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Integrin-linked kinase affects signaling pathways and migration in thyroid cancer cells and is a potential therapeutic target

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Abstract

Background—Integrin-linked kinase (ILK) is a serine-threonine kinase that regulates interactions between the cell and the extracellular matrix. In many cancers, overexpression of ILK leads to increased cell proliferation, motility, and invasion. We hypothesized that ILK functions as a regulator of viability and migration in thyroid cancer cells.

Methods—Eleven human thyroid cancer cell lines were screened for ILK protein expression. The cell lines with the greatest expression were treated with either ILK small interfering RNA (siRNA) or a novel ILK inhibitor, T315, and the effects were evaluated via Western blot and migration assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assays were performed to assess cell viability.

Results—siRNA against ILK decreased phosphorylation of downstream effectors Akt and MLC, as well as decreased migration. Treatment with T315 showed a dose-related decrease in both Akt and MLC phosphorylation, as well as decreased migration. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assays showed T315 to have an half maximal inhibitory concentration of less than 1 μ M in cell lines with high ILK expression.

Conclusion—ILK is expressed differentially in thyroid cancer cell lines. Both ILK siRNA and T315 inhibit motility of thyroid cancer cell lines, and T315 is shown to be cytotoxic at low concentrations. Altogether, our study suggests that ILK may represent an important kinase in aggressive thyroid cancers.

Thyroid cancer, in general, has an excellent prognosis with an indolent course and a high cure rate. Nevertheless, up to 30% of patients will experience in recurrence within 30 years.¹

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SUPPLEMENTARY DATA

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In addition, thyroid cancer is increasing in incidence and is projected by 2030 to be the second most common cancer diagnosed in women and the fourth most common overall.² Finally, although most patients do very well, there is a proportion, most notably those with anaplastic or other poorly differentiated forms of thyroid cancer, who succumb to their disease. In these patients, there are no treatments that improve patient survival. Thus, novel therapies are needed greatly in such cases.

Integrin-linked kinase, or ILK, is a serine-threonine kinase that under normal conditions plays a role in cell-extracellular matrix interactions. In some cancers, however, ILK often is overexpressed, leading to increased cancer growth and spread by promoting cell proliferation, migration, and epithelial-mesenchymal transition (EMT).³⁻⁵ ILK has several downstream targets for its kinase activity, most notably Akt, a protein known to play a critical role in the progression of thyroid cancer.⁶⁻⁸ Indeed, previous studies have shown increased ILK expression in poorly differentiated thyroid cancer and implied a relationship between ILK overexpression and poor prognosis.⁹

Therefore, we hypothesized that ILK, due in part to its ability to activate Akt signaling, induce migration, and facilitate EMT, could provide a viable drug target in thyroid cancer. We also wanted to evaluate the effectiveness of our novel ILK inhibitor T315 in this cancer type. T315 has been shown to inhibit the kinase activity of ILK, thereby significantly decreasing cell proliferation of breast and prostate cancer while normal breast and prostate cell lines remains resistant.^{10,11} Thus, we hypothesized that T315 could decrease thyroid cancer cell viability and ILK kinase activity in a dose-dependent manner.

MATERIALS AND METHODS

Reagents

T315, an ILK inhibitor developed in the laboratory of C.S.C., was synthesized according to an established procedure,¹⁰ and its identity and purity were confirmed by nuclear magnetic resonance spectroscopy (300 MHz), high-resolution mass spectrometry, and elemental analysis. Stock solutions of T315 were made in dimethyl sulfoxide (DMSO) and diluted in culture medium to a final DMSO concentration of 0.1%. Antibodies against various target proteins were purchased from the following commercial sources: Akt, p-473S-Akt, FOXO3a, ILK, MLC, p-18T/19S-MLC, Mammalian target of rapamycin, p-2448S-mTOR, Snail, and ZEB1 from Cell Signaling Technology, Inc. (Danvers, MA); Twist from Abcam (Cambridge, MA); and β -actin from MP Biomedicals (Irvine CA). Control small interfering RNA (siRNA) and siRNA for ILK were purchased from Cell Signaling Technology, Inc. Protein lysates were derived from 11 thyroid cancer cell lines donated generously from the laboratories shown in Supplementary Table I. DNA was isolated from the cell lines grown in our laboratory and were then sent to Dr. C. Korch at University of Colorado on a fee-for-service basis for performing DNA fingerprinting analysis using methods described by Schweppe et al.¹² Identity was then confirmed by comparing with DNA fingerprinting from the original clones described in the previous publication by Schweppe et al.

