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Original Article

Expression status of cytoskeleton regulatory protein Mena as a prognostic marker for human thyroid carcinoma

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Abstract: Objective: In recent years, Mena (Mammalian Enabled) has been reported to be highly expressed in malignant tumors. However, data on the expression pattern and clinical relevance of Mena in thyroid carcinoma are unclear. The purpose of this study was to investigate the expression of Mena and its prognostic significance in human thyroid carcinoma. Materials and Methods: *Mena* expression at the mRNA level was examined by real-time quantitative polymerase chain reaction (RT-PCR) in 8 paired thyroid carcinoma and adjacent normal tissues. Mena protein expression in clinical samples was analyzed in paraffin-embedded papillary thyroid carcinoma samples and normal thyroid tissues by immunohistochemistry (IHC). Statistical analyses were also performed to evaluate the clinicopathological significance of Mena expression. Results: The results show that expression of *Mena* mRNA is higher in thyroid carcinoma than in adjacent normal tissues in 8 paired samples. In paraffin-embedded tissue samples, the expression of Mena was higher in papillary thyroid carcinoma than normal thyroid tissues. Compared with normal thyroid tissues, overexpression of Mena was detected in 47.11% (57/121) of papillary thyroid carcinoma patients. Overexpression of Mena was significantly associated with T Stage ($P = 0.007$), capsular invasion ($P = 0.015$), lymph node metastasis ($P = 0.000$), and clinical stage ($P = 0.029$). Conclusion: Mena is up-regulated in thyroid carcinoma and is associated with expression of T Stage, lymph node metastasis, clinical stage and disease-free survival. Mena may serve as a prognostic indicator for patients with thyroid carcinoma.

Keywords: Thyroid carcinoma, Mena, overexpression, prognosis

Introduction

Thyroid carcinoma is the most common endocrine malignant tumor, ranking as the seventh of all malignant tumors in females. A recent epidemiological survey showed that the incidence of thyroid carcinoma is rising in Canada, the United States, Europe, South Korea, and China. However, to find a new treatment strategy and an effective treatment for high-risk patients with thyroid cancer is a significant issue.

Mammalian Ena (Mena, also referred to as ENAH-enabled homolog) is a member of the Ena/Vasodilator-stimulated phosphoprotein (VASP) family of actin-binding proteins, which functions in various cell types [1, 2]. Mena is a key mediator of cytoskeletal arrangement [3] that regulates cell movement by protecting actin filaments from capping proteins during polymerization [4]. Up-regulation of Mena has been reported in human breast cancer cell lines and tissues [5]. Similarly, over-expression of Mena is also observed in human hepatocellular carcinoma [6], pancreatic tumors [7], colorectal carcinoma [8], and cervix precursor lesions [9], whereas in normal tissues, expression of Mena has been reported at very low or undetectable levels [6]. However, the clinical significance of Mena in thyroid carcinoma remains unclear. In this study, we examined Mena expression in thyroid carcinoma tissue samples and reveal its clinicopathological and prognostic significance.

Table 1. Correlation of Mena expression with clinicopathologic features

Characteristics	Total (n = 121)	Mena		*P* value
		Negative (n = 64)	Positive (n = 57)	
Gender				0.939
Male	23 (19.0%)	12 (52.2%)	11 (47.8%)	
Female	98 (81.0%)	52 (53.1%)	46 (46.9%)	
Age (years)				0.409
≥ 45	61 (50.4%)	30 (49.2%)	31 (50.8%)	
< 45	60 (49.6%)	34 (56.7%)	26 (43.3%)	
T stage				0.007
T1-2	68 (56.2%)	45 (66.2%)	23 (33.8%)	
T3-4	53 (43.8%)	19 (35.8%)	34 (64.2%)	
Capsular invasion				0.015
No	87 (71.9%)	52 (59.8%)	35 (40.2%)	
Yes	34 (28.1%)	12 (35.3%)	22 (64.7)	
Lymph node metastasis				0.000
No	80 (66.1%)	56 (70.0%)	24 (30.0%)	
Yes	41 (33.9%)	8 (19.5%)	33 (80.5%)	
M stage				0.102
0	118 (97.5%)	64 (54.2%)	54 (45.8%)	
1	3 (2.5%)	0 (0%)	3 (100%)	
TNM stage				0.029
I	52 (43.0%)	33 (63.5%)	19 (36.5%)	
II	35 (28.9%)	20 (57.1%)	15 (42.9%)	
III	3 (2.5%)	1 (33.3%)	2 (66.7%)	
IV	31 (25.6%)	10 (32.3%)	21 (67.7%)	
Calcification				0.107
No	85 (70.2%)	49 (57.6%)	36 (42.4%)	
Yes	36 (29.8%)	15 (41.7%)	21 (58.3%)	

