B-Raf\textsuperscript{V600E} and thrombospondin-1 promote thyroid cancer progression

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Although B-Raf\textsuperscript{V600E} is the most common somatic mutation in papillary thyroid carcinoma (PTC), how it induces tumor aggressiveness is not fully understood. Using gene set enrichment analysis and in vitro and in vivo functional studies, we identified and validated a B-Raf\textsuperscript{V600E} gene set signature associated with tumor progression in PTCs. An independent cohort of B-Raf\textsuperscript{V600E}-positive PTCs showed significantly higher expression levels of many extracellular matrix genes compared with controls. We performed extensive in vitro and in vivo validations on thrombospondin-1 (TSP-1), because it has been previously shown to be important in the regulation of tumor angiogenesis and metastasis and is present in abundance in tumor stroma. Knockdown of B-Raf\textsuperscript{V600E} resulted in TSP-1 down-regulation and a reduction of adhesion and migration/invasion of human thyroid cancer cells. Knockdown of TSP-1 resulted in a similar phenotype. B-Raf\textsuperscript{V600E} cells in which either B-Raf\textsuperscript{V600E} or TSP-1 were knocked down were implanted orthotopically into the thyroids of immunocompromised mice, resulting in significant reduction in tumor size and fewer pulmonary metastases from the primary carcinoma as compared with the control cells. Treatment of orthotopic thyroid tumors, initiated 1 week after tumor cell implantation with PLX4720, an orally available selective inhibitor of B-Raf\textsuperscript{V600E}, caused a significant tumor growth delay and decreased distant metastases, without evidence of toxicity. In conclusion, B-Raf\textsuperscript{V600E} plays an important role in PTC progression through genes (i.e., TSP-1) important in tumor invasion and metastasis. Testing of a patient's thyroid cancer for B-Raf\textsuperscript{V600E} will yield important information about potential tumor aggressiveness and also allow for future use of targeted therapies with selective B-Raf\textsuperscript{V600E} inhibitors, such as PLX4720.

extracellular matrix | metastasis | papillary thyroid cancer | tumor microenvironment | cell invasion

Papillary thyroid cancer (PTC), with its incidence increasing by almost 5% each year, currently ranks as the eighth most common malignancy diagnosed in women (1). Neck recurrences alone are responsible for a third of thyroid cancer–related deaths. There is no effective treatment for radioiodine-resistant metastatic disease; the 10-year survival rate in these cases is only 10% (2). Molecular understanding of the aggressive clinical behavior of this subset of patients is needed to develop new therapeutic options. The T1799A (V600E) point mutation occurs in exon 15, which encodes the kinase activation domain of B-Raf. It is the most prevalent genetic alteration implicated in the initiation and progression of PTC, especially in aggressive subtypes such as the tall cell variant of PTC and in those with extrathyroidal extension (3, 4) and lymph node or distant metastases (3, 5). It has been associated with both radioactive iodine refractoriness and PTC recurrence (3). The B-Raf\textsuperscript{V600E} mutation possesses elevated kinase activity that leads to activation of the MAPK (i.e., ERK1/2) signaling pathway (6). Although it is not clearly understood how B-Raf\textsuperscript{V600E} mutation leads to more aggressive PTCs, some altered pathways have been previously described (3).

In our study, we analyzed the role of the B-Raf\textsuperscript{V600E} mutation in the expression and regulation of human genes linked to cell adhesion, migration, invasion, and metastasis. Gene set enrichment analysis (GSEA) was performed on microarray data from human PTCs with or without B-Raf\textsuperscript{V600E} mutation, as well as from human normal thyroid tissue (NT) samples. GSEA is a bioinformatic tool based on genome-wide expression profile analysis (7). It is based on the identification of gene set leading-edge genes that represent a subset of highly ranking genes mostly associated with detected differences between two experimental groups (7). We analyzed microarray data (8) to identify groups of genes (gene sets) significantly associated with B-Raf\textsuperscript{V600E} PTC that are regulated together, share the same function, or belong to the same biochemical pathway.

Using both in vitro and in vivo models of human thyroid cancer, we found that thrombospondin-1 (TSP-1), a multifunctional molecule known to have important effects on tumor stroma and endothelium, serves as a mediator of invasiveness and aggressive tumor behavior when the B-Raf\textsuperscript{V600E} mutation is present.

