

Original Article

Abnormal hedgehog signaling activation promotes lymphangiogenesis and lymph node metastasis in nasopharyngeal carcinoma

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Received November 19, 2015; Accepted September 6, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Objective: To investigate the role of abnormal Hedgehog (Hh) signaling activation in lymphangiogenesis and lymph node metastasis in nasopharyngeal carcinoma (NPC). Methods: Gli-1 protein expression and lymphatic vessel density (LVD) were detected in the nasopharyngeal epithelial tissues of 69 cases with NPC and 15 cases of nasopharyngeal normal epithelial tissues using immunohistochemistry. The relationship between Gli-1 protein expression, clinicopathologic features and LVD was discussed. Results: Gli-1 protein was upregulated in NPC tissues compared with that in normal nasopharyngeal epithelium ($P < 0.01$); Gli-1 protein expression was correlated with LVD ($r = 0.464$, $P < 0.01$) and lymph node metastasis ($P < 0.05$). Conclusion: Abnormal Hh signaling activation promotes lymphangiogenesis and lymph node metastasis in NPC.

Keywords: Hedgehog signaling pathway, Gli-1, nasopharyngeal carcinoma, lymphangiogenesis, lymph node metastasis

Introduction

A large number of patients with nasopharyngeal carcinoma (NPC) are already found to have neck lymph node metastasis upon the first diagnosis. Lymphangiogenesis is closely related to tumor invading the lymphatic vessels and lymph node metastasis in NPC [1, 2]. It is now widely recognized that Hedgehog (Hh) signaling pathway plays an important role in embryonic development, organ formation and regulation of maintenance of stem cells as well as in progenitor cell transformation and malignancy occurrence. Gli-1 is one of the three downstream nuclear transcription factors in the Hh signaling pathway (Gli-1, Gli-2 and Gli-3). It only fulfils the role of activating nuclear transcription and serves as the important target gene of Hh signaling pathway. Therefore, the expression level of Gli-1 directly reflects whether the Hh signaling pathway is activated. Excessive Gli-1 expression is related to lymph node metastasis of several malignancies [3-6]. The latest researches on the blocking of Hh signaling pathway, especially those targeting the Gli gene, have made impressive progress [7].

Though the abnormal expression of Hh signaling components has been noted [8], the role of abnormal Hh signaling activation in NPC is rarely known. The study on the role played by Hh signaling pathway in the metastasis of NPC can help find new therapeutic targets and prognostic evaluation indicators. In this experiment, we detected the Gli-1 protein expression level in NPC tissues, aiming to determine the relationship between abnormal Hh signaling activation and clinicopathologic features as well as lymphangiogenesis in NPC.

Materials and methods

Clinical data

NPC tissues from 69 patients were the paraffin-embedded specimens preserved at Department of Pathology, Nanxishan Hospital in Guangxi Zhuang Autonomous Region. The tissues were obtained by biopsies from patients hospitalized from April 2013 to April 2014 using nasopharyngofiberscope. All cases were pathologically confirmed as undifferentiated, nonkeratinizing, and squamous cell carcinoma based on speci-