Materials and methods

Clinical samples

One hundred and twenty-one papillary thyroid cancer (median age: 45 years; range: 16-79 years) and ninety benign thyroid tissue samples (relatively normal tissues from the nodular goiters) were used in this study and were histopathologically diagnosed and excised via curative resection at the First Affiliated Hospital of Sun Yat-sen University and the Third Affiliated Hospital of Sun Yat-sen University between January 1998 and December 2002. The demographic features and clinicopathologic information of these patients are summarized in **Table 1**. The papillary thyroid cancer were staged following the guidelines of the American Joint Committee on Cancer (AJCC) stage classification system.

Paired adjacent non-cancerous tissues were obtained in 8 patients (non-cancerous tissue was defined as at least 2 cm distance from the edge of the tumor). The 8 pairs of thyroid cancer and adjacent non-cancerous tissues were preserved immediately after operation for real-time PCR.

All of the patients consented to the use of the clinical specimens for research purposes and the protocol was approved by the Institutional Research Ethics Committee of Sun Yat-sen University.

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA samples were extracted from thyroid tumor materials using TRIzol reagent (Invitrogen, CA, USA). Extracted RNA was pretreated with RNase-free DNase. A total of 2 μg of RNA from each sample was used for cDNA synthesis. For the PCR amplification of Mena cDNA, an initial amplification step using Mena-specific primers was performed with denaturation at 95°C for 10 min followed by 28 cycles of denaturation at 95°C for 60 s, primer annealing at 58°C for 30 s, and primer extension at 72°C for 30 s. Upon completion of the cycles, a final extension at 72°C for 5 min was performed before the reaction mixture was stored at 4°C. Real-time PCR was then performed to determine the fold increase of Mena mRNA in each of the pairs of thyroid tumors and normal thyroid tissues from the same patient. The primer sequences were as follows: Mena sense 5'-GTGCCATTCCTAAAGGGTTGA-3', antisense 5'-GCTGCCAAAGTTGAGACCATAC-3'. GAPDH (sense 5'-TGTTGCCATCAATGACCCC-3', antisense 5'-CTCCACGACGTACTCAGC-3'). The primers were designed by Primer Express v 2.0 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and all experiments were performed in triplicate.

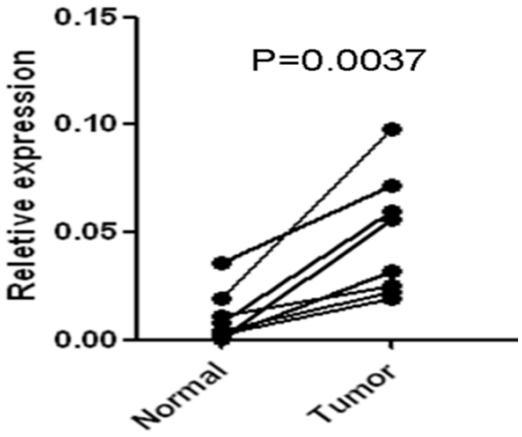

Figure 1. Expression levels of Mena mRNA in thyroid cancer and adjacent non-cancerous tissues. Expression levels of Mena mRNA in 8 paired thyroid cancer and adjacent non-cancerous tissues by real-time PCR. Normal, adjacent non-cancerous tissues. Tumor, thyroid cancer tissues.

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed to study altered protein expression in 60 human thyroid cancer tissues, and 40 normal thyroid tissues. Briefly, 4-μm-thick paraffin sections of the tissues were deparaffinized with xylene and rehydrated. Antigenic retrieval was performed by submerging the slides into EDTA antigenic retrieval buffer and microwaving. To quench endogenous peroxidase activity, the slides were treated with 3% hydrogen peroxide in methanol, and then incubated with 1% bovine serum albumin to block non-specific binding. After that, sections were incubated with rabbit polyclonal anti-Mena antibody (1: 100; BD) at 4°C over-night. Normal goat serum was used as a negative control. The tissue sections were incubated with a biotinylated anti-rabbit secondary anti-body (Abcam) after washing 3 times, followed by a further incubation with streptavidin-horseradish peroxidase complex (Abcam). Slides were immersed in 3-amino-9-ethyl car-bazole, then counterstained with 10% Mayer's hematoxylin, and finally dehydrated and mounted in Crystal Mount.

