IQGAP1 Plays an Important Role in the Invasiveness of Thyroid Cancer

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Statement of Translational Relevance

This study investigated the oncogenic role of IQGAP1 in thyroid cancer and its potential as a novel prognostic marker and therapeutic target in this cancer. The main findings include the genetic copy gain of IQGAP1 associated with increased IQGAP1 protein expression and increased invasiveness and aggressiveness of thyroid cancer. IQGAP1 copy gain was preferentially seen in aggressive types of thyroid cancer and highly associated with extrathyroidal and vascular invasion. In BRAF mutation-positive papillary thyroid cancer (PTC), IQGAP1 copy gain was particularly associated with a high tumor recurrence rate of 60%. In vitro knockdown of IQGAP1 dramatically inhibited thyroid cancer cell invasion. The clinical implication of these results is two-fold: 1) IQGAP1 copy gain can be used to predict invasiveness and aggressiveness of thyroid cancer and, when co-existing with BRAF mutation in PTC, is a particularly powerful predictor for cancer recurrence; 2) IQGAP1 represents a novel potential therapeutic target for thyroid cancer.
Abstract

Purpose: This study was designed to explore the role of IQGAP1 in the invasiveness of thyroid cancer and its potential as a novel prognostic marker and therapeutic target in this cancer.

Experimental Design: We examined IQGAP1 copy gain and its relationship with clinicopathological outcomes of thyroid cancer and investigated its role in cell invasion and molecules involved in the process.

Results: We found IQGAP1 copy number gain ≥ 3 in 1/30 (3%), 24/74 (32%), 44/107 (41%), 8/16 (50%), and 27/41 (66%) of benign thyroid tumor, follicular variant papillary thyroid cancer (FVPTC), follicular thyroid cancer (FTC), tall cell PTC, and anaplastic thyroid cancer, respectively, in the increasing order of invasiveness of these tumors. A similar tumor distribution trend of copy number ≥ 4 was also seen. IQGAP1 copy gain was positively correlated with IQGAP1 protein expression. It was significantly associated with extrathyroidal and vascular invasion of FVPTC and FTC and, remarkably, a 50-60% rate of multifocality and recurrence of BRAF mutation-positive PTC (P = 0.01 and 0.02, respectively). siRNA knockdown of IQGAP1 dramatically inhibited thyroid cancer cell invasion and colony formation. Co-immunoprecipitation assay demonstrated direct interaction of IQGAP1 with E-cadherin, a known invasion-suppressing molecule, which was up-regulated when IQGAP1 was knocked down. This provided a mechanism for the invasive role of IQGAP1 in thyroid cancer. In contrast, IQGAP3 lacked all these functions.
Conclusions: IQGAP1, through genetic copy gain, plays an important role in the invasiveness of thyroid cancer and may represent a novel prognostic marker and therapeutic target for this cancer.
Introduction

The human IQGAP protein family comprises three members, which, as scaffold proteins, play important roles in the regulation/modulation of cytoskeletal architecture and cell adhesion through binding to various effectors/regulators (1-4). Among these, IQGAP1 has been best characterized (3-6). This protein was initially identified in human osteosarcoma cells and consists of a 1,657-amino acid polypeptide with significant similarity to the Ras-related GTPase-activating (RasGAP) proteins (7). A unique structural feature of IQGAP proteins is their IQ domain, which contains about 25 amino acids and is found in diverse families of calmodulin-binding proteins. This motif is capable of effecting on intracellular Ca\(^{2+}\) signaling and a wide range of cellular activities. Some previous studies showed that IQGAP1 might be able to modulate the Ras → Raf → MEK → MAP kinase/ERK pathway (MAP kinase pathway) in certain cells, but it can also function independently of this mechanism (8-10). Over-expression of IQGAP1 was seen in certain cancers, suggesting a role of this molecule in human tumorigenesis (3,4,11-13). IQGAP3 may potentially also have such a role as it was shown to be involved in cell proliferation (2). IQGAP2 has been less well studied and may function as a tumor suppressor (4).

Follicular epithelial cell-derived thyroid cancer is a common endocrine malignancy (14). This cancer can be histologically classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC). PTC can be further classified into several variants or subtypes, including mainly conventional PTC (CPTC), follicular variant PTC (FVPTC) and tall cell PTC (TCPTC).
A striking genetic alteration in PTC is the \textit{BRAF} mutation, which plays an important oncogenic role through activation of the MAP kinase pathway (15-17). ATC is a rare but rapidly lethal cancer with aggressive invasion and metastasis. PTC and FTC are both differentiated thyroid cancers with a relatively indolent clinical course, but FTC is generally more invasive and aggressive than PTC except for certain types, such as TCPTC which is also relatively aggressive (18,19). FTC and FVPTC tend to display invasive behaviors, such as vascular invasion (20). It is not known whether IQGAP proteins play any role in thyroid tumorigenesis. In the present study, we explored such a role particularly for IQGAP1 in various types of thyroid cancers. IQGAP3 was similarly studied for comparison. Given the unique role of \textit{BRAF} mutation in promoting the aggressiveness of PTC (21,22), we also investigated its relationship with IQGAP1 in this cancer.