Hedgehog pathway and nasopharyngeal carcinoma

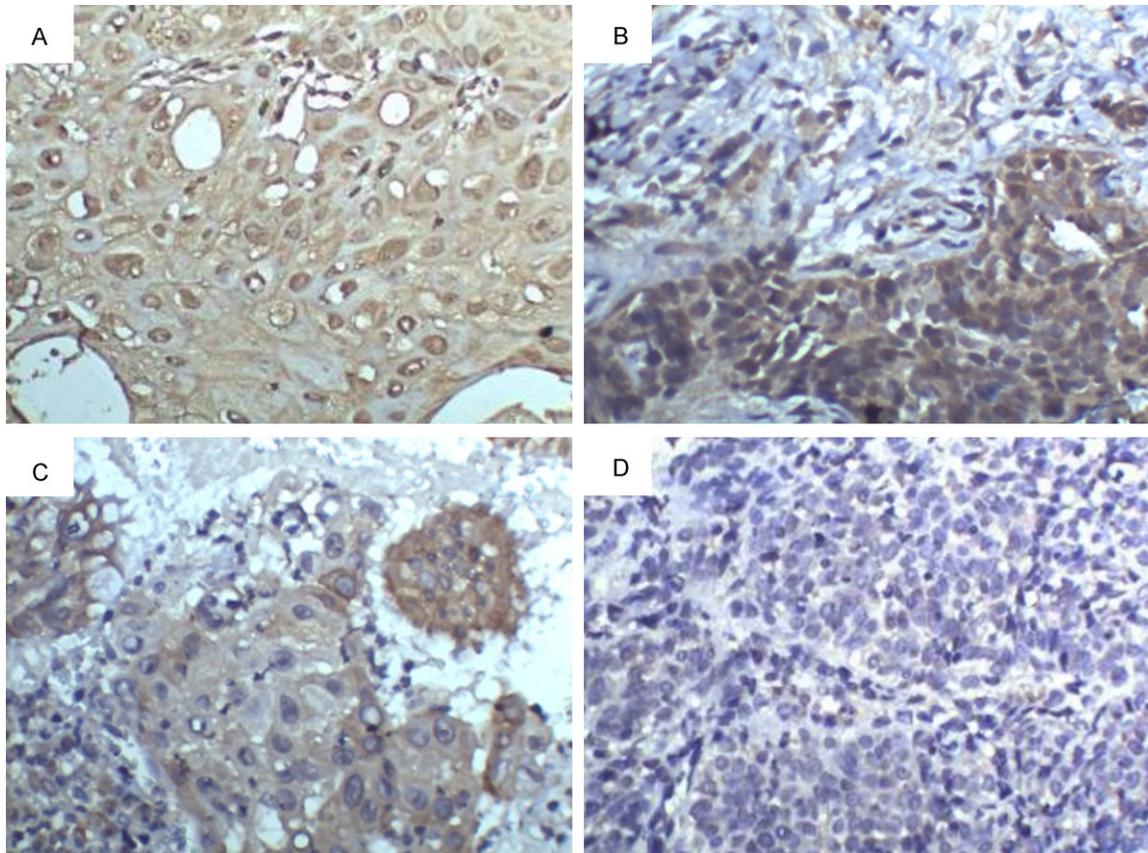


Figure 1. Immunohistochemical staining of Gli-1 protein in nasopharyngeal epithelial cells of NPC tissues. A: Strong staining of Gli-1 protein in nuclei and weak staining in cytoplasm of nasopharyngeal epithelial cells of NPC tissues (400 \times); B: Strong staining of Gli-1 protein in nuclei and cytoplasm of nasopharyngeal epithelial cells of NPC tissues (400 \times); C: Weak staining of Gli-1 protein in nuclei and strong staining in cytoplasm of nasopharyngeal epithelial cells of NPC tissues (400 \times); D: Negative control (NPC tissue, 400 \times).

mens fixed in 10% formaldehyde. All of them had complete clinical and pathological data and had never received any treatment previously. The cases included 56 males and 13 females with a median age of 52 years (32-74 years). The TNM (tumor, node, metastasis TNM) stage was determined according to the Chinese 2008 staging system for NPC: there were 8 cases of stage II, 22 cases of stage III, 33 cases of stage IVa and 6 cases of stage IVb; regarding to tumor statues of carcinoma, there were 4 cases of T1, 21 cases of T2, 23 cases of T3 and 21 cases of T4; regarding to lymph node status, there were 11 cases of N0, 21 cases of N1, 21 cases of N2, and 16 cases of N3; concerning distant metastasis, there were 63 cases of M0 and 6 cases of M1. Meanwhile, 15 cases of archived paraffin embedded normal nasopharyngeal epithelial tissue specimens during the same period were set as controls. The protocol was approved by Ethics Committee of Nanxishan Hospital of Guangxi

Zhuang Autonomous Region, and written informed consent was obtained from all patients.

