Abstract. Frequent somatic mutations in the GNA11, matrix metalloproteinase (MMP)27, FGD1, TRRAP and GRM3 genes have been reported in various types of human cancer, but whether these genes are mutated in thyroid cancer is not known. In the present study, a mutational analysis of these genes was performed in thyroid cancer cell lines and thyroid cancer samples. No GNA11 mutations were identified in the papillary thyroid cancer (PTC) cell line and follicular thyroid cancer (FTC) samples. Additionally, no mutations were identified in the MMP27 gene, although three synonymous [C351T (N117N), C1089T (S363S) and G1227A (G409G)] single nucleotide polymorphisms (SNPs) were observed infrequently in FTC. No mutations were detected in the FGD1 gene, but two infrequent synonymous [T2091C (T697T) and A2136G (P712P)] SNPs were observed in FTC. Furthermore, no mutations were identified in TRRAP and GRM3, although a frequent synonymous SNP [G1323A (G475D)] of GRM3 was observed in PTC. No mutation of these genes was observed in 12 cell lines derived from various types of thyroid cancer. The present study reports for the first time the mutational status of the GNA11, MMP27, FGD1, TRRAP and GRM3 genes in thyroid cancer. No mutations were identified in these genes in the various types and cell lines of thyroid cancer. Therefore, unlike in other types of cancer, mutations in these genes are absent or uncommon in thyroid cancer.

Introduction

Thyroid cancer is histologically classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC). Thyroid cancers frequently harbor activating mutations in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, as represented by RET/PTC, RAS and BRAF mutations in the former and by PIK3CA and PTEN mutations in the latter (3,4). As a significant mechanism for the tumorigenesis of thyroid cancer, aberrant activation of these two important signaling pathways by such mutations causes uncontrolled cell division, proliferation and survival, leading to malignancy.

High-frequency somatic mutations of the GNA11, matrix metalloproteinase (MMP)27, TRRAP and GRM3 genes have been reported in uveal melanomas and melanomas with various incidences (5-8). We previously demonstrated that FGD1 was normally maintained, hypomethylated and overexpressed by BRAF (V600E) in thyroid cancer cells (9). GNA11 activates the MAPK signaling pathway. Particularly frequent somatic mutations of the GNA11 gene at codon 209 in exon 5 and codon 183 in exon 4, resulting in mutant GNA11R209H and GNA11R183C, respectively, have been reported in uveal melanoma and blue nevi (5). The GNA11 gene encodes a G-protein α-subunit (Ga11) that mediates signals from G-protein-coupled receptors (GPCRs) to the MAPK pathway. The normal amino acid, glutamine, encoded by codon 209 of the GNA11 gene, lies within the RAS-like domain of GNA11 (corresponding to residue 61 of Ras) and is essential for GTP hydrolysis. In members of the RAS family, mutations at this site and at codon 12 cause the loss of GTPase activity with constitutive activation of Ras. The GNA11R209H and GNA11R183C mutants have been demonstrated to be able to transform 3T3 cells and form tumors in immunocompromised mice (5). MMPs are proteolytic enzymes that degrade components of the extracellular matrix and basement membranes. MMP abnormalities have been associated with the metastasis of various types of cancer (6,10-12). In particular, mutations of the MMP27 gene have been observed in melanoma. The majority of the mutations in this gene have been identified in exons 1, 2, 3, 8 and 9 (6). FGD1 gene mutations have been reported in Aarskog-Scott syndrome (AAS), or facio-digito-genital dysplasia (13). At present, 20 different FGD1 gene mutations have been reported in this syndrome (13). FGD1 is a Dbl family member that has been
shown to function as a CDC42-specific guanine nucleotide exchange factor (GEF). It has also been demonstrated that FGD1 expression is sufficient to cause tumorigenic transformation of NIH3T3 fibroblasts (14). Two studies from the same group reported that the TRRAP gene was recurrently mutated and clustered in one amino acid position S722F (7). Furthermore, a frequent mutation of the GRM3 gene has been reported, and the authors also noted that the mutant selectively regulated the phosphorylation of MEK in the activation of the MAPK signaling pathway, leading to the anchorage-independent growth and migration of cells (8).

