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Review

Cancer Research

BRAF^{V600E} and Microenvironment in Thyroid Cancer: A Functional Link to Drive Cancer Progression

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Abstract

Papillary thyroid cancer (PTC) rates continue to increase in the United States and Europe, and, although most patients do well, some recur and die of their disease. Patients with PTC harboring the BRAF^{V600E} mutation seem to display a more aggressive clinical behavior, but little is known about the role of this mutation in crucial processes in the tumor microenvironment, such as tumor adhesion, migration, invasion, and metastasis. The extracellular matrix (ECM) microenvironment is not merely a structural scaffold for the cellular elements of the epithelial and stromal microenvironment, but it also elicits a profound influence on cell behavior affecting viability, proliferation, adhesion, and motility. The effects of BRAF^{V600E} on cell surface receptors (i.e., integrins) and ECM noncellular components [i.e., thrombospondin-1 (TSP-1) and fibronectin (FN)] seem to trigger different pathologic biological processes in a cell context–dependent manner. This review focuses on the recent progress in understanding the role of BRAF^{V600E} in the regulation of some ECM noncellular components and *trans*-membrane receptors of the microenvironment in PTC in order to design novel targeted therapies directed at the BRAF^{V600E} multifaceted signaling cascades. Some of these targeted therapeutics, such as ATP-competitive BRAF^{V600E} inhibitors (i.e., orally bioavailable PLX4720 and PLX4032 compounds), are already under investigation. *Cancer Res; 71(7); 2417–22. ©2011 AACR.*

Introduction

The incidence of thyroid cancer is increasing more rapidly than other cancers in both the United States (1) and other countries (2). Papillary thyroid carcinoma (PTC) originates in the follicular cells of the thyroid and represents one of the most frequent endocrine malignancies. Well-differentiated PTCs typically have a favorable prognosis with thyroidectomy followed by thyroid hormone suppressive therapy and radioactive iodine ablation of normal thyroid tissue and any residual tumor in some (3). However, for the group of patients who fail to respond to this treatment paradigm or present initially with aggressive and refractory thyroid carcinomas, rates of neck recurrence and distance metastases are high and survival rates are very low, and rational targeted therapies are being investigated (3).

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The BRAF^{V600E} Mutation, Thyroid Cancer, and Tumor Microenvironment

Molecular targets for recurrent PTC are mostly centered on the RAS/BRAF/mitogen-activated extracellular signal regulated kinase [MAPK; i.e., extracellular signal-regulated kinase 1/2 (ERK1/2)] signaling pathway, given the prominence of this pathway as an oncogenic event in PTC progression (4). The *BRAF* gene is located on human chromosome 7q24 and encodes a cytosolic serine–threonine protein kinase that is expressed in many human cells, including thyroid follicular cells (5). The wild-type (wt) BRAF is activated at the plasma membrane through a complex process that involves RAS activity, phosphorylation events, and protein–lipid interactions. BRAF kinase exhibits a characteristic bilobar structure similar to all protein kinases. The inactive conformation of BRAF involves the simultaneous binding of 14-3-3 to phosphorylated sites S365 and S729 (6). The activated wt BRAF is phosphorylated at site S446, leading to a maximally negatively charged amino region. Extracellular signals (i.e., mitogens, hormones, and neurotransmitters) induce a tyrosine kinase receptor, act on RAS-GTP, and activate wt BRAF (6, 7). Two conserved sites (T598 and S601) of wt BRAF are oncogenic RAS-dependent phosphorylation sites. This event not only renders BRAF constitutively active but also induces ERK1/2 activation, causing cell transformation (6, 7).