Cell culture

Papillary thyroid cancer–derived KTC1 cells and the anaplastic thyroid cancer cell lines SW1736, hTh7, hTh104, and hTh112 cancer cells (Supplementary Table I) were maintained at 37°C in a humidified incubator with 5% CO₂ in either Dulbecco's modified Eagle's medium (DMEM; hTh7) or Roswell Park Memorial Institute medium (RPMI) 1640 (hTh104, hTh112) culture medium containing 10% fetal bovine serum (FBS), 1× penicillin/streptomycin, and 1× NEAA (Gibco, Grand Island, NY).

siRNA transfection

Cells (5×10^5) were plated in 6-well plates in 3.5 mL of RPMI or DMEM with 10% FBS for 24 hours. Next, they were transfected with either scrambled control or ILK siRNA with Lipofectamine 2000 (Life Technologies, Grand Island, NY), per the company protocol. Cells were treated with the siRNA/Lipofectamine mixture in 1.5 mL of RPMI or DMEM/10% FBS for 6 hours, after which the mixture was removed and changed to 3.5 mL of media. After 72 hours, cells were harvested and lysed for protein collection. Expression of ILK siRNA was assessed by immunoblotting analysis of the target proteins and quantitated with Image J software (National Institutes of Health, Bethesda, MD).

Immunoblotting

Cells were seeded in 6-cm plates (1×10^6 cells/plate), incubated in 10% FBS-supplemented medium (3.5 mL/plate) for 24 hours, and exposed to T315 at the indicated concentrations in 5% FBS-supplemented medium for 24 hours. Cells were collected by scraping followed by centrifugation, washed with cold phosphate-buffered saline, and lysed in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 50 mM Tris buffer pH 8.1, 10 mM ethylenediaminetetraacetic acid) containing cocktails of protease inhibitors (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (0.625 mM glycerophosphate, 1.25 mM NaF, 0.25 mM sodium pyrophosphate, 0.5 mM Na₃VO₄). Lysates were sonicated for 30 seconds to disrupt cellular organelles and genomic DNA, and then centrifuged at 13,200 rpm for 15 minutes. Equal amounts of proteins as determined by a colorimetric bicinchoninic acid assay (Thermo) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk for 1 hour, membranes were probed with primary antibodies at 1:1,000 dilution in Tris-buffered saline containing 0.1% Tween 20 at 4°C for 16 hours, followed by goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates at 1:5,000 and 1:3,000 dilutions, respectively, for 1 hour at room temperature. Proteins were visualized by enhanced chemiluminescence. β -actin expression was used to assess for equal loading of protein in each lane.

MTT assay

The effect of T315 on cell viability was assessed by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in 5 replicates. Cells (5×10^3 per well) were grown in 96-well plates in RPMI with 10% FBS for 24 hours and then exposed to various concentrations of T315 dissolved in DMSO in 5% FBS-supplemented medium. At various time points after T315 treatment, based on the 24- to 48-hour cell cycle of the

selected cell types, medium was removed from each well and replaced by 200 mL of 0.5 mg/mL MTT in 10% FBS-containing medium. Cells were then incubated in the CO₂ incubator at 37°C for 4 hours. Supernatants were removed from the wells, and the MTT dye was dissolved in 120 mL/well DMSO. Absorbance at 570 nm was determined on a plate reader. Cell viabilities are expressed as percentages of that in the corresponding vehicle-treated control group.