Reagents and methods

Rabbit anti-Gli-1 monoclonal antibody was purchased from Abcam (Lot: ab134906, USA). D2-40 mouse monoclonal antibody, HRP anti-mouse & rabbit universal secondary antibody (ready to use) and DAB color development kit were purchased from Fuzhou Maixin Biotech. Co., Ltd. The paraffin-embedded specimen was cut into serial slices in 5 μ m thickness, roasted at 73 $^{\circ}$ C for 15 min and at 62 $^{\circ}$ C for 2 h. After dewaxing and hydration, the slice was boiled in citrate antigen retrieval solution (Ph 6.0) for 20 min and incubated in 3% H₂O₂ for 10 min. Then the slice was washed with PBS three times for 5 min each time. The slide was wiped clean and added with rabbit anti-Gli-1 monoclonal antibody (working concentration 1:400) or D2-40

Hedgehog pathway and nasopharyngeal carcinoma

Table 1. Comparison of Gli-1 protein expression in nasopharyngeal epithelial cells of NPC tissue and normal tissue

Group	Case	Gli-1		
		Low expression	High expression	P
NPC	69	35 (50.7%)	34 (49.3%)	0.003
Normal nasopharyngeal epithelium	15	14 (93.3%)	1 (6.7%)	2.73±2.02

Note: Low expression, IRS=0-6; high expression, IRS=7-12.

Table 2. Correlation between Gli-1 protein expression and clinico-pathologic features of NPC

	Case	Gli-1		P
		Low expression (n=35)	High expression (n=34)	
Age (years)				0.398
<52	34	19 (55.9%)	15 (44.1%)	
≥52	35	16 (45.7%)	19 (54.3%)	
Gender				0.326
Male	56	30 (53.6%)	26 (46.4%)	
Female	13	5 (38.5%)	8 (61.5%)	
Clinical staging				0.259
II	8	6 (75.0%)	2 (25.0%)	
III+IV	61	29 (47.5%)	32 (52.5%)	
Tumor infiltration depth				0.096
T1+T2	25	16 (64.0%)	9 (36.0%)	
T3+T4	44	19 (43.2%)	25 (56.8%)	
Lymph node metastasis				0.024
N0	11	9 (81.8%)	2 (18.2%)	
N1-3	58	26 (44.8%)	32 (55.2%)	
Distant metastasis				0.198
M0	63	30 (47.6%)	33 (52.4%)	
M1	6	5 (83.3%)	1 (16.7%)	

Note: Low expression, IRS=0-6; high expression, IRS=7-12.

mouse monoclonal antibody. The cells were incubated at 37°C in an incubator for 2 h and washed with PBS three times for 5 min each time. The slide was wiped clean, added with secondary antibody and placed at 37°C in an incubator for 30 min. The cells were washed with PBS three times for 5 min each time. The slide was wiped clean, added with DAB solution and counterstained with hematoxylin. After dehydration through alcohol gradient and drying, the slide was sealed with neutral balsam. For the negative control, PBS was added instead of primary antibody.

Result interpretation

Assessment of Gli-1 expression: Positive Gli-1 protein expression was defined as appearance

of light yellow to dark brown particles in the cytoplasm or nuclei. The slides were reviewed by two pathologists independently, and in case of disagreement, a consensus was reached through discussion. Five high-power fields of view were randomly selected and 200 cells were counted in each field. The percentage of positive cells was scored as follows: score 0 for <5%, 1 for 5%-25%, 2 for 26%-50%, 3 for 51-75%, 4 for >75%. The degree of staining was scored as follows: score 0 for negative, 1 for light yellow, 2 for brown and 3 for dark brown. Using immunoreactive score (IRS), the Gli-1 protein expression was scored for each specimen and the score of percentage of positive cells was multiplied by the score of staining degree; low expression was defined as final

score ≤6 (IRS: 0-6), and high expression as ≥7 (IRS: 7-12).