Constitutive activation of the RAS-ERK signaling pathway is common to numerous cancers. Approximately 15% of human cancers have activating RAS mutations (8). More than 30 mutations of the *BRAF* gene associated with human cancers

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have been identified, the majority of which are located within the kinase domain (8). In 2002, Davies and colleagues (1) identified an oncogene widespread among human cancers, mutant BRAF^{V600E}. BRAF^{V600E} is expressed in different human cancer cell lines including melanoma, colorectal cancer, and thyroid cancer (4, 9). An activating mutation located on exon 15 of the B isoform of the RAF kinase gene results in a valine-to-glutamic acid substitution at amino acid 600 (BRAF^{V600E}). The V600E mutation strongly enhances BRAF kinase activity by inserting a negatively charged residue adjacent to the phosphorylation site at T598 and mimicking phosphorylation at Thr598 and Ser601 residues (7, 10), with increased ERK1/2 phosphorylation (8, 11). These molecular features render BRAF^{V600E} a unique kinase, able to elicit strong phosphorylation activity on ERK1/2, 480-fold higher than wt BRAF or other BRAF mutants (8). This mutation is very prevalent in PTC and is clearly seen much more frequently in the tumors with larger size, lymphovascular invasion or metastases, and mortality, and may play a role in the progression of PTC to anaplastic thyroid cancer (ATC; refs. 4, 9, 12, 13).

Decades of research have shown that tumorigenesis is strongly affected by nonmalignant cells (i.e., stromal cells) that compose the tumor microenvironment (14). Interestingly, a large number of genes abnormally expressed in human cancer encode secreted proteins and receptors, with paracrine and autocrine effects on other components of the tumor such as stromal cells (e.g., fibroblasts, macrophages, endothelial cells, smooth muscle cells, T lymphocytes, and monocytes), and extracellular matrix (ECM) noncellular components (15, 16). Dynamic and reciprocal interactions involving cell adhesion molecules (e.g., integrins, CD44), ECM noncellular components [i.e., thrombospondin-1 (TSP-1), fibronectin (FN)], and soluble cytokines occur between tumor epithelial cells and tumor microenvironment stroma cells (17). The degree of these interactions may represent the basis of triggering of intracellular signaling pathways that confer tissue-specific characteristics to the epithelium (17). The ECM is, therefore, a fundamental component of cell microenvironment and has been substantially expanded during the evolution of vertebrates. It provides more than mechanical support and is a locus for cell adhesion, with potential roles in basement membranes and tumors. All epithelial cells are in association with basement membranes during their lives and include the ECM. ECM composition and organization undergo radical alterations in human cancers and could affect cell survival, proliferation, adhesion, migration, and other properties of both tumor and stromal cells.

Importantly, the BRAF^{V600E} mutation has been associated with aggressive clinical behavior in some patients with PTC (4). Some data shed light on how the BRAF^{V600E} oncogene can affect the tumor microenvironment in thyroid cancer, including interactions between neoplastic thyroid follicular cells and ECM components. Deregulated pathways downstream of BRAF^{V600E} in human cancers harboring this mutation include tumor suppressor genes (i.e., *TIMP-3*), deregulation of microRNAs, and positive regulation of Skp-2 and NF-κB signaling (4). It has also been recently shown that BRAF^{V600E} expression correlates significantly with VEGF protein expression in PTCs with extrathyroidal invasion, perhaps via BRAF^{V600E} modulation of hypoxia-inducible factors (4). Mesa and colleagues have shown that BRAF^{V600E}-activated normal rat thyroid cells express genes such as matrix metalloproteinases (MMP; i.e., MMP-3, MMP-9, and MMP-13; ref. 18). Traditionally, these enzymes may promote tumor invasion by breaking down various noncellular components of the ECM. It has been observed that PTC harboring BRAF^{V600E} show a more aggressive clinical-pathologic behavior (e.g., extrathyroid extension) and a significant increase in MMP-2 and MMP-9 protein levels, thus suggesting that these proteins may play a role in PTC progression (12).

