Abstract. The present study investigated the expression of non-metastasis 23 (nm 23) in laryngeal cancer tissues and analyzed its correlation with the prognoses of laryngeal cancer patients. A total of 122 laryngeal cancer patients who were admitted to The Affiliated Hospital of Jining Medical College from June 2009 to June 2012, and 30 normal laryngeal mucosal tissues were selected as the control group. Immunohistochemical staining method was used to test the expression of nm 23 in tissues. Quantitative-polymerase chain reaction (q-PCR) and western blotting were conducted to test the expression of nm 23 in tissues at the gene and protein levels, respectively. Moreover, the prognoses of patients were analyzed. The positive expression rate (90.00%) of nm 23 in the normal laryngeal mucosal tissues was markedly higher than that in laryngeal cancer tissues (56.56%) (p<0.05). The expression of nm 23 proteins was correlated with the clinical staging of laryngeal cancer and the metastasis of lymph nodes (p<0.05). The expression of both nm 23 genes and proteins in the laryngeal cancer tissues were significantly lower than those in the normal laryngeal mucosal tissues (p<0.05). The survival rate of the positive nm 23 expression was substantially higher than that of the negative expression with a statistically significant difference (p<0.01). In conclusion, the expression of nm 23 proteins plays an important role in the development and metastasis of laryngeal cancer and may be taken as one of the indicators to evaluate the prognoses of such patients.

Introduction

Pharyngeal cancer, the most prevalent malignant tumor in head and neck, tends to attack laryngeal mucous membranes (1). Currently, the pathogenesis of pharyngeal cancer is yet to be identified. In the 1990s, scholars initially segregated and identified non-metastasis 23 (nm 23) and later it was verified that the expression of nm 23 is closely correlated with the metastasis suppression in patients (2). Studies have shown that the expression product of nm 23 can directly affect the signal transduction system in cells and furthermore, have effects on the microfilaments and microtubules involved in biological activities of cells. In addition, it inhibits metastasis of tumor cells (3,4). To date, although many scholars have reported the mechanism of nm 23 expression to inhibit metastasis of tumor cells, the study on the relation between nm 23 and prognoses of pharyngeal cancer patients remain controversial, and no agreement exists in the academic circle. In the present study, pharyngeal cancer patients diagnosed and treated from 2009 to 2012 were retrospectively analyzed. Immunohistochemical staining, quantitative-polymerase chain reaction (q-PCR) and western blotting were used to analyze the expression of nm 23 in different tissues. Besides, such patients were followed up for 5 years.

Patients and methods

Patients. A total of 122 pharyngeal cancer patients who were admitted to The Affiliated Hospital of Jining Medical College (Jining, China) from June 2009 to June 2012 were selected, and none received chemoradiotherapy preoperatively. There were 69 males and 53 females aged 39-78 years. Tumor node metastasis (TNM) staging indicated: 2 stage I cases, 21 stage II cases, 51 stage III cases and 48 stage IV cases; 23 stage T1-2 cases, 66 stage T3 cases, and 33 stage T4 cases. Histopathological grading showed: 16 stage I, 69 stage II, and 37 stage III cases. Moreover, there were 54 patients with lymphatic metastasis and 68 patients without lymphatic metastasis. Thirty normal pharyngeal mucosal tissues were taken as the control group. The patients or their family members signed the informed consent. The study was approved by the Ethics Committee of The Affiliated Hospital of Jining Medical College.

Main reagents. Rabbit anti-human nm 23 (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), goat anti-rabbit secondary antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal reference antibody.
Mix, 10 µl the foregoing reaction solution and 4 µl RNase-free
1 µl PrimeScript RT Enzyme Mix, 1 µl PrimeScript RT Enzyme
ribonuclease (RNase) free distilled water (dH₂O) eraser buffer, 1 µl gDNA eraser, 1 µg total RNA and
cDNA was synthesized using reverse transcription kit.

