Nucleotide Variants within the *IQGAP1* Gene in Diffuse-Type Gastric Cancers

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IQGAP1 is recognized as a negative regulator of cell–cell adhesion at adherens junctions in several cell types, including gastric mucosal cells. The histopathologic appearance of diffuse gastric carcinoma is defined by non- or poorly cohesive tumor cells, indicating abnormal intercellular adhesion. Hence, we screened 38 gastric cancers for activating point mutations in *IQGAP1*. In 2 of the 33 diffuse gastric cancers, there was a missense nucleotide change predicted to alter the amino acid sequence in the GAP-related domain, which includes part of the binding site for the activated small G proteins Cdc42 and Rac1. Many intronic *IQGAP1* gene changes were observed, and several occurred more frequently in diffuse-type gastric cancers than in intestinal-type gastric cancers. A highly variable pentanucleotide repeat was identified in the final intron of *IQGAP1*. The most expanded six-repeat sequence was present exclusively in diffuse-type gastric cancers. Additionally, 19 diffuse cases and two intestinal cases exhibited silent coding region nucleotide alterations. Taken together, our results suggest that *IQGAP1* coding sequence mutations are not a frequent event in gastric cancer, but do occur in a subset of diffuse-type gastric carcinomas. Additional studies analyzing other proteins involved in cell adhesion may lead to a better molecular understanding of the histopathologic appearance of diffuse gastric cancers.

INTRODUCTION

Gastric adenocarcinomas are among the leading causes of cancer-related deaths worldwide (Parkin et al., 2001). The most important genetic alterations in the development and progression of gastric cancer have yet to be defined. Gastric cancers are classified histologically into intestinal and diffuse types, with the latter type characterized by poorly cohesive cells that penetrate the mucosa, as well as by submucosa and muscularis propria. In general, the prognosis for patients with diffuse-type gastric cancer is very poor because of the advanced stage of the neoplasm at diagnosis and the lack of effective therapy for nonresectable disease.

The E-cadherin/β-catenin cell adhesion complex is a critical mechanism of cell adhesion in epithelial cells. Both E-cadherin and β-catenin have been studied extensively in carcinomas of the gastrointestinal tract. Germ-line E-cadherin (*CDH1*) gene alterations have been identified in inherited forms of diffuse gastric cancer (Guilford et al., 1998; Oliveira et al., 2002; Yabuta et al., 2002; Graziano et al., 2003). Furthermore, E-cadherin (*CDH1*) somatic alterations have been identified in approximately one-third of sporadic diffuse gastric cancers (Stone et al., 1999; Ascano et al., 2001). β-Catenin gene alterations in sporadic gastric cancers are very infrequent (Candidus et al., 1996; Park et al., 1999; Sasaki et al., 2001; Woo et al., 2001), and membrane localization of its encoded protein has been lost in only a few instances (Chan et al., 2003; Ebert et al., 2003; Tsukashita et al., 2003). Hence, it is likely that other molecular alterations are also important in gastric tumorigenesis.

Several lines of evidence suggest that IQGAP1 is involved in carcinogenesis (Clark et al., 2000), specifically in the gastric mucosa (Li et al., 2000; Sugimoto et al., 2001; Zhou et al., 2003). IQGAP1 is recognized as a negative regulator of intercellular adhesion based on its ability to interact with the E-cadherin/β-catenin cell adhesion complex at the plasma membrane (Kuroda et al., 1998; Takemoto et al., 2001; Nabeshima et al., 2002; Shimao et al., 2002). Specifically, IQGAP1 is

Abbreviations: CHD, calponin homology domain; GRD, GTPase activating protein (GAP)–related domain; G#, case designation for primary gastric tumors; X#, case designation for xenografted tumors; N#, case designation for normal gastric tissue.

