IQGAP1 Plays an Important Role in the Invasiveness of Thyroid Cancer

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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 clinicopathologic outcomes of thyroid cancer and investigated its role in cell invasion and molecules involved in the process.

Results: We found IQGAP1 copy number (CN) gain ≥3 in 1 of 30 (3%), 24 of 74 (32%), 44 of 107 (41%), 8 of 16 (50%), and 27 of 41 (66%) of benign thyroid tumor, follicular variant papillary thyroid cancer (FVPTC), follicular thyroid cancer (FTC), tall cell papillary thyroid cancer (PTC), and anaplastic thyroid cancer, respectively, in the increasing order of invasiveness of these tumors. A similar tumor distribution trend of CN ≥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 FTC (P = 0.01 and 0.02, respectively). The siRNA knockdown of IQGAP1 dramatically inhibited thyroid cancer cell invasion and colony formation. Coimmunoprecipitation assay showed direct interaction of IQGAP1 with E-cadherin, a known invasion-suppressing molecule, which was upregulated 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. Clin Cancer Res; 16(24): 6009–18. ©2010 AACR.

Introduction

The human IQGAP protein family comprises 3 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, 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 Ca2+ 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). Overexpression 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 (TCPCT). A striking genetic alteration in PTC is the 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...
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 2-fold: (i) IQGAP1 copy gain can be used to predict invasiveness and aggressiveness of thyroid cancer and, when coexisting with BRAF mutation in PTC, is a particularly powerful predictor for cancer recurrence; (ii) IQGAP1 represents a novel potential therapeutic target for thyroid cancer.

Materials and Methods

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). In brief, 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.

Analysis of IQGAP1 mutation in thyroid tumor samples

Genomic DNA sequencing was done for 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 Supplementary Table S1.

Analysis of genomic copy gain of the IQGAP genes with quantitative real-time PCR

Quantitative real-time PCR was used to analyze copy number (CN) of IQGAP1 and 3 genes using the ABI 7900HT PCR system (Applied Biosystems). This is a widely used and robust technique for genomic CN 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 IQGAP1, IQGAP3, TMEM84, and β-actin genes. The probes were labeled with 5’ fluoroscent reporter dye (FAM) and 3’ quencher dye (TAMRA). 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 Supplementary Table S1.

Analysis of IQGAP1 protein expression by immunohistostaining

Immunohistostaining (IHS) was done using a modified protocol (23). In brief, thyroid tumor tissue sections were dewaxed, soaked in alcohol, and incubated in 3% hydrogen peroxide for 15 minutes to inactivate endogenous peroxidase activity after microwave treatment in an antigen-unmasking solution (Vector Lab). Tissue sections were incubated overnight at 4°C with anti-IQGAP1 antibody (BD Biosciences). Immunostaining was done with Vectastain Universal Quick Kit (Vector Lab) following the manufacturer’s protocol. Peroxidase activity was revealed using 3,3-diaminobenzidine. Negative control was obtained by omission of primary antibodies. We classified IHS into 3 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) and Dr. Kenneth B. Ain (University of Kentucky Medical Center), respectively; and the FTC cell line FTC133 was from Dr. Georg Brabant (Christie Hospital). They were provided by Drs. Rebecca Schwepppe and Bryan Haugen (University of Colorado). 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://maidevdesigner.invitrogen.com/maixpress/setOption.do?design-Option=shrna&pid=67150486777617069736). The shRNA target sequence for IQGAP1, GCCATCCACTTACAGGATA, located from 763 to 781nt (nucleotide) 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.

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Lentiviral mediated gene knockdown

The lentiviral shRNA-expressing vector, the packaging plasmid PSPAX2, and the VSVG-G envelope coding plasmid pMD2G were cotransfected to HEK293T cells, plasmid PSPAX2, and the VSV-G envelope protein-coding vector pSicoR-PGK-puro (Addgene Corp.; ref. 27) and synthesizing through filters with pore size of 45 μm. The cellular supernatants were harvested and filtrated by the manufacturer's protocol. After transfection for 48 hours, plasmid pMD2G 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.

Cell proliferation study

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

Cell invasion assay

The invasion assay was carried out using Growth Factor Reduced Matrigel invasion chambers with 8.0-μm polycarbonate filters (BD Biosciences). In brief, 2.5 × 10^4 cells were seeded on chamber plates and cultured in routine medium. After an incubation of 22 hours at 37°C in a humidified incubator with 5% CO_2, nonmigratory 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 filter 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 × 10^4 cells/well) were plated into 6-well plates in growth medium containing 10% FBS, 1.5 mg/mL puromycin, and 0.3% agar, on top of 0.6% agar gel. After 2–3 weeks of culture, colonies were counted and photographed under a microscope (28).

