OncoTargets and Therapy

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ORIGINAL RESEARCH

DUSP4/MKP2 overexpression is associated with BRAF^{V600E} mutation and aggressive behavior of papillary thyroid cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy
15 April 2016
Number of times this article has been viewed

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Abstract: The study was performed to retrospectively analyze the correlation of dual specificity phosphatase 4 (*DUSP4*) expression with clinicopathological variables and BRAF^{V600E} mutation to better characterize the potential role of *DUSP4* as a biomarker in papillary thyroid cancer (PTC). Patients (n=120) who underwent surgery for PTC at Fudan University Shanghai Cancer Center (FUSCC) were enrolled in this study, and a validation cohort from The Cancer Genome Atlas (TCGA) database was identified to confirm the preliminary findings in our study. We investigated *DUSP4* expression at the mRNA level in PTC tissues and adjacent normal tissues using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). BRAF^{V600E} mutation analysis was also performed in PTC tissues using Sanger sequencing. Initially, we compared PTC tissues with paired normal tissues in *DUSP4* expression using Student's *t*-test, and then analyzed the correlation of *DUSP4* with clinicopathological variables and BRAF^{V600E} mutation in PTC using Mann–Whitney *U*, Kruskal–Wallis, χ^2, and Fisher's exact tests. Human-derived thyroid cell lines were also used to verify our findings. *DUSP4* was significantly overexpressed in PTC tissues compared with the adjacent normal tissues ($P<0.001$). High *DUSP4* expression showed a significant association with lymph node metastasis and extrathyroidal extension in both FUSCC and TCGA cohorts, and *DUSP4* overexpression was an independent risk factor for lymph node metastasis in multivariate analysis. Additionally, *DUSP4* expression was associated with BRAF^{V600E} mutation in both the cohorts (FUSCC: $P=0.002$, TCGA: $P<0.001$) and PTC cell lines ($P=0.023$). In conclusion, *DUSP4* was identified as a potential biomarker for aggressive behavior in PTC, and its overexpression was BRAF^{V600E} mutation-related.

Keywords: *DUSP4*/MKP2, BRAF^{V600E}, LNM, PTC

Introduction

Differentiated thyroid cancer comprises the vast majority of all thyroid cancers, which includes papillary thyroid and follicular cancer.[1] Papillary thyroid cancer (PTC) is responsible for the rapid increase in the incidence of thyroid cancer over the last few decades.[2] Though the prognosis for PTC is usually excellent with a 10-year survival rate exceeding 90%,[3] many patients suffer disease recurrence, which in some cases proves to be incurable and fatal.[4] Clinicopathological parameters such as older age at diagnosis, poor histological subtypes, extrathyroidal extension (ETE), lymph node metastasis (LNM), and advanced tumor–node–metastasis (TNM) stage are considered as prognostic factors for poor clinical outcomes. Identification of specific molecular markers for the prediction of these aggressive behaviors of PTC may assist us in evaluating disease status and prognosis for patients.

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http://dx.doi.org/10.2147/OTT.S103554

Alteration of genes encoding effectors in the mitogen-activated protein kinase (MAPK) signaling pathway is a critical characteristic of PTC.[5] MAPK phosphatases (MKPs), representing a distinct subfamily within a large group of dual-specificity protein phosphatases, act as negative regulators of MAPK activity in mammalian cells. Dual specificity phosphatase 4 (*DUSP4*), also known as MAPK phosphatase 2 (MKP2), plays a critical role as an inducible nuclear phosphatase in regulating cellular proliferation and differentiation via inactivation of ERK1/2, P38, and JNK.[6]

The behavior of *DUSP4* in carcinoma still remains a complicated puzzle. As a negative regulator of the MAPK signaling pathway via inhibition of ERK1/2, *DUSP4* represents a logical tumor suppressor. Downregulation or loss of *DUSP4* expression has been reported to be correlated with carcinogenesis in breast cancer, glioma, and lung cancer.[7–9] However, there are some studies, by contrast, suggesting that the gain of *DUSP4* expression plays a crucial role in cancer development and progression.[10–12] It is also interesting to note recent studies showing that *DUSP4* is related to drug resistance in breast cancer patients following chemotherapy.[13,14] So far, the clinical significance and biological effects of *DUSP4* have not been established in PTC. $BRAF^{V600E}$ mutation, the most common and specific gene alteration in PTC, has been considered as a poor prognostic factor for PTC.[15–17] Recently, Cagnol and Rivard[18] suggested that $BRAF^{V600E}$ mutation as well as KRAS mutation may be involved in the regulation of *DUSP4* expression in colon cancer cell lines. To better characterize the potential role of *DUSP4* as a biomarker, the present study was performed to analyze the correlation of *DUSP4* expression with the above clinicopathological variables and $BRAF^{V600E}$ mutation in PTC.