Migration assay

Cancer cells were grown in DMEM and RPMI 1640 medium containing FBS until 50–60% confluent, transfected, washed with PBS, trypsinized for 5 minutes, collected with 10% FBS DMEM and RPMI 1640 medium, and centrifuged at 300g for 5 minutes. Cells were resuspended with 0% FBS DMEM and RPMI 1640 medium and counted with a cell countess (Invitrogen, Waltham, MA). A volume of 1×10^5 cells in 300 μ L of medium was placed into the upper chamber of Boyden chamber (8 μ m pore) inserts in 24-well plates filled with 400 μ L of either DMEM or RPMI 1640 medium containing 10% FBS chemoattractant in the bottom chamber. For the rescue assays, a concentration of 0.5 μ M of T315 was added to the upper chamber and incubated for 1 hour before the chemoattractant was added. Cells were incubated at 37 °C and 5% CO₂. The cells on and under the Boyden chamber membrane were fixed with 3.7% formaldehyde containing 0.05% crystal violet for 5 minutes after they were washed with PBS. The chambers were washed with distilled water, and the excess water was eliminated. The cells on the top (nonmigrated) and bottom (migrated) sides of the membrane were collected by scraping the top and bottom of the chamber with a cotton swab, which was subsequently placed into a 1.5-mL tube. The remainder of the cells remained in the Boyden chamber. The cotton swabs containing the scraped cells and the Boyden chamber containing the non-migrated cells were incubated separately in 80% methanol, vortexed, and the extracted dye was measured at 570 nm. Migration was quantified as the ratio of the migrated cells over the total cells (nonmigrated plus remaining cells) to calculate migration rates. Experiments were performed on at least 3 separate occasions as described in the figure legends.

Transient transfection for rescue

A vector containing cDNA encoding full-length human *ILK* was purchased from Thermo Scientific (Rockville, IL). The cDNA was transfected into hTh7 and hTh112 cells by the use of Optifect Reagent (Life Technologies) when cells were 50% confluent. After an overnight incubation, the liposomes were aspirated; either RPMI 1640 medium or DMEM supplemented with 10% FBS (Life Technologies) was added to the cells for 24 hours before migration assays were performed.

Statistical analysis

Because the experiments performed on the same day are correlated, linear mixed effects models were used to take account of the correlation of the observations obtained on the same day. The outcomes including cell viability, cell migration, and invasion obtained from different treatment conditions were compared from those models. The half maximal inhibitory concentration (IC₅₀) was estimated from a 4-parameter logistic regression model. All analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

We first examined the expression levels of ILK in 11 genetically confirmed thyroid cancer cell lines (BCPAP, KTC1, TPC1, FTC133, SW1736, C643, hTh7, hTh74, hTh83, hTh104, and hTh112). The lines consisted of a mix of papillary, follicular, and anaplastic cancer cells with differing mutational status, including mutations in BRAF, RET/PTC, and NRAS (Supplementary Table I). There was a differential expression of ILK among the cell lines (Fig 1), with the greatest expression in papillary line KTC1, as well as anaplastic lines SW1736, hTh7, and hTh112 cells. There was no apparent correlation between known mutational status of the cell lines and ILK expression status. Of note, 3 lines (KTC1, SW1736, and hTh7) had high Akt phosphorylation at serine 473 (Fig 1).