\textbf{Materials and Methods}

\textit{Genomic DNA isolation from thyroid tumor specimens}

A total of 383 thyroid tumor samples were analyzed for this study, including 30 benign tumors, 205 PTC, 107 FTC and 41 ATC, which were originally obtained and prepared for genomic DNA isolation with institutional review board approvals (23). Briefly, after xylene treatment to remove paraffin, tissues were digested with sodium dodecyl sulfate-proteinase K and DNA was subsequently isolated following standard phenol-chloroform extraction and ethanol precipitation protocols.

\textit{Analysis of IQGAP1 mutation in thyroid tumor samples}
Genomic DNA sequencing was performed for \textit{IQGAP1} mutation analysis. We selected exons 19 to 23 and exon 29 for mutation analysis as they constitute the IQ domain of IQGAP1 or sometimes harbored mutations in other cancers. Genomic DNA was amplified by PCR using standard reaction conditions and the primers presented in Table S1.

\textit{Analysis of genomic copy gain of the IQGAP genes with quantitative real-time PCR}

Quantitative real-time PCR was used to analyze copy number of \textit{IQGAP1} and 3 genes using the ABI 7900HT PCR system (Applied Biosystems, Foster City, CA). This is a widely used and robust technique for genomic copy number analysis, for which we use our well-established FISH-proven protocol as detailed previously (23-26). Specific primers and probes were designed using Primer Express 3.0 (Applied Biosystem) to amplify and detect \textit{IQGAP1}, \textit{IQGAP3}, \textit{TMEM84} and \textit{β-actin} genes. The probes were labeled with 5' fluorescent reporter dye (6FAM) and 3' quencher dye (TAMRA). \textit{β-actin} was run in parallel to standardize the input DNA. Standard curve was established using normal leukocyte DNA with a quantity range of 0.02–10 ng/µl. The primers and probes used are summarized in Table S1.

\textit{Analysis of IQGAP1 protein expression by immunohistostaining (IHS)}

IHS was performed using a modified protocol (23). Briefly, thyroid tumor tissue sections were dewaxed, soaked in alcohol, and incubated in 3% hydrogen peroxide for 15 min to inactivate endogeneous peroxidase activity after microwave treatment in an antigen-unmasking solution (Vector Lab, Burlingame, CA). Tissue sections were
incubated overnight at 4°C with anti-IQGAP1 antibody (BD Biosciences, San Jose, CA). Immunostaining was performed with Vectastain Universal Quick kit (Vector Lab, Burlingame, CA) following the manufacturer’s protocol. Peroxidase activity was revealed using 3,3 diaminobenzidine. Negative control was performed by omission of primary antibodies. We classified IHS into three levels represented by scores I-III: IHS score I, staining of < 10% of cells; IHS score II, staining of 10-50% of cells; IHS score III, staining of > 50% of cells.

Thyroid cancer cell lines

Three human thyroid cell lines used in this study were as follows: the ATC cell lines SW1736 and KAT18 were originally from Dr. N. E. Heldin (University of Uppsala, Uppsala, Sweden) and Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY, USA) respectively; and the FTC cell line FTC133 was from Dr. Georg Brabant (Christie Hospital, Manchester, UK). They were provided by Drs Rebecca Schwepe and Bryan Haugen (University of Colorado, Aurora, CO, USA). Cells were cultured and maintained as described previously (26).

shRNA plasmid construction

Specific and scrambled control shRNAs were designed using an online program of Invitrogen Corp (https://rnaidesigner.invitrogen.com/rnaexpress/setOption.do?designOption=shrna&pid=-6715048677617069736). The shRNA target sequence for IQGAP1, GCATCCACTTACCAGGATA, located from 763nt to 781nt of the IQGAP1 cDNA.
(with the translation start site set as +1), was selected for IQGAP1 gene knockdown. The corresponding nonspecific shRNA duplex containing the same nucleotide composition but in random sequence (scrambled) were used as control: GCCACCCATAGAAATTTTCG. The shRNA target sequence for IQGAP3, GGACCTGAGCAAGCTAGAA, located from 4005nt to 4023nt of the IQGAP3 cDNA was selected for specific knockdown and the corresponding nonspecific scrambled shRNA duplex (GCCAACGGACGTAATGAAG) were used as control. There were two complementary oligonucleotides, T-(target or control sense)-TTCAAGAGA-(target or control antisense)-TTTTTC and TCGAGAAAAAA-(target or control sense)-TCTCTTGAA-(target or control antisense)-A, synthesized for each target or control sequence and annealed in vitro. The annealed double-stranded oligonucleotides were cloned into the HpaI and XhoI sites of shRNA lentiviral expressing vector pSicoR-PGK-puro (Addgene Corp., Cambridge, MA) (27) and confirmed by sequencing.

**Lentiviral-mediated gene knockdown**

The lentiviral shRNA-expressing vector, the packaging plasmid PSPAX2, and the VSV-G envelope protein-coding plasmid pMD2G were co-transfected to HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. After transfection for 48 h, cellular supernatants were harvested and filtrated by passing through filters with pore size of 45 μm. The lentiviral supernatants were used to infect thyroid cancer cells. For stable transfection, the transformants were selected in medium containing 1.5 mg/ml puromycin for about 2 weeks. Stable cell clones were confirmed by Western blotting analysis.
Western blotting analysis

Routine Western blotting procedures were followed as described previously (26), using various primary antibodies, including anti-IQGAP1, anti-IQGAP3, anti-E-cadherin, or anti-cdc42 (BD Biosciences, San Jose, CA); anti-phospho-ERK, anti-phospho-Akt, or anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-FAK (Invitrogen, Carlsbad, CA); or anti-phospho-Src (Cell Signaling Technology, Danvers, MA).