Materials and methods

Patients and clinicopathological data

Consecutive samples were selected from 120 patients of thyroidectomy diagnosed with PTC by pathology at the Fudan University Shanghai Cancer Center (FUSCC) from March 2012 to June 2012. The data on patients' clinical features including sex, age at diagnosis, maximum size of tumor, multifocality, Hashimoto's thyroiditis, histological types, ETE, and cervical LNM were retrospectively abstracted from patient records. All the patients were staged using the 2009 TNM classification of the American Joint Committee on Cancer/International Union Against Cancer.[19] The selected samples were subjected to repeated evaluation to confirm the diagnosis of the aforementioned histological characteristics. Each patient provided a written informed consent for his/her specimens and information to be used for research and stored in the hospital database; this study was approved by the Ethical Committee of FUSCC. All the procedures performed in our study were in accordance with the ethical standards of our institutional research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards. In addition, a validation cohort from The Cancer Genome Atlas (TCGA) database was identified to confirm the preliminary findings at FUSCC. A total of 499 primary PTC patients with detailed *DUSP4* expression, $BRAF^{V600E}$ mutation, and clinical data were collected from the updated TCGA database according to parameters in a previous study.[20] The TCGA cohort data were available on the website of Cancer Genomics Brower of California Santa Cruz (UCSC) (https://genome-cancer.ucsc.edu/), and the gene expression dataset and clinical data were obtained from the file named TCGA_THCA_exp_HiSeqV2_PANCAN.[21] The process of analysis of *DUSP4* in the TCGA cohort in detail is shown in Figure S1.

Human PTC cell lines and cell culture

Human-derived Nthy-ori-3-1, TPC-1, K1, and B-CPAP cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) at 37°C in a 5% CO_2 chamber. The Nthy-ori-3-1 cell line was obtained from normal follicular thyroid cells, and the other cell lines were derived from human PTC. $BRAF^{V600E}$ mutation status in the present cell lines has been reported in a previous study,[22] and the outcomes of $BRAF^{V600E}$ mutation in the cell lines were also found on the website of Cancer Cell Line Encyclopedia (http://www.broadinstitute.org/ccle/home). The B-CPAP and K1 cell lines were obtained from the cell bank at Chinese Academy of Sciences (Shanghai, People's Republic of China) and the Cancer Research Institute of FUSCC (Shanghai, People's Republic of China), respectively; the other cell lines were kindly provided by Professor Haixia Guan from China Medical University (Shenyang, People's Republic of China).

RNA extraction and real-time qRT–PCR analysis

Total RNA was extracted from the surgical specimens and cell lines using the QIAamp RNA Mini Kit (Qiagen, Chatsworth, California, USA) according to the manufacturer's instructions. The extracted RNA was reverse transcribed for cDNA, followed by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) as previously described.[23] The primers for *DUSP4* were

as follows: AGGCGGCTATGAGGTTTT (sense) and CACTGCCGAGGTAGAGGAAG (antisense). *GAPDH* was used as a housekeeping gene. qRT–PCR assays were performed in triplicates for each sample, and the mean value was used for the calculation of mRNA expression levels. The relative mRNA expression levels of *DUSP4* were determined by the comparative Ct ($2^{-\Delta Ct}$) method. The amount of target gene expression levels was given as ratios to *GAPDH* mRNA level.

DNA and BRAF^{V600E} mutation analysis

Genomic DNA was extracted from the aforementioned specimens using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA template was amplified for analysis of mutations in exon 15 of the *BRAF* gene using PCR protocol as previously mentioned,[24] followed by a Big Dye (Thermo Fisher Scientific) reaction for Sanger sequencing. BRAF^{V600E} mutation was recognized on sequencing electropherograms.

Statistical analysis

Categorical data were summarized with frequencies and percentages. The continuous results were expressed as mean ± standard deviation (SD). Paired and independent Student's *t*-tests were used to compare continuous variables in two groups. Associations between continuous variables and categorical variables were evaluated using Mann–Whitney *U*-test for two groups and Kruskal–Wallis test for more than two groups. χ^2 and Fisher's exact tests were used for categorical variables. To verify the associations between *DUSP4* and BRAF^{V600E} mutation and other characteristics, patients were divided into two subgroups (low expression and high expression) according to the median value of *DUSP4* expression at the mRNA level in each cohort. A nonparametric receiver operating characteristic (ROC) analysis was performed to calculate the best cutoff value for *DUSP4* expression level that would be predictive of LNM, using the GraphPad Prism 6.0 for Windows (La Jolla, California, USA). Moreover, univariate and multivariate analyses were performed to determine the risk factors for LNM in PTC in the FUSCC and TCGA cohorts using a logistic regression calculated by odds ratio (OR) and 95% confidence interval (CI). The area under a receiver characteristic curve was used to measure the relative predictability of independent factors for LNM. A *P*-value <0.05 was considered significant. Statistical analyses were performed using the GraphPad Prism 6.0 and SPSS for Windows (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological data of patients in the FUSCC and TCGA cohorts

