Biochemical and Biophysical Research Communications 421 (2012) 508–513

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Global tyrosine kinome profiling of human thyroid tumors identifies Src as a promising target for invasive cancers

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ARTICLE INFO

Article history:
Received 3 April 2012
Available online 10 April 2012

Keywords:
Src
Kinome profiling
Tyrosine kinase
Thyroid cancer

ABSTRACT

Background: Novel therapies are needed for the treatment of invasive thyroid cancers. Aberrant activation of tyrosine kinases plays an important role in thyroid oncogenesis. Because current targeted therapies are biased toward a small subset of tyrosine kinases, we conducted a study to reveal novel therapeutic targets for thyroid cancer using a bead-based, high-throughput system.

Methods: Thyroid tumors and matched normal tissues were harvested from twenty-six patients in the operating room. Protein lysates were analyzed using the Luminex immunosandwich, a bead-based kinase phosphorylation assay. Data was analyzed using GenePattern 3.0 software and clustered according to histology, demographic factors, and tumor status regarding capsular invasion, size, lymphovascular invasion, and extrathyroidal extension. Survival and invasion assays were performed to determine the effect of Src inhibition in papillary thyroid cancer (PTC) cells.

Results: Tyrosine kinome profiling demonstrated upregulation of nine tyrosine kinases in tumors relative to matched normal thyroid tissue: EGFR, PTK6, BTK, HCK, ABL1, TNK1, GRB2, ERK, and SRC. Supervised clustering of well-differentiated tumors by histology, gender, age, or size did not reveal significant differences in tyrosine kinase activity. However, supervised clustering by the presence of invasive disease showed increased Src activity in invasive tumors relative to non-invasive tumors (60% v. 0%, $p < 0.05$). *In vitro*, we found that Src inhibition in PTC cells decreased cell invasion and proliferation.

Conclusion: Global kinome analysis enables the discovery of novel targets for thyroid cancer therapy. Further investigation of Src targeted therapy for advanced thyroid cancer is warranted.

1. Introduction

Thyroid cancer is the most common endocrine malignancy in the United States with approximately 44,000 new cases diagnosed in 2010 [1]. Although most patients with thyroid cancer have favorable outcomes following conventional therapy, a small subset of patients will develop aggressive and invasive disease that is refractory to surgery, TSH suppression, and radioactive iodine (RAI) treatment. Consequently, alternative therapies are needed to target these resistant tumors.

The aberrant activation of tyrosine kinases is known to drive tumorigenesis in multiple cancers including thyroid cancer, and several clinical trials have been initiated to test the efficacy of tyrosine kinase inhibitors (TKI) in the treatment of advanced thyroid cancer [2]. Current targeted therapies, however, are biased toward a small subset of tyrosine kinases, thus limiting the range of possible therapeutic targets. In addition, unraveling the complex interactions among signaling pathways requires a comprehensive picture, rather than a narrow focus concentrating on a few known targets. Consequently, recent advances in technology have allowed for the high-throughput analysis of the full kinome array in human tumor samples.

Du et al. recently described a novel bead-based method for the global profiling of activated tyrosine kinases in cancer cell lines and patient samples [3]. They identified upregulated Src activity in primary human glioblastoma samples and validated kinome profiling as a feasible technique for the identification of potential therapeutic targets. Similarly, other groups utilized kinome array analysis to interrogate tyrosine kinase activity in pediatric brain tumors and chondrosarcoma primary cultures and cell lines [4,5]. In these studies, the authors identified increased AKT, PDGFRβ, and Src activity in their samples and performed functional assays demonstrating that Src inhibition resulted in decreased cell viability

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http://dx.doi.org/10.1016/j.bbrc.2012.04.034

and increased apoptosis. Taken together, these reports validate kinome profiling as a novel technique for the identification of a wide range of potential targets for the treatment of human cancers.

