Loss of *CPSF2* Expression Is Associated with Increased Thyroid Cancer Cellular Invasion and Cancer Stem Cell Population, and More Aggressive Disease

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Purpose: Identification of molecular factors that promote thyroid cancer progression have important clinical implications for therapy and prognostication in patients with papillary thyroid cancer (PTC). The aim of this study was to validate and determine the function of dysregulated genes that were associated increased mortality in patients with PTC.

Experiemental Design: We selected the cleavage and polyadenylation specificity factor subunit 2 (*CPSF2*) gene from the top 5 significantly dysregulated genes associated with PTC-associated mortality from our previous study. We used 86 PTC samples enriched for aggressive disease (recurrence and mortality) by quantitative RT-PCR (qRT-PCR). *In vitro* functional studies of the validated gene were performed.

Results: Decreased *CPSF2* gene expression was associated with shorter disease-free survival ($P = .03$), large tumor size (T3 and T4) ($P = .03$), tumor recurrence ($P < .01$), and mortality ($P < .01$), independent of *BRAF* V600E mutation status. *CPSF2* knockdown increased cellular invasion by 1.8- to 3.2-fold ($P < .01$) and increased markers of thyroid cancer stem cells (CD44 and CD133 expression). Immunohistochemistry showed an inverse correlation between CD44 protein expression in PTC samples and CPSF2 expression.

Conclusion: Decreased *CPSF2* expression is associated with increased cellular invasion and cancer stem cell population, and more aggressive disease in PTC. **(*J Clin Endocrinol Metab* 99: E1173–E1182, 2014)**

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for more than 80% of all thyroid cancer cases. Although most patients with PTC have an excellent prognosis, mortality associated with differentiated thyroid cancer occurs in approximately 1–2% of all cases (1). Given the increasing incidence of thyroid cancer (2), it is essential to preoperatively identify patients with PTC who might have an unfavorable oncological outcome and would likely benefit from more aggressive intervention. Furthermore, there has been an increase in thyroid cancer mortality in US with 1850 estimated deaths in 2013 (3). To tailor a treatment specific to individual tumor behavior, the identification of accurate preoperative diagnostic and prognostic molecular markers for PTC is critically important because most patients have low-risk tumors and risk being overtreated.

BRAF mutations are the most common genetic events in thyroid cancer and have been reported by some, but not all, investigators to be associated with more aggressive disease and increased mortality (4). The overall prevalence of *BRAF* mutations in PTC is approximately 45%, and almost all are point mutations in exon 15, resulting in a valine-to-glutamate transversion (V600E) (5–7). However, relatively high *BRAF* mutation rates have been reported (up to 90%) in PTC, and these rates may be higher in recently diagnosed cases, thus making it unclear

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Abbreviations: AMES, Age, Metastasis, Extent and Size; APA, alternative polyadenylation; EMT, epithelial-mesenchymal transition; FTC, follicular thyroid cancer; GWE, genome-wide expression; PTC, papillary thyroid cancer.

whether BRAF mutation testing would be an accurate prognostic marker (8). The identification of other molecular prognostic markers that may be used alone or in conjunction with *BRAF* mutation analysis not only could improve patient risk stratification and treatment strategy, but also could be explored for targeted therapy.

The objective of this study was to study the biological significance of previously identified dysregulated gene(s) associated with mortality in patients with PTC and to assess the function of the dysregulated gene(s) in thyroid cancer cells (9). Previously, we performed a genome-wide expression (GWE) analysis in PTC samples from cases with known clinicopathological data and outcomes and identified five genes (*CPSF2*, *LARS*, *AURKC*, *TRNT1*, and *BCL11A*) that were significantly differentially expressed in patients with PTC-associated mortality (9). We found *CPSF2* expression to be lower in tumor samples from patients with PTC-associated mortality. Because the role of *CPSF2* in cancer is unknown, we performed in vitro functional studies to determine the functions of *CPSF2* in cellular invasion, epithelial-mesenchymal transition, and markers of cancer stem-like cells.

Materials and Methods

The study was approved by the Office of Human Research Protections, US Department of Health and Human Services and Institutional Review Board at the National Institutes of Health. All research participants provided written informed consent.

Patients and tissue samples

Eighty-six primary PTC samples (60 samples for the initial analyses and 26 additional independent samples), 8 multinodular goiter, and 26 normal thyroid tissue samples were procured at the time of initial surgery and snap-frozen immediately after tumor removal. All tissue samples were reviewed by an endocrine pathologist to confirm the diagnosis. PTC samples containing more than 80% tumor cells were used. Patient demographics and clinical and pathological information were collected prospectively with annual patient follow-up. *BRAF* mutation testing in exon 15 was performed by direct sequencing in 77 PTC samples. The clinical characteristics of patients with PTC are summarized in Table 1.

In addition, paraffin blocks containing thyroid tissue from 12 PTC patients, 6 containing normal thyroid tissue, and 6 containing tissue from patients with locally invasive (T3 and T4) PTC were used for immunohistochemistry staining for CPSF2 and CD44.

Validation of selected genes from the GWE analysis

We selected *CPSF2*, which had the highest predictive score among 5 top-scored significantly differentially expressed genes by mortality (*CPSF2*, *LARS*, *AURKC*, *TRNT1*, and *BCL11A*) (9) for additional testing. We validated *CPSF2* expression by TaqMan Quantitative real-time PCR (qRT-PCR) using RNA from 26 additional and independent PTC samples. Six of these independent PTC 26 samples were derived from patients with PTC-associated mortality (Table 1).

