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Original Article

Ki67 and nm23 are potential prognostic markers in patients with nasopharyngeal carcinoma

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Abstract: Objective: To investigate the expression and clinical associations of Ki67 and nm23 in chronic nasopharyngitis and nasopharyngeal carcinoma (NPC). Methods: Expressions of Ki67 and nm23 were examined by immunohistochemical assay (SP method) in tissues among 23 patients with nasopharyngitis and 59 patients with NPC. Associations of Ki67 and nm23 expression with clinical parameters such as clinical stage, cervical lymph node metastasis status, and distant metastasis status and 5-year survival rate were statistically analyzed. Results: 1) Comparing to chronic nasopharyngitis, the incidence rate of positive Ki67 in NPC was significantly higher, whereas nm23 was lower ($P < 0.01$). 2) The positive incident rate of Ki67 in NPC was positively correlated with clinical stage and distant metastasis status. Patients with positive expression of Ki67 had lower 5-year survival rate. 3) The incidence rate of positive nm23 in NPC was negatively correlated with clinical stage, cervical lymph node metastasis status, distant metastasis status, but not with age and T-stage. Patients with positive expression of nm23 had higher 5-year survival rate. 4) The positive incidence rate of Ki67 and nm23 were negatively correlated. We found patients with negative Ki67 and positive nm23 had longer survival time. Conclusions: The expressions of Ki67 and nm23 were associated with disease progression, invasiveness and metastatic status of NPC. Together, the data suggest Ki67 and nm23 could provide helpful information in understanding clinical characteristics of NPC tumors, and are potential prognostic markers for NPC patients.

Keywords: Nasopharyngeal carcinoma, Ki67, nm23, cervical lymph node metastasis, distant metastasis, survival curve

Introduction

Nasopharyngeal carcinoma (NPC) is highly prevalent in southern China especially in Guangdong province, of which the incident rate can be more than 10/10,000 person per year. The definite cause of most cases of NPC is far from clear, but a spectrum of etiologies including genetic susceptibility, environmental factors, and viral infection has been proposed [1]. Early NPC is not easily detected because the nasopharynx is anatomically located deep inside the head. Most patients at the first presentation are diagnosed advanced NPC, often with lymph node metastasis already developed, making the treatment challenging. In recent years, because of the improvement of radiation therapy, the 3-year survival rate of patients with NPC can reach > 90% while the 5-year one can be 78%. Despite the efficiency of therapy, local-regional recurrence and distinct metastasis represent the leading causes of mortality among patients with NPC [2].

Ki67 protein is a cellular marker for proliferation, while nm23 is a metastasis inhibitor. Although aberrant expression of the two is believed to play roles in tumor invasion and metastasis, it is unclear whether the expression would be associated with clinicopathological parameters of NPC like clinical stage, cervical lymph node metastasis, and distant metastasis. The correlation of the two proteins in clinical patients also remains to be deciphered. As such, we employed immunohistochemistry to examine the expression of Ki67 and nm23 in NPC tissues, and evaluated their expressions in relation to clinical characteris-

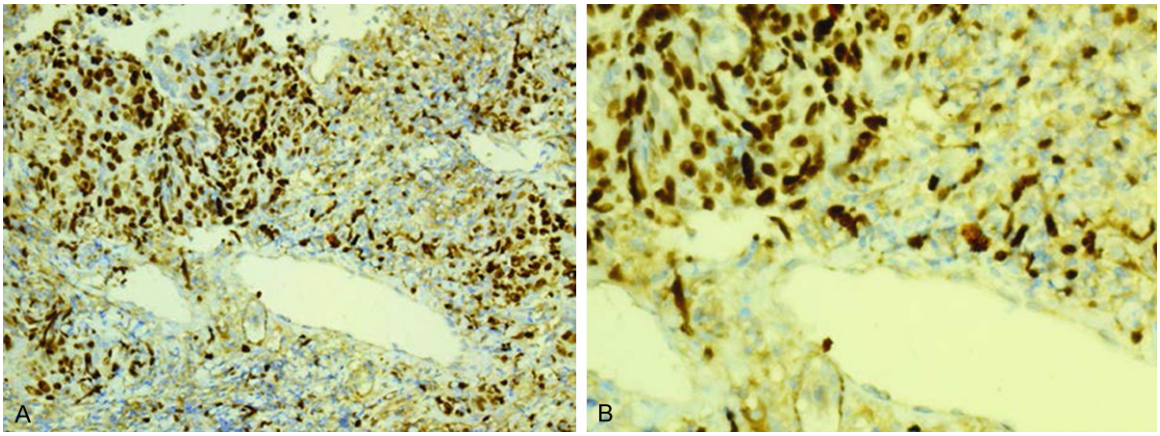

Figure 1. Immunohistochemistry showing the positive Ki67 expression in NPC tissue. A. Magnification ×200. B. Magnification ×400.

tics of NPC. We also analyzed the survival data of the patients in order to determine if Ki67 and nm23 can be used as prognostic markers.