A total of 120 patients (29 males and 91 females; median age: 44.54±1.20; range: 16–84 years) from the FUSCC were enrolled in this study. The maximum size of a tumor, on an average, was 1.37±0.94 cm. The incidence of LNM and ETE was 64.71% (77 of 119 PTC patients) and 9.20% (11 of 120 PTC patients), respectively. Conventional PTC comprised the majority of patients (98.30%), and the follicular-variant PTC was present in only two patients (1.70%).

A validation cohort of 499 PTC patients (136 males and 363 females; median age: 47.13±15.86; range: 15–89 years) was collected from the TCGA database. LNM was present in 226 of 449 (50.33%) patients, and ETE occurred in 153 of 482 (31.74%) patients. The ratio of classic, follicular, and tall-cell PTC types was 71.54%, 19.24% and 7.41%, respectively.

Comparison of *DUSP4* expression between PTC and adjacent normal tissues in the FUSCC cohort and in vitro outcomes

DUSP4 expression was detected in the carcinoma specimens and paired normal tissues from 120 PTC patients at FUSCC. As shown in Figure 1A, *DUSP4* mRNA expression was significantly elevated in PTC compared with the level in the adjacent normal tissues ($P<0.001$). We also detected *DUSP4* expression in human-derived Nthy-ori-3-1, TPC-1, K1, and B-CPAP cell lines. Increased *DUSP4* expression was significant in PTC-derived K1 ($P=0.036$) and B-CPAP ($P=0.042$) cell lines in comparison to Nthy-ori-3-1 cell line (Figure 1B). There was no significant difference in *DUSP4* expression between TPC-1 and Nthy-ori-3-1 cell lines (Figure 1B, $P>0.05$).

Clinical significance of *DUSP4* in PTC

The correlation of *DUSP4* with the clinicopathological characteristics of PTC patients in the TCGA and FUSCC cohorts was analyzed, as shown in Table 1. High *DUSP4* expression was associated with LNM and ETE in both the FUSCC and TCGA cohorts, showing a higher expression level in PTC with aggressive behavior. The TCGA cohort also indicated there was a significant difference in *DUSP4* expression among different TNM stages ($P<0.005$). The other characteristics including sex, age, tumor size,

