CDK9 is up-regulated and associated with prognosis in patients with papillary thyroid carcinoma

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Abstract

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy but shows excellent prognosis. We investigated the clinical significance of cyclin-dependent kinase 9 (CDK9) in patients with PTC. This prospective observational study included 192 patients with PTC, who visited our hospital between August 2018 and February 2020. We obtained 93 tissue samples from patients with benign thyroid disease during the same period as controls. Immunohistochemical evaluation and reverse transcription-quantitative polymerase chain reaction assay were performed to evaluate CDK9 expression. Patients’ demographic and clinical characteristics were analyzed.

Delphian lymph node (DLN) metastasis in patients with PTC was associated with clinicopathological characteristics. CDK9 expression was up-regulated in patients with PTC, and those with DLN metastasis showed higher CDK9 expression. We also observed that tumor size, capsule invasion, tumor-node-metastasis classification (TNM) stage, and multifocality were the risk factors for DLN metastasis in patients with PTC. Additionally, CDK9 expression was strongly associated with tumor size, capsule invasion, TNM stage, and multifocality and weakly associated with the number of metastatic DLN.

CDK9 is up-regulated in patients with PTC and associated with prognosis in these patients.

Abbreviations: BTD = benign thyroid disease, CDK9 = cyclin-dependent kinase 9, DLN = Delphian lymph node, LN = lymph node, PTC = papillary thyroid carcinoma, RT-qPCR = reverse transcription-quantitative polymerase chain reaction.

Keywords: CDK9, Delphian lymph node metastasis, papillary thyroid carcinoma, prognosis

1. Introduction

As one of the most prevalent endocrine malignancies and the fifth most prevalent malignant tumor in women worldwide, thyroid carcinoma accounts for 3% of all malignant neoplasms.[1]

Papillary thyroid carcinoma (PTC) is the most common histopathological type of thyroid malignancy but shows excellent prognosis. Clinicopathological characteristics, such as tumor size, lymph node (LN) metastasis, distant metastasis, extrathyroid extension, and completeness of resection serve as predictors of poor prognosis in patients with PTC.[2] It is important to establish new diagnostic and prognostic biomarkers for PTC to improve clinical outcomes.

Cyclin-dependent kinase 9 (CDK9) is a kind of kinases that form the catalytic core of the positive transcription elongation factor b, regulating the cell cycle and apoptosis. Reportedly, CDK9 is a driver of oncogenic transcription and promotes cancer cell survival and proliferation.[3] In vivo and in vitro studies show CDK9 inhibitors are widely used for the treatment of various cancers. CDKI-73 is a highly potent small-molecule inhibitor of CDK9 used for treating colorectal cancer.[4] Another study reports that AZD4573, a CDK9 inhibitor, induces apoptosis in hematological cancer cells.[5] CDK9 inhibitors that suppress cancer stem cell activity are also being used for the treatment of nonsmall cell lung cancer.[6] However, no study has illustrated the association between CDK9 expression and prognosis in patients with PTC.

The Delphian lymph node (DLN), also referred to as the prelaryngeal or cricothyroid node, is located between the cricoid and thyroid cartilages in the fascia superior to the thyroid isthmus.[7] DLN metastasis is a known predictor of extensive cervical LN metastasis, high recurrence rates, and increased mortality rates for patients with PTC.[8] Several studies have demonstrated the association between clinicopathological factors and DLN metastasis. Zheng et al revealed that DLN metastasis was significantly associated with tumor size, extrathyroid...
extension, multifocality, as well as central and lateral neck node metastases, among other variables.\(^9\) Therefore, DLN involvement requires careful evaluation in patients with PTC.

In this study, we observed that DLN metastasis was associated with clinical characteristics in patients with PTC. CDK9 was overexpressed and was also associated with clinical outcomes in patients with PTC. This study might provide a potential research target for future clinical studies in patients with PTC.

2. Materials and methods

2.1. Objective and samples

This prospective observational study included patients with PTC (37 with DLN metastasis and 155 without DLN metastasis), who underwent thyroid surgery at our hospital between August 2018 and February 2020. The diagnosis and staging of PTC was confirmed by postoperative histopathological evaluation and was based on The Union for International Cancer Control-Tumor-Node-Metastasis (TNM) classification,\(^1\)\(^0\) Inclusion criteria for PTC patients were as follows:

1. confirmed diagnosis of PTC based on postoperative histopathological evaluation,
2. no operative contraindications,
3. thyroid surgery performed for the first time and,
4. no distant metastasis.