Because there was no significant difference in *CPSF2* expression between normal thyroid tissue and multinodular goiter samples, *CPSF2* expressions in pooled benign and normal thyroid tissue samples (n = 34) were compared with combined PTC samples (n = 86).

The methods for RNA extraction and qRT-PCR used in this study were as previously described (9). The relative gene expression levels from the initial GWE samples and the additional independent samples were used to assess the prognostic significance of each gene.

To evaluate the roles of the validated gene as predictors of adverse features and outcomes, we combined the normalized mRNA expression levels of all samples (n = 86). The PCR amplification efficiency was adjusted by running samples from both the GWE group and the independent samples in the same plate. We compared mRNA expression level of the validated gene

Table 1. Clinical Characteristics of Study Cohort Patients with PTC.

Patient Characteristics	Initial Cohort (n = 60)	Independent Cohort (n = 26)	*P* Value
Median age at diagnosis	41 y	43 y	.47
Male	39.0%	18.5%	.08
Tumor size, median±SD	2.2 ± 3.1 cm	2.0 ± 2.33 cm	.17
Tumor size ≥ 4 cm	19.0%	0%	**.01**[a]
Extrathyroidal extension	36.8%	16.7%	.11
Tumor multifocality	3.6%	4.3%	1.0
Lymph node metastasis	57.4%	70.4%	.33
Synchronous distant metastasis	4.1%	4.0%	1.0
High-risk AMES score[b]	48.0%	16.7%	**.01**
BRAF V600E mutation	48.0%	55.6%	.63
PTC^{B}-associated mortality	15.3%	25.9%	.25
Tumor recurrence	34.5%	44.4%	.47
Disease-free interval, median±SD	50.0 ± 37 mo	77 ± 39 mo	.12

[a] Bold number indicates significant result.

[b] AMES score denotes all patients with distant metastasis, extrathyroidal extension, and primary tumor size >5 cm in older patients (men>40 and women >50 y).

among patients with and without unfavorable prognostic features (advanced T stage, initial lymph node and synchronous distant metastasis, extrathyroidal extension, tumor differentiation, high-risk Age, Metastasis, Extent and Size (AMES) classification, disease recurrence, and mortality). The correlation between disease-free survival and mRNA expression level was analyzed.

In vitro studies in thyroid cancer cell lines

Human PTC (TPC-1), follicular thyroid cancer (FTC-133), and Hürthle cell cancer (XTC-1) cell lines were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), Fungizone (250 ng/mL), and insulin (10 μg/mL) in a humidified 5% CO_2 atmosphere at 37°C. Cells were subcultured twice weekly. A human anaplastic thyroid cancer cell line (8505C) was maintained in Eagle's minimum essential medium (MEM) containing Hank's balanced salt solution (MEM with HBSS) supplemented with 2 mM glutamine, 1% nonessential amino acids, and 10% fetal bovine serum. The TPC-1 cell line was provided by Dr Nabuo Satoh (Japan), the FTC-133 cell line by Dr Peter Goretzki (Germany), the XTC-1 cell line by Dr Orlo H. Clark (California), and the 8505C cell line was purchased from the European Collection of Cell Cultures (Porton Down, UK).

To assess the association between *CPSF2* and *BRAF* in 8505C cells (*BRAF* V600E mutant), 8505C cells were treated with 5 μM and 7.5 μM of vemurafenib (PLX4032), a selective *BRAF*(V600E) inhibitor, for 24 and 48 h.

Whole-cell lysate was then obtained for Western blot analysis. All cell lines were characterized by short tandem repeat profiling and the authenticity of all cell lines was confirmed. All experiments were performed using cells at 25 passages or fewer.

Small interfering RNA (siRNA) transfection

Three siRNAs for CPSF2 (Silencer Select siRNA, part numbers: s28827, s28828, s28829) and a scrambled negative control (Silencer Negative Control No. 1) were purchased from Applied Biosystems (Invitrogen Corp). TPC-1, FTC-133, and XTC-1 cells were transfected with each siRNA at a final concentration of 90 nM. The transfection reagent was Lipofectamine RNAiMAX (Invitrogen Corp) diluted in Opti-MEM I Reduced Serum Medium (Invitrogen Corp) according to the manufacturer's recommendation. Two siRNAs (s28827 and s28828) showed superior knock-down efficiency by qRT-PCR and were used in subsequent experiments. After 48 h, the medium was changed to maintenance culture medium and was replaced every 2 days.

Cell invasion assay

Five days after TPC-1, FTC-133, and XTC-1 were transfected with CPSF2 siRNAs (s28827 and s28828), the cells were trypsinized, counted, and resuspended in serum-free culture media. Cellular invasion assays were performed as previously described (10). Five$\times 10^4$ cells per well were used for all cell lines. We counted all invading cells in inserts with Matrigel. Cells in control inserts were counted in 4 fields. Percentage of invasion was calculated by the number of cells in the Matrigel insert divided by the number of cells in the control insert. The experiment was repeated at least twice.