Cell proliferation study

Cells (800/well) were seeded in quadruplets into a 96-well plate and cultured with 2.5% serum. MTT assay was performed daily over a course of 5-6 days to evaluate cell proliferation as detailed previously (28).

Cell invasion assay

The invasion assay was performed using Growth Factor Reduced Matrigel™ invasion chambers with 8.0μm polycarbonated filters (BD Biosciences, San Jose, CA). Briefly, 2.5×10^4 cells were seeded on chamber plates and cultured in routine medium. After an incubation of 22 h at 37°C in a humidified incubator with 5% CO₂, non-migratory cells on the upper surface of the filter were removed by wiping with a cotton swab. Invasive cells that penetrated through pores and migrated to the underside of the membrane were stained with crystal violet and counted and photographed under a microscope.
Anchorage-independent colony formation assay

For colony formation assay to examine anchorage-independent cell growth in soft agar, cells (1 x 10^4 cells/well) were plated into 6-well plates in growth medium containing 10% fetal bovine serum, 1.5 mg/ml puromycin, and 0.3% agar, on top of 0.6% agar gel. After 2-3 wks of culture, colonies were counted and photographed under a microscope (28).

Immunoprecipitation assay

Immunoprecipitation was performed using the Protein G Immunoprecipitation Kit (Roche Applied Science, Indianapolis, IN) with slight modifications. Briefly, 1 ml cell lysates were pre-cleared with 50 ul homogeneous Protein G-agarose suspension for 6 hours. After centrifugation, the supernatants were divided into equal aliquots (about 0.5 ml of each) and incubated overnight with either mouse monoclonal anti-IQGAP1 antibody (BD Biosciences) or purified mouse IgG (Sigma-Aldrich Corp., St. Louis, MO) as control. The immune complexes were incubated with 50 ul Protein G-agarose suspension for 6 hours, followed by five washings with lysis buffer, re-suspension in SDS buffer, and analysis by SDS-polyacrylamide gel electrophoresis and Western blotting.

Statistical analysis

Chi-square test was used to analyze the relationship of IQGAP1 copy gain with immunohistostaining scores, BRAF mutation, tumor recurrence, lymph node metastasis,
multifocality, and extrathyroidal extension status. Fisher’s exact test was used when any number of the data was less than 5. Student’s t test was used to analyze the relationship of \(IQGAP1\) copy gain with patient age and tumor size. Wilcoxon rank sum test was used when data was not in normal distribution. Linear regression was used to analyze the relationship between \(IQGAP1\) copy number and patient age, tumor stage, and tumor size. The professional statistical software SPSS 10.0 (SPSS Inc., Chicago, IL) was used in these analyses. P values < 0.05 were considered significant.

**Results**

*Uncommon mutation but common genetic copy gain of the \(IQGAP1\) gene in thyroid cancers*

A M1231I \(IQGAP1\) mutation was occasionally seen in certain human cancers (29). We examined this mutation in 70 thyroid tumor samples but found only one case (1.4%) to harbor this mutation. We also examined exons 19 to 23, which constitute the functionally important IQ domain of IQGAP1, for possible mutation, and found no mutation in 70 thyroid tumor samples. Therefore, mutation of the \(IQGAP1\) gene does not seem to be a major mechanism in thyroid cancer.

As tumor-promoting genes often gain copy number in thyroid cancer (25), we analyzed \(IQGAP1\) and \(IQGAP3\) copy number in various types of thyroid cancers. As shown in Table 1, \(IQGAP1\) copy gain was common in thyroid cancers, seen in 66% and 41% of ATC and FTC, respectively, when the copy number (CN) \(\geq\) 3 was used as the cut-off value. The highest copy gain rate was seen in ATC, followed by FTC, and PTC.
regardless of whether the cut-off value of CN ≥ 3 or CN ≥ 4 was used to define copy gain.

Among the three subtypes of PTC, the most aggressive TCPTC has the highest rate of IQGAP1 copy gain when the cut-off CN ≥ 3 was used in the analysis (up to 50%). We also studied the copy number of IQGAP1 in 30 benign thyroid tumors and 30 normal thyroid tissue samples. Among all these samples, only one case of benign tumor was identified with copy gain when a cut-off CN ≥ 3 was used. Therefore, a clear trend of association between IQGAP1 copy gain and the known order of increasing aggressiveness of these thyroid cancers was demonstrated, suggesting an important role of IQGAP1 copy gain in thyroid tumorigenesis. Fifteen of those normal thyroid tissue samples had matched PTC tumor samples and 4 (27%) of these 15 matched PTC had IQGAP1 copy gain. These results demonstrated the malignancy specificity of IQGAP1 copy gain. We also examined the copy number of the TMEM84 gene, which is located at 15q23 and about 20Mb away from the IQGAP1 gene in the same chromosome. Only one out of 60 (1.7%) cases examined, including 30 PTC and 30 FTC, was found to have TMEM84 copy gain. This result suggests that IQGAP1 copy gain in these cancers represented mainly genetic amplification, not chromosome number gain. For the IQGAP3 gene, copy gain was seen only in 4/38 (10.5%) ATC, 4/39 (10.3%) FTC and 4/85 (4.7%) PTC when using CN ≥ 3 as the cut-off value, and 1/38 (2.6%) ATC, 0/39 (0%) FTC and 2/85 (2.4%) PTC when using CN ≥ 4 as the cut-off value. Thus, unlike
IQGAP1, copy gain of IQGAP3 is uncommon and does not play a major role in thyroid tumorigenesis.

