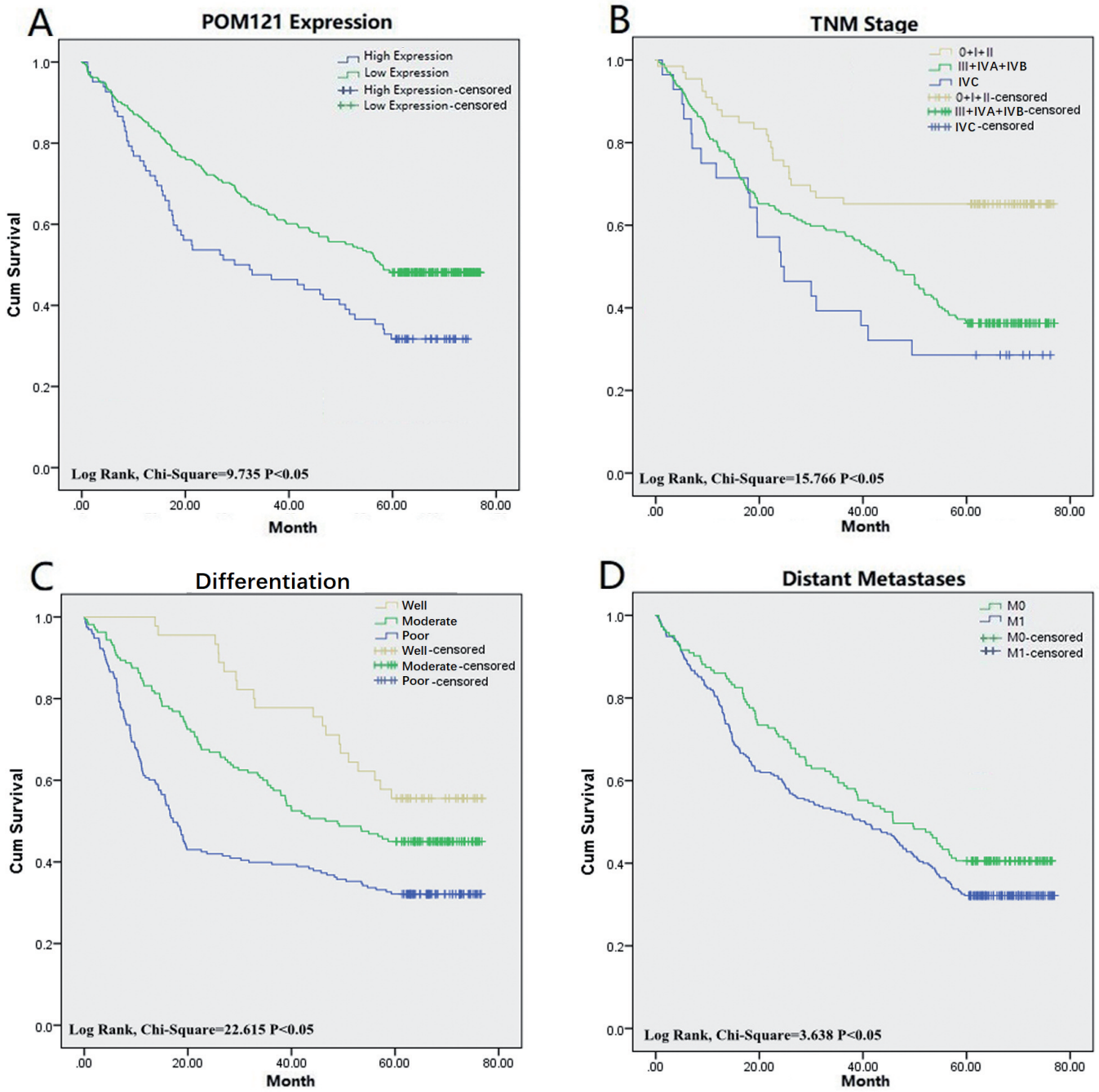

Fig. 3. Survival curves of laryngocarcinoma patients using the Kaplan-Meier method and the log-rank test. **A.** Overall survival curves for patients with high POM121 expression (blue line) and patients with low POM121 expression (green line). **B.** Overall survival curves by TNM stage, TNM 0, I and II (yellow line), TNM III, IV A and IV B (green line), IVC (blue line). **C.** Overall survival curves for patients with high differentiation (yellow line), patients with moderate differentiation (green line) and patients with poor differentiation (blue line). **D.** Overall survival curves by distant metastases, M0 (green line), M1 (blue line).

signaling pathway (Fig. 4, Table 4).