Western blot analysis

Whole-cell lysate was prepared with heated 1% SDS plus 10 mM Tris (pH 7.5) buffer. The protein concentration was determined using the BCA Protein Assay kit (Thermo Scientific). Protein samples were separated a in 4–12% SDS-PAGE (Novex NuPAGE SDS-PAGE, Life Technologies Corporation) and transferred onto a polyvinyldifluoride membrane using a dry transfer method (iBlot Dry Blotting System, Invitrogen Corp). Western blotting was performed following standard procedures using the following mouse monoclonal primary antibodies: anti-CPSF2 (SC-165983, Santa Cruz Biotechnology, Inc), 1:200 dilution; anti-CD44, 1:1000 dilution (No. 3570, Cell Signaling Technology, Inc); antivimentin 1:200 (ab28028, Abcam); and anti-glyceraldehyde-3-phosphate dehydrogenase, 1:15,000 dilution (sc-47724, Santa Cruz Biotechnology, Inc). Also used were the following rabbit monoclonal antibodies: anti-N-cadherin, 1:500 (04–1126, EMD Millipore) and antiphospho MEK1/2 (Ser217/221), 1:1000 dilution (No. 9121, Cell Signaling Technology, Inc). Signal detection was performed using an HRP-conjugated secondary antibody (SC-2005, Santa Cruz Biotechnology, Inc) at a 1:5000 dilution and the SuperSignal West Pico and SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Immunohistochemistry staining

Tissues were formalin-fixed, embedded in paraffin, and cut into 5-μm-thick sections for hematoxylin and eosin (H&E) and immunostaining. Sections were deparaffinized and rehydrated in graded alcohol. Sections were then placed in ×1 citrate buffer and epitope retrieval was performed using pressurized steam for 10 minutes at 120°C. The primary antibodies were anti-CPSF2 mouse monoclonal antibody, 1:200 dilution (SC-165983, Santa Cruz Biotechnology, Inc), and anti-CD44 mouse monoclonal antibody, 1:50 dilution (No. 3570, Cell Signaling Technology, Inc). The sections were incubated at 4°C overnight followed by incubation with a biotinylated secondary antibody, 1:150 dilution (Vector Laboratories) for 1 h at room temperature. The sections were developed using 3,3-diaminobenzidine as the chromogen (Elite ABC Kit, Vector Laboratories) and hematoxylin as the counterstain. The sections were dehydrated and mounted with VectaMount mounting medium (Vector Laboratories). The slides were viewed under an Olympus BX41 light microscope (Olympus Corporation of the Americas) and photographs were taken at ×20 magnification. A semiquantitative scoring system was used to analyze CPSF2 protein expression. The expression level in nuclei was classified as no or weak staining (0), moderate intensity staining (1), and strong intensity staining (2). Two observers, who were blinded to the tumor type, independently scored each sample. The scores were averaged to obtain the final CPSF2 expression score.

Flow cytometry

Six days after TPC-1, FTC-133 were transfected with *CPSF2* siRNAs (s28827 and s28828), the cells were trypsinized, resuspended in ×1 phosphate buffered saline, and fixed in 2% paraformaldehyde in PBS for 10 minutes at 37°C. Chilled 90% methanol was used in cell permeabilizion. Aliquoted cells were resuspended and blocked in 0.5% bovine serum albumin. Mouse monoclonal primary antibodies were anti-CD44, 1:800 dilution (No. 3570, Cell Signaling Technology, Inc) and anti-CD133, 1:400 dilution (No. MAB4399, EMD Millipore). Secondary an-

tibodies were Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Antibody, 1: 250 dilution and Alexa Fluor 594 Goat Antirabbit IgG (H+L) Antibody, 1:250 dilution (Life Technologies Corporation). A total of 20 000 cells were examined in a fluorescence-activated cell sorting system (FACS) using BD FACSCanto flow cytometer (Becton-Dickinson), and data were analyzed using FlowJo version 8.8.6 software.

Analysis of publicly available gene expression PTC datasets

We accessed the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and identified publicly available thyroid cancer datasets (GSE6004), which comprise gene expression data of 18 samples from seven patients with stage T4 PTC provided by Vasko et al. All samples were microdissected by two experienced thyroid pathologists from frozen intratumoral tissue samples procured intraoperatively. The final diagnosis was confirmed by hematoxylin-eosin staining. Paired samples were taken from the central part of the cancer (n = 7), from an intratumoral part of the invasive region close to the leading edge (n = 7), and from histologically normal tissue (n = 4). On histological examination, more than 90% of the cells were thyroid cancer epithelial cells. Affymetrix Human Genome U133 Plus 2.0 Arrays were used (11). We compared the expression levels of *CPSF2* of 7 invasive regions to those of central part of tumors from the same patients. Data were analyzed using GEO2R software available from the web site.

Statistical analyses

The *CPSF2* expression levels between groups were compared using the Mann-Whitney *U* test. The 2-tailed Pearson correlation test was used to determine the correlation between *CPSF2* expression levels and disease-free intervals. Kaplan-Meier analyses and log-rank tests were performed to compare disease-free survival to *CPSF2* expression level.

Patient characteristics within the initial screening cohort and the validating cohort were compared using Fisher's exact test and the Mann-Whitney *U* test for categorical and nonparametric continuous data, respectively. All statistical analyses were performed using SPSS for Windows version 16.0 (IBM Corp).

Results

Lower *CPSF2* expression is associated with adverse prognostic features in PTC

We validated the association of *CPSF2* expression and mortality status in 26 independent PTC samples that showed a significantly lower expression in samples from patients with PTC-associated mortality ($P = .027$) (Figure 1A) Although there were no significantly differentially expressed genes found in other adverse prognostic features in previous microarray analysis, we were interested in determining whether there were any associations between lower *CPSF2* expression and other clinical features that could be associated with mortality in a larger sample size. Indeed, *CPSF2* mRNA expression levels in all samples (n = 86) were significantly lower in patients with more advanced T stage (T3 and T4) ($P = .03$) (Figure 1B), tumor recurrence ($P < .01$) (Figure 1C), and PTC-associated mortality ($P < .01$) (Figure 1D). The *CPSF2* mRNA expression levels was associated with disease-free interval ($P = .03$) (Figure 1E). Patients whose *CPSF2* mRNA expression in tumor samples (n = 86) measured below the mean *CPSF2* mRNA expression of the entire cohort had significantly lower survival rates ($P = .046$) (Figure 1F).