Immunoprecipitation assay

Immunoprecipitation (IP) was carried out using the Protein G Immunoprecipitation Kit (Roche Applied Science) with slight modifications. In brief, 1 ml cell lysates were precleared with 50 μL 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.) as control. The immune complexes were incubated with 50 μL Protein G-agarose suspension for 6 hours, followed by 5 washings with lysis buffer, resuspension 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 HSH scores, BRAF mutation, tumor recurrence, lymph node metastasis, multifocality, and extrathyroidal extension status. Fisher’s exact test was used when the number of 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’s rank-sum test was used when data was not in normal distribution. Linear regression was used to analyze the relationship between IQGAP1 CN and patient age, tumor stage, and tumor size. The professional statistical software SPSS 10.0 (SPSS Inc.) 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 1 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 CN in thyroid cancer (25), we analyzed IQGAP1 and IQGAP3 CN 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 CN ≥ 3 was used as the cutoff value. The highest copy gain...
rate was seen in ATC, followed by FTC, and PTC regardless of whether the cutoff value of CN ≥ 3 or CN ≥ 4 was used to define copy gain. Among the 3 subtypes of PTC, the most aggressive TCPTC has the highest rate of IQGAP1 copy gain when the cutoff CN ≥ 3 was used in the analysis (up to 50%). We also studied the CN of IQGAP1 in 30 benign thyroid tumors and 30 normal thyroid tissue samples. Among all these samples, only 1 case of benign tumor was identified with copy gain when a cutoff 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 showed, 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 showed the malignancy specificity of IQGAP1 copy gain. We also examined the CN of the TMEM84 gene, which is located at 15q23 and about 20Mb away from the IQGAP1 gene in the same chromosome. Only 1 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 of 39 (10.3%) FTC, and 4 of 85 (4.7%) PTC when using CN ≥ 3 as the cutoff value, and 0 of 39 (0%) FTC, and 2 of 85 (2.4%) PTC, when using CN ≥ 4 as the cutoff 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 show 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 IHS analysis using IQGAP1-specific antibodies on 29 FTC samples that had available histopathologic slides. Figure 1 shows representative tumor samples illustrating the IHS scores and corresponding IQGAP1 CNs. Among the 9 cases without IQGAP1 copy gain (CN < 3), 6 (66.7%) had a IHS score I, 2 (22.2%) had a score II, and 1 (11.1%) had a score III, whereas among the 20 cases with IQGAP1 copy gain (CN ≥ 3), only 1 (5%) had a IHS score I, but 10 (50%) and 9 (45%) had cores II and III, respectively, with a significant difference between the 2 groups on overall analysis (P < 0.01). To take a further look at specific groups, IHS score I was seen in 6 of 9 (66.7%) of the cases with CN < 3 versus 1 of 20 (5%) of the cases with CN ≥ 3 (P < 0.001). Conversely, IHS scores II and III, in combination, were seen in 3 of 9 (33%) of the cases with CN < 3 versus 19 of 20 (95%) of the cases with CN ≥ 3 (P < 0.001). These data clearly showed an association of

![Fig. 1. IHS of IQGAP1 in thyroid cancer. Presented are representative tumor samples with various IQGAP1 CNs and corresponding IHS of the IQGAP1 protein. The brown color of staining using anti-IQGAP1 antibodies represents IQGAP1 protein. Cases A, B, and C represent IHS scores I, II, and III, respectively, as defined in the Materials and Methods section. Corresponding IQGAP1 CNs are indicated in the brackets.](image-url)
IQGAP1 copy gain with increased expression of the IQGAP1 protein in thyroid cancer, showing that this IQGAP1 copy gain was biologically relevant with respect to expected increase in IQGAP1 expression.

**Relationship of IQGAP1 copy gain with clinicopathologic 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 clinicopathologic role of IQGAP1 copy gain in thyroid cancer by investigating its relationship with clinicopathologic characteristics of thyroid cancers. As shown in Table 2, IQGAP1 copy gain was associated with extrathyroidal invasion in FTC. Specifically, 6 of 35 (17.1%) cases without IQGAP1 copy gain versus 12 of 28 (42.9%) cases with copy gain were associated with extrathyroidal invasion ($P = 0.025$) when the cutoff CN $\geq 3$ was used. This relationship marginally missed statistical significance ($P = 0.07$) when the cutoff CN $\geq 4$ was used. IQGAP1 copy gain was also associated with vascular invasion in FTC. Specifically, 12 of 35 (34.3%) cases without IQGAP1 copy gain versus 18 of 28 (64.3%) cases with copy gain were associated with vascular invasion ($P = 0.018$) when the cutoff CN $\geq 3$ was used. This relationship was still significant ($P = 0.05$) when the cutoff CN $\geq 4$ was used. In FVPTC, IQGAP1 copy gain was also associated with extrathyroidal invasion. Specifically, 3 of 65 (4.6%) cases without IQGAP1 copy gain versus 3 of 8 (37.5%; $P = 0.02$) cases with copy gain were associated with extrathyroidal invasion in FVPTC when the cutoff CN $\geq 4$ was used. When the cutoff CN $\geq 3$ 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 2 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 of 25 (12%) cases of samples with IQGAP1 copy gain versus 4 of 18 (22.2%) 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 cutoff CN $\geq 4$ 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 of 63 (22.2%) cases without IQGAP1 copy gain versus 6 of 10 (60%) cases with copy gain were associated with tumor recurrence ($P = 0.02$) when the cutoff CN $\geq 4$ was used. A significant association of IQGAP1 copy gain with tumor multifocality was observed in CPTC that harbored BRAF mutation. Specifically, 4 of 56 (7.1%) cases without IQGAP1 copy gain versus 4 of 8 (50%) cases with copy gain were associated with multifocality ($P = 0.01$) when the cutoff CN $\geq 4$ was used. These associations of IQGAP1 copy gain were not observed in BRAF mutation–negative PTC (data not shown). Patient