**Results**

GSEA was performed on microarray data (8) from 26 human PTCs with B-Raf\textsuperscript{V600E} mutation, 14 without B-Raf\textsuperscript{V600E} mutation, and 10 NT samples. Of 539 independent gene sets tested, 18 were significantly associated with PTCs-B-Raf\textsuperscript{V600E} mutation; 17 of these were up-regulated (including many genes important for the tumor microenvironment), and 1 was down-regulated (including genes involved in oxidative enzymatic reactions and cell polarity in epithelial cells), with a false-discovery rate (FDR) of< 0.20. Details about gene sets associated with B-Raf\textsuperscript{V600E}–positive human PTCs and validation of the leading-edge genes using an independent cohort of PTCs, with or without B-Raf\textsuperscript{V600E} mutation, and NT are reported in SI Text, Figs. S1 and S2, and Tables S1 and S2.

Detailed validation was undertaken for gene sets enriched in leading-edge genes known to be important in angiogenesis, invasion, and metastasis. One such gene, TSP-1, was shown to be regulated. We performed extensive in vitro and in vivo validations on thrombospondin-1 (TSP-1), because it has been previously shown to be important in the regulation of tumor angiogenesis and metastasis and is present in abundance in tumor stroma. Knockdown of B-Raf\textsuperscript{V600E} resulted in TSP-1 down-regulation and a reduction of adhesion and migration/invasion of human thyroid cancer cells. Knockdown of TSP-1 resulted in a similar phenotype. B-Raf\textsuperscript{V600E} cells in which either B-Raf\textsuperscript{V600E} or TSP-1 were knocked down were implanted orthotopically into the thyroids of immunocompromised mice, resulting in significant reduction in tumor size and fewer pulmonary metastases from the primary carcinoma as compared with the control cells. Treatment of orthotopic thyroid tumors, initiated 1 week after tumor cell implantation with PLX4720, an orally available selective inhibitor of B-Raf\textsuperscript{V600E}, caused a significant tumor growth delay and decreased distant metastases, without evidence of toxicity. In conclusion, B-Raf\textsuperscript{V600E} plays an important role in PTC progression through genes (i.e., TSP-1) important in tumor invasion and metastasis. Testing of a patient's thyroid cancer for B-Raf\textsuperscript{V600E} will yield important information about potential tumor aggressiveness and also allow for future use of targeted therapies with selective B-Raf\textsuperscript{V600E} inhibitors, such as PLX4720.
significantly altered in PTCs with B-Raf^{V600E} mutation as part of the leading-edge of the BREAST_CANCER_ESTROGEN_SIGNALLING up-regulated gene set (Fig. S1 and Tables S1 and S2).

TSP-1 became the focus of our study because it (i) is a multifunctional matricellular protein (structured with amino- and carboxy-terminal domains and three types of sequence repeats) that regulates extracellular matrix (ECM) structure during tissue genesis and tumor progression (9, 10); (ii) binds concurrently to a wide variety of integrins (involved in cellular proliferation pathways and metastasis) and nonintegrin (i.e., proteoglycans) cell surface receptors, matrix proteins (i.e., fibronectin), cytokines (i.e., TGF-β1), proangiogenic factors (e.g., VEGF), and matrix proteases (i.e., matrix metalloproteinase (MMP)-9), indicating its importance in cross-talk between surface receptors; and (iii) serves as a key regulator of tumor cell adhesion and migration, metastasis, and angiogenesis and may direct clustering of receptors to specialized domains for these biological processes (9, 10).

**TSP-1 Expression Is Regulated by B-Raf^{V600E} in 8505c Cells.** The 8505c cell line is an authenticated human papillary/anaplastic thyroid carcinoma (SI Text), which was confirmed to harbor a V600E mutation (11). Greater than 90% B-Raf^{V600E} mRNA and protein levels silencing was achieved using two shRNAs (sh#1 and sh#10 targeting B-Raf^{V600E}) (Fig. 1A and B). This silencing resulted in significant down-regulation of phospho-MEK1/2 and phospho-ERK1/2, downstream of B-Raf^{V600E}, kinase activity (Fig. 1B), as well as significant decrease in TSP-1 mRNA and protein levels (Fig. 1 B–D).