We next compared *CPSF2* expression in normal, benign thyroid tumors and PTC to determine whether its expression was only altered in PTC or in noncancerous, proliferative thyroid lesions. *CPSF2* expression was significantly higher in PTC tissue samples than in normal thyroid tissue ($P = .03$) or benign multinodular goiter samples ($P = .001$) (Supplemental Figure 1).

Semiquantitative CPSF2 protein expression analysis by immunohistochemistry showed lower expression in advanced T stage (T3 and T4) tumors compared to lower T stage (T1 and T2) tumors ($P = .03$) (Figure 2). There were also trends toward lower CPSF2 expression in PTC samples with extrathyroidal extension ($P = .07$) and lymph node metastasis ($P = .09$).There was no difference in *CPSF2* mRNA expression levels (n = 86) by sex ($P = .59$), tumor differentiation ($P = .61$), tumor multifocality ($P = .52$), or synchronous distant metastasis ($P = .96$), AMES score ($P = .23$), or *BRAF* V600E mutation status ($P = .1$). No significant association between *CPSF2* expression and tumor size was observed ($P = .13$).

We did not find any associations between the presence of *BRAF* V600E mutation and age ($P = .3$), sex ($P = .6$), advanced T stage (T3 and T4) ($P = .7$), multifocality ($P = .6$), extrathyroidal extension ($P = 1.0$), synchronous distant metastasis ($P = .6$), tumor recurrence ($P = .8$), or PTC-associated mortality ($P = .2$). There was a trend toward higher rate of lymph node metastasis in PTC with *BRAF* V600E mutation (59% vs 41%, $P = .06$).

Because we found a significantly lower protein expression of CPSF2 in patients with advanced T stage (T3 and T4) as well as a trend toward extrathyroidal extension, we were interested in determining whether there was a difference in the invasive area vs the central portion of PTC. We analyzed a publicly available gene expression dataset for 14 paired samples derived from invasive and central areas of tumors from seven patients with stage T4 PTC. We found lower *CPSF2* expression levels in the invasive area, with average log fold change of 0.11 but this difference was not statistically significant. Five of seven tumors (71.4%) had lower *CPSF2* expression in the invasive area compared with the center (Supplemental Figure 2).