Methods and materials

Study population

A total of 59 NPC cases diagnosed between January 2000 and January 2008 were recruited; among them 43 were males and 16 were females, with ages ranged from 15 to 80 years old (median at 48.4 years). All patients had no history of radiation therapy, chemotherapy or immunotherapy before enrollment. Primary tumors from these patients were formalin-fixed, paraffin-embedded, and sectioned for histology examination. All cases were confirmed as non-keratinizing NPC.

All the patients underwent endoscopic examination, nasopharyngeal CT scan, neck and abdominal ultrasound, single-photon emission computed tomography (SPECT). Three of the patients additionally had a whole body PET-CT imaging. TNM staging was defined according to the Chinese 1992 Fuzhou Staging System, and there were 24 Stage I/II and 35 Stage III/IV cases. Forty-four cases were found positive with cervical lymph node metastasis, in which 22 were N1, 17 were N2, and 5 were N3. Patients at Stage I/II received radiotherapy at the doses of NPDT 6,800-7,200 CGy. Patients at Stage III/IV received the radiotherapy and combination chemotherapy of cisplatin (DDP) and fluorouracil (5-FU). All the patients have complete follow-up information. The follow-up time ranged from 5 to 84 months, with a median follow-up time of 68 months. Specimens in the control group were obtained from 23 patients diagnosed with chronic inflammatory nasopharyngeal mucosa. Controls include 15 males and 8 females at ages ranged from 23 to 68 years old (median at 41.5).

Immunohistochemistry

Histological diagnosis was confirmed by H&E staining on the paraffin embedded sections, with Ki67 and nm23 expression studied using two-step immunohistochemistry assay. Monoclonal mouse anti-human Ki67, monoclonal nm23 antibodies, immunohistochemical SP kit and DAB reagent (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were used. The staining processes were performed in accordance to the manufacturer's instruction. For negative control, sections were probed with PBS instead of the primary antibody. Sections of known NPC-positive tissues were used as positive controls.

Scoring method

The IHC results were evaluated and scored by two senior pathologists in a double-blinded manner. In each section, 5 fields were selected randomly under high power microscope, with each field 200 tumor cells counted. Ki67 and nm23 positive signals were observed in various staining intensity ranging from light yellow to brown granules. Ki67 signal was mainly observed in nuclei (**Figure 1**), and nm23 signal was observed in cytoplasm (**Figure 2**). The per-

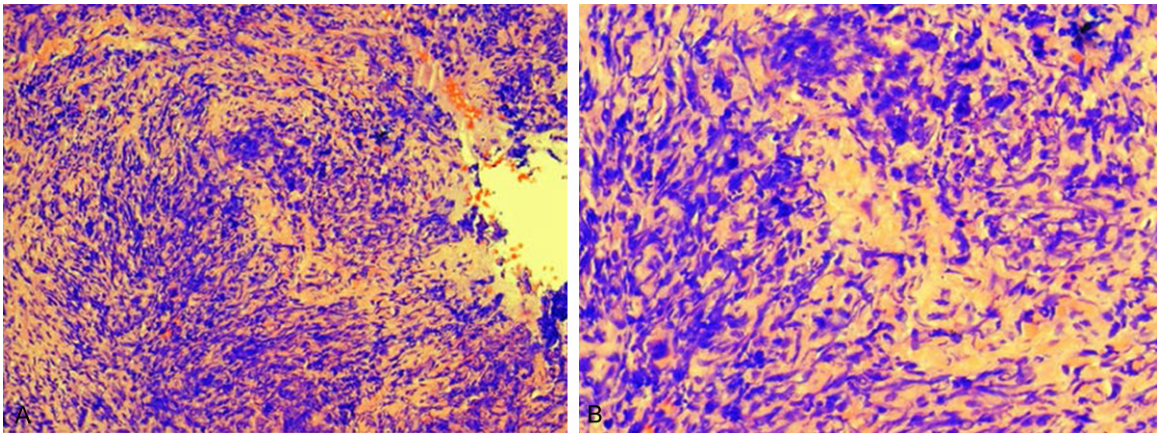

Figure 2. Immunohistochemistry showing the positive nm23 expression in NPC tissue. A. Magnification ×200. B. Magnification ×400.

centage of positive cells was calculated by: number of cells with positive signal/total number of cells counted x 100%. According to the percentages, the section was graded as "-" (< 5%), "±" (5%-25%), "+" (25%-50%), "++" (50%-70%), or "+++" (> 75%). Sections with ≤ 25% positive cells were defined as negative expression, whereas sections with > 25% positive cells were defined as positive expression.