As for evaluation of immunostaining, the degree of immunostaining was viewed and scored separately by two pathologists, who were blind to the histopathological characteristics and patient information of the samples. Scores given by the two independent pathologists were averaged for further comparative evaluation of Mena expression. The intensity of Mena staining was graded as follows: 0, no staining; 1, weak staining = light yellow; 2, moderate staining = yellow brown; 3, strong staining = brown. The percentage of stained tumor cells was scored as follows: 0, no positive tumor cells; 1, 1-25% positive tumor cells; 2, 26-50% positive tumor cells; 3, 51-75% positive tumor cells; 4, > 75% positive tumor cells.

The staining score was calculated as the product of the proportion of positive tumor cells and the staining intensity score. The expression level of Mena was defined as follows: "-" (score 0, negative), "+" (score 1-4, weakly positive), "++" (score 5-8, positive), "+++" (score 9-12, strongly positive). Cut-off values for Mena were chosen on the basis of the heterogeneity using log-rank test with respect to overall survival (OS). The optimal cut-off value was estimated as follows: a staining index score of ≥ 4 was used to define tumors with high Mena expression and < 4 indicated low Mena expression.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 software (SPSS Inc. Chicago, IL, USA). The statistical comparisons between groups were analyzed using Student's *t*-test; the results are expressed as the mean ± SEM. Paired Students' *t*-test and Chi-square test were used to analyze the mRNA and protein levels of Mena. The difference of Mena expression between thyroid cancer tissues and normal thyroid tissues was analyzed by Chi-square test. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The relationship between TARBP1 expression and other clinicopathological characteristics was analyzed by Chi-square test and Fisher's exact test, or two-sample *t*-test as appropriate. The endpoint of this analysis was disease free survival (DFS), which was defined as the length of time from the date of surgery on the primary tumor to the local or regional recurrence, the distant metastasis or death. All statistical tests were two-sided, and a *p*-value of less than 0.05 was considered as statistically significant.

Results

Mena is overexpressed in thyroid cancer tissues

To determine whether Mena is highly expressed in human thyroid cancer samples, we per-

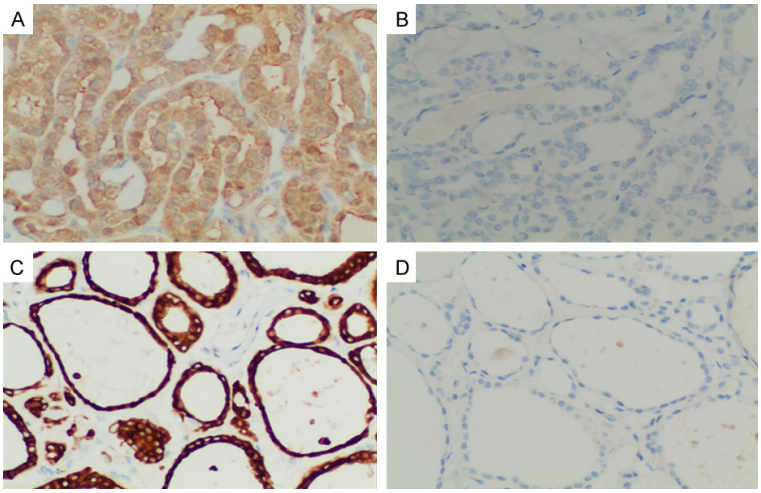

Figure 2. Expression analysis of Mena protein as determined by immunohistochemistry. Mena expression was mainly localized in the cytoplasm of thyroid tumor cells. Mena was either weak or non-existent in normal thyroid epithelial cells. A. Positive staining of Mena in thyroid carcinoma tissues. B. Negative staining of Mena in thyroid carcinoma tissues. C. Positive staining of Mena in normal thyroid tissues. D. Negative staining of Mena in normal thyroid tissues.

formed RT-PCR analyses on 8 thyroid tumor samples and adjacent non-cancerous tissues. As illustrated in **Figure 1**, Mena mRNA was expressed at higher levels in all of the 8 thyroid cancer tissues than in adjacent non-cancerous tissues, with the differential expression level ranging from 4.7- to 49.4-fold.