**Silencing of B-Raf^{V600E} in 8505c Cells Results in Decreased Proliferation, Adhesion, Migration, and Invasion.** Silencing of B-Raf^{V600E} in 8505c thyroid cancer cells decreased BrdU (marker of cell proliferation) incorporation from 50.1% ± 5.1% in the controls to 35.6% ± 2.6% (P < 0.05) and 30.3% ± 2.5% (P < 0.01) by sh#1 and sh#10, respectively (Fig. 2A). There was also a reduction in the fraction of cells in S-phase from 43.5% ± 1.1% to 36.4% ± 3.3% (sh#1, P < 0.01) and 32.1% ± 1.3% (sh#10, P < 0.001), and an increase in G1 from 38.4% ± 1.6% in control 8505c cells to 49.3% ± 5.2% (P < 0.01) by sh#1 and 57.7% ± 3.5% (P < 0.01) by sh#10. B-Raf^{V600E} silencing did not lead to apoptosis (absence of sub-G1 cell population) according to flow cytometric analysis.

We found sh control 8505c cells to possess significant adhesiveness properties (97 ± 1.4 cells per field) on type I collagen after 2 h of incubation. By contrast, 8505c cells with B-Raf^{V600E} silenced by sh#1 (22.2 ± 3.8 cells per field, P < 0.001) and sh#10 (26.2 ± 3.0 cells per field, P < 0.001) showed a consistent and statistically significant reduction of adhesion (Fig. 2B).

B-Raf^{V600E} silencing by sh#1 and sh#10 unequivocally reduced migration of 8505c cells on type I collagen and invasion into Matrigel. Migration was reduced by approximately 3-fold (from 76 ± 5.6 to 25.2 ± 2.5 cells per field by sh#1 and to 27 ± 3.3 cells per field by sh#10, P < 0.001) and invasion by >20-fold (from 69 ± 4.2 to 3.5 ± 1.2 cells per field by sh#1 and to 3.2 ± 0.9 cells per field by sh#10, P < 0.001) (Fig. 2C). Conversely, WT B-Raf silencing in the papillary human thyroid carcinoma cell line TPC-1, which harbors a receptor tyrosine kinase translocation (RET/PTC-1) did not affect TSP-1 mRNA levels nor cellular proliferation, adhesion, migration, or invasion (SI Text).

**TSP-1 Silencing Results in Decreased Proliferation, Adhesion, Migration, and Invasion of 8505c Cells.** TSP-1 silencing using two sh reduced in 90% (sh#7) and 75% (sh#8) reduction of TSP-1 mRNA and protein expression levels (Fig. 3A), as well as a 60% reduction in phospho-ERK1/2 levels (Tnr202/Tyr204) (Fig. 3A). TSP-1 silencing also decreased BrdU incorporation (from 50.0% ± 5.2% in controls to 13.7% ± 1.4% by sh#7, P < 0.001) (Fig. 3B); a reduction of the fraction of cells in S-phase from 43.5% ± 1.1% to 33.1% ± 5.6% by sh#7 (P < 0.05) was seen, as well as an increase of cells in G1 from 38.4% ± 1.6% in controls to 44.8% ± 0.1% (P < 0.01). TSP-1 silencing did not lead to apoptosis (absence of sub-G1 cell population), as demonstrated using flow cytometric analysis.

![Fig. 1. TSP-1 expression in B-Raf^{V600E}-positive 8505c cells.](figure1.jpg)

![Fig. 2. Silencing of B-Raf^{V600E} and functional effects in 8505c cells.](figure2.jpg)
analysis. TSP-1 mRNA silencing in 8505c cells reduced tumor cell adhesion, migration, and invasion (Fig. 3 C and D).

The 8505c thyroid cancer cells treated with sh targeting TSP-1 showed a decrease in the number of cells that adhered to type I collagen compared with control levels (97 ± 1.4 cells per field in controls vs. 13 ± 2.1 cells per field by sh#7 and 42.5 ± 5.2 cells per field by sh#8, P < 0.001) (Fig. 3C). TSP-1 silencing also reduced migration on type I collagen and invasion into Matrigel. Migration was reduced from 77.2 ± 4.9 to 25.2 ± 2.5 (sh#7, P < 0.001) and 62.5 ± 2.8 cells per field (sh#8, P < 0.01) (Fig. 3D). Invasion was reduced from 64 ± 4.1 to 27 ± 2.5 by sh#7 and to 36.2 ± 4.5 cells per field by sh#8 (P < 0.001) (Fig. 3D). Similar results were obtained with an additional authenticated human papillary thyroid carcinoma cell line (i.e., BCPAP) harboring the V600E mutation (SI Text and Fig. S3) (11). To confirm the role of B-RafV600E in cell adhesion, migration, and invasion, we stably overexpressed B-RafV600E in normal human foreskin BJ fibroblasts. B-RafV600E overexpression in BJ cells was found to increase cell adhesion, migration, and invasion, as well as TSP-1 and phospho-ERK1/2 levels (SI Text and Fig. S4 A and B).