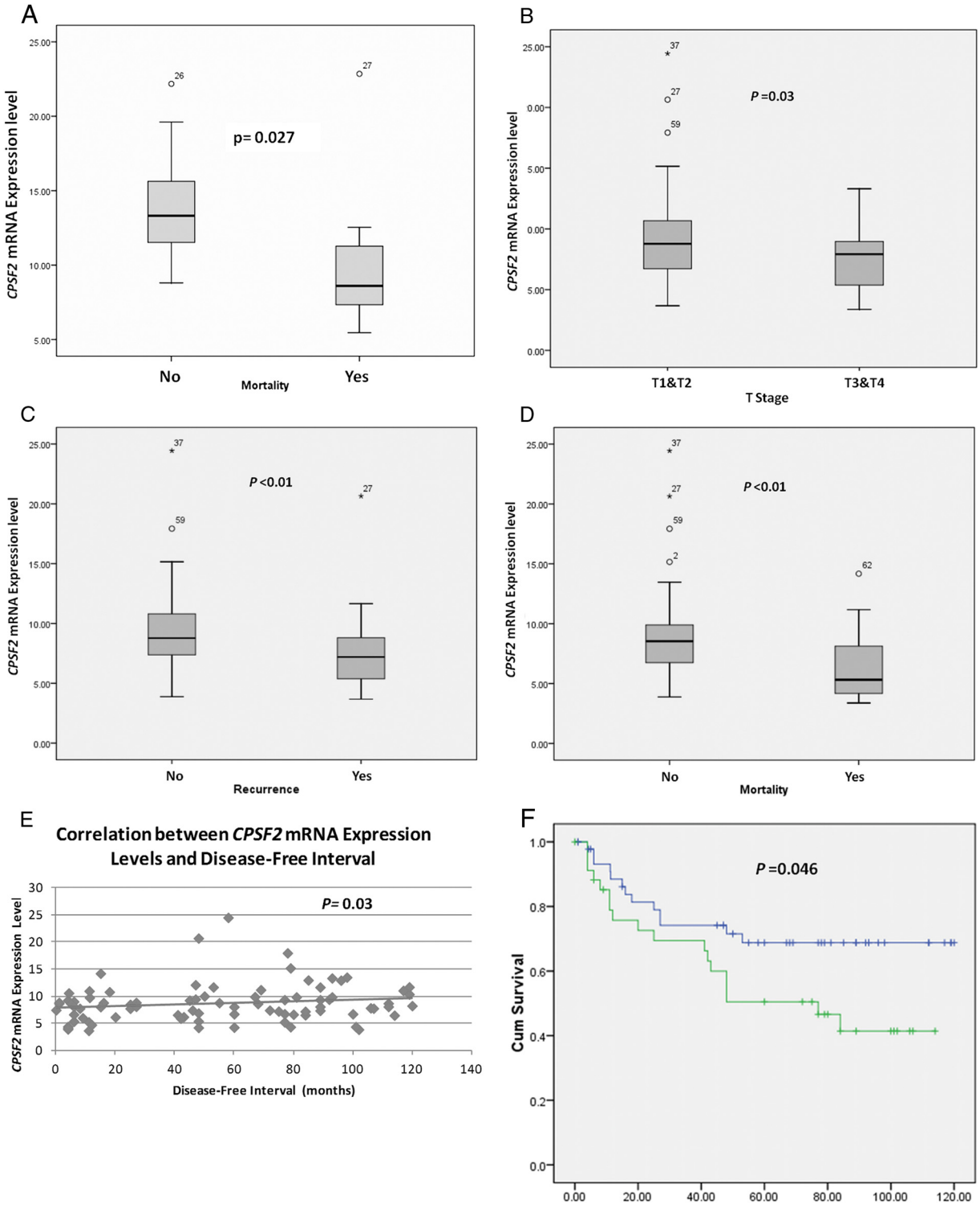

Figure 1. *CPSF2* mRNA expression was significantly lower in PTC-associated mortality using additional independent samples (n = 26) (A) *CPSF2* mRNA expression levels of combined samples (n = 86) by clinical characteristics. Significantly lower *CPSF2* mRNA expression in PTC with advanced T stage (T3 and T4) (B), tumor recurrence (C), mortality (D). Outliers are shown in small circles and stars. Y axis indicates the mRNA expression levels and X axis indicates adverse clinical status of patients with PTC. There was a significant correlation between *CPSF2* mRNA expression levels and disease-free interval (E) and a significantly shorter disease-free survival was observed in patients with lower *CPSF2* mRNA expression than average expression of the cohort, shown in green graph. Blue graph represents disease-free survival of patients with *CPSF2* mRNA expression above than average expression of the cohort. Y-axis represents cumulative survival and X-axis represents disease-free interval in months (F).

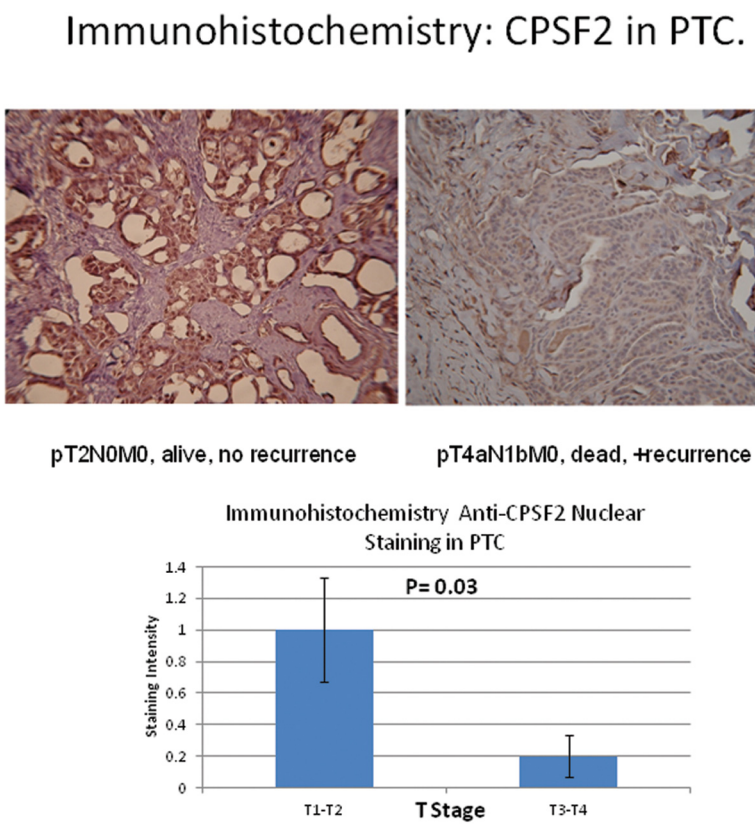

Figure 2. Immunohistochemistry stain of PTC samples showed a significantly lower expression of CPSF2 protein in PTC at advanced T stage (T3 and T4) than early T stage (T1 and T2). Pathological TNM (Tumor, Node, Metastasis) cancer staging system was used. Error bars indicate SEM.

CPSF2 knockdown increases cellular invasion and stem cells population, and is not a target gene of BRAF activation

We evaluated basal expression of *CPSF2* in 4 thyroid cancer cell lines (TPC-1, FTC-133, XTC-1, and 8505C) by qRT-PCR and Western blot analysis and found it to be expressed in all 4 cell lines. The mRNA expression levels were similar among the cell lines, with a ΔC_t ranging from 3.5–4.6 relative to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. There was good knockdown of CPSF2 protein expression with both siRNAs (s28827 and s28828) for at least 11 days (Supplemental Figure 3).

CPSF2 knockdown was associated with increased cellular invasion in all 3 cell lines. Specifically, we found an increase in the number of invading cells when TPC-1 cells were treated with siRNAs s28827 (2.43-fold, $P < .01$) and s28828 (3.25-fold, $P < .01$). *CPSF2* knockdown using both siRNAs also resulted in a significantly larger number of invading cells in the FTC-133 and XTC-1 cell lines (FTC-133, 1.8- and 2.1-fold; $P < .01$; XTC-1, 1.8- and 2.1-fold; $P < .01$ for s28827 and s28828, respectively) (Figure 3).

Because patients with recurrent PTC and PTC-associated mortality had significantly lower *CPSF2* expression in their tumors, we hypothesized that lower *CPSF2* expression was associated with increased thyroid cancer stem-like cell population, which can result in tumor recurrence and metastasis. Thus, we evaluated the effect of *CPSF2* knockdown on CD44 and CD133 expression because these 2 cell surface proteins have previously been reported as markers of thyroid cancer stem-like cells (12–14).