Association of IQGAP1 copy gain with increased expression of IQGAP1 protein in thyroid cancers

To demonstrate the biological relevance of genetic copy gain of the IQGAP1 gene in thyroid cancer, we investigated the impact of this genetic alteration on the expression of IQGAP1 protein by immunohistostaining (IHS) analysis using IQGAP1-specific antibodies on 29 FTC samples that had available histopathological slides. Fig 1 shows representative tumor samples illustrating the IHS scores and corresponding IQGAP1 copy numbers. Among the nine cases without IQGAP1 copy gain (CN < 3), six (66.7%) had a IHS score I, two (22.2%) had a score II, and one (11.1%) had a score III, while among the 20 cases with IQGAP1 copy gain (CN ≥ 3), only one (5%) had a IHS score I, but 10 (50%) and nine (45%) had cores II and III, respectively, with a significant difference between the two groups on overall analysis (P < 0.01). To take a further look at specific groups, IHS score I was seen in 6/9 (66.7%) of the cases with CN < 3 vs. 1/20 (5%) of the cases with CN ≥3 (p < 0.001). Conversely, IHS scores II and III, in combination, were seen in 3/9 (33%) of the cases with CN < 3 vs. 19/20 (95%) of the cases with CN ≥3 (p < 0.001). These data clearly showed an association of IQGAP1 copy gain with increased expression of the IQGAP1 protein in thyroid cancer, demonstrating that this IQGAP1
copy gain was biologically relevant with respect to expected increase in IQGAP1 expression.

*Relationship of IQGAP1 copy gain with clinicopathological characteristics of thyroid cancers*

The preferential association of *IQGAP1* copy gain with invasive and aggressive types of thyroid cancer presented above prompted us to explore further the clinicopathological role of *IQGAP1* copy gain in thyroid cancer by investigating its relationship with clinicopathological characteristics of thyroid cancers. As shown in Table 2, *IQGAP1* copy gain was associated with extrathyroidal invasion in FTC. Specifically, 6/35 (17.1%) cases without *IQGAP1* copy gain vs. 12/28 (42.9%) cases with copy gain were associated with extrathyroidal invasion (p = 0.025) when the cut-off CN ≥ 3 was used. This relationship marginally missed statistical significance (p = 0.07) when the cut-off CN ≥ 4 was used. *IQGAP1* copy gain was also associated with vascular invasion in FTC. Specifically, 12/35 (34.3%) cases without *IQGAP1* copy gain vs. 18/28 (64.3%) cases with copy gain were associated with vascular invasion (p = 0.018) when the cut-off CN ≥ 3 was used. This relationship was still significant (p = 0.05) when the cut-off CN ≥ 4 was used. In FVPTC, *IQGAP1* copy gain was also associated with extrathyroidal invasion. Specifically, 3/65 (4.6%) cases without *IQGAP1* copy gain vs. 3/8 (37.5%) (p = 0.02) cases with copy gain were associated with extrathyroidal invasion in FVPTC when the cut-off CN ≥ 4 was used. When the cut-off CN ≥ 3 was used, a marginal association was noted (p = 0.09).
We did not find a significant association of *BRAF* mutation with *IQGAP1* copy gain either in the total PTC cohort or within the subgroup of CPTC or FVPTC, suggesting that *IQGAP1* copy and *BRAF* mutation were two independent genetic events (Table 2). TCPTC could not be analyzed due to the relatively small number of cases. Similarly, we did not find an association of *Ras* mutations with *IQGAP1* copy gain. Specifically, we found *Ras* mutations in 3/25 (12%) cases of samples with *IQGAP1* copy gain vs. 4/38 (11%) cases of samples without *IQGAP1* copy gain (p = 0.9). Although not statistically significant, a strong trend for the association of *IQGAP1* copy gain with recurrence of PTC (overall cases) was noted when the cut-off CN ≥ 4 was used (Table 2). Interestingly, when we divided the PTC samples into *BRAF* mutation-positive and – negative groups, *IQGAP1* copy gain was significantly associated with tumor recurrence in the *BRAF* mutation-positive group. Specifically, 14/63 (22.2%) cases without *IQGAP1* copy gain vs. 6/10 (60%) cases with copy gain were associated with tumor recurrence (p = 0.02) when the cut-off CN ≥ 4 was used. A significant association of *IQGAP1* copy gain with tumor multifocality was observed in CPTC that harbored *BRAF* mutation. Specifically, 4/56 (7.1%) cases without *IQGAP1* copy gain vs. 4/8 (50%) cases with copy gain were associated with multifocality (p = 0.01) when the cut-off CN ≥ 4 was used. These associations of *IQGAP1* copy gain were not observed in *BRAF* mutation-negative PTC (data not shown). Patient age and gender, tumor size, and lymph node metastasis were not significantly associated with *IQGAP1* copy gain (data not shown).
Effects of shRNA knockdown of IQGAP proteins on the signaling of MAP kinase and PI3K/Akt pathways and proliferation of thyroid cancer cells