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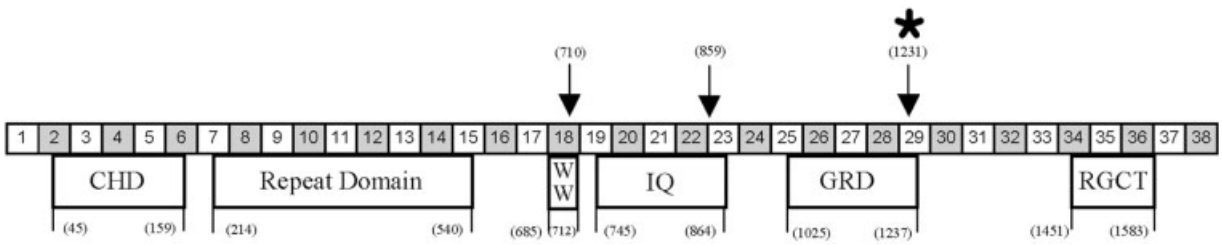

Figure 1. Functional domains and location of coding sequence changes in *IQGAP1*. Characterized functional domains of *IQGAP1* and their corresponding exons are depicted above. The amino acid at which the respective domain begins and ends according to the NCBI Conserved Domain Summary is shown in parentheses below the diagram. Arrows indicate the locations of altered nucleotides identified in coding regions, with an asterisk highlighting the missense change [CHD, calponin homology domain; Repeat Domain, coiled-coil repeat domain; WW, polyproline-binding motif; IQ, CaM- and Ca^{2+}/CaM-binding domain; GRD, Ras GTPase-activating protein (GAP)–related domain; RGCT, RasGAP-like C-terminal domain.

reported to bind β-catenin directly (Kuroda et al., 1998), so that α-catenin, which tethers the actin cytoskeleton to the cadherin±catenin complex, dissociates from β-catenin (Kuroda et al., 1998; Takemoto et al., 2001). Cell–cell adhesion is thereby destabilized, promoting dissociation.

Genomic studies have shown that the *IQGAP1* gene, on chromosome segment 15q26.1, was amplified in two diffuse-type gastric cancer cell lines (Sugimoto et al., 2001). In addition, a region of chromosome 15 near the *IQGAP1* gene was found to contain a newly recognized duplicon on a BAC/PAC contig map (Pujana et al., 2001), indicating the potential for chromosomal aberrations at this locus. At the posttranscriptional level, *IQGAP1* mRNA was enhanced >3-fold in an oligonucleotide-array screen of gene expression in mouse pulmonary metastases compared to that in poorly metastatic tumor cells (Clark et al., 2000). Moreover, in vitro and in vivo protein studies have substantiated the involvement of IQGAP1 in gastric tumorigenesis. The IQGAP1 protein was found to be overexpressed in gastric cancer cell lines (Sugimoto et al., 2001). In immunohistochemical analyses of gastric (Takemoto et al., 2001) and colorectal (Nabeshima et al., 2002) carcinomas, IQGAP1 was localized at cell membranes, specifically at the invasive front of tumor cells.

Notably, the only reported phenotype of *Iqgap1* knockout mice showed a significant increase in late-onset gastric hyperplasia, indicating increased adhesion and inability for cells in the stomach to separate properly and slough off into the lumen (Li et al., 2000). Loss of *IQGAP1* expression appears to increase cell adhesion, specifically in the stomach, leading to the formation of serrated hyperplastic lesions, as opposed to *IQGAP1* overexpression, which negatively regulates intercellular adhesion. Thus, dysregulation of *IQGAP1* results in abnormal cell adhesion in the gastric mucosa, promoting cellular dissociation when overexpressed and adhesion when lost.