We used Western blot analysis to assess CD44 protein expression in TPC-1 and FTC-133 cells with and without CPSF2 knockdown and found increased CD44 protein expression with knockdown (Figure 4A). We also observed increased CD44- and CD133-positive TPC-1 cells (CD44, negative control, 2.2%; s28827, 8.7%; and s28828, 24.3%. CD133, negative control, 3.3%; s28827, 12.5%; and s28828, 6.2%) and FTC-133 cells (CD44, negative control, 2.1%; s28827, 11.1%; and s28828, 15.4%. CD133, negative control, 2.5%; s28827, 17%; and s28828, 29%) with *CPSF2* knockdown (Figure 4B). Furthermore, we found more CD44-positive cells in locally advanced PTC samples with low CPSF2 expression by immunohistochemistry. There were also no CD44-positive PTC samples in samples with high CPSF2 expression by immunohistochemistry (Figure 4C).

Because we observed increased cellular invasion and increased CD44 expression with CPSF2 knockdown in the thyroid cancer cells, which have been associated with epithelial-mesenchymal transition (EMT) (15), we evaluated the effect of CPSF2 knockdown on EMT markers (vimentin, N-cadherin, and integrin alpha 3). However, using Western blot analysis, we found no difference in vimentin, N-cadherin, or integrin alpha 3 protein expression with CPSF 2 knockdown.

Because of the high prevalence of *BRAF* mutation in PTC, which has been shown to be associated with aggressive disease, we were interested in determining whether *CPSF2* regulated BRAF or was a downstream target of BRAF. To assess whether *CPSF2* was a downstream target, we treated *BRAF* V600E mutant thyroid cancer cells (8505C) with the BRAF inhibitor, vemurafenib, for 24

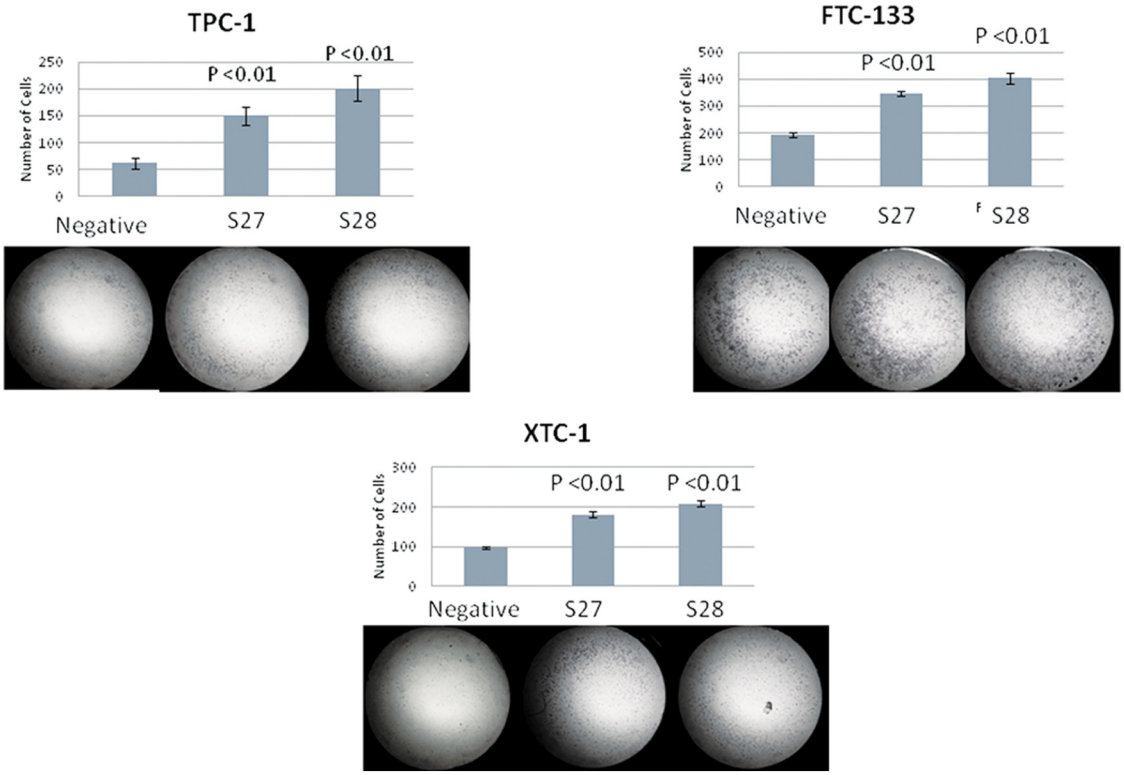

Figure 3. *CPSF2* knockdown significantly increased cell invasion in TPC-1 (s28827 and s28828 *CPSF2* siRNAs), FTC-133, and XTC-1 (both *CPSF2* siRNAs). Negative control is shown on the first bar from the left and two groups treated with *CPSF2* siRNAs (s28827 [s27] and s28828 [s28]) are shown on the right. Error bars represent SEM.

and 48 h. As expected, we observed a reduction in phospho-MEK 1/2 protein in the treated cells; however, there was no difference in CPSF2 protein expression between control and treated cells (Figure 5A). To determine whether CPSF2 altered the downstream targets of BRAF pathway, we used CPSF2 knockdown in the *BRAF* wild type cell line TPC-1. We found no difference in phospho-MEK1/2 protein expression between TPC-1 cells treated with CPSF2 siRNAs and control (Figure 5B). Therefore, CPSF2 protein expression was independent of BRAF inhibition.