As MAP kinase and PI3K/Akt pathways played an important role in promoting thyroid cancer cell proliferation (15-17), we next examined whether IQGAP proteins were involved in these signaling pathways in thyroid cancer cells. We used the lentiviral-mediated specific shRNA-expression system to stably knock down the expression of IQGAP proteins in SW1736, KAT18, and FTC133 cells that have been documented to be unique thyroid cancer cell lines (30). Although there was no IQGAP1 or IQGAP3 copy gain in these cells (data not shown), we reasoned that if IQGAP proteins normally had a significant influence on these pathways, alteration in their expression should affect their signaling. As shown in Fig S1A, IQGAP1 expression was nearly completely knocked down in all the three cells while the control cells stably transfected with non-specific shRNA maintained normal expression of IQGAP1. The p-ERK level was only slightly reduced in FTC133 cells and was not changed at all in SW1736 and KAT18 cells with IQGAP1 knockdown. There was no change in p-Akt in any of these cells after IQGAP1 knockdown. Using this approach, we also successfully and stably knocked down the IQGAP3 in these cells and observed no effect on p-ERK and p-Akt (Fig S1B). Therefore, IQGAP proteins did not seem to play a significant role in the MAP kinase and PI3K/Akt pathways in thyroid cancer. Consistent with this observation was, as shown in Fig S2, the lack of significant effects of knockdown of IQGAP1, as well as IQGAP3, on the proliferation of these cells.
Effects of shRNA knockdown of IQGAP proteins on the invasion of thyroid cancer cells

Since IQGAP1 copy gain was associated with invasiveness and aggressiveness of thyroid cancer in this study (Tables 1 and 2), we next used the Matrigel™ in vitro system to functionally test whether IQGAP1 played a role in thyroid cancer cell invasion. As shown in Fig 2, knockdown of IQGAP1 dramatically inhibited the invasion of all the three thyroid cancer cells while, in contrast, knockdown of IQGAP3 did not have effect on the invasion of these cells. Thus, these data, consistent with the results of clinicopathological correlation studies (Tables 1 and 2), functionally demonstrate and confirm the specific and important role of IQGAP1 in the invasiveness of thyroid cancer.

Effects of shRNA knockdown of IQGAP proteins on anchorage-independent colony formation of thyroid cancer cells

Cell colony formation and expansion on soft agar requires cells to invade and migrate peripherally. We therefore reasoned that, unlike cell proliferation in normal culture medium (Fig S2), colony formation on soft agar could be affected by interfering with IQGAP1 if the latter was important to thyroid cancer cell invasion. Indeed, as shown in Fig 3, cells with specific IQGAP1 knockdown formed anchorage-independent colonies in soft agar with smaller size or number compared with cells transfected with control shRNA expressing scrambled nucleotides. This was seen both in SW1736 and FTC133 cells, somehow with a more profound effect seen in the former cells. In contrast, specific knockdown of IQGAP3 showed no effect on colony formation of the two cells (Fig 3). KAT18 cells, whether wild-type, control or specific shRNA transfection, did not grow on soft agar under our conditions (data not shown).
Direct interaction of IQGAP1 with E-cadherin in thyroid cancer cells

We also investigated the relationship of IQGAP1 with several molecules that had been known to be involved in cancer cell adhesion, invasion and progression. Among these was E-cadherin, a calcium-dependent adhesion molecule, which was well known for its important role in cell-cell adherence and its deficiency caused cell migration and invasion in cancers (31). As shown in Fig 4A, the expression of E-cadherin was normal in SW1736 cells but naturally silenced in KAT18 and FTC133. Interestingly, the protein level of E-cadherin in SW1736 cells was significantly up-regulated with knockdown of IQGAP1, suggesting that IQGAP1 normally interacts with E-cadherin to down-regulate it and weaken the cell adherence system, thus promoting cell migration and invasion. In contrast, the expression of cdc42 was not affected by knockdown of IQGAP1 (Fig 4A), suggesting that the change in E-cadherin was specific. Similarly, IQGAP1 knockdown had no effect on the phosphorylation of focal adhesion kinase (FAK) and Src, which were both previously suggested to play a role in thyroid cancer cell invasion and migration (32) (Fig 4A).

To demonstrate a direct interaction of IQGAP1 with E-cadherin, we performed co-immunoprecipitation assay for the two in SW1736 cells. As shown in Fig 4B, E-cadherin showed up in the immunoprecipitates when immunoprecipitated with IQGAP1 antibodies but not with control non-specific IgG antibodies, demonstrating that these two proteins directly interact and complex with each other in thyroid cancer cells. In contrast, co-immunoprecipitation using IQGAP1 antibodies failed to show a band of cdc42, suggesting the lack of direct interaction between IQGAP1 and cdc42 in thyroid cancer.
cells (Fig 4B). This result also suggests that the interaction of IQGAP1 with E-cadherin was a specific event. However, the results on the relationship between IQGAP1 and cdc42 do not rule out the possibility that the activity of cdc42, not the protein level, could be affected by IQGAP1.