In this study, we sought to characterize the kinome profile of human thyroid tumors to identify potentially novel targets for therapy. Using the Luminex immunosandwich assay, we analyzed the phosphorylation status of seventy-one unique tyrosine kinases in twenty-six thyroid tumors. Based on these results, we also performed functional studies in papillary thyroid cancer (PTC) cells using the Src inhibitor dasatinib to identify Src as a promising target for the treatment of invasive thyroid cancers. To our knowledge, this is the first application of kinome profiling in thyroid tumors and further validates this technique as a high-throughput assay for characterizing aberrant signaling networks in human cancers.

2. Materials and methods

2.1. Human thyroid tissue samples

Twenty-six human thyroid tumors and normal adjacent thyroid tissue were obtained from surgical specimens resected from patients treated at the Brigham and Women's Hospital between October 2008 and October 2009. Upon removal from patients, specimens were immediately frozen in liquid nitrogen and stored frozen until the time of protein extraction for kinome array profiling. This study has been approved by the institutional review boards of Brigham and Women's Hospital.

2.2. Reagents

Dasatinib was obtained from the Broad Institute (Cambridge, MA) and was synthesized as previously described [6]. Rabbit anti-human c-Src antibody and anti-phospho-Src at tyrosine residue 416 (Y416) were purchased from Cell Signaling (Beverly, MA). Mouse anti-phosphotyrosine antibody was purchased from BD Bioscience (San Diego, CA).

2.3. Luminex immunosandwich assay

The Luminex assay was performed as previously described [3]. Briefly, antibody-coupled beads were incubated with cellular protein lysates to capture tyrosine kinases. A biotinylated phosphotyrosine antibody and streptavidin R-phycoerythrin (SAPE) were added sequentially to bind to the phosphotyrosine residues.

The mixture was analyzed with a Luminex 100 instrument (Luminex), whereby each microsphere is analyzed with 2 lasers – one to detect the bead color and thereby the identity of the tyrosine kinase, and the other to detect the R-phycoerythrin signal reflecting the tyrosine phosphorylation levels on the bead. Values were considered positive if they were threefold over the background and represented by log-2 transformation of the folds over background. Negative values were threshold to −5. The preprocessed data were converted to. gct files and analyzed with GenePattern 3.0 (The Broad Institute). For normalization, sample and antibody backgrounds were subtracted from the raw values. The ratios between normalized values from paired tumor and normal samples were calculated. The processed data were converted to. gct files and analyzed with GenePattern 3.0 (The Broad Institute).

2.4. Immunoprecipitation

Tissue specimens or treated cells were lysed with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing protease inhibitors cocktail (Sigma–Aldrich) and 2 mM Na vanadate. Lysates were centrifuged at 5000 rpm for 10 min at 4 °C. Antibodies used in the individual experiments (1:100 dilution) were added to 0.5 ml of supernatants and incubated for 24 h at 4 °C. Immunocomplexes were then separated by incubating with 30 μl of Protein G Plus/Protein A Agarose Suspension (Calbiochem) for 4 h at 4 °C followed by centrifugation at 5000 rpm for 5 min at 4 °C. The pellets were mixed with non-reducing sample buffer and processed for Western blotting.

2.5. Western blotting

Equal amounts of immunoprecipitated products were separated by 4–10% SDS–PAGE and electrophoretically transferred to Immobilon® membranes (Millipore, Bedford, MA). The transferred membranes were blocked with blocking buffer (PBS + 0.1% Tween 20 with 1% BSA) at room temperature for 1 h. The membranes were immunoblotted with appropriate primary antibody for 2 h, and then washed in washing buffer (PBS + 0.1% Tween 20, without 1% BSA) once for 15 min followed by two washes for 5 min. The membranes were blocked again in new blocking buffer for 1 h at room temperature and then immunoblotted with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. Membranes were subsequently washed in washing buffer once for 15 min followed by two washes for 5 min each. Proteins on each immunoblot were visualized with Renaissance® Western blot chemiluminescence reagent (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ).