Exclusion criteria for PTC patients were as follows:

1. incomplete data regarding T or N staging,
2. a history of thyroidectomy for thyroid cancer,
3. diagnosis of concomitant diffuse benign thyroid disease (BTD) except lymphocytic thyroiditis,
4. unavailability of data regarding histopathological evaluation of the thyroid gland and,
5. a history of cervical radiation or a family history of thyroid tumors.

Ninety three tissue samples from patients with BTD was obtained as controls. This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Anhui Medical University and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

2.2. Immunohistochemical evaluation

Immunohistochemical (IHC) evaluation using an SP reagent kit (Tiangen, Beijing, China) was performed to analyze CDK9 expression in tissue samples obtained from patients with PTC. CDK9 expression in BTD tissue was also detected. Briefly, tissues were heated for 2 hours at 60°C, and the histological sections were deparaffinized using xylene solution and rehydrated using graded ethanol and distilled water. Target retrieval solution was used for antigen retrieval in slides, and the specimens were incubated in 3% hydrogen peroxide for 10 minutes. The slides were blocked by application of 5% goat serum and incubated with human CDK9 primary antibody (ab76320, 1100, Abcam, Cambridge, MA) overnight in a humidified chamber at 4°C. Subsequently, the slides were washed thrice using TBST and SignalStain Boost Detection Reagent and SignalStain diamino-benzidine (DAB) (all reagents from Cell Signaling Technology) were used to detect the bound antibody on the array. Finally, the slides were counterstained using hematoxylin (Vector Laboratories) and mounted using VectaMount AQ (Vector Laboratories) for long-term preservation when the DAB reaction was terminated. IHC markers were used for cytoplasmic staining. The CDK9 staining intensity and pattern were assessed semi-quantitatively based on the following scale: 0 staining: none of the cells were immunopositive, 1+: <25% immunopositive cells, 2+: 25%–50% immunopositive cells and, 3+: 50%–100% immunopositive cells. Likewise, 0, 1+, 2+, and 3+ were defined as immunonegative, weakly immunopositive, moderately immunopositive, and strongly immunopositive results, respectively. In the present study, 0 staining and 1+ staining were defined as low expression, and 2+ staining and 3+ staining as high expression.

2.3. Reverse transcription-quantitative polymerase chain reaction assay

A reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay was performed to evaluate CDK9 expression. Total RNA was extracted from tissue samples using TRizol reagent (Invitrogen, Carlsbad, CA). RNA was converted into cDNA using an iScript cDNA synthesis kit (Takara, Shiga, Japan). Subsequently, a LightCycler480 system (Roche, Mannheim, Germany) with SYBR Premix Ex Taq\(\text{TM}\) (Takara, Dalian, China) was used to perform quantitative real-time PCR under the following conditions: 95°C for 2 minutes, 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 20 seconds for a total of 40 cycles. The following primers were used for PCR: F 5’-GGAGACAGGG-CATTGTAGTTA-3’ and R 5’-ATAGGATTGCGTTGAGT- GAG-3’ for CDK9, F 5’-AAGGTAAGGTCGGATTCAAC-3’ and 5’-GGGTTGATTGACTCAAATATA-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression level of mRNA was calculated using the 2\(^{-\Delta\Delta Ct}\) method and normalized to the level of GAPDH used as the internal control.

2.4. Data collection

We analyzed patients’ demographic characteristics, including age, sex, and body mass index and clinical characteristics, including tumor size, capsule invasion, multifocality, DLN and nonDelphian central LN metastasis, and TNM stage.

2.5. Statistical analysis

Continuous data are presented as means ± standard deviation. The Student t test was used for comparison between 2 groups. The one-way analysis of variance (ANOVA) test followed by Tukey post hoc test was used for comparison between 3 or more groups. The Chi-Squared or Fisher exact test was used for comparison of rates. Pearson correlation coefficient was used to analyze correlations. Logistic regression analysis was performed using a stepwise method. P value <.05 was considered statistically significant. All statistical analyses were performed using the SPSS software, version 18.0 (SPSS Inc., Chicago).