We then selected the high-ILK-expressing cell lines for further experiments. For analysis of protein expression, hTh7, KTC1, and SW1736 cells were treated for 24 hours with increasing doses of ILK inhibitor T315 and then lysed to examine protein expression of ILK as well as direct downstream targets. Several direct targets of ILK kinase activity, including Akt phosphorylation site serine 473 and MLC phosphorylation sites threonine 18 and serine 19,^{13,14} were decreased with increasing doses of T315 (Fig 2, A). Additionally, expression of FOXO3a, a protein we have shown previously to be a downstream target of ILK,⁵ was decreased in the hTh7 and KTC1 lines. Markers of EMT tested, including Twist, Snail, and ZEB1, however, had no significant change in expression with increasing doses of T315 in 2 of 3 cell lines tested, with decreased Snail expression only in the SW1736 line.

To confirm that decreased ILK activity was leading to these changes, we next performed transient transfection with siRNA against ILK versus control scrambled siRNA. Successful knockdown of ILK expression was shown by Image J analysis of Western blot, with >50% decrease in ILK protein expression in all lines tested. ILK knockdown recapitulated several of the findings from T315 treatment, with decreased phosphorylation of Akt and MLC, as well as decreased FOXO3a expression in the hTh7 line (Fig 2, B); however, the effects of ILK knockdown on EMT markers were cell-line specific. A modest decrease in Twist and ZEB1 expression was noted in the hTh7 line, whereas Snail expression decreased, with an increase in Twist and ZEB1 expression in KTC1 cells and increased Snail and ZEB1 expression in SW1736 cells.

Given the finding that ILK suppression led to decreased MLC phosphorylation and overall MLC expression, we next performed migration assays to assess functional consequences. Transfection with ILK siRNA led to a decrease in the high-ILK-expressing cell lines hTh7 (49% vs 25%, $P < .01$), hTh112 (49% vs 28%, $P < .01$), KTC1 (68% vs 18%, $P < .05$), and SW1736 (62% vs 32%, $P < .05$) (Fig 3, A). Similarly, treatment with a sublethal dose of T315 (0.5 μ M) decreased migration in these cell lines (for hTh7 52% vs 34%, $P < .01$, for KTC1 57% vs 31%, $P < .01$, for SW1736 70% vs 43%, $P < .01$, and for hTh112 60% vs 40%, $P < .05$) (Fig 3, C). MTT assays performed in parallel showed no difference in cell viability in treatment types for these experiments (Fig 3, B and D). To see whether ILK inhibition by T315 was its reason for migration suppression, the cell lines were transfected transiently with constitutively active ILK before T315 treatment, showing abrogation of the effects of T315 on migration in both cell lines (Fig 3, E and F).

Next, cell viability of 2 anaplastic thyroid cancer cell lines, high-ILK-expressing hTh7 and low-ILK-expressing hTh104, was assessed after T315 treatment. By MTT assay, both hTh7 and hTh104 cell lines were found to be sensitive to T315, and this sensitivity persisted at up to 72 hours after treatment (Fig 4, *A* and *B*). At all time points, the IC₅₀ for hTh7 cells was less than 1 μ M, whereas the IC₅₀ for hTh104 was greater than 1 μ M. When we assessed all time points, hTh7 cells were more sensitive to T315 than hTh104 at concentrations of 2 μ M ($P = .02$ at 24 hours and $P < .001$ each at 48 and 72 hours) and 3 μ M ($P = .005$ at 24 hours, $P = .048$ at 48 hours, and $P < .001$ at 72 hours). By the 4 μ M dose, cell viability was <6% at all time points in both cell lines. In both selected lines, there was no significant difference in cell viability for each time point (24, 48, and 72 hours). For this reason, we performed MTT assays at earlier time points (1, 3, 6, 12 hours), which showed a modest effect of T315 at even the earliest time point (data not shown). MTT assays for the other high-ILK-expressing lines KTC1, SW1736, and hTh112 showed similar sensitivity to the hTh7 line (Fig 4, *C–E*).