Discussion

NPC is the only turnstile embedded in eukaryotic nuclear membrane. NPC regulates the material communication between cytoplasm and nucleus, endowing it with the ability to maintain many cellular functions (Lim and Wong, 2018). According to the existing literature, NPC participates in cell cycle control (Kumar et al., 2018), gene expression regulation (Raices and D'Angelo, 2017), DNA repair (Géli and Lisby, 2016) and chromatin modulation (Manuel and Hiroyuki, 2015). With in-depth study, some nucleoporins are found to be associated with cancer formation and progression, such as Tpr, Nup98, Nup62, Nup88 and Nup214 (Kin-Hoe et al., 2012; Singer et al., 2012; Dan and Rout, 2014; Xu et al., 2016).

Table 4. The main enriched KEGG pathways in laryngeal tissues with high POM121 expression.

Name	ES	NES	P-value	FDR q-value
KEGG_SPHINGOLIPID_METABOLISM	0.689	1.670	0.004	0.219
KEGG_LYSOSOME	0.454	1.597	0.008	0.161
KEGG_FATTY_ACID_METABOLISM	0.559	1.556	0.028	0.161
KEGG_RIBOSOME	0.418	1.528	0.026	0.148
KEGG_NUCLEOTIDE_EXCISION_REPAIR	0.576	1.496	0.029	0.187
KEGG_PPAR_SIGNALING_PATHWAY	0.504	1.442	0.030	0.197