For immunostaining results, overexpression of Mena was observed in 47.11% (57/121) of papillary thyroid cancer patients. Mena protein staining was weak or there was no staining in the normal thyroid tissues (**Figure 2**). Overexpression of Mena was observed in only 7.78% (7/90) of normal thyroid tissues. The difference between the thyroid cancer group and normal thyroid tissues group was statistically significant ($X^2 = 37.779$, $P < 0.001$).

Mena correlates with clinicopathological features of papillary thyroid cancer

For a better understanding of the potential roles of MENA in papillary thyroid cancer development and progression, we investigated the status of MENA expression in 121 paraffin-embedded archived papillary thyroid cancer tissues by immunohistochemical staining, including 53 stage I tumors, 35 stage II tumors, 3 stage III and 31 stage IV tumors. Among 121 samples, MENA protein expression was detected in 57 samples (47.11%), and no staining was observed in 64 tumor samples (52.89%, **Table 1**). As shown in **Figure 3**, MENA was highly expressed in papillary thyroid cancer tissues. In contrast, no signals or only weak staining were detected in adjacent normal tissues. The subcellular location of MENA was mainly in the cytoplasm.

We further analyzed the correlation between MENA expression and other clinicopathological characteristics of staining patients. As is summarized in **Table 1**, there was no significant correlation between the expression level of MENA protein and patient age, gender, M stage and calcification. However, MENA expression was markedly associated with T Stage ($P = 0.007$), capsular invasion ($P = 0.015$), lymph node metastasis ($P = 0.000$), and clinical stage ($P = 0.029$).

Association between MENA expression and patient disease-free survival

The survival analysis showed a clear negative correlation between MENA protein expression level and disease-free survival of papillary thyroid cancer patients ($P < 0.001$, **Figure 3A**). We further analyzed the prognostic value of MENA in selective patient subgroups stratified by N Stage, T Stage and clinical stage. Expression of MENA was strongly associated with DFS of the patients in both T1-2 subgroups (log-rank test, $P = 0.049$) and T3-4 subgroups (log-rank test, $P = 0.000$) (**Figure 3D** and **3E**). It was also strongly associated with DFS of the patients in both Stage I-II subgroups (log-rank test, $P = 0.038$) and Stage III-IV subgroups (log-rank test, $P = 0.000$) (**Figure 3F** and **3G**). However, when it was evaluated according to the N Stage, the impact on outcome associated with MENA expression was favorable neither in the N0 subgroup (**Figure 3B**, log-rank test, $P = 0.799$), nor in the N1 subgroup (**Figure 3C**, log-rank test, $P = 0.064$).

Discussion

Thyroid carcinoma is the most prevalent endocrine malignant tumor. According to the data