Discussion

We for the first time report common genomic IQGAP1 copy gain, associated with over-expression of IQGAP1 protein, in thyroid cancer, with a preferential association with invasive types of thyroid cancer, such as ATC, TCPTC, and FTC. The IQGAP1 gene is located at 15q26, a chromosome area that is frequently amplified in many human malignancies. IQGAP1 itself is also amplified in other cancers (4) and was associated with IQGAP1 protein over-expression in gastric cancer cells (11). The IQGAP1 copy gain was a specific event in thyroid cancer as IQGAP3 did not show copy gain. Moreover, we saw only rare copy gain of TMEM84, a gene that is located at 15q23, about 20 Mb from IQGAP1 in the same chromosome, in striking contrast with the common copy gain of the IQGAP1 gene. Therefore, IQGAP1 copy gain in thyroid cancer was most probably due to the amplification of the gene but not aneuploidy. The fact that IQGAP1 copy gain was associated with protein expression and poorer clinicopathological outcomes provides strong support for the functional relevance of this genetic alteration. This also suggests that IQGAP1 copy gain in thyroid cancer is sufficient to play a significant amplifying role in the pathway cascade involving IQGAP1.

It is interesting that IQGAP1 copy gain-associated tumor invasiveness occurred mainly in FTC and FVPTC. It is well known that invasion, including vascular invasion,
is a particularly strong feature of these follicular types of thyroid cancer. Since the main finding of the present study was the invasive role of IQGAP1 copy gain in thyroid cancer, it seems to make good sense to see association of IQGAP1 copy gain with tumor invasiveness particularly in these cancers. The association of IQGAP1 copy gain with thyroid cancer invasion and aggressiveness is also consistent with previous reports of over-expression of IQGAP1 in the invasion fronts of other cancers, such as colorectal cancer (12) and ovarian cancer (13). Our in vitro studies using ATC- and FTC-derived cancer cell lines further support a role of IQGAP1 in thyroid tumorigenesis by showing remarkable inhibition of cell invasion on in vitro assays and colony formation on soft agar of thyroid cancer cells by IQGAP1 knockdown. These in vitro data are consistent with and support the clinicopathological correlation findings of IQGAP1 in thyroid cancers in the present study. The contrasting infrequent copy gain of the IQGAP3 gene and the lack of a role of IQGAP3 in cell colony formation and invasion suggest that the role of IAGAP1 in thyroid tumorigenesis is a specific one.

The present study suggests that promotion of cell growth and proliferation may not be a major event in IQGAP1-promoted pathogenesis of thyroid cancer. This is consistent with the inability of IQGAP1, shown in the present study, to interact with the MAP kinase and PI3K/Akt pathways in thyroid cancer cells, which are major signaling pathways that promote thyroid cancer cell proliferation (15-17). In this regard, thyroid cancer cells are different than some other cancer cells in which IQGAP1 could interact with the MAP kinase pathway (8-10). However, up-regulated IQGAP1 system through IQGAP1 copy gain and aberrant activation of the MAP kinase pathway through BRAF mutation may synergistically promote a poor clinical course of PTC in that in BRAF
mutation-positive PTC, but not in *BRAF* mutation-negative PTC, *IQGAP1* copy gain was associated with tumor multifocality and recurrence. As *BRAF* mutation has a well established role in the aggressiveness of PTC (22), it makes great logic to see its synergism with the invasiveness-promoting *IQGAP1* copy gain in promoting aggressiveness and poor clinical outcomes of PTC. This phenomenon is similar to the previously reported association of *PIK3CA* gene copy gain with increased recurrence of thyroid cancer when co-existing with *BRAF* mutation (33). The prevalence of *BRAF* mutation in PTC is relatively high, around 45% on average (21, 22). This may practically limit its use as a prognostic marker in tailoring aggressive treatment of PTC as many patients would have this mutation. Given the present study, it may be possible to use the combination of the two genetic markers to identify a small group of PTC patients with a particularly high risk of recurrence for more aggressive treatments.

The transmembrane protein E-cadherin was demonstrated to directly interact with the *IQGAP1* protein in thyroid cancer cells in the present study, as similarly seen in some other cells (34). It is interesting to see an up-regulation of E-cadherin protein after *IQGAP1* knockdown in thyroid cancer cells, suggesting that *IQGAP1* normally functions to negatively regulate E-cadherin in thyroid cancer cells. Over-expression of *IQGAP1* through *IQGAP1* copy gain may thus be expected to cause down-regulation of E-cadherin in thyroid cancer cells although we could not directly test this as we were unable to succeed in exogenously over-expressing the large 1,657-amino acid protein of *IQGAP1* in our *in vitro* cell system (data not shown). An important function of E-cadherin is to enhance cell adhesion and prevent proteolytic degradation of surrounding tissue and cell motility, thus preventing cancer cells from invading and metastasizing (31). E-cadherin
was also reported to be able to suppress transformation of colorectal cells (35), consistent with the inhibition of colony formation of thyroid cancer cells on soft agar induced by IQGAP1 knockdown in the present study. Thus, disruption of E-cadherin may be an important mechanism underlying the association of IQGAP1 copy gain with the invasiveness and aggressiveness of thyroid cancers and, conversely, the inhibition of cell invasion and anchorage-independent colony formation of SW1736 cells by IQGAP1 knockdown in the present study. Obviously, other mechanisms remain to be also identified.