Proinvasive Role of B-RafV600E in 8505c Cells Is Mediated by TSP-1. To provide further mechanistic insight into the link between B-RafV600E and TSP-1, we assayed the behavior of 8505c thyroid cancer cells in a reconstituted 3D cell culture-based ECM assay in the presence of 5% FBS (SI Text and Figs. S5 A–D and S6A). In this assay, 8505c cells with B-RafV600E mutation showed invasive properties (dendritic-like morphology with filopodia-like structures) and high growth rate (large cell aggregates) (SI Text and Fig. S5 A–D). The invasive properties disappeared and the cells organized as small spheroids with knockdown of B-RafV600E or TSP-1 (Fig. 4 B and C and Figs. S5 A–D and S6B). Different stable protein components of TSP-1 (Fig. 4A), including amino-terminal domain [thought to be important in cell adhesion/migration through α3β1 (ITGA3B1) and α6β1 (ITGA6B1) integrin-binding domains], 3TSR (type I repeats known to be important in angiogenesis through the CD36), and carboxy-terminal domain (also important in cell adhesion and migration) were then added to the 8505c cells to determine whether the invasive phenotype could be rescued (Fig. 4 B and C and Fig. S6 B–D). Addition of 0.6 μM full-length human TSP-1 protein (Fig. 4B) or 2 μM of recombinant versions of the amino-terminal domain significantly restored the invasive phenotype in 8505c cells with B-RafV600E knockdown in this 3D Matrigel assay (Fig. 4B and Fig. S6C). Treated cells increased their growth rate and transformed to a dendritic-like morphology by day 9 (Fig. 4B and Fig. S6C). Treatment with 3TSR caused a slight reversion of the invasive phenotype (Fig. 4B and Fig. S6C). The 8505c cells with TSP-1 knockdown also transformed to a dendritic-like morphology upon the addition of amino-terminal or 3TSR domains (Fig. 4C and Fig. S6D). Treatment with the carboxy-terminal domain did not revert the morphology to the control phenotype for either B-RafV600E or TSP-1 knockdown cells (Fig. 4B and C and Fig. S6 C and D). Together, these results indicate that

![Image](https://via.placeholder.com/150)

**Fig. 3.** TSP-1 silencing and functional effects in 8505c cells. (A) Western blot analysis shows a decrease in TSP-1 protein levels by shRNAs (sh) targeting TSP-1 in 8505c cells. TSP-1 knockdown reduces endogenous levels of phosphorylated p-ERK1/2 (Thr202/Tyr204). Real-time RT-PCR analysis shows a significant decrease of TSP-1 relative mRNA levels, 33.3-fold less by sh#7 [fold change (FC) = 0.03 ± 0.006] and 6.25-fold less by sh#8 (FC = 0.2 ± 0.013) vs. sh control cells (FC = 1.04 ± 0.1). (B) Flow cytometric analysis shows a significant reduction of BrdU incorporation in S-phase in 8505c cells sh#7 TSP-1 vs. sh control cells. (C) Cell adhesion and (D) migration and invasion assays show that knockdown of TSP-1 by both sh#7 and sh#8 significantly reduces the number of 8505c cells adherent to type I collagen substrate and the number of migrating and invading 8505c cells. These data represent the average ± SD of three independent experiments. **P < 0.01; ***P < 0.001.