The BRAF^{V600E} Mutation and Extracellular Matrix Noncellular Components

We have recently investigated the role of this single mutation in the gene expression patterns of PTC and ATC cells and in the tumor microenvironment, leading to a better understanding in order to design future targeted therapies directed at BRAF^{V600E} signaling cascades (19). Our results suggest that the BRAF^{V600E} pathway plays an important role in PTC progression through proteins crucial for the ECM remodeling processes including tumor cell adhesion, migration, invasion, and metastasis (19). Using both *in vitro* and *in vivo* models (i.e., orthotopic mouse models) of human thyroid cancer, we found that 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 BRAF^{V600E} mutation is present. Using a novel technique based on genome-wide expression profiling and designed to look at alterations in gene sets (gene set enrichment analysis), we identified 17 upregulated gene sets that were significantly associated with PTC with BRAF^{V600E} when compared with PTCs without the mutation or in normal thyroid tissue. Many of these altered gene sets are involved in the composition and remodeling of ECM such as TSP-1, TGF-β1, integrin-α3, -α6, -β1, FN, CD44, cathepsin-B (CTS-B), and cathepsin-S (CTS-S). These genes seem to be either targeted or affected by the BRAF^{V600E} mutation in PTCs (19). They might act in concert and elicit important biological cross-talk during tumor cell adhesion, migration, and invasion processes involving tumor microenvironment, and ultimately trigger thyroid cancer progression (Fig. 1). Furthermore, our data showed that decreasing mutant BRAF with knockdown, or using a drug (PLX4720) designed to selectively deactivate BRAF^{V600E} in those thyroid cancer cells with at least one copy of mutant BRAF, results in reversal of tumor migration and invasion and metastasis, which is translatable to decreased tumor volume in mice with orthotopic thyroid cancers 5 weeks after tumor implantation (19).

For those familiar with the broad range of TSPs and their important role in development, angiogenesis, and tumor stroma, it is not surprising to find them implicated in BRAF-mediated tumor progression. TSPs are a family of 5 secreted proteins that play distinct roles in development and physiology, with TSP-1 and TSP-2 playing potential roles in tumors. TSP-1 is not only the most abundant protein in