Calculation of lymphatic vessel density (LVD)

Immunohistochemistry using D2-40 was performed and positive expression was defined as appearance of yellow or brown particles in the cytoplasm or the envelope. Lymphatic vessels were single layers of flattened epithelium without the aggregation of red blood cells. LVD was calculated using the method reported in literature [1].

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. The mean scores of the groups were compared using t-test. χ^2 test of Fisher's

Hedgehog pathway and nasopharyngeal carcinoma

Table 3. Comparison of LVD in NPC tissue and normal nasopharyngeal epithelium

Group	Case	LVD		
		Median (scope)	$\bar{x} \pm s$	P
NPC tissue	69	8.33 (1.00~25.67)	9.38±5.64	0.014
Normal nasopharyngeal epithelium	15	2.33 (1.00~14.57)	5.33±5.26	

exact test was used for inter-group comparison, and the correlation between the measurements was tested by Spearman's rank correlation coefficient. $P < 0.05$ indicated statistical significance.

Results

Expression of Gli-1 protein in NPC tissue and normal nasopharyngeal epithelium

The cytoplasm of normal nasopharyngeal epithelial cells and the cytoplasm and nuclei of epithelial cells of NPC tissue were stained differently due to different Gli-1 protein expression; Gli-1 protein expression was the highest in the nuclei of epithelial cells of NPC tissues (**Figure 1**). By immunohistochemistry, the final score was 6.26 ± 3.91 for 69 cases with NPC (median 8, 1-12). Among them, 35 cases (50.7%) had low expression (IRS: 0-6) and 34 cases (40.3%) had high expression (IRS: 7-12). The final score was 2.73 ± 2.02 for 15 normal cases (median 2, 1-8). Among the 15 normal cases, 14 cases (93.3%) had low expression and 1 case (6.7%) had high expression. The Gli-1 protein expression was significantly higher in NPC tissue than in normal nasopharyngeal epithelium ($P < 0.01$) (**Table 1**).

Correlation between Gli-1 protein expression and clinicopathologic features of NPC patients

The 69 cases with NPC were stratified by low expression (IRS: 0-6) and high expression (IRS: 7-12) of Gli-1 protein, and the correlation between Gli-1 protein expression and clinicopathologic features was analyzed. It was found that Gli-1 protein expression was significantly different between cases with and without lymph node metastasis ($P < 0.05$). However, it was not significantly correlated with distant metastasis, clinical staging, age and gender (**Table 2**).

Correlation between Gli-1 protein expression and lymphangiogenesis in NPC

LVD was obviously higher in NPC tissues than in normal nasopharyngeal epithelium ($P < 0.05$),

which indicated excess lymphangiogenesis in NPC (**Table 3**). According to Spearman's rank correlation coefficient, the immunohistochemistry score of Gli-1 protein expression in NPC tissues was significantly positively correlated with LVD ($r = 0.464$, $P < 0.01$). LVDs for NPC tissues with high and low Gli-1 protein expression were 12.20 ± 5.37 and 6.65 ± 4.47 , respectively ($P < 0.01$), indicating significant correlation between Gli-1 protein expression and lymphangiogenesis.

Discussion

Once Hh signaling pathway is activated, the cytoplasmic Gli-1 protein will enter the nucleus to bind to specific sequences of the target genes belonging to cyclins family (B, D, E, P^{21}), TGF- β family, β -catenin family, MMPs family and FGF family. This directly results in the activation of target gene expression, etc, thereby inhibiting cell apoptosis, promoting cell proliferation, migration, angiogenesis and lymphangiogenesis and finally leading to tumor occurrence and progression. There are plenty of clinical and pathological evidences demonstrating the involvement of Hh signal transduction in the occurrence of malignancies [9, 10]. However, the role of Hh signaling pathway in the occurrence and metastasis of NPC is rarely known. Experiments show that the tumor cells can massively produce factors that promote lymphangiogenesis (eg., VEGF-C/D and LYVE-1), which in turn accelerates lymph node metastasis [11]. The upregulation of members of Hh signaling pathway (eg., Shh, Gli-1) can promote tumor cell migration, invasion and metastasis through lymphatic vessels, thereby affecting the prognosis of patients with epithelial malignancies [4, 12, 13]. We also observed the correlation between upregulation of Gli-1 and lymphangiogenesis as well as lymph node metastasis in NPC.

