Invasion in follicular thyroid cancer cell lines is mediated by EphA2 and pAkt

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Background. EphA2 is a tyrosine kinase receptor that is overexpressed in many cancers and is associated with poor prognosis and increased metastasis. Phosphorylated Akt (pAkt) plays a role in the regulation of thyroid cancer invasion and metastasis. We investigated the role of EphA2 and Akt in FTC-133 and FTC-238, 2 closely related human cell lines with differing invasive phenotypes.

Methods. Western blot was used to measure the total protein expression in cell lines, and immunohistochemistry was performed on thyroid tissue microarrays. Thyroid cell lines were transfected with siRNA or cDNA. Invasion assays were performed using Matrigel chambers, and invaded cells were assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT).

Results. EphA2 protein was expressed in thyroid cancer cell lines and in benign and malignant human thyroid tumors but not in normal thyroid. Compared with FTC-133, FTC-238 expressed fivefold more EphA2 protein and had a fivefold increase in invasion (P < .001). In FTC-238, EphA2 siRNA decreased EphA2 levels and reduced invasion, with a decrease in pAkt protein. Overexpression of EphA2 in FTC-133 increased invasion and increased pAkt protein. Akt siRNA and Akt inhibitors decreased pAkt levels and invasion without changing EphA2 levels.

Conclusion. EphA2 is expressed in human thyroid cancer and mediates invasion in the follicular thyroid cell lines FTC-133 and -238. Phosphorylated Akt (pAkt), an important regulator of thyroid cancer metastasis, is attenuated by EphA2 knockdown, providing evidence that EphA2 may act through pAkt to mediate invasion. EphA2 and pAkt may be candidates for targeted therapy against metastatic thyroid cancer. (Surgery 2012;152:1218-24.)

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Although most patients with thyroid cancer have good long-term survival rates, those who do not survive usually succumb to distant metastases, local invasion, or both. Conventional chemotherapy, radical surgery, and radiotherapy have limited effectiveness in advanced thyroid cancer. Targeted therapy against advanced thyroid cancer holds significant promise, and clinical trials have demonstrated encouraging results. It is therefore important to continue to work in identifying novel candidates for targeted therapies.

EphA2 is a membrane-bound tyrosine kinase receptor that is a member of the Eph receptor family. Eph receptor signaling is important in cell-cell repulsion or adhesion, and the family and its ligands, ephrins, play an important role in embryonic development. In cancer, EphA2 is overexpressed in a variety of tumors, including breast, prostate, lung, esophageal, cervical, ovarian, renal, and colon cancers. Elevated EphA2 expression in cancer has been correlated with higher stage of disease and decreased survival as well as metastases. The role of EphA2 in thyroid cancer has not been described previously.

Akt is a family of serine/threonine kinases that are involved in cell signaling in metabolism, cell proliferation, apoptosis, transcription, and migration. In cancer, it is associated with tumor cell survival, proliferation, and invasiveness. Increased Akt activation has been noted especially in follicular and poorly differentiated...
thyroid cancers.\(^5\) Phosphorylated Akt is thought to play an important role in thyroid cancer cell migration and invasion, and higher levels of pAkt were found in the invasive fronts of primary thyroid cancers.\(^4\) Recently, EphA2 was found to have opposing ligand-dependent and ligand-independent effects. Receptor binding with ephrin A1 decreased invasion, whereas ligand-independent activation increased invasion. Akt was found to be a key regulator of this phenomenon.\(^5,6\)

We previously found that EphA2 was overexpressed in the FTC-238 cell line, when compared with FTC-133. These are 2 follicular thyroid cancer cell lines derived from the same patient. FTC-133 was established in culture from a lymph node and FTC-238 from a lung metastasis.\(^7\) FTC-238 was shown to be more invasive than FTC-133.\(^8,9\) The goal of this study was to investigate whether EphA2 had a role in the invasion of these cell lines.