2.6. Cell culture

The human papillary thyroid cancer cell line TPC-1 was kindly provided by Dr. Orlo H. Clark (University of California at San Francisco, San Francisco, CA). The human papillary thyroid cancer cell line 8505-C was obtained from Dr. Sareh Parangi (Massachusetts General Hospital, Boston, MA). Another papillary thyroid cell line KTC-1 was obtained from Dr. James A. Fagin (Memorial Sloan Kettering Cancer Institute, New York, NY). Cells were cultured as previously described [7–9]. All cells were subcultured at 80% confluency by trypsinization (in a 0.5% (v/v) trypsin solution, supplemented with 0.2% (v/v) EDTA).

2.7. Clonogenic survival assay

Eight hundred cells were plated at the appropriate dilutions and surviving colonies were stained with crystal violet ten days later by standard clonogenic survival assay methods, as previously described [10].

2.8. Boyden chamber invasion assay

Cellular invasion was determined by using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber was used according to the manufacturer's instructions (BD Biosciences, Bedford, MA). 5% FBS was added to lower chambers as a chemoattractant. Cells were co-incubated with DMSO or 100 nM of Dasatinib in the absence or presence of 10% FBS for 48 h. Cells were trypsinized and then cell suspensions in serum-free medium containing 5×10^4 cells/ml were seeded onto Matrigel-coated filters. After 22 h of incubation, the filters were stained using the Diff-Quik kit (BD Biosciences). Images were acquired under phase-contrast microscope (40X) and the number of cells that had invaded through the filter was counted in randomly selected high-power fields.

Table 1
Histopathological characteristics of well-differentiated thyroid cancer specimens.

Case No.	Extension	CI	LVI	Histology
1	+	–	–	PTC, classical variant
2	–	–	–	PTC, follicular variant
3	–	–	–	PTC, follicular variant
4	–	–	–	PTC, follicular variant
5	–	–	–	PTC, follicular variant
6	+	–	+	PTC, follicular variant
7	–	–	–	PTC, follicular variant
10	–	–	–	PTC, mixed classical and follicular variant
11	–	+	–	Follicular carcinoma
12	–	–	+	PTC, mixed classical and follicular variant
22	–	–	+	PTC, follicular variant
23	–	–	–	PTC, classical variant

Abbreviations: CI, capsular invasion; LVI, lymphovascular invasion; PTC, papillary thyroid cancer.

2.9. Overexpression vectors (cloning), transfection, and viral infection

To introduce wild type (WT) and mutant Src (SRC_T341I) into 8505-C cells, we used the pLenti6/V5 Directional TOPO Cloning kit (Invitrogen). Construction of pLenti-SRC-WT was described previously [3]. The SRC_T341I point mutation was introduced into pLenti-SRC-WT by using QuikChange II XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). pLenti6/UbC/V5-DEST/lacZ (Invitrogen) was used as a control. Viral transduction of the cells was done following the manufacturer's instructions. Briefly, the 8505-C cells were transiently transfected with viral vector together with ViraPower (Invitrogen). Cells were plated in 6-well dish plates (0.5×10^5 cells/well) and infected with 200 µl of LacZ control, SRC_WT and mutant tyrosine kinase lentivirus. Infections were done in triplicates. On day 2, cells were treated with various concentrations of dasatinib. Clonogenic survival assay was assessed at 96 h after dasatinib treatment.

