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Original Article

Clinicopathological significance and prognostic value of NKX2.2 expression in papillary thyroid carcinoma

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Abstract: Objectives: This study aims to investigate NKX2.2 protein expressions in papillary thyroid carcinoma (PTC) patients, and discuss its correlation with PTC clinicopathological features and prognosis. Methods: A sum of 187 PTC patients treated in our hospital between October 2011 and September 2012 were randomly selected as the case group. Another 124 volunteers receiving health examination in our hospital were taken as the control group. A few thyroid cell groups were obtained by vivo fine needle aspiration and fresh surgical PTC specimens were collected. NKX2.2 protein expressions in specimens were detected by immumohistochemical staining and Western Blot. Follow up was conducted for 3 years to study the relationship between NKX2.2 and postoperative recurrence rates. Results: Immunohistochemical staining showed that NKX2.2 protein showed different levels of expression in different staged PTC samples. Compared with the control group, positive ratios of NKX2.2 in the case group were significantly lower (all $P < 0.05$). As PTC worsened, positive ratios of NKX2.2 decreased. Western Blot showed that as PTC worsened, NKX2.2 protein expression declined. Maximum diameter, lymph node metastasis, distant metastasis and neoplasm invasiveness were negatively related to positive ratios of NKX2.2 and NKX2.2/GAPDH protein expression. Follow up analysis showed that as NKX2.2 protein expressions and positive ratios of NKX2.2 increased, postoperative recurrence rates declined. Conclusion: NKX2.2 protein is associated with PTC clinicopathological features and prognosis thus can be an indicator for PTC diagnosis and prognosis evaluation.

Keywords: NKX2.2 protein, papillary thyroid carcinoma, immunohistochemical staining, clinicopathological features, correlation, prognosis, recurrence rate

Introduction

Thyroid cancers are common endocrine cancers in human [1]. According to statistics, 4.9 to 14.3 in every 100,000 people suffer from thyroid cancers in the past 30 years, among which 3.4 to 12.5 people were diagnosed with papillary thyroid carcinoma (PTC) [2]. Median size of PTC is 7 mm, and this disease often occurs on the basis of thyroid diseases, thereby tending to cause missed diagnosis in clinical treatment [3]. The formation of tumors is a biological behavior of multiple factors, which is likely to be related to various oncogenes and anti-oncogenes [1]. At present, although studies on PTC are increasingly thorough both at home and abroad, detailed and accurate explanation about its formation mechanism still remains insufficient [4]. Therefore, in the early diagnosis of PTC, it is vital to probe into the expression of various genes in thyroid cancer and to find related molecular markers.

Thyroxine, mainly produced by thyroid, is one of the hormones involved in the growth and development process of mammals, with significant regulating effect. *Homeobox* gene is a sort of polypeptide found in fruit flies which consists of 180-183 base codes and 60-61 amino acid bases and can regulate the expression of other genes [5]. NKX family, a sort of *homeobox* gene, is modules widely expressed in various regions of brain tissues, and it is associated with the development of different brain regions [6]. Current studies indicates that the functions of thyroid are related to the levels of NKX2.1 and NKX6.1 [7, 8] in brain tissues, yet research on NKX2.2 mainly focuses on fields such as pancreatic functions and its impact on oligodendrocyte and visceral neural differentiation [9, 10].

In this regards, we hypothesized that PTC was accompanied with weakened thyroid functions, and there might be corresponding changes in NKX2.2 expression. This study collected specimens of clinical PTC patients to perform statistical analysis on the correlation of NKX2.2 expression with clinicopathological features and prognosis of PTC patients.

Materials and methods

Ethical statement

This study was carried out in conformity to medical ethical standards, and was approved by the Ethics Committee of our hospital. Informed consents were obtained from all participants or their family members.