<table>
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<tr>
<th>Tumor types</th>
<th>Clinicopathologic characteristics</th>
<th>Cutoff CN $\geq 3.0$</th>
<th>Cutoff CN $\geq 4.0$</th>
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</thead>
<tbody>
<tr>
<td>FTC</td>
<td>Extradithyoidal invasion</td>
<td>12/28 (42.9)</td>
<td>12/51 (23.5)</td>
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<tr>
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<td>Vascular invasion</td>
<td>18/28 (64.3)</td>
<td>21/51 (41.2)</td>
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<td>Cancer multifocality</td>
<td>2/29 (6.9)</td>
<td>2/51 (3.9)</td>
</tr>
<tr>
<td>FTC</td>
<td>Tumor recurrence</td>
<td>3/29 (10.3)</td>
<td>9/51 (17.6)</td>
</tr>
<tr>
<td>FTC</td>
<td>BRAF mutation</td>
<td>30/71 (42.3)</td>
<td>75/177 (42.4)</td>
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<tr>
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<td>23/71 (32.4)</td>
<td>52/177 (29.4)</td>
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<td>28/71 (39.4)</td>
<td>72/177 (40.7)</td>
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<tr>
<td>FTC</td>
<td>Tumor recurrence</td>
<td>12/60 (20)</td>
<td>25/149 (16.8)</td>
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<tr>
<td>FTC</td>
<td>BRAF mutation</td>
<td>19/39 (48.7)</td>
<td>56/149 (37.1)</td>
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<tr>
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<td>15/39 (38.5)</td>
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<td>43/149 (28.6)</td>
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<td>FTC</td>
<td>Tumor recurrence</td>
<td>6/31 (19.4)</td>
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<td>BRAF mutation</td>
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<td>FTC</td>
<td>Cancer multifocality</td>
<td>10/19 (52.6)</td>
<td>4/56 (7.1)</td>
</tr>
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Abbreviations: $n$, the number of cases positive for the indicated clinicopathologic characteristics; $N$, the number of cases examined.
were not significantly associated with age and gender, tumor size, and lymph node metastasis.

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 Supplementary Figure S1A, IQGAP1 expression was nearly completely knocked down in all the 3 cells whereas the control cells stably transfected with nonspecific 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 (Supplementary 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, as shown in Supplementary Figure S2, was 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

As 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 Figure 2, knockdown of IQGAP1 dramatically inhibited the invasion of all the 3 thyroid cancer cells whereas, in contrast, knockdown of IQGAP3 did not have effect on the invasion of these cells. Thus, these data, consistent with the results of clinico-pathologic correlation studies (Table 2), functionally show 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 (Supplementary 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 Figure 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 in both SW1736 and FTC133 cells, somehow with a more profound effect was seen in the former cells. In contrast, specific knockdown of IQGAP3 showed no effect on colony formation of the 2 cells (Fig. 4). 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 Figure 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 upregulated with knockdown of IQGAP1, suggesting that IQGAP1 normally interacts with E-cadherin to downregulate 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 (ref. 32; Fig. 4A).

To show a direct interaction of IQGAP1 with E-cadherin, we carried out co-IP assay for the two in SW1736 cells. As shown in Figure 4B, E-cadherin showed up in the precipitates when immunoprecipitated with IQGAP1 antibodies but not with control nonspecific IgG antibodies, showing that these 2 proteins directly interact and complex with each other in thyroid cancer cells. In contrast, co-IP 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 overexpression 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 clinicopathologic 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. As the main finding of this 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 overexpression 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 clinicopathologic correlation findings of IQGAP1 in thyroid cancers in this 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 IQGAP1 in thyroid tumorigenesis is a specific one.

**Fig. 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 × 10⁴ cells/well, were seeded on Matrigel invasion chamber plates and cultured in routine medium for 22 hours. 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 3 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.
This 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 this 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, upregulated 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 coexisting 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 in this study, it may be possible to use the combination of the 2 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 shown to directly interact with the IQGAP1 protein in thyroid cancer cells in this study, as similarly seen in some other cells (34). It is interesting to see an upregulation 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. Overexpression of
IQGAP1 through IQGAP1 copy gain may thus be expected to cause downregulation of E-cadherin in thyroid cancer cells although we could not directly test this as we were unable to succeed in exogenously overexpressing 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 the 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 this 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 showed 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.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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