2.10. Statistical analysis

The data was analyzed using GraphPad Prism 3.0 software (GraphPad, San Diego, CA). Differences between treatment groups were evaluated with a two-tailed independent Student's *t* test or Fisher's exact test, as appropriate. Each assay was performed in triplicate, and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Src activity is upregulated in invasive thyroid cancer specimens

We collected twenty-six thyroid tumors and matched normal tissue from the operating room at Brigham and Women's Hospital. Histological analysis of specimens confirmed twelve well-differentiated tumors (classical variant of PTC, follicular variant of PTC, follicular carcinoma), nine tumors with intermediate differentiation (tall cell, diffuse sclerosing, solid, columnar variants of PTC), and five benign adenomas. Tumor status regarding extrathyroidal extension, capsular invasion, and lymphovascular invasion (LVI) for all twelve well-differentiated tumors is described in Table 1. Tyrosine kinome profiling demonstrated upregulation of nine tyrosine kinases in tumors relative to matched normal thyroid tissue: EGFR, PTK6, BTK, HCK, ABL1, TNK1, GRB2, ERK, and SRC (Fig. 1A). Supervised clustering of well-differentiated tumors by histology, gender, age, or size did not reveal significant differences in tyrosine kinase activity. However, supervised clustering of well-differentiated tumors by the presence of invasive disease (extrathyroidal extension, lymphovascular invasion, capsular invasion) showed increased Src activity in invasive tumors relative to non-invasive tumors (60% v. 0%, $p < 0.05$) (Table 2).

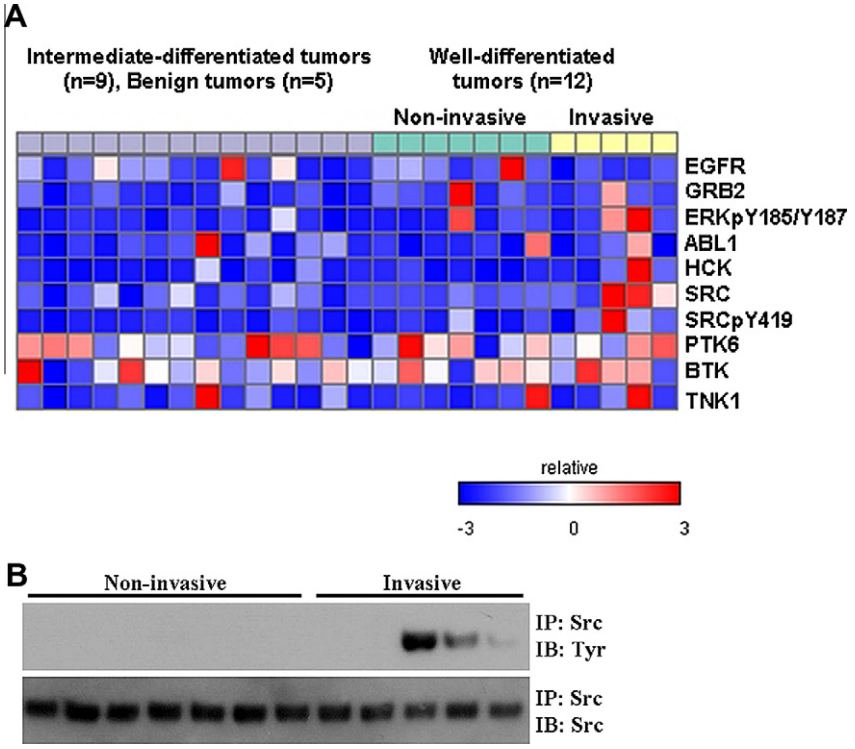

Fig. 1. Src is activated in invasive thyroid cancer specimens. (A) Twenty-six paired normal and thyroid tumor protein lysates were collected and subjected to kinome array analysis. Tyrosine kinase phosphorylation levels were tested by single or multiple antibodies. The raw data were processed and positive signals were normalized to fold-over-backgrounds. The kinase phosphorylation value of each tumor specimen was divided by the matched normal specimen. The normalized readings were log-transformed and converted into a .gct file and the HeatMap diagram was generated using the GenePattern 3.0 software. (B) Immunoprecipitation for c-Src and immunoblot for tyrosine-phosphorylated c-Src in well-differentiated thyroid tumor specimens was performed.

Table 2
Presence of Src activation and invasion in well-differentiated thyroid cancers.