DISCUSSION

In the present study, we evaluated ILK expression patterns in thyroid cancer cell lines and assessed possible roles for ILK in proliferation and motility thyroid cancer cell lines. In screening 11 distinct cell lines, we found that ILK overexpression occurs in cell lines derived from both differentiated and anaplastic cancers. Of note, high ILK expression did not appear to correlate with BRAF V600E, RET/PTC, or NRAS oncogenes in the limited number of cell lines examined. Next, using both siRNA against ILK and our ILK inhibitor T315, we were able to show that Akt phosphorylation at Ser 473 and MLC phosphorylation in high-ILK expressing lines were dependent on ILK expression. Interestingly, the effects of ILK inhibitors on EMT markers were variable, consistent with the known complex regulation of EMT-related proteins.¹⁵ Nonetheless, we found that ILK inhibition led to a significant decrease in cell migration. Finally, we found that T315 can significantly decrease cell viability in several thyroid cancer cell lines. The efficacy of the compound was similar in cell lines with higher or lower ILK levels, suggesting either that the levels of ILK in each cell line are sufficient to regulate survival or that the compound may have activities at that are also ILK-independent. Nonetheless, the line with high ILK expression, hTh7, was more sensitive than the hTh104 line at several dose levels.

There are several implications to our findings. First, that ILK plays a role in Akt activation is important in that many studies have shown the importance of the Akt signaling pathway in thyroid cancer, particularly in invasive and recurrent tumors.⁷ Once activated by phosphorylation, Akt increases cell proliferation and protein synthesis, and it inhibits apoptosis through a variety of mechanisms.¹⁶ Enhanced levels of phosphorylated Akt have been reported in the invasive fronts of aggressive thyroid cancers,⁷ activating mutations in Akt1 have been reported in recurrent thyroid cancer tissues,¹⁷ and mutations and epigenetic changes resulting in activation of PI3K are common in anaplastic thyroid cancers¹⁸; together, they suggest a key role for this signaling pathway in the behavior of aggressive thyroid cancer. Although the threonine 308 phosphorylation site on Akt is known to be a target of PDK-1, several candidate proteins have been implicated as the kinase that phosphorylates Akt at serine 473, the so-called “PDK-2” kinase, including ILK.¹¹ Our data

are consistent with a possible role for ILK as a PDK2 enzyme in the tested thyroid cancer cell lines.

Second, in various cancer cell types, including nonsmall cell lung, colon, and renal cell cancer, ILK regulates Akt-independent pathways that enhance cell invasion and migration through the initiation of EMT.^{19–21} Additionally, ILK has been shown previously to activate MLC through phosphorylation,^{13,14} thereby affecting cell adhesion and migration. Studies have demonstrated previously that ROCK kinase also regulates MLC phosphorylation; thus, MLC may serve as an important signaling node in regulating cancer cell motility, potentially being targeted by T315.²² The role of ILK in the motility of thyroid cancer was strengthened further by our findings that suppression of ILK expression, both by RNAi and T315, led to a significant decrease in cell migration. In the present study, ILK inhibition resulted in variable changes in the expression of several EMT markers. As such, further studies must be completed to understand more completely the effect of ILK on EMT in thyroid cancer as well as its role in MLC phosphorylation vs. ROCK kinase.

Targeting ILK may be an important regulator for tumor-stroma interactions. Although not studied in the present report, studies in other cancer types have shown that ILK facilitates TGFβ1-related communication in ovarian cancer²³ as well as IL-6-related communication in melanoma.²⁴ ILK also has been shown to play an integral role in integrating cytokine cell signaling in both dermal fibroblasts²⁵ and hepatic stellate cells.²⁶ Thus, inhibition of ILK pharmacologically has the potential to alter not only the tumor, but the dysregulated stroma as well, adding additional novelty and potential therapeutic efficacy to this drug target.