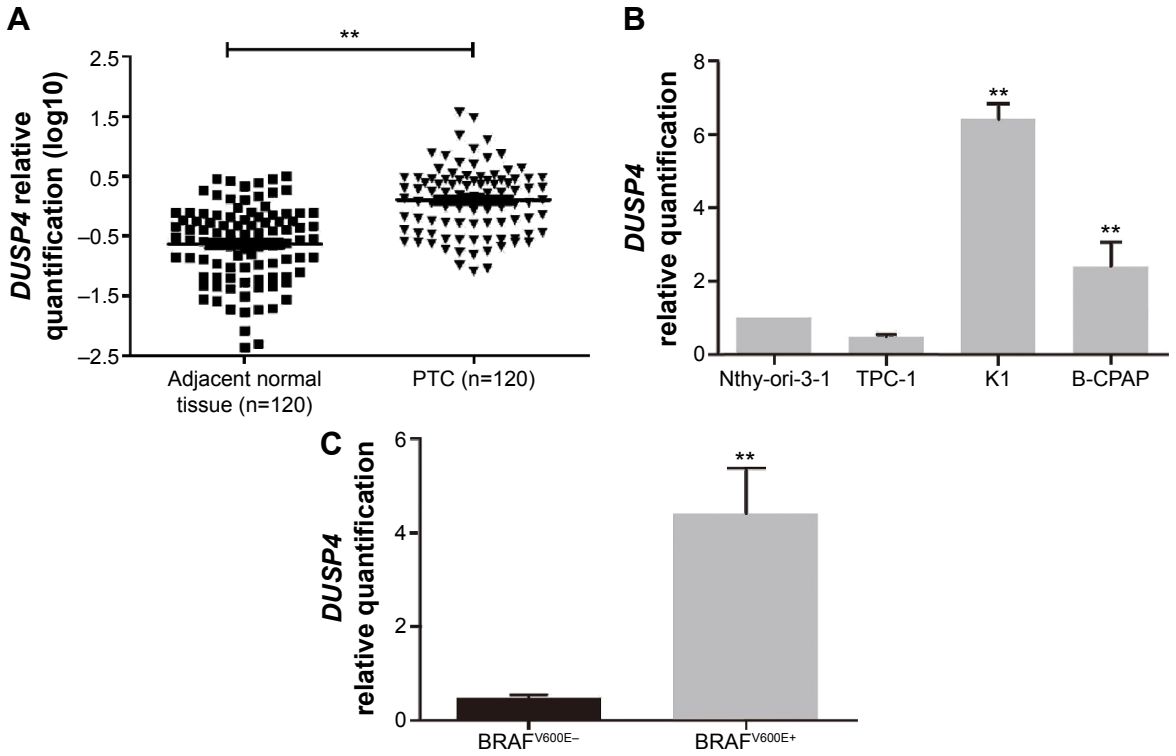

Figure 1 A comparison of *DUSP4* expression between PTC and adjacent normal tissues in the FUSCC cohort and in vitro outcomes.
Notes: (**A**) Shows Comparison of *DUSP4* expression between PTC and adjacent normal tissues. *DUSP4* mRNA expression was significantly elevated in PTC compared with the level in the adjacent normal tissues (**$P<0.001$). *DUSP4* mRNA expression was normalized for the *GAPDH* mRNA level ($2^{-\Delta Ct}$). The relative quantification of *DUSP4* expression in PTC (n=120) and adjacent normal tissues (n=120) was measured as log10 values. (**B**) Shows *DUSP4* expression in human-derived thyroid cell lines. Increased *DUSP4* expression was significant in PTC-derived K1 (**$P=0.036$) and B-CPAP (**$P=0.042$) cell lines in comparison to Nthy-ori-3-1 cell line. *DUSP4* expression difference between the TPC-1 and Nthy-ori-3-1 cell lines was not significant ($P>0.05$). The relative quantification of *DUSP4* expression in PTC cell lines was measured as folds to Nthy-ori-3-1 cell line. (**C**) Shows *DUSP4* expression in $BRAF^{V600E}$ wild-type and -mutated thyroid cell lines. A higher level expression of *DUSP4* was present in $BRAF^{V600E}$-mutated cell lines (**$P=0.023$). The relative quantification of *DUSP4* expression in $BRAF^{V600E}$-mutated cell lines was measured as folds to $BRAF^{V600E}$ wild-type cell line.
Abbreviations: *DUSP4*, dual specificity phosphatase 4; PTC, papillary thyroid cancer; FUSCC, Fudan University Shanghai Cancer Center.

multifocality, histological types, and Hashimoto's thyroiditis failed to correlate with *DUSP4* expression.

A further analysis was performed to determine whether increased *DUSP4* expression was an independent risk factor for LNM in PTC in the two cohorts. ROC analysis was used to identify the best cutoff of *DUSP4* expression levels that were predictive of LNM. Table 2 shows tumor size between 2 and 4 cm (OR =3.113, 95% CI 1.035–22.375, $P=0.045$), and *DUSP4* expression greater than the cutoff value (OR =4.064, 95% CI 1.632–10.124, $P=0.003$) was a risk factor for LNM in univariate analysis in the FUSCC cohort. In total, 29 (48.3%) of 60 patients aged ≥45 years had LNM in comparison with 48 (81.4%) of 59 patients less than 45 years, showing that age ≥45 years (OR =0.219, 95% CI 0.091–0.524, $P=0.001$) was a protective factor for LNM. After adjusting for age, tumor size, and *DUSP4*, *DUSP4* expression greater than the cutoff value (OR =4.215, 95% CI 1.565–11.347, $P=0.004$) was found to be an independent risk factor for LNM, and age ≥45 years as a protective factor remained significant (OR =0.195, 95% CI 0.076–0.501, $P=0.001$) in multivariate analysis. The TCGA cohort was analyzed to confirm the significance of *DUSP4* overexpression, which showed that *DUSP4* expression greater than the cutoff value (OR =1.741, 95% CI 1.074–2.820, $P=0.024$), age ≥45 years (OR =0.456, 95% CI 0.287–0.724, $P=0.001$), and ETE (OR =3.214, 95% CI 1.948–5.303, $P<0.001$) were independent factors for LNM in a multivariate analysis. Though $BRAF^{V600E}$ mutation and female sex were shown to be correlated with LNM in a univariate analysis, they failed to be independent factors after adjusting sex, age, ETE, $BRAF^{V600E}$, and *DUSP4* (Table 3).