	Src (+)	Src (−)	*p* Value
Invasive	3	2	
Non-invasive	0	7	0.045

3.2. Kinome profiling is a valid technique for identifying activated kinases in human cancers

In order to validate the results of the Luminex assay, we performed immunoprecipation for c-Src in all twelve well-differentiated tumors and examined the expression level of tyrosine-phosphorylated c-Src by immunoblot (Fig. 1B). Consistent with results from our kinome profile, we found that invasive tumors demonstrated increased levels of phosphorylated Src relative to non-invasive tumors (60% v. 0%, $p < 0.05$). This result confirms the feasibility of global kinome profiling as a novel technique for characterizing activated tyrosine kinases and signaling pathways in human cancers.

3.3. Src inhibition decreases cell proliferation in papillary thyroid cancer cells

Oncogenic activation of Src family kinases (SFK) plays a critical role in driving a variety of cellular events including proliferation, survival, and invasion [11]. To investigate the role of Src inhibition as a potential therapeutic target, we treated the papillary thyroid cancer (PTC) cell line 8505-C with increasing doses of the Src inhibitor dasatinib for 48 h and showed that tyrosine phosphorylation of c-Src decreased with increasing doses of dasatinib, most notably at 100 nM (Fig. 2A). We then treated three PTC cell lines with 100 nM dasatinib and subjected harvested cells to a clonogenic survival assay (Fig. 2B). We found that dasatinib was effective in decreasing cellular proliferation in treated cells relative to controls ($p < 0.05$). Moreover, dasatinib-treated PTC cells demonstrated decreased levels of phosphorylated Src relative to untreated controls, suggesting that activated Src plays a role in promoting cell survival in PTC (Fig. 2C).

3.4. Src inhibition decreases cell invasion in papillary thyroid cancer cells

We next investigated the effect of Src inhibition on cell migration by performing a Boyden chamber invasion assay on PTC cells following treatment with 100 nM of dasatinib. PTC cells treated with dasatinib showed decreased cellular invasion relative to controls, suggesting that Src inhibition may provide a promising target for therapy in invasive thyroid cancers (Fig. 3).