![Image](https://via.placeholder.com/150)

**Fig. 4.** Proinvasive role of B-RafV600E in 8505c cells grown in 3D cultures. (A) Structural and functional domains of full-length human TSP-1 protein. A single subunit is depicted as a series of structural domains based on the amino acid sequence. The vertical lines that are adjacent to the amino-terminal domain are the interchain disulfide bonds. (B) 3D cell culture-based ECM assay shows that treatment with 0.6 μM full-length human TSP-1 protein or 2 μM of recombinant amino-terminal domain of TSP-1 protein rescues the invasive phenotype (dendritic-like structures with filopodia; arrows) of 8505c cells that underwent knockdown of B-RafV600E (sh#1) (day 9). (C) 3D assay shows that treatment with 2 μM of recombinant amino-terminal or 3TSR domains of TSP-1 rescues the invasive phenotype (dendritic-like structures with filopodia; arrows) of 8505c cells with that underwent TSP-1 knockdown (sh#7) (day 9).
B-RafV600E 8505c thyroid cancer cells have higher potential for invasion and dissemination than 8505c cells with knockdown of either B-RafV600E or TSP-1. Amino-terminal and 3T3R domains of TSP-1 may cooperate mechanistically through a positive feedback with B-RafV600E in 8505c cells to activate ERK1/2 to p-ERK1/2 and promote tumor invasion and metastases.

**Knockdown of Either B-RafV600E or TSP-1 Decreases Cell Growth and Metastasis in Vivo.** Given our in vitro data, we wanted to evaluate the role of mutant B-RafV600E and TSP-1 in tumor growth and metastasis in vivo. We previously developed an orthotopic mouse model using 0.5 x 10^6 8505c human thyroid cancer cells in SCID mice (12). In this orthotopic model [Green Fluorescent Protein (GFP) engineered], all mice reliably developed palpable thyroid carcinomas by 5 weeks after injection of 8505c cells into the right thyroid, and necropsy showed large (Fig. Sbi–vi) and highly proliferative (Fig. S7A) thyroid tumors with tracheal compression similar to the behavior of aggressive human thyroid carcinoma (Fig. Sbi–vi). All sh control 8505c tumor-bearing mice had multiple histologically confirmed micrometastases to lungs (also visible by detection of GFP signal in vivo) (Fig. 5 Aii and Cii–iii). Fifty percent (3 of 6) of the sh control 8505c control mice had cervical lymph node metastasis (12). Orthotopic tumor-bearing mice were never found to have bone or liver metastases.

Here implantation of 8505c cells with knockdown of B-RafV600E (sh##1) into the right thyroid resulted in significantly smaller thyroid tumors at 5 weeks, with no evidence of cervical lymph node metastasis [mean orthotopic tumor volume was 0.50 ± 0.20 mm^3 (n = 6) vs. 350.1 ± 90 mm^3 for control tumor (n = 6) (P < 0.001)] (Fig. 5 Ai and Bii–aix). Importantly, in those animals implanted with 8505c cells with B-RafV600E knockdown, the number of metastasis to the lungs from the primary orthotopic carcinoma was reduced from 18.5 ± 1 metastases per section of whole lung for the control tumors to 2 ± 0.8 metastases per section of whole lung for those with B-RafV600E knockdown (Fig. 5 Aii and Cii–vi). Implantation of 8505c cells with TSP-1 knockdown (sh##7) also produced significantly smaller tumors at 5 weeks (mean tumor volume was 151.2 ± 28.5 mm^3, P < 0.001) (Fig. 5 Ai and Bii–aix) when compared with control, with fewer metastases (7.75 ± 1.15 metastases per section of whole lung for the 8505c tumors with TSP-1 knockdown compared with control, P < 0.001) (Fig. 5 Aii and Cii–vi). In addition, proliferative rate, as measured by Ki-67 nuclear expression, was slightly lower in the small orthotopic carcinomas from 8505c cells in which either B-RafV600E or TSP-1 was knocked down (Fig. S7 B and C). Finally, given the known importance of TSP-1 to angiogenesis, we analyzed some angiogenic markers (e.g., CD31, VEGF, and MMP-2 and -9) in these tumors at 5 weeks after tumor implantation (SI Text and Fig. S7 D–I).

**PLX4720 Inhibits Tumor Growth and Lung Metastasis in Vivo.** Immunocompromised mice (n = 16) were orthotopically implanted with 8505c and B-RafV600E human thyroid cancer cells, and 1 week after injection half were randomized to treatment with an orally absorbed compound PLX4720 (Plexxikon) that selectively inhibits B-RafV600E kinase activity (13). PLX4720 binds in the cleft between the N and C lobes of the B-RafV600E kinase domain near the hinge region, which overlaps with the ATP-binding site (13). PLX4720 treatment by oral gavage (30 mg/kg per day for 21 days) caused a significant reduction in tumor growth (>90%, P < 0.001) compared with control mice treated with vehicle, and dramatically decreased lung metastases (Fig. 5D), from 16 metastases per section of whole lung in the control group to 2 metastases per section of whole lung (P < 0.001), with no evidence of toxicity.