Additionally, preoperative independent factors for LNM including age and *DUSP4* were evaluated to see if *DUSP4* could influence the predictive effect for LNM in the TCGA cohort. As shown in Figure 2, a comparison of predictability for LNM as measured by area under the ROC curve among age, *DUSP4*, and their combination showed that the predictive effect size for LNM increased from 0.571 to 0.643 after *DUSP4* was added ($P=0.014$). The difference in predication between age (0.571) and *DUSP4* (0.637) alone was not significant ($P=0.075$).

Table 1 Correlation between *DUSP4* expression and clinicopathological characteristics in PTC in the FUSCC and TCGA cohorts

Variables	FUSCC cohort (N=120)				TCGA cohort (N=499)			
	N	Low	High	*P*-value	N	Low	High	*P*-value
Sex				0.242				0.075
Male	29	12 (41.4%)	17 (58.6%)		136	77 (56.6%)	59 (43.4%)	
Female	91	49 (53.8%)	42 (46.2%)		363	173 (47.7%)	190 (52.3%)	
Age (years)				0.584				0.503
<45	60	29 (48.3%)	31 (51.7%)		229	111 (48.5%)	118 (51.5%)	
≥45	60	32 (53.3%)	28 (46.7%)		270	139 (51.5%)	131 (48.5%)	
Maximum size of tumor (cm)				0.733				0.250
≤2	99	51 (51.5%)	48 (48.5%)		85	50 (58.8%)	35 (41.2%)	
2–4	18	8 (44.4%)	10 (55.6%)		131	73 (55.7%)	58 (44.3%)	
>4	3	2 (66.7%)	1 (33.3%)		131	63 (48.1%)	68 (51.9%)	
Multifocality				0.458				0.225
Unifocal	91	48 (52.7%)	43 (47.3%)		263	124 (47.1%)	239 (52.9%)	
Multifocal	29	13 (44.8%)	16 (55.2%)		226	119 (52.7%)	107 (47.3%)	
Histological type				0.981				0.901
Classical PTC	118	60 (50.8%)	58 (49.2%)		357	181 (50.7%)	176 (49.3%)	
Follicular PTC	2	1 (50.0%)	1 (50.0%)		96	45 (46.9%)	51 (53.1%)	
Tall-cell PTC	–	–	–		37	19 (51.4%)	18 (48.6%)	
Other types	–	–	–		9	5 (55.6%)	4 (44.4%)	
Coexistent HT				0.886				0.058
Yes	23	12 (52.2%)	11 (47.8%)		35	12 (34.3%)	23 (65.7%)	
No	97	49 (50.5%)	48 (49.5%)		406	207 (51.0%)	199 (49.0%)	
ETE				0.023*				0.024*
Yes	11	2 (18.2%)	9 (81.8%)		153	65 (42.5%)	88 (57.5%)	
No	109	59 (54.1%)	50 (45.9%)		329	176 (53.5%)	153 (46.5%)	
LNM				0.006*				0.003*
N0	42	29 (69.0%)	13 (31.0%)		226	128 (56.6%)	98 (43.4%)	
N1	77	33 (42.9%)	44 (57.1%)		223	95 (42.6%)	128 (57.4%)	
Nx	1				50			
T stage				0.076				0.132
T1–T2	106	57 (53.8%)	49 (46.2%)		307	162 (52.8%)	145 (47.2%)	
T3–T4	14	4 (28.6%)	10 (71.4%)		192	88 (45.8%)	104 (54.2%)	
TNM stage				0.547				0.005*
I	92	50 (54.3%)	42 (45.7%)		284	148 (52.1%)	136 (47.9%)	
II	3	1 (33.3%)	2 (66.7%)		53	34 (64.2%)	19 (35.8%)	
III	16	6 (37.5%)	10 (62.5%)		108	51 (47.2%)	57 (52.8%)	
IV	9	4 (44.4%)	5 (55.6%)		54	17 (31.5%)	37 (68.5%)	
$BRAF^{V600E}$				0.002*				<0.001*
Mutation	57	21 (36.8%)	36 (63.2%)		249	93 (37.3%)	156 (62.7%)	
Wild-type	63	41 (65.1%)	22 (34.9%)		167	113 (67.7%)	54 (32.3%)	

Note: *Statistically significant.

Abbreviations: *DUSP4*, dual specificity phosphatase 4; PTC, papillary thyroid cancer; FUSCC, Fudan University Shanghai Cancer Center; TCGA, The Cancer Genomics Atlas; HT, Hashimoto's thyroiditis; ETE, extrathyroidal extension; LNM, lymph node metastasis; Nx, evaluation not available; TNM, tumor–node–metastasis.