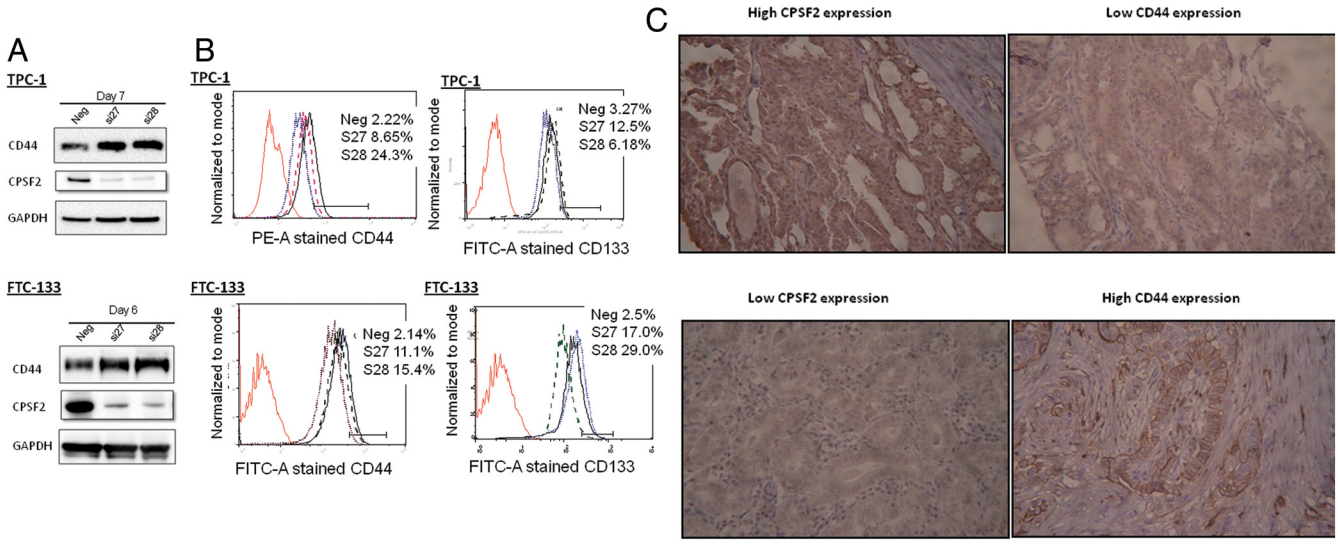

Figure 4. CPSF2 expression was increased in TPC-1 and FTC-133 cells treated with CPSF2 siRNAs for 7 and 6 d, respectively (A). The population of CD44- and CD133- enriched thyroid cancer cells was increased when TPC-1 and FTC-133 cells were treated with CPSF2 siRNAs for 6 d (B). Unstained cells are shown red solid lines, cells treated with negative control (Neg), s28827 siRNA (s27) and s28828 (s28) siRNA are shown in dotted, dashed, and solid black lines, respectively. CD44 expression (membranous stain) in PTC samples inversely correlated with CPSF2 expression (nuclear and cytoplasmic stain) (C).

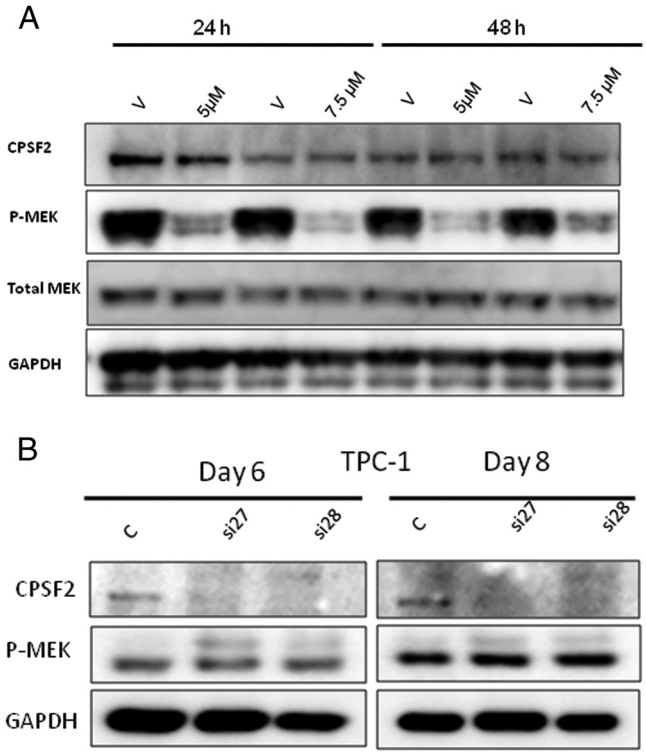

Figure 5. CPSF2 protein expression in 8505C cells treated with selective BRAFV600E inhibitor (PLX4032) (A). Phospho-MEK 1/2 protein expression when TPC-1 cells were treated with CPSF2 siRNAs for 6 and 8 d (B).