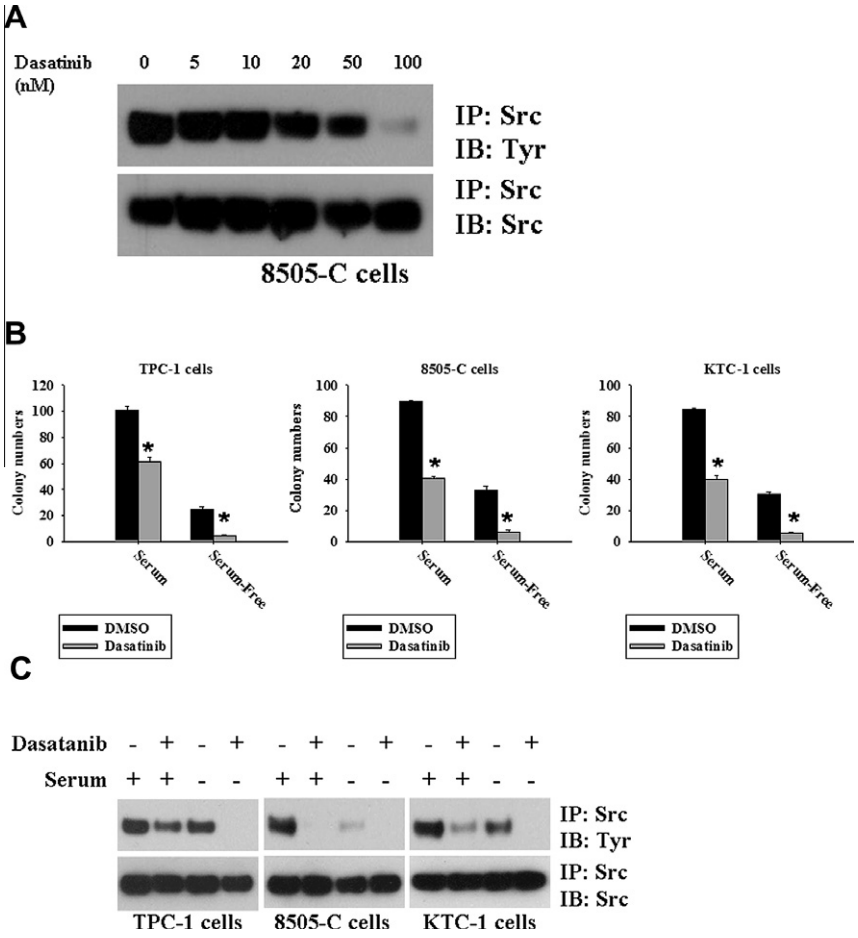

Fig. 2. Src inhibition decreases cell proliferation in PTC cells. (A) 8505-C cells were first treated with increasing doses of the Src inhibitor dasatinib and tyrosine phosphorylation status of c-Src was determined by immunoprecipitation. (B) TPC-1, 8505-C, and KTC-1 cells were then treated with DMSO or 100 nM of dasatinib in the presence or absence of 10% FBS serum for 48 h. Treated cells were harvested and then subjected to the clonogenic survival assay. Each bar of the histogram represents a minimum of three experiments, and the data are presented as the mean ± SD. Asterisk (*) represents a *p* value of <0.05, indicating a significant statistical difference between DMSO control and dasatinib treatment groups. (C) Immunoprecipitation for c-Src and Western blot analysis for tyrosine-phosphorylated c-Src in DMSO control and dasatinib-treated PTC cells was performed.

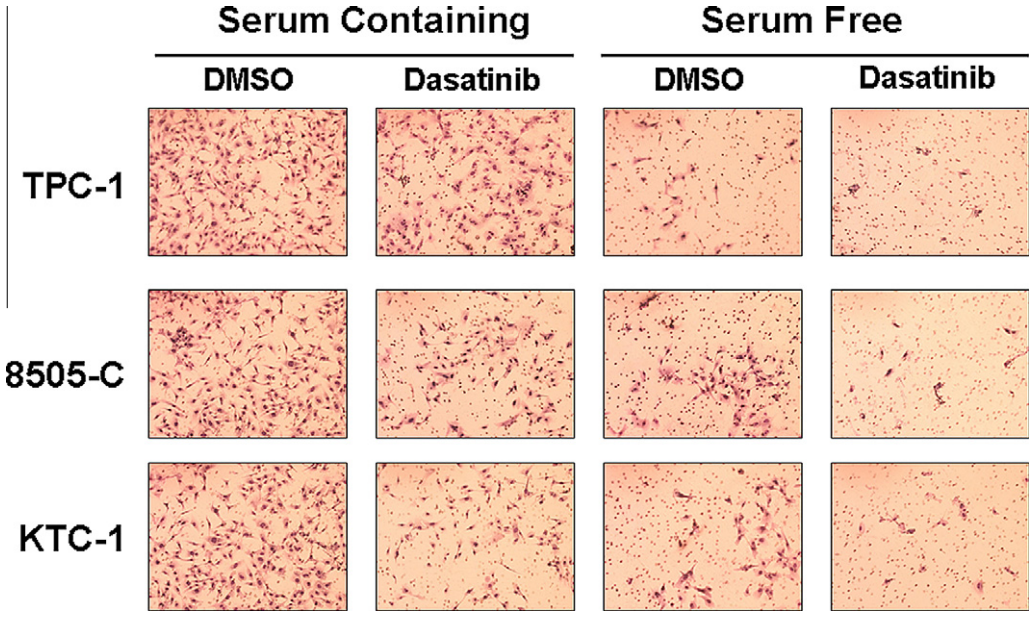

Fig. 3. Src inhibition decreases cell invasion in PTC cells. TPC-1, 8505-C, and KTC-1 cells were treated with DMSO or 100 nM of the Src inhibitor dasatinib in the presence or absence of 10% FBS serum for 48 h. Treated cells were harvested and then subjected to Boyden chamber invasion assay. Five percentage FBS was loaded in the lower chamber as a chemotaxis inducer. 500 μl of cells at 5 × 104/ml were plated in the upper chamber and were allowed to migrate through 8-μm porous filters coated with Matrigel for 22 h. Non-invading cells were removed, and the filter was fixed and stained with crystal violet. Images were visualized by phase contrast microscopy (40×).