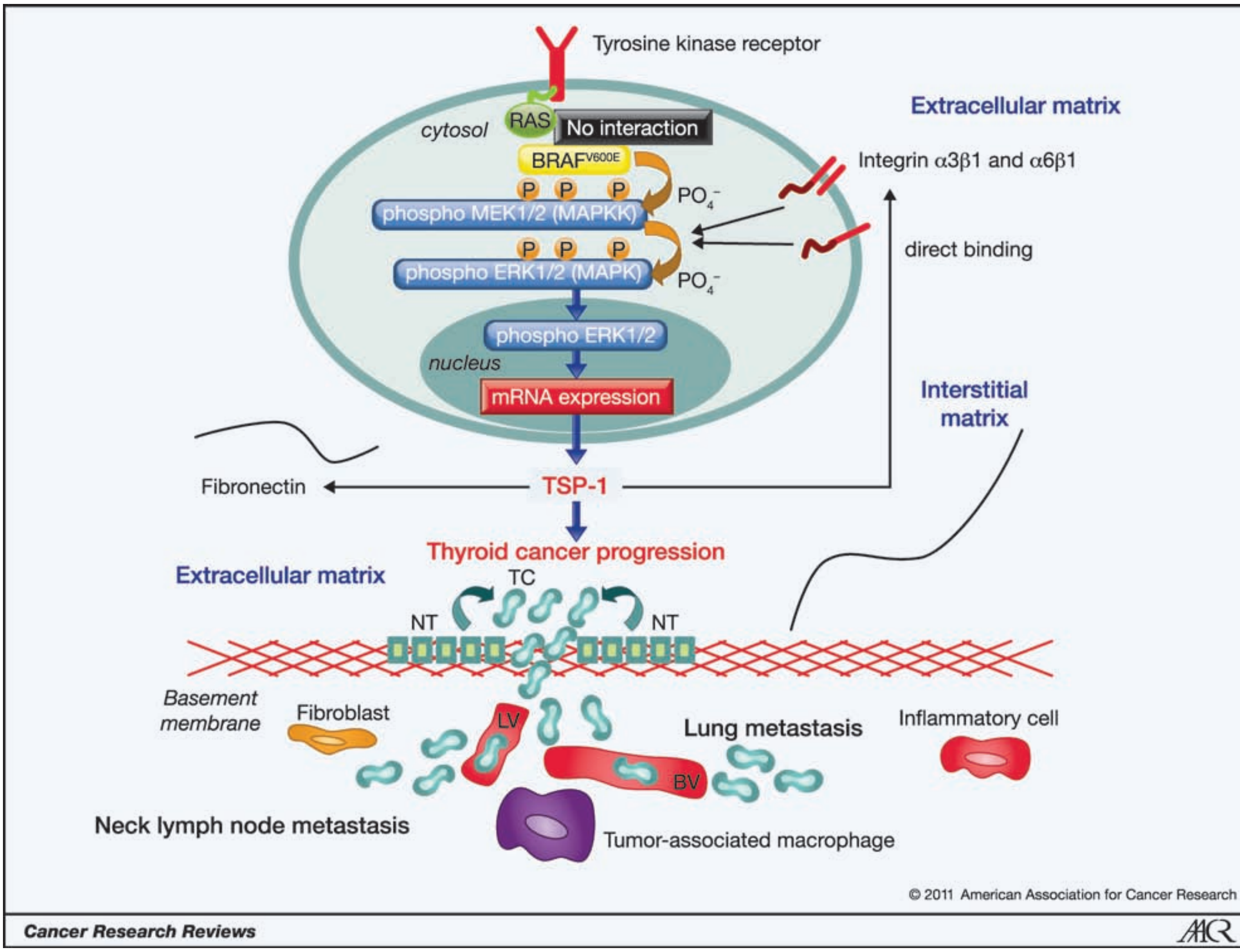

Figure 1. The BRAF^{V600E} oncoprotein activates the phospho-ERK1/2 signaling pathway and regulates the expression of ECM noncellular components. The BRAF^{V600E} oncoprotein dramatically elicits increased kinase activity and activates phospho-ERK1/2. This oncoprotein is constitutively active and does not require RAS signaling. The BRAF^{V600E}-activated phospho-ERK1/2 pathway is able to transform normal thyroid cells (NT) to thyroid cancer cells (TC). It upregulates some ECM noncellular components (i.e., TSP-1, FN) and cellular *trans*-membrane receptors (e.g., integrins), which together may increase the levels of phospho-ERK1/2 through a positive feedback driven by TSP-1. These pathologic processes trigger ECM remodeling and determine tumor cell invasion into the lymphatic (LV) and/or blood vessels (BV) through the basement membrane, causing thyroid cancer progression.

α-granules of platelets but is also expressed in tumor stroma (20). TSP-1 binds to a wide variety of integrins and nonintegrin (i.e., proteoglycans) cell surface receptors, matrix proteins (i.e., FN), cytokines (i.e., TGF-β1), proangiogenic factors (e.g., VEGF), and matrix proteases (i.e., MMP-9), indicating its importance in cross-talk between surface receptors; 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. TSP-1 influences VEGF activity by inhibiting the activation of MMP-9 and suppressing the release of VEGF from the ECM and is also a major activator of TGFβ1 (20).

Whereas the role of TSP-1 as an antiangiogenic factor is well documented (20), its biological action in tumor progression and metastasis is still controversial. Yee and colleagues have recently shown that TSP-1 can promote metastasis to lungs in a transgenic mouse model of breast cancer (21). We have shown that TSP-1 knockdown in aggressive human thyroid cancer cells harboring BRAF^{V600E} prevents the progression of this cancer by resulting in decreased phospho-ERK1/2 protein levels and reduced cell proliferation, adhesion, migration, and invasion, as well as metastasis *in vivo* (19). In addition, the G_1 arrest of these cells shows that TSP-1 promotes cell cycle progression (19). Overall, these results suggest that TSP-1 can be considered a regulator of the thyroid cancer microenvironment, eliciting promigratory and proinvasive roles in thyroid cancer cells harboring BRAF^{V600E} (19). By contrast, mutated RAS is able to repress TSP-1 expression via the c-myc oncogene or activation by the RAF/ERK pathway in human breast cancer models (22), and loss of p53 function has been shown to correlate with reduction in TSP-1 expression and a switch to a proangiogenic phenotype in fibroblasts derived from a patient with Li-Fraumeni syndrome (20). Overall, varied TSP-1 expression is regulated differently depending on the genetic context of the cells.