Using the ILK inhibitor QLT0267, the authors of a recent publication⁹ evaluated the role of ILK in anaplastic thyroid cancers. Although they did show increased ILK expression in anaplastic as well as Hürtle cell thyroid cancers in comparison with normal thyroid tissues, there were several limitations to this study. First, for their in vitro studies wherein they screened 6 cell lines for ILK expression, 4 of the lines were found subsequently through genetic testing to be lines of other cancer types.¹² Many of the in vitro results and all of the in vivo results relied on the DRO cell line, which has been confirmed to be a melanoma-derived line. Regardless of the cell type, the potency of QLT0267 was less than T315, with 20% of cells remaining viable at the greatest doses tested. Further, the human tissues evaluated were from core biopsies and likely did not have adequate sampling of the leading edge, adjacent normal, or stromal tissues to compare expression. They also were unable to correlate expression with clinical outcomes. Despite these drawbacks, the study was able to provide some initial evidence that ILK has a functional role in aggressive thyroid cancers. This previous work along with our own provide evidence that treatment with an ILK inhibitor such as T315 should be performed in an in vivo model of thyroid cancer to further investigate therapeutic potential.

In summary, ILK is expressed in human thyroid cancer cell lines. Inhibition of ILK molecularly and with the novel kinase inhibitor T315 led to disruption of Akt and MLC phosphorylation and decreased cell migration in thyroid cancer cells. T315 was cytotoxic in thyroid cancer cells in vitro. Thus, further studies of ILK and also T315 in aggressive thyroid cancers are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Western blot evaluating ILK protein expression in 11 thyroid cancer lines. Four lines with the greatest ILK expression included KTC1 (papillary cancer, BRAF mutant), SW1736 (anaplastic cancer, BRAF mutant), hTh7 (anaplastic cancer, BRAF wild type, NRAS mutant), and hTh112 (anaplastic cancer, BRAF wild type).

Fig 2.

(A) Western blot analysis of protein expression in hTh7, KTC1, and SW1736 cells showing decreased Akt phosphorylation at 2 μM dose of T315 as well as decreased MLC and mammalian target of rapamycin (mTOR) phosphorylation. There were no significant changes in EMT markers Snail and Twist with compound treatment. (B) Knockdown of ILK with siRNA transfection in hThh7, KTC1, and SW1736 cells recapitulated the findings of decreased Akt, MLC, and mTOR phosphorylation. Expression levels of EMT markers Snail, Twist, and ZEB1 were altered in a cell-line specific manner. *Scrb*, Scrambled siRNA.

Fig 3.

ILK inhibition decreased cell migration in thyroid cancer cell lines. The hTh7, hTh112, KTC1, and SW1736 cell lines were either (A) transfected with an ILK-specific siRNA or (B) treated with 0.5 μ M T315. Both treatments reduced cell migration in both cell lines compared with controls. (C) and (D) MTTs showed no statistical difference between cells transfected with ILK siRNA or treated with T315 compared with controls. (E) Rescue experiments showed that decreased thyroid cell migration in the tested cell lines through ILK inhibition with 0.5 μ M T315 could be rescued with constitutively active ILK (CA ILK). (F) MTT assays demonstrate no statistical difference between the different combinations of treatments. Values are mean $\pm$ SD.

Fig 4.

MTT assay evaluating cell viability after treatment with ILK inhibitor T315 in thyroid cancer cell lines. (A) In the hTh7 line, T315 had an IC₅₀ of 0.85 μM at 24 hours, 0.77 μM at 48 hours, and 0.88 μM at 72 hours. (B) In the low-ILK-expressing hTh104 line, IC₅₀ was 1.56 μM at 24 hours, 1.27 μM at 48 hours, and 1.52 μM at 72 hours. (C–E) MTT assays in the additional high-ILK-expressing lines hTh112 (IC₅₀ of 0.56, 0.57, 0.32 μM at 24, 48, 72 hours), KTC1 (IC₅₀ of 0.88, 0.75, 0.66 μM at 24, 48, 72 hours), and SW1736 (IC₅₀ of 0.84, 0.85, 0.74 μM at 24, 48, and 72 hours) revealed sensitivities similar to the high-ILK-expressing hTh7 line. Values are mean ± SD.