Statistical analysis

Statistical analysis was performed using SPSS 11.5. Clinicopathological parameters were analyzed as categorical data using Chi-square test. Correlation between Ki67 and nm23 expressions in cancerous tissues was determined using Pearson correlation test. Survivals of NPC patients with differential expressions of Ki67 and nm23 were analyzed using Kaplan-Meier analysis.

Results

Expression of Ki67 and nm23 in NPC

Ki67 was positively expressed in 4.35% (1/23) of tissues with chronic inflammation of nasopharyngeal mucosa and in 71.19% (42/59) of tissues with NPC. The results showed a significant higher positive rate of Ki67 in NPC (χ^2 = 27.0232, P < 0.01). nm23 was positively expressed in 91.3% (21/23) of tissues with chronic inflammation of nasopharyngeal mucosa and expressed in 49.15% (29/59) of tissues with NPC, indicating a significantly lower positive rate of nm23 in NPC (χ^2 = 8.3255, P < 0.01).

Association of Ki67 and nm23 expression and clinical parameters

Higher positive rate of Ki67 was found in patients of advanced stages (stage III/IV vs. stage I/II, P < 0.05). The expression, however, had no significant association with gender, age, cervical lymph node metastasis, and T-stage of tumor. Expression of nm23 was negatively correlated with clinical stage: lower rate of nm23expression was found in stage III/IV than in stage I/II (P < 0.05). The expression was also negatively correlated with lymph node metastasis stage: lower rate of nm23 expression was found in N2/N3 than in N0/N1 (P < 0.01). Expression of nm23 had no significant association with sex, age, and T-stage of tumor (**Table 1**).

Prognosis of Ki67 and nm23 expressions

Higher rate of positive Ki67 was found in tissues obtained from patients with distant metastasis, whereas lower rate of positive nm23 was found in these patients (P < 0.05). Patients with positive Ki67 had lower 5-year survival rate. In contrast, patients with positive nm23 had higher 5-year survival rate (P < 0.05) (**Table 1**).

Correlation of Ki67 and nm23 expressions

We found the expression of Ki67 was negatively correlated with expression of nm23 in NPC tissues (P < 0.05, Pearson correlation coefficient, r = 0.2632) (**Table 2**).

Table 1. Correlation of tumor Ki67 and nm23 expressions with clinicopathological parameters of patients with NPC

Clinicopathological Parameters	Case	Positive Ki67		χ^2	Positive nm23		χ^2
		Case	Rate (%)		Case	Rate (%)	
Gender							
Male	43	29	67.44	1.0840	20	46.51	0.4425
Female	16	13	81.25		9	56.25	
Age (years)							
< 48.4	32	24	75.00	0.4958	15	46.88	0.1451
≥ 48.4	27	18	66.67		14	51.58	
Clinical stage							
I + II	24	13	54.17	5.7136[a]	16	66.67	4.9654[a]
III + IV	35	29	82.86		13	37.14	
T stage							
T1 + T2	33	23	69.70	0.0810	18	54.55	0.8714
T3 + T4	26	19	73.08		11	42.31	
N stage							
N0 + N1	37	25	67.57	0.6336	24	64.86	9.8016[b]
N2 + N3	22	17	77.27		5	22.73	
Distinct metastasis							
Present	28	24	85.71	5.4835[a]	9	32.14	6.1692[a]
Absent	31	18	58.06		20	64.52	
Survival (years)							
< 5	26	22	84.62	4.0870[a]	8	30.77	6.2856[a]
≥ 5	33	20	60.61		21	63.64	

Remark: [a]$P < 0.05$; [b]$P < 0.01$.

Table 2. Correlation of Ki67 and nm23 expressions in NPC tissues

Protein expression		nm23		χ^2 value	r value
		Negative	Positive		
Ki67	Negative	5	12	4.3905	0.2632
	Positive	25	17		

Ki67 and nm23 expression in relation to survival time of patients

A Kaplan-Meier analysis showed that survival time of patients with positive Ki67 (58.00± 10.18 months) was shorter than patients with negative Ki67 (71.00±6.86 months) ($P < 0.05$, **Figure 3A**). On the other hand, survival time of patients with positive nm23 expression (83.00 months) was longer than the patients with negative nm23 (52.00±10.25 months) ($P < 0.05$, **Figure 3B**). Since the expressions of Ki67 and nm23 were negatively correlated, we performed a multivariate regression analysis to examine the association between the expression of the two proteins and survival time. We found patients with negative Ki67 and positive nm23 had a median survival time of 70.00±5.51 months, while patients with positive Ki67 and negative nm23 had a median survival time of 43.00.00±7.62 months, the difference was statistically significant ($P < 0.05$, **Figure 3C**).