**Discussion**

Patients with B-RafV600E-positive PTCs have a higher risk of recurrent and persistent disease and a less favorable outcome (3, 14). In this study, we identified the gene expression signature of B-RafV600E by applying GSEA to microarray data from human PTCs with and without BRAF mutation. The B-RafV600E signature was validated in another independent cohort of human PTCs samples with comparisons performed in WT B-Raf PTCs, mutant B-Raf PTCs, or NT samples. Previous gene expression profiling studies on the effect of B-RafV600E focused on identifying individual genes that were up- or down-regulated in melanoma (15) and thyroid cancer (8). Although useful, they fail to detect biological processes affecting sets of genes that act in concert, such as metabolic or oncogenic pathways, which are distributed across an entire network of genes but are subtle at the level of individual genes (7). GSEA is a recent bioinformatic approach to genome-wide expression profiling that evaluates microarray data at the level of gene sets instead of single genes and overcomes some important limitations of traditional differential gene expression analysis (7).

In our GSEA analysis, B-RafV600E PTCs identified only one down-regulated gene set, which included genes involved in cell polarity in epithelial cells, thus suggesting B-RafV600E's importance in cell dedifferentiation, loss of cell polarity, and tissue morphogenesis. We identified 17 up-regulated gene sets significantly associated with B-RafV600E in PTCs, including mainly those genes involved in the regulation of the tumor ECM, such as TSP-1, TGF-β1, ITGA3, ITGA6, ITGB1, FN, CD44, CTS-B, and CTS-S, which are identified as potential genes either targeted or affected by the B-RafV600E mutation in PTCs. These genes may act in concert and elicit important biological cross-talk and regulate the tumor microenvironment.

The role of B-RafV600E in tumor progression has been previously demonstrated in melanoma tumor cells (16, 17). To determine whether B-RafV600E could affect ECM composition and drive thyroid cancer progression, we validated various genes linked to invasion and metastasis and focused our in vitro and in vivo validations on TSP-1. TSP-1 and its target TGF-β1 are crucial regulators of epithelial cell growth, motility, polarity, and morphogenesis (9, 10) and are important in stromal/epithelial interaction and angiogenesis. Whereas the role of TSP-1 as an inhibitor of tumor angiogenesis is well documented, its biological action in tumor progression and metastasis requires further study (9, 10). In this study, TSP-1 was significantly increased in B-RafV600E-PTCs vs. NT and compared with WT B-Raf PTCs. In our study, the results of B-RafV600E knockdown in 8505c cells radiobiologically linked to increased cell proliferation, adhesion, migration, and invasion. We also found a significant though not complete decrease in TSP-1 mRNA and protein levels in the cells with B-RafV600E silencing, thus suggesting that this oncogene can affect TSP-1 levels by either transcriptional or posttranscriptional regulation.

TSP-1 knockdown in 8505c cells resulted in decreased phospho-ERK1/2 protein levels and reduced cell adhesion, migration, and invasion, as well as cell proliferation. In addition, the G1 arrest demonstrates that this protein promotes cell cycle progression. A number of studies have previously shown that TSP-1 is a mitogen-inducible immediate–early response gene (18) and is intimately involved in the regulation of cellular proliferation through effects on integrin pathways (19). Furthermore, consistent with our results, TSP-1 has been shown to be involved in the integrin pathways that can affect tumor cell adhesion (20) or migration/invasion (9).

There are few previous data on the role of TSP-1 in thyroid cancer progression and no previous studies demonstrating a link to B-Raf mutations. TSP-1 is able to enhance follicular thyroid cancer cell invasion by an increase of urokinase expression (21). In corroboration with a potential role of TSP-1 in thyroid cancer progression, microarray analysis of poorly differentiated or undifferentiated thyroid cancer subtypes has recently revealed up-regulation of TSP-1 in these aggressive human thyroid cancers; however, neither in vitro nor in vivo functional validations for TSP-1 were performed, nor was there any attempt to make a link to B-Raf mutation status in this study (22).
B-Raf\textsuperscript{V600E} overexpression in normal human BJ fibroblasts confirmed up-regulation of TSP-1 mRNA and protein levels by B-Raf\textsuperscript{V600E}. BJ-B-Raf\textsuperscript{V600E} cells also acquired an invasive phenotype compared with controls, thus reinforcing our findings regarding the importance of B-Raf\textsuperscript{V600E} mutation in altering TSP-1 expression and the invasive program of the cell. By contrast, our data indicate that WT B-Raf mRNA silencing in TPC-1 cells (RET/PTC-1) does not affect TSP-1 expression levels, indicating that TSP-1 expression is regulated differently in various cell types and is dependent on genetic context.