RNA extraction kit. The complementary deoxyribose nucleic
Institute with the sequences shown in Table I. The total RNA in

1-4 points, (+); 5-8 points, (++) and 9-12 points, (+++).

percentages of positive cells - (1,10), (10,25), (25,50), (50,75)

light yellow, brownish-yellow and tan, respectively. Scoring
positively expressed was calculated as the number of positive

field to be observed was randomly selected to count 100 cells,

were added with DAB developer for color development,

foregoing sections were washed by PBS. Then, the sections

antibody solution (diluted by 1:50) was added for incubation

primary antibody solution (diluted by 1:50), containing

was used to wash the resulting product and then, the secondary

was added with DAB developer for color development,

washed by distilled water, counterstained and sealed for

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GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was adopted for the survival analysis. \( \alpha = 0.05 \) was taken as the statistical verification standard.

**Results**

**Immunohistochemical staining.** The immunohistochemical staining results showed that the positive expression rate of nm 23 in pharyngeal cancer tissues was 56.56% (69/122), while that in the normal laryngeal mucosal tissues was 90.00% (27/30). Differences between the two groups were statistically significant (\( p < 0.05 \)) (Fig. 1 and Table II).

**Relationship between nm 23 expression and clinical features of pharyngeal cancer.** The comparison of the nm 23 expression in pharyngeal cancer tissues with different pathological grades showed no obvious difference, without statistical significance (\( p > 0.05 \)). In the the clinical staging, the expression rate (86.96%) in stage I-II cases was significantly higher than that (48.48%) in stage III-IV cases, with a statistically significant difference (\( p < 0.05 \)). Similarly, that (75.00%) in cases without lymphatic metastasis was also obviously higher than that (37.78%) in cases with lymphatic metastasis, and the difference presented the statistical significance (\( p < 0.05 \)) (Table III).

**q-PCR results.** The relative expression level of nm 23 mRNA in tissues was tested via q-PCR, and the results showed that the expression level of nm 23 mRNA in the normal pharyngeal mucosal tissues was significantly higher than that in normal pharyngeal mucosal tissues.

### Table II. Comparison of nm 23 expression in pharyngeal cancer tissues and normal pharyngeal mucosal tissues.

| Groups                              | No. of cases | - | +  | ++ | +++ | Positive rate (%) | P-value |
|-------------------------------------|--------------|---|----|----|-----|-------------------|--------|
| Laryngeal cancer tissues            | 122          | 53 | 36 | 27 | 6   | 56.56             | 0.026  |
| Normal pharyngeal mucosal tissues   | 30           | 3  | 7  | 5  | 15  | 90.00             |        |

### Table III. Relations between the nm 23 expression and the clinical features of pharyngeal cancer.

| Groups                      | No. of cases | Positive nm 23 expression (n (%)) | t-value | P-value |
|-----------------------------|--------------|-----------------------------------|---------|---------|
| Sex                         |              |                                   |         |         |
| Male                        | 69           | 45 (65.22)                        | 2.841   | 0.177   |
| Female                      | 53           | 23 (43.40)                        |         |         |
| Age (year)                  |              |                                   |         |         |
| ≤60                         | 50           | 30 (60.00)                        | 3.115   | 0.082   |
| >60                         | 72           | 38 (52.78)                        |         |         |
| Clinical staging            |              |                                   |         |         |
| I-II                        | 23           | 20 (86.96)                        | 8.452   | 0.037   |
| III-IV                      | 99           | 48 (48.48)                        |         |         |
| Lymphatic metastasis        |              |                                   |         |         |
| Yes                         | 45           | 17 (37.78)                        | 10.793  | 0.021   |
| No                          | 68           | 51 (75.00)                        |         |         |
| Pathological grading        |              |                                   |         |         |
| I                           | 16           | 11 (68.75)                        | 2.361   | 0.202   |
| II                          | 69           | 39 (56.52)                        |         |         |
| III                         | 37           | 18 (48.65)                        |         |         |

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was adopted for the survival analysis. \( \alpha = 0.05 \) was taken as the statistical verification standard.
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The pharyngeal cancer tissues, with a statistically significant difference ($p<0.05$) (Fig. 2).

**Western blotting.** Western blotting was conducted to detect the expression of nm 23 proteins. According to the result, the nm 23 proteins were significantly highly expressed in the normal pharyngeal mucosal tissues, and the difference from that in the pharyngeal cancer tissues was statistically significant ($p<0.05$) (Figs. 3 and 4).