We sought to determine whether activating gene alterations of *IQGAP1* were present in gastric cancers, particularly in those of the diffuse subtype. The total genomic region of *IQGAP1* exceeds 110 kb and includes 38 exons that total about 4.5 kb. The 190-kDa IQGAP1 protein contains several characterized domains including an N-terminal actin-binding calponin homology domain (CHD; Erickson et al., 1997; Fukata et al., 1997; Ho et al., 1999; Mateer et al., 2002), a series of six consecutive coiled-coil repeats thought to function in dimerization (Mateer et al., 2002), a WW domain that may be a site of interaction for proline-rich ligands (Macias et al., 2002), four consecutive IQ repeat motifs that are sites of calmodulin (CaM) and Ca^{2+}/CaM binding (Hart et al., 1996; Joyal et al., 1997; Ho et al., 1999; Mateer et al., 2002), a GTPase-activating protein (GAP)–related domain (GRD) that lacks intrinsic GTPase ability but is a part of the binding site for Cd42 and Rac1 (Hart et al., 1996; Kuroda et al., 1996; Joyal et al., 1997; Ho et al., 1999; Swart-Mataraza et al., 2002), and a RasGAP C-terminal (RGCT) domain that is necessary for binding β-catenin (Briggs et al., 2002) and E-cadherin (Kuroda et al., 1998; Li et al., 1999; Fig. 1). Here we report genomic sequencing results for *IQGAP1* in 38 human gastric carcinomas.

MATERIALS AND METHODS

Specimens

Resected primary gastric adenocarcinomas and paired normal tissue were collected from patients between 1994 and 2003 at the University of Virginia and Johns Hopkins University. Collection of these tissues was done in accordance with internal review board–approved protocols. Tumor-node metastasis

staging of resected cancers was assessed according to the consensus criteria adopted by the American Joint Committee on Cancer (Sobin and Fleming, 1997). Histopathology of the principal sample set used to screen the entire *IQGAP1* gene was composed of 38 gastric cancer samples assessed according to the Lauren classification: 33 tumors were classified as diffuse type and 5 as intestinal type. Twenty-eight additional intestinal-type tumor samples were assessed as controls specifically for the nucleotide variant change identified in the diffuse cases. Cryostat sections were stained with H&E, and manual dissection of frozen-tissue blocks was performed in order to enrich for >70% neoplastic cells (Vogelstein et al., 1988). High-molecular-weight genomic DNA was extracted from tumor and normal samples by standard organic methods.

Xenografts

Several of the primary gastric tumor tissues were used for xenografting enrichment of neoplastic cells as previously described (Hahn et al., 1995). Briefly, primary tumor tissue was embedded in matrigel (Collaborative Biomed Research, Bedford, MA) and implanted subcutaneously into flanks of immunodeficient mice (nu/nu from Harlan, Indianapolis, IN, or SCID from Charles River Laboratories, Wilmington, MA). The resulting xenografts were harvested when their growth reached approximately 1 cm. DNA was prepared as described above.

Genomic Sequencing Analysis

All 38 exons of the *IQGAP1* gene were amplified from genomic DNA and sequenced for each of the principal sets of gastric cancer samples cited above. The amplified regions included the intron/exon borders and the entire coding sequence of each exon. The representative *IQGAP1* sequence to which these samples were compared was obtained from UCSC Genome Bioinformatics (www.genome.ucsc.edu) and cross-referenced with PAC clone pDJ443n8, on 15q26.1, GenBank accession number AC004587 (Pujana et al., 2001). The bases that were not in agreement from these two sources were avoided for the purposes of primer design. Amplimers and sequencing primers are available from the authors on request.

Amplifications were performed by polymerase chain reaction (PCR) under the following conditions: in a 25-μl reaction volume, 50 ng of genomic DNA, 1× PCR buffer, 4 mM $MgCl_2$, 5.2% DMSO, 1 mM dNTPs, 175 ng of forward and reverse primers, and 0.25 μl (1.25 U) of Platinum Taq polymerase (Invitrogen, Carlsbad, CA) were combined and subjected to a standard thermocycle reaction for 40 cycles (A detailed description of conditions is available on request.) PCR-amplified products were purified by treatment with shrimp alkaline phosphatase and exonuclease according to the manufacturer's instructions (Amersham, Piscataway, NJ). PCR-amplified products were sequenced by use of the appropriate sequencing primer and a Thermo Sequenase Radiolabeled Terminator Sequencing Kit (USB, Cleveland, OH) according to the manufacturer's instructions. Sequencing reaction products were then electrophoresed on a 6% denaturing polyacrylamide gel and visualized by autoradiography. Each missense change was confirmed by independent PCR amplification and sequence analysis.