Subjects

A total of 187 patients performed PTC excision in our hospital between October 2011 and September 2012 were randomly selected as the case group. Staging criteria in the sixth edition of American Joint Committee on Cancerajcc (AJCC) was set as a standard [11]. Inclusion criteria: 1. Confirmed as PTC by clinical manifestation and histopathological examination of fine needle aspiration thyroid specimens; 2. Without merging with other tumors; 3. Without receiving any chemotherapeutics or radiotherapy before research. Exclusion criteria: 1. Patients with severe heart, hepatic and renal dysfunction; 2. Merged with immunological and endocrine system diseases; 3. Receiving chemical therapy before operation. Another 124 volunteers receiving health examination in this hospital were taken as the control group. A small amount of thyroid cell groups were obtained by fine needle aspiration in vivo to perform immumohistochemical staining.

Immunohistochemical staining

EnVision two-step method was adopted in immumohistochemical staining. Fresh surgical specimens of PTC were collected, fixed with 4% of formaldehyde and embedded in paraffin, followed by conventional dewaxing. The specimens were treated with 3% of hydrogen peroxide for 1 h to inhibit the activity of endogenous peroxidase, and then washed by poly butylenes succinate (PBS) solution (1.9 mM monopotassium phosphate, 8.1 mM dipotassium phosphate and 75 mM sodium chloride, pH 7.4) for 3 times, each time for 2 min. Primary mouse anti-human NKX2.2 (NK2 homeobox 2) monoclonal antibodies (1:100, purchased from ZSGB Biotechnology Co., Ltd, Beijing) were added drop by drop. The specimens were incubated for 1 h at 37°C, and then taken out to be washed by PBS solution for 3 times, 2 min in each time. Universal secondary antibodies EnVision (ZSGB Biotechnology Co., Ltd, Beijing) were added drop by drop to the specimens, which were then incubated for 30 min at room temperature and washed by PBS solution for 3 times, 2 min in each time. DAB color developing agent (ZSGB Biotechnology Co., Ltd, Beijing) was applied for coloration. The reaction, observed under a microscope, was terminated when claybank sediments appeared. After the coloration was terminated, the cell nucleus was counter stained with hematoxylin. Following washing and bluing, the specimens were dehydrated by conventional graded ethanol. Dimethylbenzene was transparent, and neutral balsam was used for sealing. Staining grade was calculated in accordance with positive cells rate. Three regions in each specimen were randomly selected within the field of view of 10X. Ratios of positive cells in total cells were calculated, and their average value was taken as the staining grade of the specimens.

Protein expression detection by Western blot

A total of 0.1 g fresh surgical PTC specimens were collected. After the specimens were cut up, 0.5 mL PBS solution were added. Cell suspension underwent disruption by an ultrasonic processor (Ningbo Scientz Biotechnology Co., Ltd) for 5 min in total, with 1 s of pause after 1 s of work. Cell debris was removed through centrifuging at 12000 rpm/min for 10 min. Bradford method was applied to detect total protein concentrations in supernate, and the total protein concentrations of various samples were adjusted to be equal. Loading quantities of samples were the same. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% of separating gel concentration was applied to separate proteins, which were then transferred to polyvinylidene fluoride (PVDF) membranes by semi-dry trans-blot (Bio-Rad, USA). The PVDF membranes were at first soaked with methyl alcohol for 15 s, and then soaked in transfer buffer (25 mM Tris, 192 mM