Table 2 Clinicopathological and molecular factors associated with LNM in PTC in the FUSCC cohort

Variables	Univariate analysis			Multivariate analysis		
	P-value	OR	95% CI for OR	*P*-value	OR	95% CI for OR
Female	0.094	0.422	0.154–1.159			
Age ≥45 years	0.001#	0.219	0.091–0.524	0.001#	0.195	0.076–0.501
Tumor size (cm)						
2–4	0.045#	3.113	1.035–22.375	0.062	4.740	0.927–24.290
>4	0.841	1.283	0.112–14.702			
Multifocality	0.431	1.484	0.556–3.960			
HT	0.113	0.450	0.168–1.208			
ETE	0.120	5.311	0.646–43.664			
$BRAF^{V600E}$	0.394	1.424	0.632–3.208			
DUSP4 (greater than cutoff)*	0.003#	4.064	1.632–10.124	0.004#	4.215	1.565–11.347

Notes: #Statistically significant; **DUSP4* expression level greater than the best cutoff value for the prediction of LNM.

Abbreviations: LNM, lymph node metastasis; PTC, papillary thyroid cancer; FUSCC, Fudan University Shanghai Cancer Center; OR, odds ratio; CI, confidence interval; HT, Hashimoto's thyroiditis; ETE, extrathyroidal extension; *DUSP4*, dual specificity phosphatase 4.

Table 3 Clinicopathological and molecular factors associated with LNM in PTC in the TCGA cohort

Variables	Univariate analysis			Multivariate analysis		
	P-value	OR	95% CI for OR	*P*-value	OR	95% CI for OR
Female	0.037#	0.643	0.424–0.974	0.064	0.626	0.381–1.027
Age ≥45 years	0.010#	0.610	0.420–0.887	0.001#	0.456	0.287–0.724
Tumor size (cm)						
2–4	0.343	1.340	0.732–2.455			
>4	0.127	1.593	0.876–2.895			
Multifocality	0.107	1.362	0.935–1.982			
HT	0.058	1.987	0.976–4.043			
ETE	<0.001#	2.919	1.919–4.440	<0.001#	3.214	1.948–5.303
$BRAF^{V600E}$	<0.001#	2.168	1.415–3.321	0.076	1.549	0.955–2.511
DUSP4 (greater than cutoff)*	<0.001#	2.513	1.689–3.738	0.024#	1.741	1.074–2.820

Notes: #Statistically significant; **DUSP4* expression level greater than the best cutoff value for the prediction of LNM.
Abbreviations: LNM, lymph node metastasis; PTC, papillary thyroid cancer; TCGA, The Cancer Genomics Atlas; OR, odds ratio; CI, confidence interval; HT, Hashimoto's thyroiditis; ETE, extrathyroidal extension; *DUSP4*, dual specificity phosphatase 4.

Relationship between *DUSP4* and $BRAF^{V600E}$ mutation in PTC

The positive rates of $BRAF^{V600E}$ mutation in the FUSCC and TCGA cohorts were 47.50% (57/120) and 59.86% (249/416), respectively. *DUSP4* expression was associated with the $BRAF^{V600E}$ mutation status in PTC in the two cohorts (FUSCC: P=0.002, TCGA: P<0.001), showing a higher expression level in $BRAF^{V600E}$-mutated PTC than the level in $BRAF^{V600E}$ wild-type PTC (Table 1). *DUSP4* expression analysis was also performed in the $BRAF^{V600E}$ wild-type cell line (TPC-1) and $BRAF^{V600E}$-mutated cell lines (K1 and B-CPAP). As shown in Figure 1C, a higher level of *DUSP4* expression was present in $BRAF^{V600E}$-mutated cell lines (P=0.023). A multivariate logistic regression analysis was performed to investigate factors that could affect *DUSP4* expression in both the FUSCC and TCGA cohorts, and the outcomes indicated that $BRAF^{V600E}$ mutation was an independent risk factor with strong effect size (FUSCC: OR =3.366, 95% CI 1.377–8.228, P=0.008; TCGA: OR =2.794, 95% CI 1.753–4.452, P<0.001, Table 4).

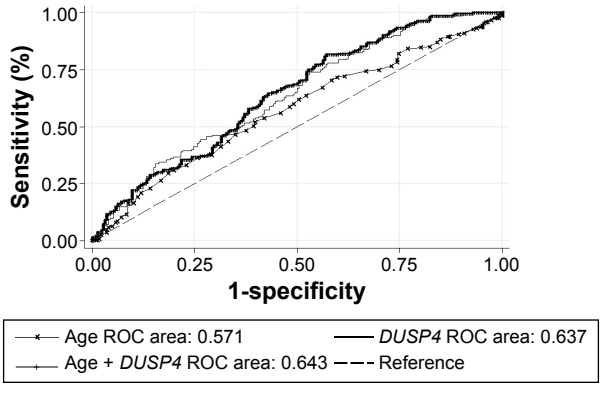

Figure 2 A comparison of predictability for LNM as measured by area under the ROC curve among age, *DUSP4*, and their combination in the TCGA cohort.
Notes: The predictive effect size for LNM increased from 0.571 to 0.643 after *DUSP4* was added (P=0.014). The difference in predication between age (0.571) and *DUSP4* (0.637) alone was not significant (P=0.075).
Abbreviations: ROC, receiver operating characteristic; *DUSP4*, dual specificity phosphatase 4; LNM, lymph node metastasis; TCGA, The Cancer Genome Atlas.