RESULTS

Thirty-eight patients who had undergone gastric cancer resection during the last two decades were enrolled in our study. The tissue samples selected from these cases included 22 diffuse-type primary gastric cancers, 11 diffuse-type xenografted human gastric cancers, and 5 intestinal-type xenografted human gastric cancers. Both early and advanced gastric carcinomas at each of the four TNM stages were represented in both the intestinal-type and diffuse-type cases. High-molecular-weight genomic DNA was extracted from histologically confirmed microdissected tissue samples and processed for nucleotide sequencing.

The entire *IQGAP1* coding region and surrounding intron/exon borders of the 38 gastric cancer samples were sequenced. Coding-region nucleotide changes in *IQGAP1* are depicted in Figure 1 and listed in Table 1. A missense nucleotide change occurred at *IQGAP1* codon 1231 in two diffuse-type cases. A heterozygous transition from guanine to adenine in cases G30 and X73 was predicted to change the amino acid from methionine to isoleucine. This nucleotide change occurred at an allelic frequency of 2.6% (2 of 76). Codon 1231 resides in the GRD, which mediates binding of *IQGAP1* to activated Cdc42 and Rac1. Both the normal and neoplastic tissues were altered in case G30, revealing this to be a germ-line alteration. Although normal tissue was not available for case X73, the primary adenocarcinoma from which the xenografted tumor was derived also demonstrated that nucleotide change (Fig. 2). To confirm that the guanine-to-adenine nucleotide change at codon 1231 was associated with the diffuse-type gastric cancer samples and was not a common

TABLE 1. Nucleotide Variants In *IQGAP1* Coding Region: Frequency, Location, and Result

Nucleotide Change (wt/△)	Frequency[a]	Location	Result
ATG/ATA	97.4%/**2.6%**	Exon 29, codon 1231	Met-Ile
CCT/CCC	62%/38%	Exon 18, codon 710	Silent
A■agTC/A■agTT	98.7%/**1.3%**	Exon 23, codon 859	Silent

[a]Frequencies are based on the number of alleles in 38 samples of gastric cancer. The changes that occurred only in diffuse-type cases are in boldface.