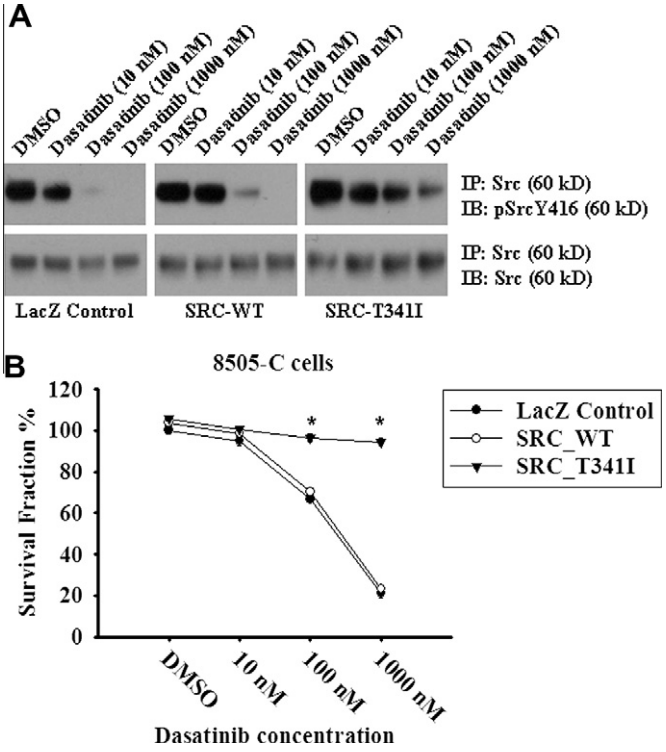

Fig. 4. Dasatinib specifically inhibits Src phosphorylation and cell survival in PTC cells. (A) 8505-C cells were infected by control LacZ, SRC_WT and SRC_T341I lentivirus and then treated with dasatinib at concentrations as indicated. The cell lysates were immunoprecipitated with a c-Src antibody and immunoblotted with either the pY419SRC antibody or a total Src antibody. (B) Infected cells were co-incubated with dasatinib at various concentrations and then subjected to clonogenic survival assay. Each bar of the histogram represents a minimum of 3 experiments, and the data are presented as the mean ± SD. Asterisk (*) represents a p value of <0.05, indicating a significant statistical difference between LacZ control and SRC_T341I treatment groups.

3.5. T341I mutation blocks dasatinib's effects on Src phosphorylation and cell survival

To test the specificity of dasatinib for Src activity, we generated a dasatinib-resistant Src kinase with the point mutation T315I. This specific mutation of a conserved threonine in the kinase domain has been identified in patients with CML who develop imatinib or dasatinib resistance [12,13]. We found that the T315I mutation abrogated dasatinib-decreased Src phosphorylation in 8505-C cells in a dose-dependent manner relative to controls (Fig. 4A). It also rescued dasatinib effects on cell survival at 100 and 1000 nM compared to controls, suggesting that dasatinib is a specific Src inhibitor in PTC cells (Fig. 4B).

4. Discussion

In this report, we characterized the tyrosine kinome profile of twenty-six human thyroid tumors using a high-throughput bead-based system. This is the first study to describe kinome profiling in human thyroid tumors for identifying novel therapeutic targets in thyroid cancer. We found several activated tyrosine kinases in our tumor samples relative to matched normal tissue; interestingly, supervised clustering of thyroid tumors by invasiveness demonstrated increased Src activity in tumors relative to matched normal tissue.