In summary, we demonstrated an important role of IQGAP1 in the invasiveness and aggressiveness of thyroid cancer through genomic IQGAP1 copy gain, which is consistent with the role of IQGAP1 in thyroid cancer cell invasion and anchorage-independent colony formation as well as the direct interaction of IQGAP1 with E-cadherin and the negative impact of the former on the latter. IQGAP1 copy gain can also synergize with BRAF mutation to promote a poorer clinical outcome of PTC. Thus, IQGAP1 copy gain represents a new molecular mechanism in thyroid tumorigenesis and a potentially novel prognostic marker and therapeutic target for this cancer.

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Conflict of interest

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
The authors declare no conflict of interest.

**Supplementary information is available at Clinical Cancer Research’s website.**
References


32. Schweppe RE, Kerege AA, French JD, Sharma V, Grzywa RL, Haugen BR. 
Inhibition of Src with AZD0530 reveals the Src-Focal Adhesion kinase complex as a 
novel therapeutic target in papillary and anaplastic thyroid cancer. J Clin Endocrinol 
Metab. 2009;94:2199-203.

cancer with PIK3CA alterations in a Middle Eastern population. J Clin Endocrinol Metab 
2008;93:611-8.

34. Kuroda S, Fukata M, Nakagawa M, et al. Role of IQGAP1, a target of the small 
GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. 

35. Gottardi CJ, Wong E, Gumbiner BM. E-cadherin suppresses cellular transformation 
2001;153:1049-60.
Figure Legends

**Figure 1. Immunohistostaining of IQGAP1 in thyroid cancer** – Presented are representative tumor samples with various IQGAP1 copy numbers and corresponding immunohistostaining of the IQGAP1 protein. The brown color of staining using anti-IQGAP1 antibodies represents IQGAP1 protein. Cases A, B, and C represent immunohistostaining (IHS) scores I, II, and III, respectively, as defined in the Materials and Methods. Corresponding IQGAP1 copy numbers are indicated in the brackets.

**Figure 2. Effects of shRNA knockdown of IQGAP1 or IQGAP3 on the invasion of thyroid cancer cells** – SW1736, KAT18 and FTC133 cells, at a density of $2.5 \times 10^4$ cells/well, were seeded on Matrigel invasion chamber plates and cultured in routine medium for 22 hr. Invasive cells that penetrated through pores and migrated to the underside of the membrane were stained by crystal violet and photographed under a microscope. **A**, Representative results on the invasion assay of three thyroid cancer cells. **B**, Histogram plots of the number of invading cells corresponding to **A** for each cell type. “C”, cell transfection with shRNA containing scrambled nucleotides; “S”, cell transfection with specific shRNA to knock down IQGAP1 or IQGAP3. (**P < 0.01**)
**Figure 3.** Effects of shRNA knockdown of IQGAP1 and IQGAP3 on anchorage--independent colony formation of thyroid cancer cells -- SW1736 and FTC133 cells stably transfected with control shRNA or specific shRNA to knock down IQGAP1 or IQGAP3 were seeded in six-well plates in 0.3% agar over a bottom layer of 0.6% agar and treated with routine medium plus 1.5 mg/ml puromycin. After 2-3 weeks, cell colony numbers were counted under microscope and cell colonies were photographed. A, Representative results of colony formation of SW1736 and FTC133 cells. B, Histogram plots of colony numbers corresponding to A for each cell type. Only cell colonies containing more than 50 cells were counted. KAT18 cells did not grow on soft agar. “C”, cell transfection with shRNA containing scrambled nucleotides; “S”, cell transfection with specific shRNA to knock down IQGAP1 or IQGAP3. (*P<0.05; **P<0.01)

**Figure 4.** Western blotting and immunoprecipitation analysis of the relationship of IQGAP1 with other signaling molecules in thyroid cancers -- Thyroid cancer cell lines with stable transfections were lysed for western blotting after 24 hr of serum starvation as described in Fig S1. Shown in Fig 4A is the Western blotting analysis of the levels of IQGAP1, E-cadherin, cdc42, phosphorylated FAK (pFAK), phosphorylated Src (pSrc), and β-actin before and after IQGAP1 knockdown. Specific and virtually complete knockdown of IQGAP1 was seen in all the three cells. All other indicated proteins were expressed in all the cells except for E-cadherin that was only expressed in SW1736 cells. An interesting up-regulation of E-cadherin after the knockdown of IQGAP1 was seen in SW1736 cells. Shown in Fig 4B is the immunoprecipitation of cell lysates from normal
wild-type SW1736 cells using anti-IQGAP1 antibodies, followed by Western blotting of the immunoprecipitates with antibodies against the indicated proteins. Specific co-immunoprecipitation of E-cadherin, but not cdc42, with IQGAP1 was seen. This only occurred with specific monoclonal anti-IQGAP1 antibodies but not non-specific control mouse IgG. “C”, cell transfection with shRNA containing scrambled nucleotides; “S”, cell transfection with specific shRNA to knock down IQGAP1. “Input” using 1/100 of the amount of lysates used for immunoprecipitation was directly run for immunoblotting analysis as control to show the proteins of interest.