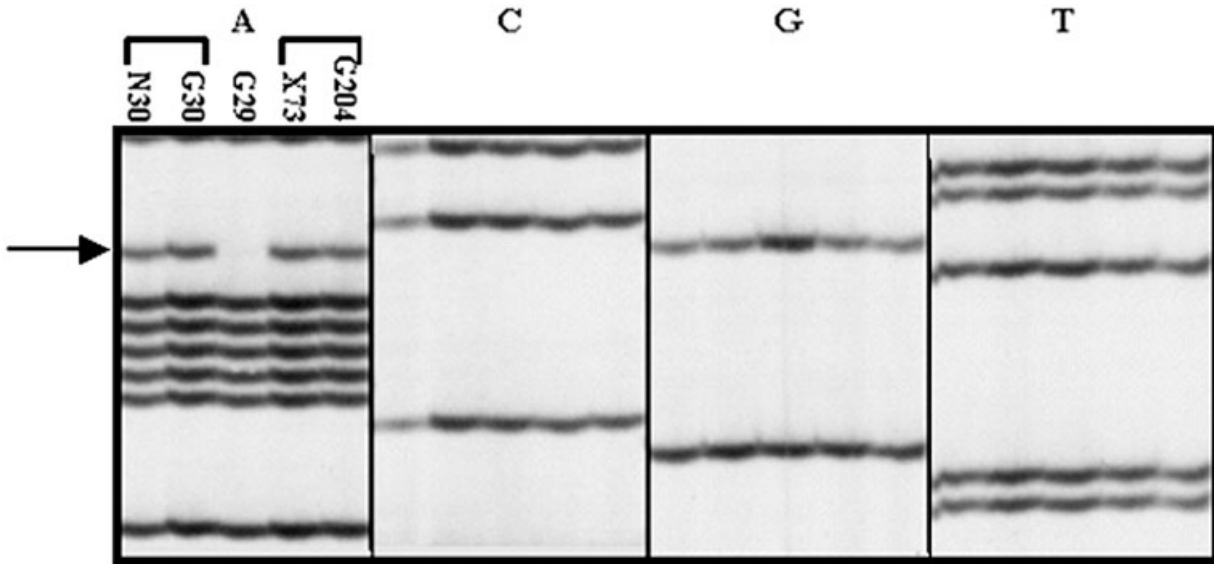

Figure 2. Missense nucleotide change of *IQGAP1* in diffuse-type gastric cancers. A heterozygous guanine-to-adenine transition (arrow) in the GAP-related domain of *IQGAP1* results in a amino acid substitution of methionine to isoleucine at codon 1231. Diffuse gastric cancer cases G30 and X73 were sequenced with their corresponding normal and primary gastric tissues, respectively. Both the normal (N30) and primary (G30) samples contained the guanine-to-adenine transition, indicating the germ-line nature of this mutation. G204, the primary sample corresponding to xenograft X73, also contained the purine transition. Case G29 (center lane) represents the wild-type *IQGAP1* sequence, as indicated by the concomitant absence of the adenine base and the strength of the guanine base.

polymorphism, we sequenced this exon in an equivalent number of intestinal-type tumor samples, all of which were found not to contain this nucleotide variant in the *IQGAP1* gene.

Another change that occurred exclusively in diffuse-type cases was the six-repeat expansion of a pentanucleotide sequence in intron 37, which precedes the final exon of the *IQGAP1* gene. The pentanucleotide sequence (gtttt) at the intron 37/exon 38 boundary was found to be repeated 3, 4, 5, and 6 times and in various heterozygous combinations (see Table 2). The number of repeats in both the intestinal and the diffuse types varied widely. Interestingly, the six-microsatellite repeat was found in 20% of the alleles and was found exclusively in 10 of the diffuse-type cases.

A third nucleotide change found exclusively in diffuse-type gastric cancers was identified in the coding region but was not predicted to affect the amino acid sequence. A cytosine-to-thymine transition at codon 859 was found at the second nucleotide position of exon 23 of diffuse-type gastric cancer G110. This substitution occurred at an allelic frequency of 1.3% (1 of 76) and was not predicted to alter the encoded isoleucine.

Another silent change in the IQGAP1 coding region occurred at a relatively high frequency in both diffuse (19 cases) and intestinal (2 cases) gastric cancer samples. A heterozygous transition from thymine to cytosine at codon 710 in the WW domain was not predicted to alter the encoded proline. A thymine-to-cytosine transition occurred in 30% of the intestinal-type (3 of 10) and 39% of the diffuse-type (26 of 66) alleles. Nucleotide variations in the coding region and the corresponding allelic frequencies, gene locations, and effects on the amino acid sequence are reported in Table 1; intronic nucleotide changes, allelic frequencies, and gene locations are reported in Table 2.

DISCUSSION

Loss of epithelial cell adhesion occurs in cancer progression and metastasis. Dysregulation of cell adhesion molecules underlies, at least in part, the

TABLE 2. Nucleotide Variants In *IQGAP1* Introns: Frequency and Location

Nucleotide change (wt/△)	Frequency[a]	Location
cacg/catg	59%/41%	5'UTR –116
ccgc/cccc	98.7%/**1.3%**	5'UTR –58
ttca/ttta	98.7%/1.3%	5'UTR –36
ggtgcagG/tgtgcagG	50%/50%	Intron 2 –7
tctagG/ttctagG	98.7%/**1.3%**	Intron 3 –5
22nt(2)/22nt(1)	93.4%/**6.6%**	Intron 4 –96
gatt/gact	84%/16%	Intron 6 –28
tttgt/ttttt	98.7%/**1.3%**	Intron 7 +20
gt(7)g/gt(8)g	98.7%/**1.3%**	Intron 7 –15
t(11)agT/t(10)agT/t(9)agT	96%/2.5%/1.5%	Intron 7 –3
aattg/aatgg	83%/17%	Intron 15 –86
gtat/gtgt	84%/16%	Intron 15 –56
cctg/cccg	74%/26%	Intron 15 –41
ggag/gggg	96%/4%	Intron 21 +33
Ttcttctttt/ttcttcttctttt	63%/37%	Intron 21 –17
tgtgctg/tgtg	78%/**22%**	Intron 32 –86
tccgtagG/tgcgtagG	80%/**20%**	Intron 33 –6
tttgc/tttcc	98.7%/**1.3%**	Intron 36 +11
gtttt(3)/gtttt(4)/ gtttt(5)/gtttt(6)	51%/21%/8%/**20%**	Intron 37 –8