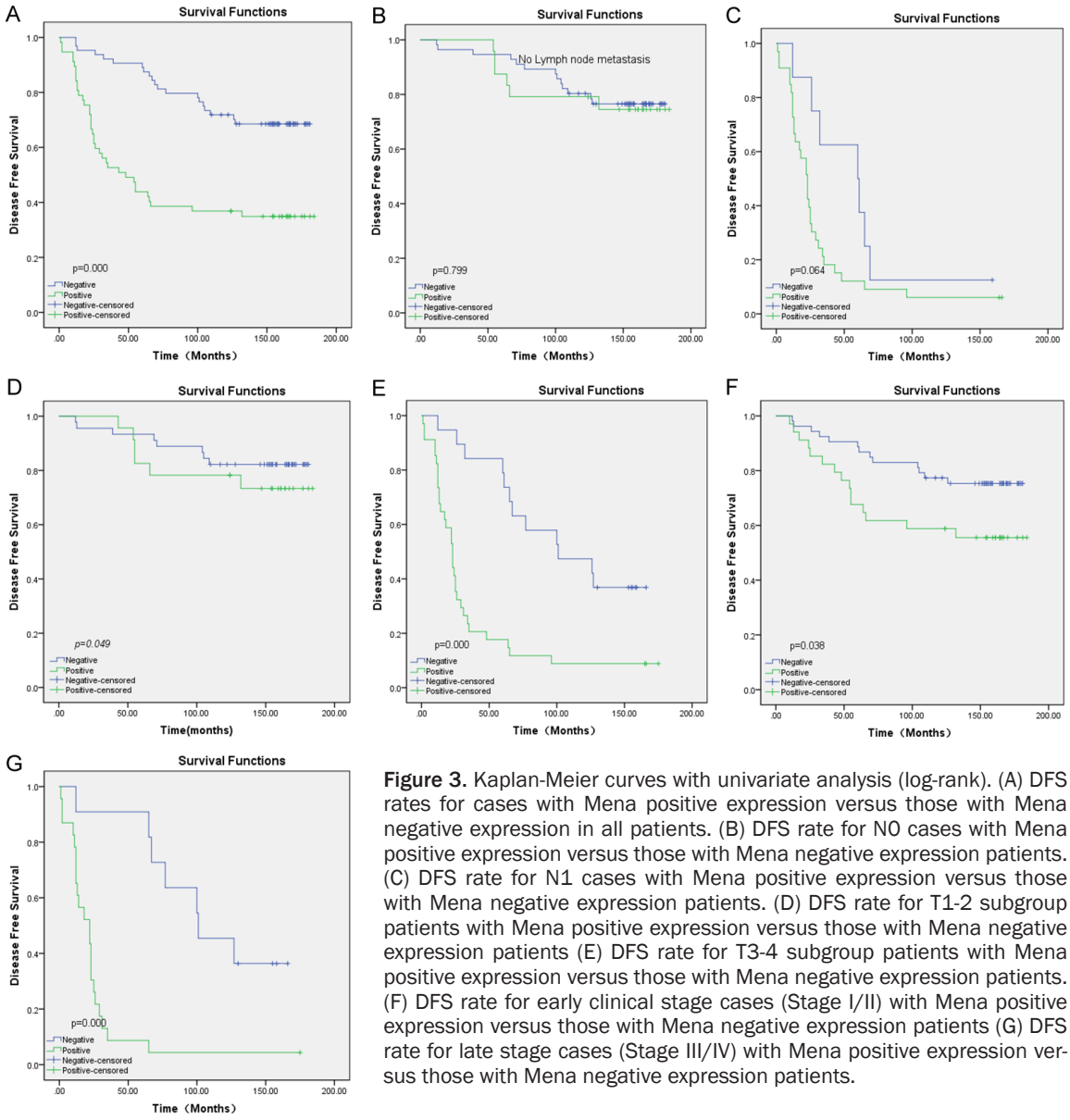

Figure 3. Kaplan-Meier curves with univariate analysis (log-rank). (A) DFS rates for cases with Mena positive expression versus those with Mena negative expression in all patients. (B) DFS rate for N0 cases with Mena positive expression versus those with Mena negative expression patients. (C) DFS rate for N1 cases with Mena positive expression versus those with Mena negative expression patients. (D) DFS rate for T1-2 subgroup patients with Mena positive expression versus those with Mena negative expression patients (E) DFS rate for T3-4 subgroup patients with Mena positive expression versus those with Mena negative expression patients. (F) DFS rate for early clinical stage cases (Stage I/II) with Mena positive expression versus those with Mena negative expression patients (G) DFS rate for late stage cases (Stage III/IV) with Mena positive expression versus those with Mena negative expression patients.

of Surveillance Epidemiology and End Results (SEER) database, its incidence has increased year by year in the USA between 1975 and 2012, and in 2015 it rose to an estimated 62,450 new cases and 1950 deaths. The rising incidence has also been recorded in many other countries [10]. Thyroid carcinoma also has a relatively high rate of recurrence and lymph node or distant metastases; estimates range between 20 to 30% [11, 12], while distant metastases develop in approximately 15% of cases [13]. Prognosis primarily depends on the recurrence site and the subsequent treatment strategy. Therefore, early diagnosis of the recurrence is of most importance [14].

The lack of universally accepted biomarkers to define aggressive thyroid carcinoma further confounds the difficulty in determining which patients with thyroid carcinoma have a poor prognosis. There is an urgent need to discover reliable biomarkers that can aid in distinguishing patients with aggressive thyroid carcinoma. The early classification of patients with poor prognosis would enable doctors to choose the adaptive treatment strategies that match more closely to the biological characteristics of the carcinoma, thereby avoiding excessive treatment, improving survival rates, and the quality of patients' lives.