Discussion

Tumor markers could be used for identifying tumor types, and some may also indicate proliferative activity of tumors. Ki67 gene, located on the long arm of chromosome 10 (10q25), has two mRNA isoforms. It is highly expressed during mitosis and has been widely used as a cell-proliferation marker. Its cellular function, however, remains elusive. It has been suggested that Ki67 functions primarily in mitosis, and may also be involved in DNA synthesis [3]. Ki67 protein composed of an amino acid sequence that is rich in proline, glutamic acid, serine, and threonine. They together form 40 weak PGST areas and 10 strong PGST areas. These PGST regions are highly sensitive to protease and are easily degraded, which might explain the short half-life (1 hr or less) of Ki67 [4]. Along the cell cycle process, Ki67 expression is minimal in late G1 and early S phase. It is accumulated and predominantly expressed in M phase, followed by rapid degradation and is absent in G0 phase [5]. These characteristics make Ki67 an excellent marker for cell proliferation.

Studies have shown that high expression of Ki67 was associated with malignant properties of cancers, and was prognostic to tumor metastasis [6, 7]. Gabusi et al. found 35% of NPC tissues had positive Ki-67 [8]. In this study, we

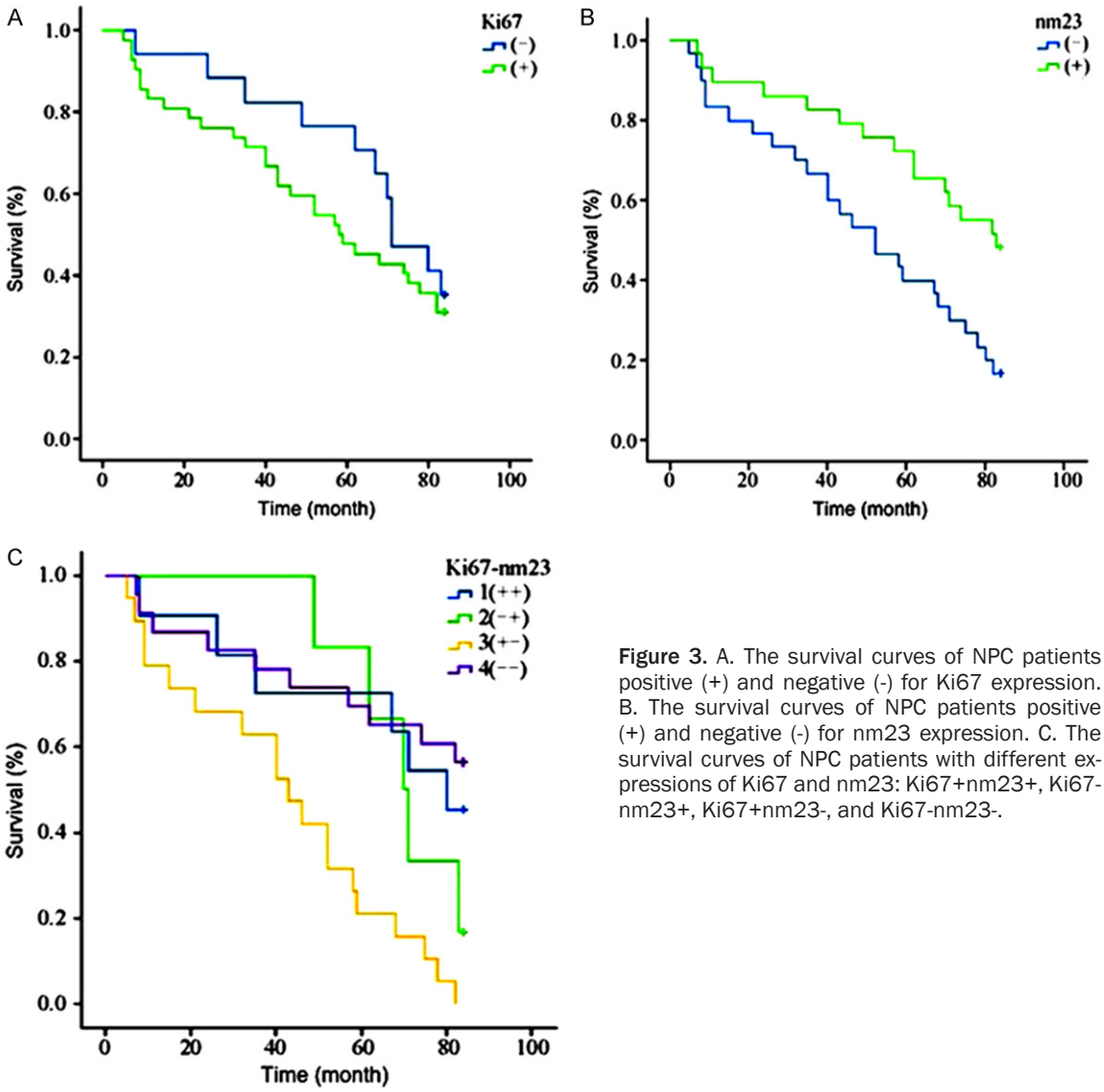

Figure 3. A. The survival curves of NPC patients positive (+) and negative (-) for Ki67 expression. B. The survival curves of NPC patients positive (+) and negative (-) for nm23 expression. C. The survival curves of NPC patients with different expressions of Ki67 and nm23: Ki67+nm23+, Ki67-nm23+, Ki67+nm23-, and Ki67-nm23-.

found 71.19% of NPC tissues had positive Ki-67. The higher positive incidence rate observed here may attribute to the differences in clinical characteristics (e.g. clinical stage, cervical lymph node metastasis status) of patients enrolled in the two studies. Nevertheless, higher incident rate of positive Ki-67 was observed in NPC when compared to chronic nasopharyngitis, suggesting the expression of Ki-67 reflect malignant transformation and proliferation activity in NPC. The marker may be used for distinguishing malignant from benign tumors.

There was no significant association between Ki67 expression and cervical lymph node metastasis or T-stages of tumor, indicating cell proliferation is an inherent characteristic of NPC and is not related to the size of the primary tumor and lymph node metastasis. Further analysis revealed that the rate of positive Ki-67 was associated with clinical staging. This is in accordance with the findings from Zheng et al. [9]; they showed higher rate of positive Ki67 in Stage III/IV than in Stage I/II of patients with NPC. Some researches, however, believe that Ki-67 expression is not clinically relevant in squamous cell carcinoma of the head and neck [10]. In the present study, higher rate of positive Ki67 was found in patients with distant metastasis and in patients with lower 5-year survival rate. The positive expression of Ki-67