[a]Frequencies are based on the number of alleles in 38 samples of gastric cancer. The changes that occurred only in diffuse-type cases are in boldface. The changes that occurred only in intestinal-type cases are underlined.

morphologic difference between intestinal-type (glandular) and diffuse-type (infiltrative) gastric cancer. Although the E-cadherin/β-catenin cell adhesion complex is a predominant mechanism of cell adhesion in epithelium-derived cells, its alteration occurs in only a minority of gastric cancers. Thus, for the majority of diffuse-type gastric cancers, the molecular alterations remain to be determined. Our interest in the intercellular adhesion pathway led us to investigate *IQGAP1* as a potential regulator of cell detachment in diffuse-type gastric cancers.

The results of the *IQGAP1* genomic sequence analysis in 38 gastric cancers indicate that genomic alterations of the coding sequence are not a frequent event in gastric tumorigenesis. However, of 33 diffuse-type and 33 intestinal-type cancers, two diffuse-type cases were found to contain a conservative missense *IQGAP1* nucleotide change in the GAP-related domain. Our data indicate that, although this nucleotide alteration occurs infrequently, it appears to occur specifically in diffuse-type gastric cancers. One tumor had the change in both the neoplastic and corresponding normal samples. The individual from whom these samples were taken was only 51 years old, and the cancer had already progressed to a late stage, indicating a possible predisposition to gastric cancer. The missense change found in a gastric cancer xenograft was confirmed in the corresponding sample from the primary tumor. Unfortunately, normal tissue for this case was not available for sequence analysis. Previous studies of each of these tumors showed loss of heterozygosity at the E-cadherin (*CDH1*) gene locus, but no functional mutations in the E-cadherin (*CDH1*) gene were found. Thus, E-cadherin and β-catenin are expected to be functional in these tumors and regulated by the altered *IQGAP1* gene that we observed.

The GAP-related domain is required for IQGAP1 binding to the Rho-family small G-proteins Cdc42 and Rac1 (Mataraza et al., 2003). There have been reports that IQGAP1 binds activated forms of Cdc42 and Rac1, which induces the F-actin crosslinking activity of IQGAP1 (Fukata et al., 1997) and that activated Cdc42 and Rac1 binding prevent IQGAP1 from binding to E-cadherin and β-catenin at adherens junctions (Kuroda et al., 1998). Thus, Cdc42 and Rac1 may balance cellular adhesion and motility through regulation of IQGAP1. The amino acid substitution observed in the GAP-related domain specifically in diffuse-type gastric cancers may disrupt this balance, rendering the cells less cohesive. Further studies are planned to determine the functional effects of this *IQGAP1* amino acid change.

The presence of the missense change observed in one xenografted tumor was confirmed in the corresponding human primary gastric cancer sample.

This is further evidence that xenografted tumors have genetic changes similar to those in the corresponding primary tumors from which they were derived (Hahn et al., 1995).

Another notable nucleotide alteration found in this screen was the variable pentanucleotide repeat in intron 37 that occurred eight nucleotides from the start of exon 38. The number of repeats ranged from three to six, with various combinations observed. An interesting observation is that the six-pentanucleotide repeat occurred only in diffuse-type gastric cancers. It can be speculated that this expansion of the pentanucleotide repeat may affect the final gene product. RT-PCR analysis of these cancers did not identify a splice alteration of the *IQGAP1* gene product (data not shown). Additional studies of other signaling molecules in the biochemical pathway involving IQGAP1 may lend insight into the underlying mechanisms leading to the infiltrative phenotype of diffuse-type gastric cancers.

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