3. Results

3.1. Patients’ demographic and clinical characteristics

The study included 192 patients with PTC; 37 patients showed DLN metastasis. Table 1 shows patients’ basic characteristics. DLN metastasis was significantly associated with tumor size,
capsule invasion, nonDelphian central LN metastasis, multifocality, and TNM stage. No signifi-
cant intergroup difference was observed in age, sex, body mass index, and the rate of
Hashimoto’s thyroiditis. These results indicated an association between DLN metastasis and clinicopathological features in patients with PTC.

3.2. Risk factors associated with Delphian lymph node metastasis in patients with papillary thyroid carcinoma

Logistic regression analysis showed that tumor size, capsule invasion, TNM stage, and multifocality were risk factors for DLN metastasis, except for non-Delphian central LN metastasis (Table 2), which suggested that tumor size, capsule invasion, TNM stage, and multifocality serve as risk factors for DLN metastasis in patients with PTC.

3.3. CDK9 expression was up-regulated in patients with papillary thyroid carcinoma

Subsequently, CDK9 expression was detected in different groups. Figure 1A-B shows the histopathological findings. Most patients with PTC showed moderate to strong expression of CDK9; however, IHC evaluation revealed negative to weakly low and focally weak CDK9 expression in BTD specimens. Table 3 shows the IHC results in each group. The ratio of patients with high CDK9 expression was higher in the total PTC group (163 of 192 patients, 84.90%) than in the BTD group (21 of 93 patients, 22.58%), in addition, PTC patients with DLN metastasis had higher ratio of high CDK expression than those PTC patients without DLN metastasis. RT-qPCR assay results revealed that CDK9 expression was significantly higher in PTC tissue samples than in BTD tissue samples, besides, patients with DLN metastasis had higher expression of CDK9 than patients without DLN metastasis (Fig. 2). These results indicated that CDK9 expression was up-regulated in patients with PTC, and those with DLN metastasis showed higher CDK9 expression.

3.4. CDK9 expression was associated with clinical outcomes in patients with papillary thyroid carcinoma

We further investigated the association between CDK9 expression and clinical outcomes in patients with PTC. As shown in Table 4, patients with high CDK9 expression had a higher ratio

Table 1

| Variable                        | PTC with DLN metastasis (N = 37) | PTC without DLN metastasis (N = 155) | P   |
|--------------------------------|----------------------------------|------------------------------------|-----|
| Age, year                      |                                  |                                    |     |
| <45                            | 23 (62.16)                       | 89 (57.42)                         | .599|
| ≥45                            | 14 (37.84)                       | 66 (42.58)                         |     |
| Sex, female (%)                |                                  |                                    |     |
| Female                         | 34 (91.89)                       | 131 (84.52)                        | .246|
| BMI, kg/m²                      | 22.68 ± 2.93                     | 23.52 ± 2.70                       | .094|
| Hashimoto’s thyroiditis, n (%)  | 21 (56.76)                       | 93 (60.00)                         | .719|
| Tumor size                     |                                  |                                    |     |
| ≤1cm                           | 14 (37.84)                       | 119 (76.77)                        | <.001|
| >1cm                           | 23 (62.16)                       | 36 (23.23)                         |     |
| Capsule invasion               |                                  |                                    |     |
| Yes                            | 12 (32.43)                       | 8 (5.16)                           | <.001|
| No                             | 25 (67.57)                       | 147 (94.84)                        |     |
| Metastasis number of DLN, (n)  | 0.40 ± 0.99                      |                                    |     |
| Non-Delphian central lymph node metastasis |                   |                                    |     |
| Positive                       | 26 (70.27)                       | 60 (38.71)                         | .001|
| Negative                       | 11 (29.73)                       | 95 (61.29)                         |     |
| TNM stage                      |                                  |                                    |     |
| I-II                           | 20 (54.05)                       | 110 (70.97)                        | .048|
| III-IV                         | 17 (45.95)                       | 45 (29.03)                         |     |
| Multifocality                  |                                  |                                    |     |
| Yes                            | 25 (67.57)                       | 45 (29.03)                         | <.001|
| No                             | 12 (32.43)                       | 110 (70.97)                        |     |