Human ortholog of murine Mena (hMena), a member of the Ena/VASP protein family, which is a key regulatory molecule that controls cell shape, movement and actin organization on cadherin adhesion contacts, is frequently affected following malignant transformation [1, 15]. The family members include Mena, VASP, and Evl in mammalians, which are actin polymerization regulatory proteins that are involved in assembling and dynamics of cytoplasmic actin networks [16]. Changes in the cellular actin network play an important role in malignant transformation and tumor progression. Ena/VASP family members are localized at the tips of protruding filopodia, lamellipodia, and adhesion foci. They almost contain a conserved domain structure, including the NH2-terminal EVH1 (Ena/VASP Homology) domain, which plays an important role in the intracellular protein localization through interacting with FP4 motifs [17]. The LIM3 domain of the tumor suppressor, Tes, specifically binds to Mena EVH1 and competes with proteins containing FP4 motifs for interaction with Mena [18]. The central proline-rich region mediates interaction with proteins that contain WW and SH3 domains and with the actin monomer binding protein, profilin [4]. The COOH-terminal EVH2 domain binds to G-actin and F-actin and contains a coiled-coil, which mediates formation of stable tetramers [19]. The interaction of the EVH2 domain with the growing ends of actin filaments is essential for the targeting of Ena/VASP to lamellipodia and filopodia [20].

The Mena gene is located on chromosome 1 and encodes the 570-aa Mena protein [21]. As a member of the Ena/VASP family, Mena regulates membrane protrusion and cell movement in different cell types and contexts by influencing the geometry and assembly of actin filament networks [16, 22-25] through binding of both G-actin and F-actin, as well as profilin. Mena enhances tumor cell migration toward EGF in part by interfering with the activity of the inhibitory capping proteins and increasing actin filament elongation, thereby promoting actin polymerization [16, 26, 27]. The anti-capping activity of Mena is proposed to amplify the barbed end output of cofilin and Arp2/3 complex pathways, particularly in response to epidermal growth factor (EGF), which is important in the metastatic potential of mammary tumors [16, 22, 28].

Up-regulation of Mena has been reported in both mouse and rat invasive mammary cancer [29], which is consistent with the observation that the expression of Mena protein increased in human breast cancer cell lines and tissues [5]. Di Modugno et al. [21] showed that Mena is overexpressed in 75% of primary breast cancers, consistent with this observation, in breast cancer patients, high Mena levels are associated with poor clinical outcome [21, 30]. Similarly, over-expression of Mena is also observed in human hepatocellular carcinoma [6], pancreatic tumors [7], colorectal carcinoma [8], and cervix precursor lesions [9]. In this report, we present new evidence that the up-regulation of Mena is associated with poor prognosis in papillary thyroid carcinoma patients. Our results clearly show that papillary thyroid carcinoma lesions display higher Mena expression at the mRNA and protein levels compared with normal tissues. Therefore, we consider Mena as an important molecular marker of thyroid carcinoma that could help with precise diagnoses. However, further studies are necessary to understand the precise signaling pathways of Mena in thyroid carcinoma.

We further analyzed the relationship between Mena expression and other clinical characteristics of patients with papillary thyroid carcinoma. MENA expression was markedly associated with T Stage, capsular invasion, lymph node metastasis, and clinical stage, which strongly suggested that overexpression of Mena would be useful as an independent biomarker for the identification of subsets of papillary thyroid carcinoma patients with a more aggressive disease. Meanwhile, there were no significant correlations between the expression levels of MENA protein with patient age, gender, M stage, and calcification in patients with papillary thyroid carcinoma.

Reports have proved the prognostic value of Mena in human malignant cancers. Many researchers have determined that high Mena levels are associated with poor clinical outcome in breast cancer patients [21, 30]. However, the prognostic implication of Mena in thyroid carcinoma has not been investigated. In our study, survival analysis showed a clear negative correlation between MENA protein expression levels and the disease-free survival of pa-

pillary thyroid cancer patients. Interestingly, sub-group analysis revealed that the expression of MENA was strongly associated with DFS of the patients in both T1-2 subgroups and T3-4 subgroups. The expression of MENA was also strongly associated with DFS of the patients in both Stage I-II subgroups and Stage III-IV subgroups.

In conclusion, to the best of our knowledge this is the first report addressing Mena expression and its clinicopathological and prognostic significance in papillary thyroid cancer. Our findings suggest that Mena is up-regulated in papillary thyroid cancer and associated with T Stage, capsular invasion, lymph node metastasis, and clinical stage. Therefore, testing the expression levels of Mena protein may be helpful for stratifying thyroid cancer patients for a novel therapeutic strategy and establishing new rational treatment selection criteria for papillary thyroid cancer patients. Further investigation is still needed to investigate the molecular mechanism of Mena involvement in the development and progression of thyroid cancer.

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Disclosure of conflict of interest

None.

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