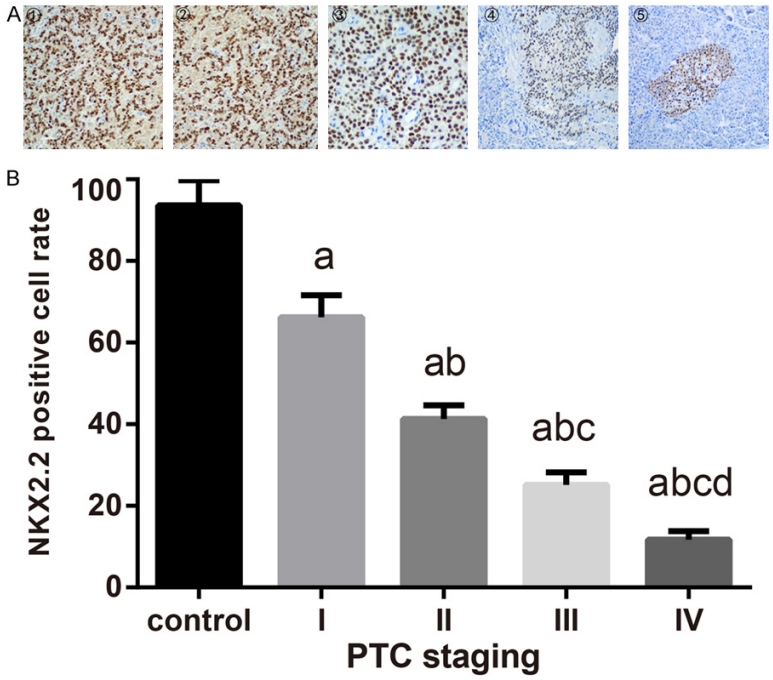

Figure 1. Differences of positive ratios of NKX2.2 in PTC patients at different stages. Note: A. Immunohistochemical staining; brownish-yellow cells referred to positive NKX2.2 cells; ①, stage I; ② stage II; ③, stage III; ④, stage IV; ⑤, normal thyroid cells. B. Grading of immunohistochemical staining; a referred to $P < 0.05$ compared with the control group; b referred to $P < 0.05$ compared with I; c referred to $P < 0.05$ compared with II; d referred to $P < 0.05$ compared with III; PTC, Papillary thyroid carcinoma.

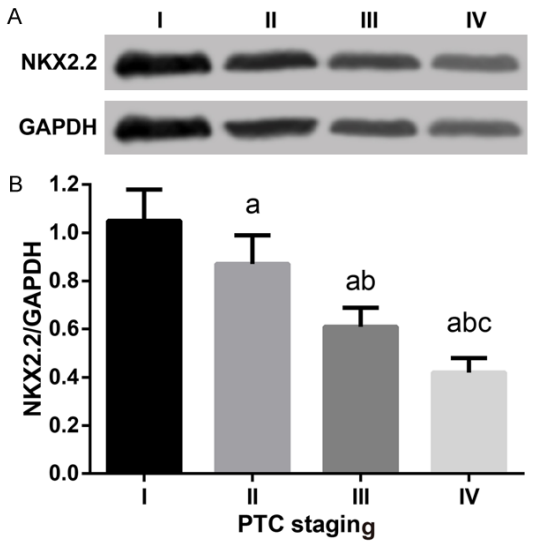

Figure 2. Differences in NKX2.2 protein expression level of PTC patients at different stages. Note: A. Western blot; B. NKX2.2/GAPDH ratio; a referred to $P < 0.05$ compared with I; b referred to $P < 0.05$ compared with II; c referred to $P < 0.05$ compared with III; PTC, Papillary thyroid carcinoma.

glycine and 20% (v/v) methyl alcohol) for 10 min with polyacrylamide gel. Conditions of membrane transfer: (1) Constant pressure of 17 V; (2) Duration of transfer for 30 min. After membrane transfer was finished, the transferred PVDF membranes were sealed with 6% (m/v) of skim milk powder (dissolved in the PBS solution) at room temperature for 2 h, and then taken out for wash by PBST buffer solution (0.1% (w/v) Tween-20 added into the PBS solution) for 3 times, 3 min in each time. After that, the PVDF membranes were hybridized with mouse anti-human NKX2.2 monoclonal antibodies (ZSGB Biotechnology Co., Ltd, Beijing) at room temperature for 1 h, and washed by PBST buffer solution for 5 times, each time for 3 min. Then the membranes were hybridized with goat anti mouse IgG (ZSGB Biotechnology Co., Ltd, Beijing) marked by horse radish peroxidase (HRP) for 1 h at room temperature, and washed by PBST buffer solution for 5 times, 3 min in each time. HRP substrate (Bio-Rad, USA) was used for coloration of target proteins.

GAPDH protein was chosen as the internal reference protein. Primary antibody was mouse anti-human GAPDH protein (ZSGB Biotechnology Co., Ltd, Beijing). Image-Pro Plus6.0 was applied to detect gray values of the NKX2.2 and GAPDH target band regions in the Western Blot result figure, with ratio of NKX2.2/GAPDH as the relative amount of NKX2.2.