Our results also point to the importance of certain key integrins (i.e., α3β1 and α6β1) that showed significantly higher mRNA levels in $BRAF^{V600E}$-positive PTC compared with wt BRAF PTC or normal thyroid tissue and may mediate thyroid tumor cell migration and invasion (19). Some integrins mediate tumor cell–ECM adhesion and provide both the connection to the adhesive substrate and cellular signaling (known as "outside-in" signaling or extracellular to intracellular), crucial for cell proliferation, migration, and invasion (17). Some integrins (e.g., α2β1 in breast cancer cells) are decreased as tumors progress, thus suggesting that α2β1 could function as a tumor suppressor, whereas elevated expression of integrin β3 seems to be closely associated with melanoma progression (23). Interestingly, α5β1 integrins may become activated upon p53 mutation and reflect an enhanced FN-binding integrin (24). We also found a potential link between FN and $BRAF^{V600E}$ in our analysis (19); $BRAF^{V600E}$-positive PTCs showed significantly higher FN mRNA levels compared with wt BRAF PTC or normal thyroid tissue. Our data support the hypothesis that FN overexpression may be involved in cancer progression harboring $BRAF^{V600E}$ and may influence the control of metastasis by mediating integrin-associated signaling pathways. Human cells mediate FN matrix assembly through integrins binding to the RGD cell-binding domain. Integrin α5β1 is the primary receptor for FN matrix assembly, which binds to the RGD sequence (Arg-Gly-Asp; ref. 17). Importantly, melanoma cells overexpress FN, which controls many fundamental pathobiological processes. There is strong evidence that overexpression of FN is tightly correlated with the acquisition of invasive and metastatic behavior of melanoma cells by constitutive $BRAF^{V600E}$/ERK kinase signaling (25). FN binding to integrin induces receptor clustering, which brings together cytoplasmic molecules such as focal adhesion kinase (FAK), Src kinase, paxillin, and others to form protein-rich focal complexes that activate polymerization of actin filaments and intracellular signaling through kinase cascades (26). FAK activation by integrin–ligand interactions promotes PI3K signaling, which is essential in promoting cancer invasion. In addition, Shibue and Weinberg have recently shown that integrin β1 is also fundamental in activating the FAK signaling axis to control the initial proliferation of micrometastatic mouse breast cancer cells disseminated in the lungs (27).

In addition to FN–integrin interactions, TSP-1–integrin interaction also contributes to initiate "outside-in" signal transduction events that modulate gene expression, cell proliferation, migration, and invasion (17). TSP-1 has many important functional interactions through its various domains, some of which (3TSR domain or termed as type-1 repeats) play an important role in activation of TGF-β1 *in vivo* (20). Our data showed that the *N* terminus of TSP-1 seems to be the critical element involved in the BRAF-mediated invasion in thyroid cancer cells (19). Chandrasekaran and colleagues (28) also showed a critical role for the TSP-1 *N*-terminal domain in breast cancer cell invasion via putative binding site (s) to integrin α3β1, which has an important role for tumor cell migration and invasion. Sumimoto and colleagues (29) have shown that $BRAF^{V600E}$ knockdown downregulated phospho-ERK1/2 protein levels and inhibited Matrigel invasion of melanoma cells, accompanied with a decrease of MMP activity and integrin β1 expression. These results clarify that the mutated $BRAF^{V600E}$ is essentially involved in a malignant phenotype of melanoma cells by regulating genes involved in ECM remodeling through ERK1/2 activation and would, thus, serve as an attractive molecular target for melanoma treatment.

TSP-1 also binds FN directly (30) or indirectly through TSG6 (also called TNF-stimulated gene 6, a secreted protein that is produced during inflammation processes; ref. 31). The binding by FN to TSP-1 induces conformational changes in TSP-1 that enhance the ability of TSP-1 to be recognized by integrin α3β1 (30); such interactions seem to enhance FN matrix assembly and increase adhesive properties of TSP-1 to integrins. In addition, Decker and colleagues have shown that FN can form a complex with integrin α4β1 and TSP-1; the α4β1/FN/TSP1 complex increased adhesion of osteosarcoma cells (32).