**METHODS**

**Cell culture.** The human thyroid cancer cell lines FTC-133, -236, -238, KAT 18, TPC1, and WRO (generous gifts from Dr Michael Demeure, TGen, Phoenix, AZ, and Dr Sylvia Asa, Toronto, ON, Canada) were cultured in Dulbecco modified Eagle medium (DMEM) (#11965, Gibco, Grand Island, NY) containing 10% fetal bovine serum, and 100 U/mL each of penicillin and streptomycin. Cell passages from 30 to 40 were used. Cells were grown in a humidified cell culture incubator with 5% CO\(_2\) at 37°C.

**Immunoblotting.** Cells were rinsed with PBS and lysed by a 1% SDS solution. Cell lystate was collected into an Eppendorf tube and heated at 90°C for 30 minutes. The protein content of each sample was determined by the bicinchoninic acid protein assay (#23221, #23224, Thermo Scientific, Rockford, IL). Samples (30 to 50 \(\mu\)g of protein) were mixed 1:1 with Tris (1.25 M, pH 6.8); 20% glycerol; 4% SDS; 10% 2-mercaptoethanol; and 0.05% bromophenol blue. Proteins were separated using SDS-PAGE and transferred to a PVDF membrane overnight at 30 V. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween (TBST) for 1 hour and incubated with the primary antibody (1:1,000 dilution) for 4 hours or overnight. The blot was washed with TBST and incubated with a 1:10,000 dilution of the secondary antibody. The immunoreactive protein was detected by an enhanced chemiluminescence detection kit (Pierce #34080, Thermo Fisher Scientific, Rockford, IL).

**Immunohistochemistry.** Tissue arrays were constructed from cases of thyroid carcinoma, benign thyroid nodules, and normal thyroid tissue. Triplicate 0.2 cm samples were included from each lesion. Staining for immunohistochemistry using antibodies against the protein EphA2 (Santa Cruz Biotechnology, Santa Cruz, CA) was performed on 5 \(\mu\)m-thick sections with appropriate controls and was interpreted in a blinded fashion by a pathologist (RAK) using a 0 to 3 scoring system.

**Invasion assays.** The 24-well BD BioCoat Matrigel Invasion Chambers (8\(\mu\m\) pores) (#354480, BD Biosciences, San Jose, CA) were used according to manufacturer’s guidelines. Cells were detached with Accutase (#AT104, Innovative Cell Technologies, San Diego, CA), centrifuged, and resuspended in DMEM; they were then counted, and 20,000 to 80,000 cells were plated in the chambers for 20 hours at 37°C, 5% CO\(_2\). Noninvaded cells were removed from the upper chamber, and the cells adhering to the bottom surface of the inserts were incubated in 0.5 mL a solution of (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (#M2128, Sigma-Aldrich, St. Louis, MO) 0.5 mg/mL) for 1 hour at 37°C, 5% CO\(_2\). Air-dried filters were cut off from inserts; the blue compound in the cells adhering on the membrane was dissolved in 130 \(\mu\)L 2-propanol, and the absorbance was measured immediately at 550 nm. A standard curve was generated from the absorbance intensity of MTT and the number of cells plated. The invaded cell number was calculated according to the standard curve.

**EphA2 transfection.** Human EphA2 cDNA (ATCC#MGC-29717) was cloned into the mammalian expression vector pcDNA 3.1 (+) (#V870-20, Invitrogen, Life Technologies, Grand Island, NY) with a hygromycin resistance cassette using HindIII at the 5’ end and XhoI at the 3’. FTC 133 cells were transfected using lipofectamine with control pcDNA or EphA2 pcDNA in OPTI-MEM media (#31985, Gibco) for 2 days, then in regular culture media DMEM with hygromycin 200\(\mu\)g/mL (#10687-010, Invitrogen).

**Treatment with siRNA and Akt inhibitor.** Cells were cultured in 6-well plates to 90% confluence. We replaced the regular culture medium (DMEM, 10% fetal bovine serum, penicillin, and streptomycin) with OPTI-MEM (#31985, Gibco). Small interfering RNA (siRNA) (EphA2) (#4392420, Applied Biosystems, Carlsbad, CA), Akt (#6211) and control (Cell Signaling Technology, Danvers, MA) were added to cells with incubating time of 48 hours. Perifosine (#S1037, Selleck Chemicals, Houston, TX) was dissolved in H\(_2\)O, added to the cell culture media with the final
concentration of 20 µM, and incubated with cells overnight.