Follow-up visit

Followed-up visit was conducted to all patients who left hospital for 3 years, and tumor recurrences of the patients in the case group were observed. Follow-up visit, mainly in the form of hospital reexamination and telephone calls, started from the time when the diagnosis of patients were confirmed and ended on September 31st 2015, with follow-up intervals

Table 1. Correlation between NKX2.2 protein expression and clinicopathological features of PTC patients

Clinicopathological features	n	Positive ratios of NKX2.2 (%)	*P* value	NKX2.2/GAPDH	*P* value
Gender			0.631		0.106
Male	41	43.9		0.73 ± 0.33	
Female	146	39.73		0.79 ± 0.16	
Age (years old)			0.378		0.198
< 43	101	43.56		0.80 ± 0.22	
≥ 43	86	37.2		0.76 ± 0.20	
Maximum diameter of tumor (cm)			< 0.001		< 0.001
< 1	48	66.67		0.86 ± 0.28	
1~3	95	38.89		0.82 ± 0.16	
> 3	44	20.45		0.60 ± 0.11	
Lymphatic metastasis			0.001		< 0.001
With	106	26.42		0.72 ± 0.24	
Without	81	59.26		0.86 ± 0.13	
Distant metastasis			0.018		0.01
With	35	22.86		0.70 ± 0.22	
Without	152	44.74		0.80 ± 0.20	
Neoplasm invasiveness degree			0.005		< 0.001
Inside envelope invasion	98	51.02		0.86 ± 0.20	
Outside envelope invasion	32	37.50		0.72 ± 0.09	
Thyroid lobe invasion	29	34.48		0.76 ± 0.16	
Invasion in surrounding tissues and organs	28	14.29		0.58 ± 0.23	

Note: PTC, papillary thyroid carcinoma.

of 3 months. There was no case losing follow-up.

Statistical methods

Data was performed using SPSS 21.0 software (SPSS Inc, Chicago, IL, USA). Measurement data were presented as means ± standard deviation (SD), with data between groups examined by *t* test. χ^2 test was applied for comparison on count data. The association between risk factors and PTC was confirmed by unconditioned logistic regression analysis. $P < 0.05$ was considered statistically significant.

Results

General features of the included subjects

There were 187 cases in the case group, including 41 men and 146 women ranging from 16~74 years old, with an average age of 41.6 ± 9.22 years old. Thyroid cancers were divided into stage I (n = 77), stage II (n = 31), stage III (n = 26) and stage IV (n = 53). There were 106 patients with lymph node metastasis, 35 patients with distant metastasis, 98 patients with inside envelope invasion, 32 patients with outside envelope invasion, 29 patients with thyroid lobe invasion and 28 patients with invasion in surrounding tissues and organs. The control group consisted of 124 healthy controls, including 59 men and 65 women aging from 19 to 65 years old, with an average age of 39.2 ± 8.15 years old. There was no statistical difference in age composition and gender ratio between the case group and the control group (both $P > 0.05$).

Immunohistochemical staining results of NKX2.2

According to the immunohistochemical staining of cells, positive NKX2.2 proteins were located in cell nucleus, and NKX2.2 protein in thyroid cell groups of normal people showed strong positive expression (brownish-yellow cells in **Figure 1A**). NKX2.2 proteins showed different degrees of expression in PTC specimens of all stages (**Figure 1A**). Positive ratios of NKX2.2 at

Table 2. Unconditional logistic analysis results of various factors and papillary thyroid carcinoma

Factors	Regression coefficient	*P* value	EXP(B) (95% CI)
Maximum diameter of tumors	0.083	0.669	1.086 (0.744-1.586)
Lymphatic metastasis	-0.853	0.114	0.426 (0.148-1.226)
Distant metastasis	-0.515	0.509	0.597 (0.129-2.758)
Neoplasm invasiveness degree	0.303	0.163	1.353 (0.885-2.070)
Expression level of NKX2.2	-2.970	0.023	0.051 (0.004-0.662)

Note: OR referred to odds ratio; CI referred to confidence interval.