Discussion

The goal of this study was to validate dysregulated PTC genes from our earlier study according to mortality status. Moreover, because these genes were novel, we were interested in characterizing their expression in other types of thyroid tumors and understanding their biologic function. We demonstrated in vitro that CPSF2 knockdown resulted in significantly increased cellular invasion and increased cancer stem cell population, which was also confirmed in PTC tissue samples. Increased CD44 expression has been associated with unfavorable features and outcomes in cancers such as colon, gastric, breast, and osteosarcoma (16–19). An increase in CD44-positive cells plays an important role in tumor recurrence and metastasis because this subpopulation of self-renewing cells is more resistant to chemo- and radiotherapy than the primary cancer cell population (20). CD133-expressing thyroid cancer cells are more radioresistant with increase survivability after radiotherapy (14). These findings are consistent with the increased tumor recurrence and higher PTC-associated mortality seen in patients with lower *CPSF2* expression. Furthermore, we found lower *CPSF2* expression in the invasive fronts of locally aggressive PTC compared with central area when we analyzed GWE data of locally advanced PTC (11). Studies in breast and colorectal cancer suggest that heterogeneous expression of genes in center part of tumor compared with tumor-invasive fronts. Leading edge of tumor has an increased EMT markers and activated Wnt pathway resulting in tumor invasion and metastasis (21, 22). Because of increased EMT in the invasive fronts of PTC compared with the central area of tumors suggested by Vasko et al (11) and increased in vitro cellular invasion, we evaluated the association between CPSF2 and EMT markers. Although we did not find the association between EMT markers and CPSF2, increased CD44-positive cells are associated with enhanced cellular invasion by non-EMT mechanism. This is because the extracellular domain of CD44 can bind to and interact with several extracellular matrix components including hyaluronan and collagen, CD44-positive tumor cells invade by upregulating hyaluronan and collagen-degrading enzymes (23, 24).

To our knowledge, our findings are the first to identify a novel gene, *CPSF2*, as a prognostic marker in patients with PTC. These data could have significant clinical ramifications as they may help improve risk stratification in patients with PTC and allow better management of patients with low-risk tumor. We found that low *CPSF2* mRNA and protein expression were associated with more advanced PTC. Patients with lower *CPSF2* mRNA expression had a higher rate of tumor recurrence, shorter disease-free survival, and higher PTC-associated mortality. Although we did not find decreased *CPSF2* expression to be significantly associated with extrathyroidal extension or lymph node metastasis, we observed trends toward lower *CPSF2* in PTC samples with these features, suggesting type II error. Additional validation in a larger cohort is warranted. When compared with normal and benign thyroid tissue samples, PTC had significantly higher *CPSF2* expression.

CPSF comprises four subunits (160, 100, 73, and 30 kDa), where CPSF2 is the 100 kDa subunit. CPSF is one of the multisubunit protein complexes involved in endonucleolytic cleavage and the synthesis of polyadenylate tails onto upstream cleavage products to mature the 3′ ends of pre-mRNA molecules. Other proteins involved in pre-mRNA 3′-end maturation are cleavage stimulation factor, cleavage factor I and II, poly(A) polymerase, polyA binding protein 2, and RNA polymerase II (25, 26). Polyadenylation is involved in many aspects of mRNA metabolism, such as translation efficiency, mRNA stability, and export of mRNA to cytoplasm. Changes in polyadenylation, known as alternative polyadenylation (APA), are widely used to regulate gene expression. More than 50% of human genes have multiple poly(A) sites and are the targets of APA (27). APA affects gene expression by 1) producing different protein isoforms, or 2) controlling the

length of the 3′ UTR, which often harbors microRNA binding sites, thus altering the amount of protein generated by mRNA (28, 29).

The role of APA in cancer biology remains poorly understood. Morris et al reported an increased expression of mRNA 3′-end processing factors, including CPSFs, during the progression from colorectal adenoma to cancer, which resulted in mRNA 3′UTR shortening (30). We found significantly higher *CPSF2* mRNA expression in PTC samples compared with normal and benign tissue samples, which would suggest an increase in mRNA 3′-end processing factors. The increase in *CPSF2* expression in PTC samples may suggest a response to enhance regulatory mechanisms in the presence of cancer cells. A decrease in tumor-suppressive functions in PTC with low *CPSF2* expression can result in increased tumor invasion and increased thyroid cancer stem-like cells, which is consistent with the clinical findings of higher recurrence rate and mortality. In this study, we did not investigate the mechanism of *CPSF2* expression but given the reduced levels in aggressive PTC, this warrants future investigation. There are several plausible mechanisms involved in decreased *CPSF2* expression in aggressive PTC, which include dysregulated microRNA targeting *CPSF2* or epigenetic changes, which have been shown to be altered in PTC.

Although the *BRAF* V600E mutation has been studied extensively and used clinically as a poor prognostic marker for PTC, (4, 31) only a few studies showed an association with mortality (32, 33). In the current study cohort, *BRAF* V600E was not associated with mortality. The increased prevalence of PTC with the *BRAF* V600E mutation may limit the use of *BRAF* V600E mutation status as a prognostic marker. Our study is the first to report that lower expression of a novel gene, *CPSF2*, is associated with PTC-related mortality, independent of *BRAF* V600E mutation status. The lack of associations between *BRAF* V600E mutation and adverse clinical features in this study is consistent with other large cohort studies (34, 35) and suggests that the expression level of *CPSF2* is independent of *BRAF* V600E mutation in PTC. Because the lack of association between lower *CPSF2* expression and *BRAF* V600E mutation status does not exclude activation of the MAPK pathway, we examined the downstream molecule (phospho-MEK) of this pathway with *CPSF2* knockdown in PTC cells. In addition, we confirmed the lack of association in vitro between *CPSF2* and *BRAF* by demonstrating unchanged CPSF2 protein expression when *BRAF* mutant cells were treated with BRAF inhibitor. The findings from this study suggest future studies evaluating *CPSF2* as a preoperative prognostic marker to identify high-risk patients is warranted.

In addition to its potential clinical application as a prognostic marker, the effects of *CPSF2* on thyroid cancer cell lines suggest that APA may have a role in thyroid cancer progression and should be further explored.

In summary, *CPSF2* knockdown increased cellular invasion in three thyroid cancer cell lines and increased CD44 and CD133 thyroid cancer stem-like markers.

Lower *CPSF2* expression was associated with advanced T stage, tumor recurrence, shorter disease-free survival, and PTC-associated mortality.

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