RESULTS

EphA2 protein is expressed in thyroid cancer cell lines and human thyroid tissue. EphA2 protein was expressed in the thyroid cancer cell lines FTC-133, -236, -238, KAT 18, TPC1, and WRO by Western analysis (Fig 1, A). FTC-133, -236, -238, and WRO are follicular thyroid cancer cell lines; KAT 18 and TPC1 are anaplastic and papillary thyroid cancer cell lines, respectively. Compared with FTC-133, FTC-238 had a greater than fivefold expression of EphA2 by densitometry and had the highest EphA2 level of all the cell lines tested. In a human thyroid tissue microarray, cytoplasmic EphA2 expression was found in papillary, follicular, and medullary thyroid cancer, as well as in Hurthle cell tumors and follicular nodules. However, there was little to no expression detected in normal thyroid tissue (Fig 2).

Matrigel invasion of thyroid cancer cell lines correlates with EphA2 expression and loss of PTEN. FTC-238 had a fivefold increase in invasion compared with FTC-133 (P < .001) (Fig 1, B). The ability to invade appeared to correlate with the amount of EphA2 protein expressed by the FTC cell lines. The KAT 18, TPC1, and WRO cell lines had low levels of invasion in the Matrigel assay despite EphA2 protein expression. These cell lines retained PTEN expression, unlike the FTC cell lines (Fig 1). PTEN is a tumor suppressor gene important in the Akt activation pathway. These findings may indicate that PTEN loss is important for EphA2-mediated invasion in thyroid cancer cells.

The ligand ephrin A1 decreases EphA2 protein expression but does not alter invasion in FTC cell lines. In a pancreas cancer cell line, treatment with ephrin A1 ligand causes a decrease in EphA2 protein expression via receptor degradation and is accompanied by a decrease in invasion. Treatment with ephrin A1 also decreased EphA2 expression in FTC-133 or FTC-238 (Fig 3, A) but had no effect on invasion (Fig 3, B), indicating that ligand-dependent downregulation of EphA2 does not decrease invasion in this cell type. The levels of endogenous ephrin A1 in the FTC cell lines were similar (Fig 1, C).

EphA2 suppression decreased invasion in FTC cell lines. Treatment with EphA2 siRNA decreased EphA2 expression fourfold and reduced invasion by 50% (P < .001) in FTC-238 cells (Fig 4). Similar findings were also seen in FTC-133, though the effect was less pronounced because both baseline EphA2 expression and invasion were lower in that cell line. Protein levels of pAkt were also significantly decreased following EphA2 siRNA treatment in both FTC-238 (20%; P < .001) and FTC-133 (20%; P < .05), indicating that EphA2 regulates Akt activation (Fig 4). With EphA2 siRNA treatment, the protein levels of E-cadherin, α-catenin, and p-120, involved in cell-to-cell junctions were unchanged, and neither were levels of P44/42 MAPK, pJNK, or p-MLK3, other enzymes involved in cell signaling pathways (data not shown).

Overexpression of EphA2 increased invasion and levels of pAkt protein. To evaluate the effect of EphA2 overexpression, the cell line FTC-133 was stably transfected with an EphA2 expression plasmid. EphA2 levels were increased at least fivefold (Fig 5), approaching the level of EphA2 expression in FTC-238. Invasion on Matrigel was increased fourfold (P < .001), confirming the role of EphA2 in invasion (Fig 5). Phosphorylated Akt protein levels were increased significantly (P < .001) as the result of EphA2 overexpression, again indicating that EphA2 promotes Akt activation (Fig 5).

The effect of Akt suppression. Treatment with Akt siRNA decreased pAkt levels and invasion without changing EphA2 levels in both FTC-133 and 238 (Fig 4). There was no additive effect of Akt siRNA and EphA2 siRNA treatment on FTC-238 Matrigel invasion. Treatment with the Akt inhibitor perifosine completely abrogated invasion by FTC-238 cells (Fig 4).