Table 3. Postoperative recurrence rates of PTC patients

Tumor staging	Protein expression level	187	Without recurrence (171)	Recurrence (16)	Recurrence rates (%)
I	0.94 ± 0.13	77	73	4	5.19%
II	0.85 ± 0.13	31	29	2	6.45%
III	0.65 ± 0.12	26	23	3	11.54%
IV	0.57 ± 0.16	53	46	7	13.21%

Note: PTC, papillary thyroid carcinoma.

various PTC stages were compared, and it could be seen that positive ratio of NKX2.2 for thyroid cell groups of normal people and stage I-IV of PTC were 93.60%, 66.20%, 41.30%, 25.10% and 11.70%, respectively. The positive ratios of NKX2.2 in the case group were significantly lower than those in the control group, showing statistical significance (all $P < 0.05$). As PTC worsened, positive ratios of NKX2.2 decreased (**Figure 1B**).

Detection results of NKX2.2 by Western blot

The results of the Western Blot showed that NKX2.2/GAPDH protein ratios in PTC specimens at stage I-IV were 1.05 ± 0.13, 0.87 ± 0.12, 0.61 ± 0.08 and 0.42 ± 0.06, respectively. Statistical significance was found in NKX2.2 protein expressions at various PTC stages (all $P < 0.05$) (**Figure 2**). The result showed that as PTC deteriorated, the expression of NKX2.2 proteins declined.

Association between NKX2.2 expressions and PTC clinicopathological features

Maximum diameter, lymph node metastasis, distant metastasis and neoplasm invasiveness were negatively related to positive ratios of NKX2.2 and NKX2.2/GAPDH protein expression (all $P < 0.05$). There was no significant relationship between NKX2.2 protein expressions and patients' gender and age (**Table 1**).

Logistic regression analysis

Logistic regression analysis found that the maximum diameter of tumors, lymph node metastasis, distant metastasis and the degree of neoplasm invasiveness were not significantly associated with the recurrences of PTC, while NKX2.2 protein expressions were related with PTC recurrences ($P < 0.05$), thus being an independent hazard for the recurrences of thyroid cancer. See **Table 2**.

Association between NKX2.2 expression and recurrences of PTC

Follow-up visit was conducted on 187 patients, and postoperative neoplasm recurrences were found in 16 patients 4 to 45 months after their operation, with average recurrence time of 37.5 months. Relapsing patients consisted of stage I patients (n = 4), stage II patients (n = 2), stage III patients (n = 3) and stage IV patients (n = 7) (**Table 3**). Based on NKX2.2 protein expressions of PTC patients at various stages, the relationship between NKX2.2 protein expressions and the recurrence rates of PTC was analyzed, which indicated that NKX2.2 protein expressions was associated with the postoperative PTC recurrence rates. Namely, with the increase of NKX2.2 protein expressions, postoperative PRC recurrence rates declined.

The value of NKX2.2 in forecasting prognosis of PTC

The ROC curve (**Figure 3**) showed that area under the curve of PTC diagnosed by NKX2.2 protein expressions was 0.866 [95% *CI* (0.826, 0.906)]. Maximization of Youden index was taken as the basis for selection, and the best dividing value in the diagnosis of PTC by NKX2.2 protein expressions was 0.65 (sensitivity = 74.1%, specificity = 86.3%).

Discussion

In this study, NKX2.2 protein expressions in 187 PTC patients were investigated so as to