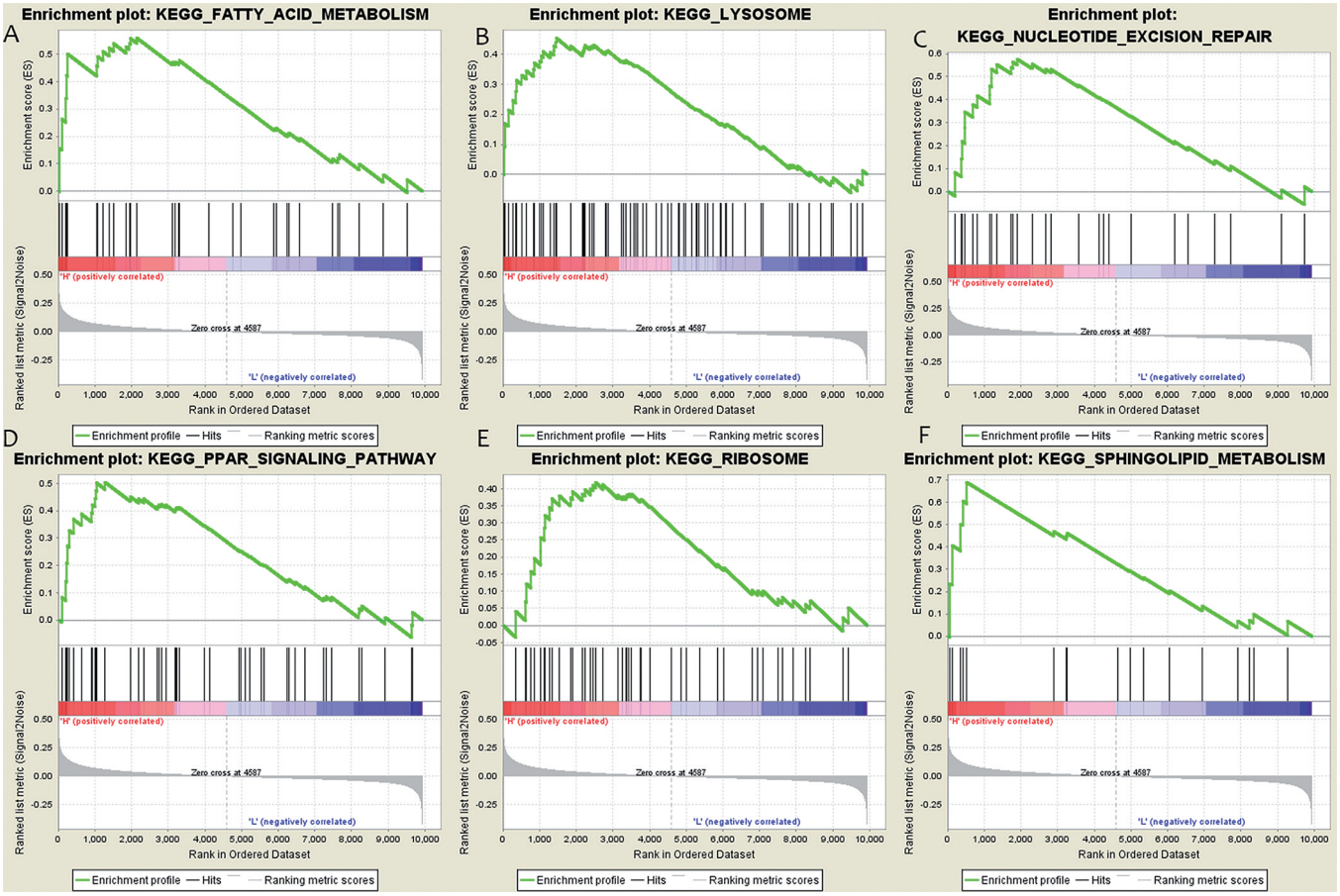

Fig. 4. The main enriched KEGG pathways. The association of POM121 levels and laryngocarcinoma related gene sets were presented by GSEA. Gene sets "sphingolipid metabolism", "lysosome", "fatty acid metabolism", "ribosome", "nucleotide excision repair" and "PPAR signaling pathway" were enriched in POM121 high expression phenotype.

As an important part of NPC, POM121 has been reported to overexpress and work as an independent prognostic factor in oral squamous cell carcinoma and colorectal cancer (Ma et al., 2019; Wang et al., 2020). In this study, we confirmed that mRNA and protein levels of POM121 expression in laryngocarcinoma tissues were both higher than those in noncancerous tissues through qRT-PCR and IHC. Furthermore, we analyzed POM121 expression and clinical parameters in laryngocarcinoma patients to identify the role of POM121 in the progression of laryngeal cancer. The results verified that POM121 expression had a positive correlation with differentiation, distant metastases, TNM stage and treatment methods. High expression of POM121 in laryngocarcinoma may promote tumor growth and metastasis, and regulate cancer cell differentiation. Kaplan-Meier survival curves showed that POM121 expression in laryngocarcinoma was negatively connected with patient's OS, and the patients expressing higher POM121 level had poorer survival. According to multivariate analyses, POM121 expression was an independent prognostic factor for patients with laryngeal cancer.

Differential expression in malignant and normal laryngeal tissue provides POM121 with the potential to be a candidate for targeted drugs. POM121 may also work as an additional marker for laryngocarcinoma progression due to positive association between POM121 expression and clinicopathologic features. Furthermore, the relationship between POM121 and OS confirmed the prognostic value of POM121 that cannot be ignored.

To preliminarily explore the working mechanism of POM121 in laryngeal cancer, we identified the genetic differences between laryngocarcinoma tissues with high and low POM121 expression. The result showed that differential genes were significantly enriched in sphingolipid metabolism, lysosome, fatty acid metabolism, ribosome, nucleotide excision repair and PPAR signaling pathway. Sphingolipids have conflicting influence on cancer cell apoptosis and survival (Ogretmen, 2018). Lysosomes take part in cell death, metabolism deregulation, immune surveillance evasion and drug resistance, which are important for tumor development (Piao and Amaravadi, 2015). Fatty acids regulate the synthesis of membranes and signaling molecules that are crucial for cellular proliferation, and low fatty acid availability can inhibit cancer cell proliferation (Currie et al., 2013). Ribosome biogenesis affects cell cycle progression and an up-regulated ribosome expression can accelerate neoplastic transformation (Derenzini et al., 2017). Nucleotide excision repair prevents mutagenesis in the genome to avoid cancer predisposition (Marteijn et al., 2014). High PPAR expression is associated with the reduction of cell proliferation, invasion and migration, and downregulation of PPARγ has been confirmed in many cancers (Alexandre and Yves, 2018). These pathways undoubtedly regulate biological functions of cancer cells, and GSEA demonstrated that POM121 has a significant impact on these pathways. Therefore, POM121 may promote carcinogenesis, progression and metastasis of laryngeal cancer through the above pathways .

This study is the first to confirm POM121 levels in laryngeal cancer and reveal the potential value of POM121 in diagnosis and prognosis of laryngocarcinoma. Synthetical analysis of POM121 expression and patients' clinical information suggested that POM121 may promote cell growth, differentiation and invasion. However, the regulating role of POM121 on the malignant biological behavior of laryngocarcinoma needs further tests and verification in vitro and vivo. What is more, further experiments are necessary to confirm the tentative study on mechanism of POM121 function by GSEA.

In summary, POM121 overexpression is associated with progression and poor prognosis in laryngeal cancer. POM121 appears to be a potential indicator for prognosis of laryngocarcinoma. Further investigations into the connections between POM121 and the pathogenesis and development of laryngeal carcinoma are encouraged.

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Conflicts of interest. The authors have declared that no competing financial interests exist.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication. Consent for publication was obtained from all authors.

Ethical statement. This study was performed in accordance with medical ethical standards and was approved by the Ethics Committee of Bayannur Hospital. Written informed consents were obtained from all study participants.

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