**Figure S1.** Western blotting analysis of shRNA knockdown of IQGAP1 and IQGAP3 and its effect on the signaling of MAPK and PI3K/Akt pathways – Upon stable transfection and selection of the three indicted thyroid cancer cell lines, a cell preparation of each type was lysed for western blotting after 24hr serum starvation. A. The protein levels of IQGAP1, phosphorylated ERK (p-ERK), phosphorylated Akt (p-Akt), and β-actin (for quality control) were analyzed with Western blotting using specific primary antibodies as described in the Materials and Methods. B. The effects of IQGAP3 knockdown were similarly examined. “C”, cell transfection with shRNA containing scrambled nucleotides; “S”, cell transfection with specific shRNA to knock down IQGAP1 or IQGAP3.

**Figure S2.** Effects of shRNA knockdown of IQGAP1 and IQGAP3 on proliferation of thyroid cancer cells -- Cells were seeded in quadruplets into 96-well plates and cultured with 2.5% FBS. MTT assay was performed daily over a time course of 5-6 days to evaluate cell proliferation as described in the Materials and Methods. No statistical
difference in cell proliferation was found between IQGAP1 knockdown and control cells. Similar MTT assay was performed for IQGAP3 knockdown and no effect was seen. “C”, cell transfection with shRNA containing scrambled nucleotides; “S”, cell transfection with specific shRNA to knock down IQGAP1 or IQGAP3.
Fig 2
Fig 3
Fig 4
Table 1. *IQGAP1* and *IQGAP3* copy gain in thyroid cancers [n/N (%)]

<table>
<thead>
<tr>
<th></th>
<th><em>IQGAP1</em> Copy #</th>
<th></th>
<th><em>IQGAP3</em> Copy #</th>
</tr>
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<tr>
<td></td>
<td>≥ 3.0</td>
<td>≥ 4.0</td>
<td>≥ 3.0</td>
</tr>
<tr>
<td>ATC</td>
<td>27/41 (65.9)</td>
<td>14/41 (34.1)</td>
<td>4/38 (10.5)</td>
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<tr>
<td>FTC</td>
<td>44/107 (41.1)</td>
<td>24/107 (22.4)</td>
<td>4/39 (10.3)</td>
</tr>
<tr>
<td>PTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>72/205 (35.1)</td>
<td>27/205 (13.2)</td>
<td>4/85 (4.7)</td>
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<tr>
<td>CPTC</td>
<td>40/115 (34.8)</td>
<td>17/115 (14.8)</td>
<td>2/54 (3.7)</td>
</tr>
<tr>
<td>FVPTC</td>
<td>24/74 (32.4)</td>
<td>8/74 (10.8)</td>
<td>2/25 (8)</td>
</tr>
<tr>
<td>TCPTC</td>
<td>8/16 (50)</td>
<td>2/16 (12.5)</td>
<td>0/6 (0)</td>
</tr>
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</table>

Note: n, the number of cases with the indicated *IQGAP1* copy #: N, the number of cases examined
Table 2. Relationship of *IQGAP1* copy gain with clinicopathological outcomes of thyroid cancers [n/N (%)]

<table>
<thead>
<tr>
<th>Tumor Types</th>
<th>Clinicopathological Characteristics</th>
<th>Cut-off Copy # ≥ 3.0</th>
<th>Cut-off Copy # ≥ 4.0</th>
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<td></td>
<td></td>
<td>Copy Gain (+)</td>
<td>Copy Gain (-)</td>
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<td>6/35 (17.1)</td>
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<td></td>
<td>Vascular Invasion</td>
<td>18/28 (64.3)</td>
<td>12/35 (34.3)</td>
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<td>Cancer Multifocality</td>
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<td>1/35 (2.9)</td>
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<td></td>
<td>Tumor Recurrence</td>
<td>3/29 (10.3)</td>
<td>8/35 (22.9)</td>
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<td>PTC (all)</td>
<td>Extrathyroidal Invasion</td>
<td>30/71 (42.3)</td>
<td>56/132 (42.4)</td>
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<td>Vascular Invasion</td>
<td>23/71 (32.4)</td>
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<td>Cancer Multifocality</td>
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<td><em>BRAF</em> Mutation</td>
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<td></td>
<td>Tumor Recurrence</td>
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<td>4/44 (9.1)</td>
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<tr>
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<td>Tumor Recurrence</td>
<td>9/25 (36)</td>
<td>11/48 (22.9)</td>
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<td>CPTC (w/BRAF)</td>
<td>Cancer Multifocality</td>
<td>10/19 (52.6)</td>
<td>18/45 (40)</td>
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</table>

Note: n, the number of cases positive for the indicated clinicopathological characteristics; N, the number of cases examined.
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