According to our results, Gli-1 was upregulated in NPC, and the patients with lymph node metastasis had a much higher Gli-1 expression than those without lymph node metastasis.

This implied the role of abnormal Hh signaling activation in NPC progression. We found that the cytoplasm of normal nasopharyngeal epithelial cells was weakly stained for Gli-1 protein, except 1 normal case scoring 8 in IRS. This may be explained by the role of Gli-1 in the maintenance of stem cells in nasopharyngeal epithelium, repair and regeneration of local tissues, consistent with Gli-1 expression in the oral cavity and gastric mucosa [4-6]. According to report, EB viruses codes EBNA1, LMP1 and LMP2A, which can induce the expression of Shh ligand in nasopharyngeal epithelial cells [14]. The latter will further cause the overexpression of Gli-1 by activating Hh signal transduction through autocrine secretion. Some scholars observed that the expression of Hh signaling pathway components correlates to the histological type of lung cancer and Gli-1 is mainly expressed in lung squamous cell carcinoma [15]. The reason and molecular basis for such difference are unclear, and they are supposed to be related to embryogenesis and malignant behavior of tumors. Since all our cases were undifferentiated, nonkeratinizing, and squamous cell carcinoma, we did not compare Gli-1 expression by histological type and differentiation degree. Such comparison can be performed using large sample size.

This study indicated there is significant correlation between Gli-1 expression and LVD ($r=0.464$, $P<0.01$). LVD was significantly higher in patients with high expression than that in patients with low expression. The mechanism of Hh signaling pathway promoting lymphangiogenesis is unclear. Asai *et al.* [16] reported that Hh signaling activation promoted wound healing and induced angiogenesis, typically endothelial progenitor cell proliferation, migration and adhesion and tubular formation. Bailey *et al.* [17] observed the reduction of LYVE-1-positive cells and inhibition of lymph node metastasis after the use of Shh neutralizing antibodies. Hh signaling pathway can induce tumor migration and lymphangiogenesis through PI3K/Akt signaling pathway and the use of pathway blockers would inhibit epithelial-mesenchymal transition (EMT) and matrix metalloproteinases (MMP) activation and lymphangiogenesis [4]. Moreover, Hh signaling regulates the expression of LYVE-1 and VEGF-D in non-small cell lung cancer [15]. We observed previously that LVD was related to lymphatic

vessel invasion in NPC [1]. The above findings suggest that lymphangiogenesis is an important step in early lymphatic vessel invasion and local lymph node metastasis. The effect of Hh signaling pathway on tumor invasion and metastasis is mediated by other processes, such as EMT and MMP activation. Hh signaling activation regulates EMT in epithelium-derived tumors by inhibiting E-cadherin expression [6]. In addition, Gli-1 upregulate the expression of transcription factor SIP1, which in turn promotes the expression of 2 EMT-related transcription factors, TWIST2 and SNAIL2. This is the mainly mechanism of Hh-EMT signaling promoting tumor invasion and metastasis [18]. We speculated that it is also the mechanism responsible for the regulation of lymph node metastasis mediated Hh signaling pathway in NPC.

Tumor cell proliferation and lymphangiogenesis induced by overexpression of factors related to Hh signaling pathway are important reasons for poor prognosis. The mechanism of Hh signaling activation is highly complex, and the interference of autocrine and (or) paracrine secretion related to this pathway may produce varying effect. By understanding the expression of factors related to Hh signaling pathway and the effect of Hh pathway inhibitors on NPC cells and stromal cells, we can find the therapeutic targets for inhibiting lymph node metastasis in tumors.

Acknowledgements

This work was supported by funds from the appropriate technology research and development project of Guangxi medicine and health technology (Grant No. S201408-01).

Disclosure of conflict of interest

None.

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Hedgehog pathway and nasopharyngeal carcinoma

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