The aberrant activation of Src family kinases (SFK) plays a critical role in tumorigenesis by driving a number of cellular events including proliferation, adhesion, survival, and invasion [11]. Multiple signaling pathways converge upon Src, which in turn acts as a central mediator that can interact with a myriad of downstream effectors to drive cancer progression. Indeed, SFKs have been shown to promote cellular proliferation through interactions with the Ras/ERK/MAPK pathway [14–16]. SFKs can also regulate gene transcription by activating transcription factors such as Stat3, which plays a role in determining apoptosis and cell cycle progression [17]. Furthermore, activated Src interacts with adhesion proteins and can disrupt the cadherin/catenin complex leading to an invasive phenotype [18].

Given its prevalent role in tumorigenesis, Src offers an attractive therapeutic target for the treatment of a wide variety of cancers. Several small molecule SFK inhibitors are currently under investigation and show promise in early preclinical studies [19]. Schweppe et al. recently reported that Src inhibition via AZD0530 decreased proliferation and invasion in papillary and anaplastic thyroid cancer cells, perhaps through the inhibition of substrates such as focal adhesion kinase (FAK) [20]. Cichini et al. also showed that Src plays a key role in TGFβ-induced epithelial-to-mesenchymal transition in hepatocytes through its interaction with FAK [21]. In our study, we found that 60% of well-differentiated thyroid tumors with an invasive phenotype demonstrated increased Src activity. We also showed that treatment with the Src inhibitor dasatinib decreased cell invasion and proliferation compared with controls. Taken together, our results suggest that Src plays a critical role in tumor invasion and metastatic potential and that Src inhibition is clinically relevant in the treatment of invasive thyroid cancers.

Despite these promising results, it would be short-sighted to focus on a single molecular target when treating cancers, especially given the propensity for tumors to mutate and develop resistance in response to monotherapy. To this end, it is necessary to understand the complex interplay of signaling pathways driving tumorigenesis and target the network from multiple directions. As such, kinome profiling is a valid and feasible technique for identifying an array of upregulated kinases and potentially novel targets for therapy. Furthermore, this method identifies activated kinases that may not otherwise be recognized by genomic mutation studies or conventional techniques examining copy number or protein levels. Additionally, Roorda et al. employed kinome analysis to investigate alterations in signaling pathways in response to drug treatment, thereby demonstrating another strategy for utilization of this technique [22].

Kinome profiling is not without its limitations–variations in tissue processing may affect cell viability during procurement from the operating room. Although efforts are made to freeze tissue immediately upon specimen removal, tissues can become devascularized during a lengthy operation before the tumor is removed in its entirety. Subsequently, tyrosine phosphorylation status may be altered in such a manner that does not accurately reflect activated pathways in a viable tumor. In addition, the Luminex assay is limited by the sensitivity and specificity of antibodies for activated kinases and their substrates; for this reason, we validated our results with alternative methods and performed functional assays to verify our hypotheses. The combination of kinome profiling and conventional techniques offers a unique opportunity for innovative discovery that may ultimately lead to advances in the field of cancer treatment.

In conclusion, global kinome analysis enables the discovery of novel targets for thyroid cancer therapy. Our results show that tyrosine kinome profiling of thyroid tumors identified upregulation of Src activity in the majority of invasive thyroid cancers from our study set. Functional experiments confirm that Src inhibition is effective in decreasing proliferation and invasion in human PTC cell lines. Future studies will include tumor specimens from collaborations with other institutions and correlate kinome profiling with clinical outcomes. Further investigation of Src targeted therapy and elucidation of its interaction with other activated kinases in invasive thyroid cancer is warranted.

Acknowledgement

Grant support: Eleanor and Miles Shore/Robert T. Osteen Junior Faculty Research Grant from the Department of Surgery at Brigham and Women's Hospital.

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