BMI = body mass index, DLN = Delphian lymph node, PTC = papillary thyroid carcinoma, TNM = tumor node metastasis.
of tumor size >1 cm, capsule invasion, multifocality, TNM stages III–IV, and a higher number of DLN metastasis; however, no obvious differences were observed with regard to metastatic non-Delphian central LN. Pearson analysis showed weakly positive correlation between CDK9 expression level and DLN metastasis, whereas no significant linear correlation was observed between CDK9 expression and tumor size (Table 5). These results suggested that CDK9 expression was associated with clinical outcomes and with the metastatic number of DLN in patients with PTC.

### 4. Discussion

Several recent studies have investigated PTC,[11] and despite advances in the diagnostic and therapeutic approach to PTC, novel biomarkers and research targets are warranted for PTC. An association between CDK9 and various cancers, such as gastric cancer,[12] pancreatic cancer,[13] and prostate cancer[14] is increasingly being reported in recent years; however, few studies have described the clinical significance of CDK9 in patients with PTC. In this study, we observed that DLN metastasis was associated with clinical characteristics. Moreover, CDK9 was up-regulated in patients with PTC and was strongly associated with tumor size, capsule invasion, and TNM stage and weakly with the metastatic number of DLNs.

Reportedly, DLN metastasis is associated with unfavorable clinicopathological characteristics.[15] A previous study showed that DLN metastasis was associated with tumor location and LN metastasis.[16] Another study reported that DLN metastasis promoted lymphovascular invasion and central and lateral LN metastasis.[17] A systematic review and meta-analysis demonstrated that DLN positivity was a risk factor for central LN metastasis in patients with PTC.[18] Consistent with these studies, we also observed that DLN metastasis was associated with non-Delphian central LN metastasis and multifocality. Li et al revealed that DLN metastasis was obviously associated with sex and age, tumor size, multifocality, and central and lateral neck lymph node metastasis, which were risk factors for DLN metastasis.[19] Nevertheless, Tan et al suggested DLN metastasis positively correlated to tumor size and capsular invasion but not for age and sex.[20] Most researches showed DLN metastasis in PTC was associated with tumor development and predicted a

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**Table 3**

The immunohistochemistry results of four groups.

| CDK9 expression | Low | 1+ | 2+ | 3+ | P  |
|-----------------|-----|----|----|----|----|
| BTD (n=93)      | 55  | 17 | 10 | 11 | <.001 |
| Total PTC (n=192) | 5   | 24 | 81 | 82 | .020 |
| PTC with DLN metastasis (n=37) | 0   | 1  | 13 | 23 | .020 |
| PTC without DLN metastasis (n=155) | 5   | 22 | 69 | 59 | .020 |

*P: Comparison of patients with high CDK9 expression in BTD group and total PTC group.
†P: Comparison High CDK9 expression in PTC with DLN metastasis and PTC without DLN metastasis.

0 staining and 1+ staining was defined as low expression, while 2+ staining and 3+ staining was defined as high expression.

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**Table 4**

The association of CDK9 expression with clinic outcomes of PTC patients.

| Variable                        | High CDK9 N=163 | Low CDK9 N=29 | P       |
|--------------------------------|-----------------|---------------|---------|
| Tumor size (cm)                |                 |               |         |
| ≤1cm                           | 60 (36.81)      | 22 (75.86)    | <.001   |
| >1cm                           | 103 (63.19)     | 7 (24.14)     |         |
| Capsule invasion               |                 |               |         |
| No                             | 55 (33.74)      | 16 (55.17)    | .028    |
| Yes                            | 108 (66.26)     | 13 (44.83)    |         |
| Non-Delphian central lymph node|                 |               |         |
| Positive                       | 115 (70.55)     | 18 (62.07)    | .362    |
| Negative                       | 48 (29.45)      | 11 (37.93)    |         |
| Metastasis number of DLN       | 0.466±0.106     | 0.035±0.186   | <.001   |
| TNM stage                      |                 |               |         |
| I-II                           | 61 (37.42)      | 17 (58.62)    | .032    |
| III-IV                         | 102 (62.58)     | 12 (41.38)    |         |
| Multifocality                  |                 |               |         |
| No                             | 52 (31.90)      | 21 (72.41)    | <.001   |
| Yes                            | 111 (68.10)     | 8 (27.59)     |         |