**Analysis of prognosis.** The survival rate of patients with nm 23 protein positively expressed was higher than that of patients with the nm 23 protein negatively expressed. Especially in terms of 3-year survival rate, the survival rate of patients with the negative nm 23 protein expression was decreased to 12.96%, while that of patients with the positive expression reached up to 82.35% and began slowly decreasing. The difference between the two groups of patients presented a markedly statistical significance ($p<0.01$) (Fig. 5).

**Discussion**

Since nm 23 was discovered in the 1990s, scholars all over the world have made a large number of systematic studies on the effects of nm 23 on various cancers (5,6). Pharyngeal cancer patients often die of metastasis, and the metastasis of tumor is a very complex process, starting from the primary tumor cells infiltrating into the surrounding cytoplasmic matrix (7,8).

Currently, it is known that non-metastasis genes can participate in several steps of tumor cell metastasis process through various pathways and serve as one of natural defenders against the tumor metastasis in the body (9,10). Pathological studies have shown that the lymphatic metastasis is one of the earliest incidents during metastasis and diffusion of most solid tumors in organic bodies and also one way of tumor metastasis (11,12).

As a metabolic suppressor gene, nm 23 has been verified through many *in vitro* and *in vivo* experiments. Marioni et al. (13) found that nm 23 can significantly suppress the cancerization of melanoma cells, such as inhibiting cell activity, tumor cell growth and distal infiltration and metastasis. Fang et al. (14) reported according to the clinical examinations that the expression of nm 23 in the patients is significantly negatively correlated with the invasion and metastasis of tumor cells and has a marked effect on the prognoses of patients. According to the findings of He et al. (15), the nm 23 mRNA expression level is significantly decreased in highly metastatic breast cancer tissues, but Han et al. (16) studied the expression of nm 23 in thyroid tumors with the same technical method, and the results showed that although nm 23 in coding region is not mutated, there is no obvious negative correlation between the expression level of nm 23 and metastasis in thyroid tumor tissues. Therefore, it was inferred that the effects of nm 23 in different cancer tissues may vary to some degree. However, the effect of the nm 23 expression on pharyngeal cancer tissues remains controversial. It was found by Tong et al. (17) that the lowly expressed nm 23 is closely correlated with the metastasis of pharyngeal cancer, while Fu et al. (18) reported that the expression of nm 23 protein is not significantly correlated with the growth position and pathological grading of laryngeal cancer, but negatively correlated with lymphatic metastasis and clinical staging. On the contrary, some scholars held that the expression of nm 23
is positively correlated with lymphatic metastasis, but has no relation with clinical staging (19). The retrospective analysis results of this study showed that the positive expression rate (90.00%) of nm 23 in the normal pharyngeal mucosal tissues was significantly higher than that (56.56%) in the pharyngeal cancer tissues, with a statistically significant difference (p<0.05). The positive expression percentage (86.96%) of nm 23 in stage I-II cases was markedly higher than that (48.48%) in stage III-IV cases, with a statistically significant difference (p<0.05). Similarly, that (75.00%) in cases without lymphatic metastasis was also obviously higher than that (37.78%) in cases with lymphatic metastasis, and there was a statistically significant difference (p<0.05). This indicates that the expression of nm 23 proteins is significantly correlated with clinical staging of pharyngeal cancer and lymphatic metastasis. At the same time, it was indicated that at the gene and protein levels, the expression of nm 23 in the pharyngeal cancer tissues was significantly lower than that in the normal pharyngeal mucosal tissues (p<0.05), which is consistent with the result of the study by Yang et al (20). According to the postoperative 5-year prognosis follow-up results of pharyngeal cancer patients, the survival rate of patients with nm 23 positive expression was significantly higher than that of patients with negative expression, and the difference showed a statistical significance (p<0.01). Especially in terms of three-year survival rate, the survival rate of patients with nm 23 proteins negatively expressed was decreased to 12.96%, while that of patients with nm 23 proteins positively expressed reached up to 82.35%, indicating that the higher the positive expression of nm 23 is, the better the prognoses of pharyngeal cancer patients are.

In conclusion, the expression of nm 23 protein plays an important role in the occurrence, development and metastasis of pharyngeal cancer and may be regarded as one of the indicators to evaluate the prognoses of such patients.

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

Authors' contributions
WW and XW contributed to immunohistochemical staining and PCR. XL helped with western blotting. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of The Affiliated Hospital of Jining Medical College (Jining, China) The patients or their family members signed the informed consent.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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