Targeting $BRAF^{V600E}$-Positive Human Cancers with Orally Available Selective Inhibitors (PLX4720 and PLX4032)

Recent advances in understanding the molecular changes that take place in human tumorigenesis have led to the development of novel therapeutic strategies that are based on various molecular targets. Pharmacologic targeting of $BRAF^{V600E}$ may provide selective and rational advantages for treatment of patients with PTC harboring this mutation. Two Plexxikon compounds, PLX4720 and PLX4032, are novel, orally available selective small-molecule inhibitors of $BRAF^{V600E}$ that have been specifically designed to insert into the ATP-binding site and trap oncogenic $BRAF^{V600E}$ in an inactive conformation (33, 34). Consistent with their high degree of selectivity for the mutant $BRAF^{V600E}$, these compounds inhibit $BRAF^{V600E}$ kinase activity both in melanoma and colorectal cancer cells, leading to the inhibition of ERK1/2 phosphorylation and G_1-phase cell cycle arrest (33, 34). PLX compounds show efficacy against either homozygous or heterozygous $BRAF^{V600E}$-mutated cell lines and animals with tumor implantation (i.e., melanoma, colorectal cancer, or ATC; refs. 19, 33, 34).

It has been shown that these ATP-competitive RAF inhibitors could have unexpected effects in some cell and genotype contexts (e.g., presence of wt BRAF along with mutated RAS), because of ERK1/2 hyper-phosphorylation by dimerization between wt BRAF with another RAF isoform, CRAF (35–37). The results from these aforementioned studies highlight the importance of individualized genomic profiling to guide patient selection for inclusion in targeted therapy trials.

Recently, phase I and II clinical trials in patients with $BRAF^{V600E}$-positive melanomas have shown a partial or complete response to PLX4032, even though duration of

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the response to this inhibitor is yet unknown (38). PLX4032 induced complete or partial tumor regression in 81% of patients who had melanoma with the BRAF^{V600E} mutation. Responses were observed at all sites of disease, including the bone, liver, and small bowel (38). Most side effects related to PLX4032 seemed to be proportional to the dose and exposure to the drug. Cutaneous side effects, fatigue, and arthralgia were the main clinical problems in the treated patients. Thirty-one percent of these patients treated with PLX4032 developed skin lesions described as cutaneous squamous cell carcinomas and keratoacanthoma. These skin lesions generally appear within a few months of treatment initiation in sun-exposed areas of skin, suggesting that preexisting oncogenic mutations may potentiate the RAF inhibitor effects (34). This drug-induced effect is of particular interest because other RAF inhibitors such as sorafenib (used in clinical trials, either alone or in combination with chemotherapy that has not had significant antimelanoma effects) also caused these skin lesions in a subset of treated patients (38, 39). The ability of PLX4032 to cause tumor regression in a large proportion of patients with BRAF^{V600E}, advanced-stage, metastatic melanoma provides strong support for the hypothesis that the BRAF^{V600E} protein is a dominant driver of tumor growth and maintenance. However, in some patients with BRAF^{V600E} mutation–positive melanoma, the tumors showed resistance without evidence of an early response, and the mechanism of this primary resistance (refractory state) is still unknown. Importantly, results from an advanced human thyroid cancer preclinical model using PLX4720 (19, 40) suggest that these inhibitors might be an effective therapy in clinical trials for the treatment of patients with BRAF^{V600E}-positive thyroid cancers that are refractory to conventional therapy.

Conclusions and Perspectives

In summary, the BRAF^{V600E} mutation may affect the expression of tumor ECM noncellular components and alters the microenvironment in PTC. The molecular action of this oncogene seems to affect both the migratory and invasive properties of the thyroid cancer cell itself as well as components of the tumor ECM microenvironment. Knowledge about these new downstream targets of BRAF may help identify biomarkers (i.e., secreted factors) and/or targets for innovative therapeutic strategies in BRAF^{V600E}-positive human cancers. Therapeutic strategies aimed at modulating the host microenvironment (i.e., ECM cellular and noncellular components) may offer a complementary perspective for the treatment of patients with these types of cancers. It will be of considerable interest, therefore, to reveal the spectrum of molecular mechanisms underlying the signaling cross-talk of the tumor microenvironment and to determine the extent to which they participate in the aberrant behavior of BRAF^{V600E}-positive human cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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