may indicate high invasiveness of the tumor that lead to the higher chance of having distant metastasis and shorter survival time.

The nm23 gene has been proposed as a metastasis suppressor gene [11]. There are two homologous genes, nm23-H1 and nm23-H2, which encode the A and B subunits of nucleoside diphosphate kinase (NDPK). It has been proposed that NDPK play a role in microtubule assembly and G-protein signaling, which are essential in maintaining normal cell division and suppressing metastasis [12]. Liu et al. showed that expression of nm23 was negatively correlated with distant metastasis and lymph node metastasis in breast cancer [13]. Krause et al. also demonstrated that nm23 expression was positively correlated to tumor differentiation and negatively correlated to staging and lymph node metastasis in bladder cancer [14]. Our data showed that 49.15% of the NPC tissues had positive expression of nm23, which is much lower that the expression in chronic inflammation of nasopharyngeal mucosa. The data suggested that nm23 had different expression levels between benign and malignant lesions. Lesions with low expression of nm23 may have higher chance of malignant transformation. We found lower rate of positive nm23 expression in advanced clinical stage (III/IV) and advanced lymph node metastasis stage. The lower rate of positive nm23 was also observed in patients with no distant metastasis, suggesting the suppressive role of nm23 in NPC metastasis.

The expressions of Ki-67 and nm23 were negatively correlated. The Kaplan-Meier analysis showed that patients with negative expression of Ki-67 and positive expression of nm23 had longer survival time. It is unclear whether the two proteins are involved in the same of related molecular mechanisms. It is possible that the characteristics of malignant tumors are highly proliferative and prone to spread to other body parts; therefore, the expressions of the two proteins appear associated. Further investigation will be needed to understand this phenomenon.

In conclusion, we revealed that expressions of Ki67 and nm23 were associated with the clinical characteristics of NPC. Patients with high Ki67 and low nm23 expressions are likely to have metastatic malignant tumors and shorter survival time. With further investigations, they may be used as prognostic markers and also markers for predicting clinical characteristics of the NPC tumors.

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Disclosure of conflict of interest

None.

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