DISCUSSION

The role of receptor kinase EphA2 in thyroid cancer has not been previously described. In this study, we show that it is expressed in human thyroid cancer cell lines and in human thyroid tumors but not in normal human thyroid glands. The effect of EphA2 on invasion was investigated in FTC-133 and FTC-238, 2 closely related follicular thyroid cancer cell lines with different invasive phenotypes. We showed that not only is EphA2 expressed highly in the more invasive cell line FTC-238, but also that suppression of EphA2 expression by siRNA decreased invasion in these cell lines. It is interesting that downregulation of EphA2 by its ligand ephrin A1 did not affect invasion. We also showed that EphA2 appears to interact with pAkt, a molecule known to be involved in thyroid cancer invasion. Phosphorylated Akt levels correlated with EphA2 expression and invasion, and EphA2 suppression decreased both pAkt levels and invasion. Overexpression of EphA2 increased pAkt levels and invasion by FTC-133 cells, whereas inhibition of pAkt using siRNA or the pAkt inhibitor perifosine inhibited invasion without affecting EphA2.
expression. These results suggest that EphA2’s role in invasion may be pAkt dependent.

The molecular mechanisms of thyroid neoplasia, invasion, and metastasis have been reviewed extensively. Multiple genes and signaling pathways are implicated. In follicular thyroid cancer, Ras mutations and PPARγ rearrangements occur commonly.

Using tissue microarray, we determined that EphA2 is expressed in both benign and malignant thyroid tumors but not in normal thyroid tissue. This suggests that EphA2 plays a role in the development of both benign and malignant thyroid tumors and is not a useful marker for determining malignancy. Hurthle cell tumors had the

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**Fig 1.** Invasion and protein expression in thyroid cancer cell lines. (A) Expression of EphA2, Akt, pAkt, PTEN, and GAPDH in thyroid cancer cell lines by immunoblotting. (B) Invasion of thyroid cancer cell lines in Matrigel. (C) Expression of ephrin A1 ligand in the FTC cell lines.

**Fig 2.** Expression of EphA2 protein in a human thyroid tissue microarray (TMA). (A) Graphical representation of immunohistochemistry staining on a TMA containing 60 thyroid tissue samples with 2 mm tumor cores arrayed in triplicate, with standard deviation calculated from the average score of each specimen in triplicate. The 0 to 3 scale was based on percent of positive cells and staining intensity. N is the number of each tumor type examined. There was little or no EphA2 staining of normal thyroid tissue. (B) Expression of EphA2 plus a normal rabbit serum negative (NRS) control by immunohistochemistry of a single tumor core (follicular variant of papillary thyroid cancer).
highest expression level of the protein, and both follicular cancer and nodules had similar levels of expression. Papillary thyroid cancers had variable levels of expression. The stage of the malignant tumors and long-term follow-up on this tissue microarray series is not available, therefore the correlation of EphA2 with invasion and metastases in human thyroid cancer has yet to be determined.

EphA2 was recently shown to mediate negative feedback inhibition of the Ras-PI3K-Akt pathway. PTEN is a tumor suppressor gene that is lost in Cowden’s syndrome, a multiple hamartoma syndrome that includes thyroid neoplasia as part of its phenotype. Reduction in PTEN levels occurs in follicular thyroid tumors through various mechanisms. Loss of PTEN is known to increase pAkt activity through activation of the PI3K signaling pathway, and re-expression of PTEN in FTC-133 was shown to block pAkt and EGF stimulated invasion. In our study, thyroid cancer cell line invasion correlated with the absence of PTEN. The cell lines that expressed PTEN, KAT18, TPC-1, and WRO, did not invade Matrigel. However, a direct relationship between PTEN- and EphA2-mediated invasion has not yet been shown. Recent studies in prostate cancer and glioma cell lines showed that EphA2 has 2 opposing roles in the regulation of cell migration via pAkt. Ligand-dependent EphA2 activation inhibited migration but ligand-independent EphA2 overexpression–promoted migration required the phosphorylation of EphA2 on serine 897 by pAkt, indicating Akt-EphA2 crosstalk. In the FTC cell lines, invasion is correlated with EphA2 and pAkt expression levels. EphA2 knockdown by siRNA reduces both pAkt

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**Fig 3.** (A) Ephrin A1 ligand (3 μg/mL) reduced Eph A2 protein expression in FTC238 after 8 hours, yet had no effect on invasion in either FTC133 or FTC 238. (B) PBS and IgG (3 μg/mL) also had no effect.