TSP-1 has many functional domains that interact with a variety of cell surface receptors and can trigger different biological effects in a cell context-dependent manner (9, 10). The 3TSR domain plays an important role in activation of TGF-β1 in vivo (9, 10). Others are certainly involved in several processes of the ECM microenvironment (i.e., amino-terminal domain) by the binding to integrins (i.e., α3β1 or α9β1) (23), which have an important role for tumor cell migration and invasion (24, 25). Here, treatment with recombinant amino-terminal domain and to a lesser extent 3TSR resulted in restoration of invasive behavior in 8505c cells that had undergone knockdown of B-Raf\textsuperscript{V600E} or TSP-1. Only these TSP-1 domains were able to restore the aggressive phenotype of the 8505c cells grown in 3D cultures, implying a link between the B-Raf\textsuperscript{V600E} action and the amino-terminal or 3TSR domains. Our observation is supported by Chandrasekaran et al. (23), who showed a critical role for the TSP-1 amino-terminal domain in human breast cancer cell adhesion, migration, and invasion via putative binding sites for α3β1 integrin. Integrins are receptors for cellular adhesion molecules such as TSP-1 (9, 10), and they initiate “outside-in” signal transduction events that modulate gene expression, cell proliferation, migration, and invasion (26). Importantly, our GSEA analysis detected an increase in α3, α6, and β1 integrin levels in B-Raf\textsuperscript{V600E}-positive PTCs vs. WT B-Raf PTCs or NT (SI Text). B-Raf\textsuperscript{V600E} oncprotein contributes to the invasive behavior of an aggressive thyroid cancer cell line in vitro, perhaps through an interaction of the amino-terminal or 3TSR domains of TSP-1 with these integrins.

We proceeded with in vivo testing of our hypothesis using an orthotopic model of thyroid cancer with 8505c cells, a unique cell line that is not cross-contaminated or redundant, harboring B-Raf\textsuperscript{V600E} (11), and originating from a patient with papillary thyroid carcinoma with anaplastic features (27). Our in vivo testing revealed that 8505c cells with B-Raf\textsuperscript{V600E} or TSP-1 knockdown resulted in significantly smaller orthotopic thyroid carcinomas and showed a dramatic decrease in the occurrence of lymph node and lung micrometastases. TSP-1 has been proposed to have both prometastatic and antimetastatic properties (9, 10). Yee et al. (9) have recently demonstrated that TSP-1 can promote metastasis to lungs in a transgenic mouse model of breast cancer. Additionally, previous in vitro studies suggested that TSP-1 promotes tumor cell adhesion, migration, and invasion (28). Interestingly, it has been