Discussion

DUSP4 is a mitogen- and stress-inducible nuclear MKP, which displays a substrate preference for ERK, p38, and JNK; it actually binds to ERK and p38 with higher affinity than to JNK.[25] Several authors have shown in studies of the ERK pathway how *DUSP4* may cooperate to regulate MAPK signaling. Caunt et al[26] indicated that *DUSP4* is employed in a sustained MEK stimulus-specific manner and regulates nuclear dephosphorylation and accumulation of ERK in loss-of-functional experiments using siRNA knockdown of MKPs. Cagnol and Rivard[18] reported that *DUSP4* is induced in response to oncogenic activation of the ERK pathway in colon-derived cancer cells, which correlates with nuclear accumulation of dephosphorylated ERK, and revealed it as a potential regulator of ERK-driven tumor cell proliferation. The correlation between *DUSP4* and cancer progression has been established regardless of the loss or gain of *DUSP4* expression in other cancer types. Saigusa et al[23] showed that decreased *DUSP4* expression is associated with tumor progression, especially distant metastasis, in colorectal cancer. Balko et al[27] reported that the activation of the MAPK pathway due to *DUSP4* loss promotes cancer stem cell-like phenotype in basal-like breast cancer. By contrast, Kim et al[12] suggested that *DUSP4* is frequently upregulated in breast malignancy and may be a marker of adverse prognosis. The effects of *DUSP4* in cancer progression still remain a puzzle. There

Table 4 Multivariate analysis of factors that could affect *DUSP4* expression in PTC in the FUSCC and TCGA cohorts

Variables	FUSCC			TCGA		
	P-value	OR	95% CI for OR	*P*-value	OR	95% CI for OR
Female	0.337	0.485	0.111–2.126	0.066	1.589	0.970–2.602
Age ≥45 years	0.533	0.727	0.267–1.980	0.012*	0.399	0.195–0.816
ETE	0.069	4.768	0.883–25.743	0.867	0.956	0.566–1.616
LNM	0.417	1.714	0.467–6.292	0.555	1.163	0.704–1.921
TNM stage	0.276	1.480	0.731–2.999	0.007*	1.632	1.147–2.323
$BRAF^{V600E}$	0.008*	3.366	1.377–8.228	<0.001*	2.794	1.753–4.452

Note: *Statistically significant.

Abbreviations: *DUSP4*, dual specificity phosphatase 4; PTC, papillary thyroid cancer; FUSCC, Fudan University Shanghai Cancer Center; TCGA, The Cancer Genomics Atlas; OR, odds ratio; CI, confidence interval; ETE, extrathyroidal extension; LNM, lymph node metastasis; TNM, tumor–node–metastasis.

is no adequate evidence and research report regarding the role of *DUSP4* in PTC.

In the present study, *DUSP4* expression was significantly elevated in PTC tissues compared with adjacent normal tissues, which was consistent with the findings in previous studies.[5,28] In addition, our report has suggested for the first time that increased *DUSP4* expression is associated with aggressive behavior of PTC such as LNM and ETE. It was also indicated in the TCGA cohort that high *DUSP4* expression correlates with advanced TNM stage. In the FUSCC cohort, *DUSP4* expression was high in 15 (60.0%) of 25 patients in the stage III and stage IV compared with 44 (46.3%) of 95 patients in the stage I and stage II, but the outcome failed to be statistically significant. The effect size could be affected by the limited sample size and big difference in sample distribution from stage I to stage IV in our cohort.

LNM is a critical parameter for physicians in determining whether performing lymph node dissection and providing the following I^{131} therapy, and it also assists in evaluating the regional recurrence and prognosis for PTC patients. Cervical LNM is very common in PTC, and the sensitivity of preoperative detection of LNM by ultrasound imaging and computerized tomography is relatively low.[29] Though the presence of $BRAF^{V600E}$ mutation identifies a part of PTC patients with LNM, the $BRAF^{V600E}$ mutation status taken in isolation cannot specifically identify PTC with extrathyroidal spread.[30] Therefore, the identification of specific predictors for LNM in PTC is crucial for surgeons to increase preoperative sensitivity of LNM detection. We performed a further analysis of LNM risk factors. *DUSP4* overexpression was confirmed to be an independent risk factor for LNM in PTC, and *DUSP4* combined with age could improve predictive value for LNM. The abovementioned outcomes may support that *DUSP4* plays a positive role in promoting the development and progression of PTC.