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**Figure 2.** CDK9 was up-regulated in PTC patients. CDK9 expression was detected by RT-qPCR in PTC tissue samples and BTD tissue samples. BTD: n=93; PTC: n=192; PTC with DLN metastasis: n=37; PTC without DLN metastasis: n=155.
Table 5
Pearson’s analysis for correlation between CDK9 expression with metastasis number of DLN and tumor size.

| Metastasis number of DLN | Tumor size |
|--------------------------|------------|
| CDK9 expression Pearson correlation | 0.364 | −0.085 |
| P | .027 | .618 |


dead of A375 melanoma cells.\[26\] A variety of in vivo studies expression and enhanced p53 activity, in cancer cells, namely, CDK9 suppression decreased MDM4 was associated with the over expression of the MDM4 oncogene /C0 CDK9 expression Pearson correlation 0.364

patients with advanced TNM stage cancer.\[30\] Moreover, the clinical outcomes in patients with prostate cancer, particularly in illustrated that CDK9 inhibitors could improve the positive tumor.

Emerging evidence indicated CDK9 was a regulator of the growth and cell cycle progression. Franco et al reported that CDK9 over expression is an important mechanism involved in cell survival and subsequent cancer development.\[23\] CDK9 inhibition significantly induced cell apoptosis in various leukemia and solid tumor cell lines, meanwhile it showed potent antitumor growth efficacy in preclinical tumor model.\[24,25\] CDK activity was associated with the over expression of the MDM4 oncogene in cancer cells, namely, CDK9 suppression decreased MDM4 expression and enhanced p53 activity, finally resulting in the death of A375 melanoma cells.\[26\] A variety of in vivo studies illustrated the essential role of CDK9 in different cancers. Emerging evidence indicated CDK9 was a regulator of the differentiation program of neuron and astrocyte, besides, neuroblastoma, and PNET tumor samples verified that up-regulated CDK9 was closely correlated to high differentiation of tumor.\[27\] In a recent study, Re et al reported that elevated levels of thymidine kinase 1 (TK1) and CDK9 in plasma-derived exosomes were associated with clinical resistance in patients with metastatic breast cancer.\[28\] Wang et al observed that CDK9 was overexpressed in human ovarian cancer cell lines and up-regulated in metastatic and recurrent ovarian tumor tissue, additionally, elevated CDK9 was significantly associated with poor prognosis in patients with ovarian cancer.\[29\] A review illustrated that CDK9 inhibitors could improve the positive clinical outcomes in patients with prostate cancer, particularly in patients with advanced TNM stage cancer.\[30\] Moreover, the anti-tumor effect of CDK9 also has been discussed in brain tumor,\[31\] hepatocellular carcinoma,\[32\] endometrial cancer\[33\] and so on. However, no study has investigated the role of CDK9 in the pathological progression of PTC. In this study, we found that CDK9 expression was associated with tumor size, capsule invasion, multifocality, and TNM stage and was positively associated with the metastatic number of DLNs in patients with PTC. Since DLN metastasis was closely associated with tumor aggressiveness, CDK9 overexpression predicted a poor prognosis for patients with PTC.

Following are the limitations of this study:
1. The small sample size is a limitation and,
2. the molecular mechanism underlying the role of CDK9 in PTC remains unclear.

5. Conclusion
This prospective observational study highlights that DLN metastasis is associated with clinicopathological characteristics in patients with PTC. Moreover, this is the first study that showed over expression of CDK9 in PTC and confirmed that CDK9 expression was strongly associated with clinical outcomes and weakly correlated with metastatic number of DLN in patients with PTC. Additionally, tumor size, capsule invasion, TNM stage, and multifocality were identified as risk factors for DLN metastasis in patients with PTC. This study provides clinical evidence to support the role of CDK9 over expression in PTC. However, further large-scale studies are warranted to confirm the molecular mechanism of CDK9 in PTC progression.

Author contributions
Conceptualization: Tao Guo.
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