![Fig. 5. Knockdown of either B-Raf\textsuperscript{V600E} or TSP-1 in an in vivo model of thyroid carcinoma. (A) Volume of orthotopic thyroid carcinomas (OTC) formed by sh control 8505c cells, sh#1 B-Raf\textsuperscript{V600E} cells, or sh#7 TSP-1 8505c cells. (Aii) Number of metastatic 8505c cells foci found in each mouse that was averaged per group (n = 6). These data represent the average ± SEM. (Bi-iii) Gross images of a large OTC (arrow) of sh control 8505c cells. (Magnification, x10.) Bi (phase contrast) and Bii (GFP excitation): tracheal impingement. (Biii) Histological sections (H&E). (Biv) OTC within the right thyroid, showing histopathologic features of a high-grade neoplasm: mitoses (arrows), necrosis (asterisk), and marked cellular pleomorphism. (Magnification: x40.) (Bv) OTC invading the neck muscles (arrows) and fat (arrowhead). (Magnification: x40.) (Bvi) adjacent tracheal wall (asterisk) and blood vessels (arrow). (Magnification: x60.) (Bvii-ix) Gross image of an sh#1 8505c cells small OTC (arrow). (Magnification: x10.) Bvii: phase contrast; Bviii: GFP excitation. (Bx and Bxi) Small OTC (asterisk) within the right thyroid (Bxii) with no tracheal wall invasion (asterisk). (Magnification: Bx, x20; Bxiv, x60; Bxv, x60.) (Bxiii) Gross picture of a sh#7 8505c cells small OTC (arrow). (Magnification: x10.) (Bxv and Bxvi) Small OTC within the right thyroid with no tracheal wall invasion (arrow and asterisk). (Magnification: Bxiv, x40; Bxv, x40). (C) Gross lung image from a mouse with metastatic sh control 8505c cells OTC. (Magnification: x10.) (Ci) GFP excitation reveals miliary multifocal lung micrometastases. (Magnification: x10.) (Cii) H&E stain of numerous lung micrometastasis of sh control 8505c cells (arrows). (Magnification: x60.) (Ciii) Gross lung image from a mouse injected with sh#1 B-Raf\textsuperscript{V600E} 8505c cells. (Magnification: x10.) (Civ) GFP excitation and (Cv) H&E stain do not reveal lung micrometastasis of sh#1 8505c cells small OTC. (Magnification: Cv, x10; Cv, x40.) (Cvi) Gross lung image from a mouse injected with sh#10 B-Raf\textsuperscript{V600E} 8505c cells. (Magnification: x10.) (Cvii) H&E stain from a mouse injected with sh#7 TSP-1 8505c cells. (Magnification: x20.) (Di) Gross image of a large OTC of 8505c cells (arrow) in mice treated with the vehicle (control group). (Dii) GFP excitation shows a large right OTC (arrow) with extension to the left side (control group). (Diii) GFP excitation reveals miliary multifocal lung micrometastases (arrows) in mice treated with vehicle (control group). (Dv) Small OTC of 8505c cells (arrow) in mice treated with PLX4720 30 mg/kg per day for 21 days. (Dvi) GFP excitation shows a small right mass (arrow) upon PLX4720 treatment. (Dvii) Significant decrease of the number of lung micrometastases in mice treated with PLX4720. (Arrow). (Magnification: Di-iv, x10.) *P < 0.05; ***P < 0.001.}
reported that plasma TSP-1 levels are higher in patients with advanced breast cancer vs. patients with early breast cancer, and these levels are significantly higher than in normal controls (29). TSP-1 is a potent inhibitor of angiogenesis by multiple mechanisms, including direct interaction with VEGF and inhibition of MMP-9 activation (10, 30). By contrast, other activities of TSP-1 seem to promote tumor cell migration, invasion, and metastasis (10). Overall, the effects of TSP-1 on any given tumor’s metastatic potential probably depends on genetic and/or epigenetic changes in the tumor cells themselves, as well as its angiogenic effects on endothelial cells. In our study, we found that the protein levels of some angiogenic factors (e.g., VEGF) were higher in the orthotopic thyroid tumors with B-RafV600E mutation as compared with the tumors with B-RafV600E knockdown (SI Text). Orthotopic thyroid carcinomas with TSP-1 knockdown showed an increase of VEGF and MMP-9 protein levels (SI Text). These results are consistent with the observations that TSP-1 mediates the uptake and clearance of both VEGF and MMP-9 (10).

In summary, we have identified a robust B-RafV600E-associated gene expression profile in human PTCs that includes genes that share the same function and/or signaling pathway. Our results indicate that B-RafV600E may affect tumor aggressiveness through the positive regulation of TSP-1 expression. The N-terminal domain and a less abundant TSR domain (cTS1 and cTS2) integrin-binding domains of TSP-1 may cooperate with B-RafV600E in 8505c cells to promote tumor invasion and metastases. PLX4720, an orally available B-RafV600E-selective inhibitor displays activity against advanced human thyroid cancer in an animal model, which reproduces the clinical–pathologic behavior of this type of cancer.

Materials and Methods
Details regarding antibodies, cell culture, RNA, real-time RT-PCR, primers sequence, protein assays (Western blotting), transient transfections, lentivirus


15. Kannengiesser C, et al.; EORTC Melanoma group (2008) Gene expression signature of some angiogenic factors (e.g., VEGF) were higher in the orthotopic thyroid tumors with B-RafV600E mutation as compared with the tumors with B-RafV600E knockdown (SI Text). Orthotopic thyroid carcinomas with TSP-1 knockdown showed an increase of VEGF and MMP-9 protein levels (SI Text). These results are consistent with the observations that TSP-1 mediates the uptake and clearance of both VEGF and MMP-9 (10).