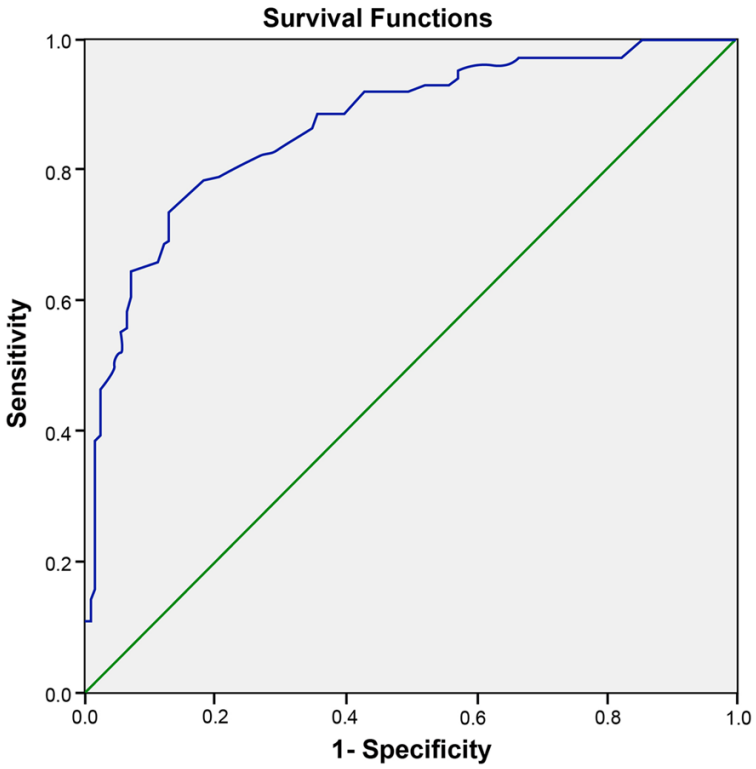

Figure 3. ROC curve analysis on NKX2.2 protein expressions in the diagnosis of PTC. Note: PTC, Papillary thyroid carcinoma; ROC, Receiver operation curve.

further discuss the relationship between NKX2.2 proteins and PTC clinicopathological features as well as prognosis with results found that NKX2.2 protein was related to the clinicopathological features and NKX2.2 protein had an impact on the postoperative recurrence rates, which indicated that NKX2.2 protein might be an indicator for the diagnosis as well as prognosis judgment of PTC.

This study also found that low NKX2.2 expressions were related to the further increase of tumor size and lymph node metastasis. Therefore, low NKX2.2 expressions could be seen as an implication for worsened PTC. Relevant studies confirm that NKX2.2 can inhibit the proliferation of tumors and the further development of cancers. Namely, NKX2.2 expression, which is highly related to the Olig2 factor [12], shows relatively high expressions in spinal cord tissues and ventricular fibers [13, 14] and the expression of these two factors can regulate cells differentiation. The Oligo2 factor plays an important role in regulating the proliferation and division of plasmacytoid dendritic cell precursors [15], neuronal cells, fibrocytes and spongiocytes [16-18], while the NKX2.2 factor mainly regulates the further differentiation and maturity of cells [12]. As a result, the synergism between Oligo2 and NKX2.2 decides the final differentiation morphology of the cells [19, 20]. A previous study shows that NKX2.2 in tumors tends to induce cells to differentiate into normal gliocytes rather than tumor cells [21]. Therefore, NKX2.2 plays a significant role in inhibiting cancers: with the decrease of NKX2.2 expressions, normal cells might be further activated to become tumor cells, thereby leading to cancer deterioration.

On the other hand, this study found that NKX2.2 expressions were associated with the postoperative recurrences of patients. That is, when NKX2.2 expressions were low, PTC recurrence rates would be high. Meanwhile, it was suggested that NKX2.2 also plays a significant role in regulating the proliferation and development of cells. Relevant studies show that *NKX2.2* gene deletion will cause massive reduction and injuries of pancreas cells [22] and dysplasia in nervous system of mouse [23]. As a result, NKX2.2 cannot only inhibit the generation of tumor cells, but also be a key regulatory factor in the development process of normal cells. When NKX2.2 expressions decrease, it is likely to influence the postoperative recovery state of patients and increase risks of recurrence. A study also confirms that as a diagnostic indicator of cancers, NKX family has relatively high sensitivity and low misdiagnosis rate as well as missed diagnosis rate [24]. Therefore, it is relatively feasible to take NKX2.2 as a candidate indicator in the early diagnosis of PTC.

In conclusion, NKX2.2 expressions are highly associated with the pathological process of PTC, especially in terms of inhibiting tumor formation and regulating cells proliferation, thus

allowing itself as a reference index in the early diagnosis of PTC. Admittedly, there remained some obstacles in this experiment. Firstly, there were fairly few clinical specimens collected in this experiment, making the conclusion lack of high accuracy. Secondly, in the analysis of prognosis, only recurrence rates were counted, and long-term follow-up statistics of patients' survival condition were insufficient, which led to the lack of evidences for further summarizing the functions of NKX2.2. Therefore, this conclusion requires further improvement and support by future clinical studies.

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Disclosure of conflict of interest

None.

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