**Fig 4.** Following treatment with EphA2 siRNA, protein levels of pAkt were significantly decreased in both FTC-238 (20%, \( P < .001 \)) and FTC-133 (20%, \( P < .05 \)). Treatment with Akt siRNA decreased pAkt levels and invasion without changing EphA2 levels in both FTC-133 and -238. There was no additive effect of Akt siRNA and EphA2 siRNA combined on the suppression of invasion in FTC-238. Treatment with the Akt inhibitor perifosine completely abrogated invasion in FTC-238. **\( P < .001 \), ***\( P < .0001 \).**
and invasion, and EphA2 overexpression increased pAkt and invasion. Suppression of pAkt resulted in inhibition of invasion, suggesting that EphA2 invasion is pAkt mediated. Interestingly, ligand-dependent downregulation of EphA2 occurred with ephrinA1, but neither pAkt nor invasion was inhibited. These results suggest that in this tumor type, with an abundance of overexpressed and activated EphA2, ligand-dependent EphA2 downregulation alone may not lead to a decrease in pAkt-dependent invasion, and that ephrinA1-based therapy may not be effective.

Targeted therapy for patients with advanced thyroid cancer is an exciting recent development. Multikinase inhibitors against tyrosine kinases RET, B-Raf, and VEGFRs are being tested in Phase I, II, and III trials, with promising results. Selective inhibitors are also being tested, possibly with even better response rates. With these compounds, partial responses and disease stabilization can occur, but increased survival has been elusive. Recently, an Akt-specific inhibitor showed activity against thyroid cancer cell lines harboring mutations activating the PI3K/Akt pathway, including FTC-133. EphA2 has been investigated as a therapeutic target. Approaches have included using soluble receptors to block EphA2 signaling, siRNAs, EphA2-activating monoclonal antibodies, an adeno-viral ephrin A1 that promotes EphA2 receptor endocytosis, ephrin mimetic peptides, and EphA2 peptides that can induce a specific antitumor response. In our study, both Akt and EphA2 siRNAs were able to block invasion, suggesting that these are promising targets for therapy.

In conclusion, we have identified EphA2 to be an important mediator of invasion by the human follicular cancer cell lines FTC-133 and FTC-238. EphA2 appears to function through the phosphorylation and activation of Akt. EphA2 is expressed in multiple thyroid cancer cell lines and in human thyroid neoplasms, but its clinical significance awaits additional analysis of a larger number of human specimens with clinical outcomes.

REFERENCES


DISCUSSION

Dr Kepal Patel (New York, NY): Do you think the role of EphA2 is specific for follicular cancer, as opposed to papillary cancer? Because your tissue microarray kind of shows expression that’s variable through all the different histologies.

And a corollary to that question is, When did your tissue microarrays, did you exclude the follicular variant of papillary thyroid cancer from your papillary thyroid cancer TMA? Because that may change the expression of this protein.

Dr Sonia Sugg (Iowa City, IA): I also am very curious about that. I don’t think we have enough specimens to really answer that question. Our papillary thyroid cancers also included the follicular variant of papillary thyroid cancer from your papillary thyroid cancer TMA? Because that may change the expression of this protein.

Dr Jennifer Rosen (Boston, MA): My question: rather than having to use siRNA, what do you know about the tyrosine kinase inhibitors and their effect on EphA2? Maybe I am just portraying my ignorance, but I’m not sure about their effect on it.

Dr Sonia Sugg (Iowa City, IA): We have not looked at that in our experiments. It certainly would be a logical next step.

Dr Jennifer Rosen (Boston, MA): Because there would be some information, if they’ve done genomewide studies, about whether EphA2 was affected or not, at least at the RNA level.

Dr Sonia Sugg (Iowa City, IA): Right. We need to look at that.