Moreover, our findings also showed that $BRAF^{V600E}$ mutation significantly correlated with increased *DUSP4* expression. Oncogenic RAS and $BRAF^{V600E}$ mutations have been reported to activate the MEK/ERK pathway, resulting in *DUSP4* expression upregulation and further ERK1/2 inhibition in colorectal cancer cells.[18] A previous study also suggested that $BRAF^{V600E}$ mutation can drive intensive downstream signaling of the MAPK pathway via blocking a feedback loop from ERK to BRAF, but actually $BRAF^{V600E}$-mutated tumor cells do not have higher levels of ERK activity than cells of other ERK-dependent tumor types.[31] A promising explanation suggests that $BRAF^{V600E}$-mutated tumors are likely to have higher levels of MKPs, which could to some extent compensate for the loss of ERK to BRAF feedback.[31] Integrated genomic characterization of PTC[5] suggests that PTC could be divided into two subgroups based on molecular landscape: $BRAF^{V600E}$-variant-like PTC and RAS-variant-like PTC; the overexpression of *DUSP4*, 5, and 6 is relatively exclusive for $BRAF^{V600E}$-variant-like PTCs, which is consistent with our findings. These findings may reveal that *DUSP4* is induced by the $BRAF^{V600E}$ mutation-activated MAPK pathway in PTC.

Despite an observation of the role of *DUSP4* overexpression in PTC in this study, the mechanism of the effects of *DUSP4* has not yet been elucidated. According to the study by Cancer Genome Atlas Research Network, *DUSP4* overexpression is interpreted to represent the high output of the ERK transcriptional program caused by robust activation of MAPK pathway signaling in $BRAF^{V600E}$-mutated PTC cases.[5] Hasegawa et al[32] found that the inhibition of *DUSP4* attenuates the in vitro and in vivo proliferation of thyroid cancer cells from transgenic mice, which is mediated by the suppression of cyclin B1 expression. Furthermore, Lee et al[33] confirmed elevated expression of *DUSP4* in thyroid cancer tissues, and their observation on the methylation status of *DUSP4* suggests that this tumor-suppressor gene does not

actually suppress tumor growth. The logical interpretations here may be that elevated expression of *DUSP4* in PTC enables it to play a positive role in tumor progression rather than a suppressor in a manner dependent on the upstream oncogenic mutation.

Conclusion

In conclusion, we identify *DUSP4* as a potential biomarker for aggressive behavior, especially for LNM, in PTC and that its overexpression is $BRAF^{V600E}$ mutation-related.

Acknowledgment

The research was supported by the Science and Technology Commission of Shanghai Municipality (14ZR1407300).

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

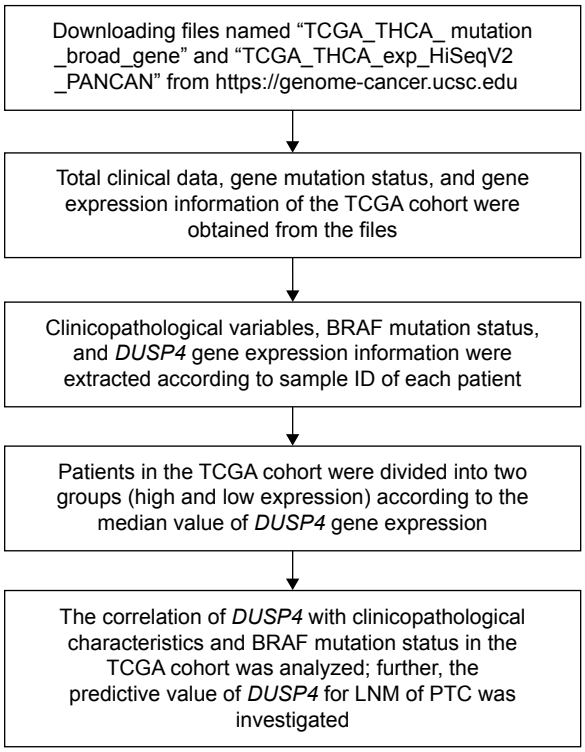

Figure S1 A flow graph of analysis of *DUSP4* expression in the TCGA cohort.
Abbreviations: TCGA, The Cancer Genome Atlas; *DUSP4*, dual specificity phosphatase 4; LNM, lymph node metastasis; PTC, papillary thyroid cancer.