The mutation status in the GNA11, MMP27, FGD1, TRRAP and GRM3 genes has not been studied in thyroid cancer. The present study was conducted to investigate the mutational status of these genes in thyroid cancer.

Materials and methods

Cell lines, tumor samples and DNA extraction. A total of 89 samples, consisting of 12 thyroid cancer cell lines and 77 thyroid tumor samples were used for the mutational analysis of the GNA11 gene. For the MMP27 mutational analysis, 29 samples consisting of 12 thyroid cancer cell lines and 17 ATC samples were used. The FGD1, TRRAP and GRM3 genes were analyzed in 28 samples, including 12 thyroid cancer cell lines and 16 PTC samples. The thyroid cancer cell lines and tumor samples were used as described previously and with institutional review board (IRB; The Johns Hopkins University School of Medicine, Baltimore, MD, USA) approval (15). The cell lines were authenticated as described previously (16). With the exception of the FTC133 cells cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, all tumor cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/ml), penicillin (100 U/ml) and 2 mM glutamine. Genomic DNA from the cell lines and tumors was isolated by standard phenol-chloroform extraction using MaXtract high-density gel tubes (Qiagen, Valencia, CA, USA) (15).

Table I. Primer sets used for PCR amplification of the MMP27 and FGD1 genes.

| Exon | Forward | Reverse |
|------|---------|---------|
| MMP27 | GCAATTCACTGACGTCTCAC | GAAAATATGCAACTGGCTCAGG |
| 1-2 | GAACGGGCTTCAGCTGAAGAGGA | CAATTCTCGCAAAAGATGTCTG |
| 2-1 | CCTGGAGGAGATTCTTCCTC | GATGGGCTACACCCTCCT |
| 2-2 | TCTTTTGTGTAGCCGATCTC | ATCATGAGACACCCAGGTT |
| 2-3 | CTCACACAGTCTTCTCCTTG | GTATGAGCTGACATGAG |
| 2-4 | TGGATCATGAGAGACTCG | CATTCAATTGACTGACACTT |
| FGD1 | GACCTTGAGGACGAGCAGAGTGG | AACAAGAACCCCTCCAGTAC |
| 1 | AGGACAGGATGTGTTGAGGCC | GTTGGTACTTCTGAGTCAT |
| 2 | AGTCCTAACTTTAACCCTCTGT | GTTGGTACTTCTGAGTCAT |
| 3 | TCTCAAGTCTGAAAGATCGT | GAGCTTATTAGTGAGAGAG |
| 4 | AGGGGAGGTGTTGAGGGCTC | GTTGGTACTTCTGAGTCAT |
| 5 | AGTCCTAACTTTAACCCTCTGT | GTTGGTACTTCTGAGTCAT |
| 6 | TCTCAAGTCTGAAAGATCGT | GAGCTTATTAGTGAGAGAG |
| 7 | GACCTTGAGGACGAGCAGAGTGG | AACAAGAACCCCTCCAGTAC |
| 8 | AGGACAGGATGTGTTGAGGCC | GTTGGTACTTCTGAGTCAT |
| 9 | AGTCCTAACTTTAACCCTCTGT | GTTGGTACTTCTGAGTCAT |
| 10 | TCTCAAGTCTGAAAGATCGT | GAGCTTATTAGTGAGAGAG |
| 11 | GACCTTGAGGACGAGCAGAGTGG | AACAAGAACCCCTCCAGTAC |
| 12 | AGGACAGGATGTGTTGAGGCC | GTTGGTACTTCTGAGTCAT |
| 13 | AGTCCTAACTTTAACCCTCTGT | GTTGGTACTTCTGAGTCAT |
| 14 | TCTCAAGTCTGAAAGATCGT | GAGCTTATTAGTGAGAGAG |
| 15 | GACCTTGAGGACGAGCAGAGTGG | AACAAGAACCCCTCCAGTAC |
| 16 | AGGACAGGATGTGTTGAGGCC | GTTGGTACTTCTGAGTCAT |
| 17 | TCTCAAGTCTGAAAGATCGT | GAGCTTATTAGTGAGAGAG |
| 18 | GACCTTGAGGACGAGCAGAGTGG | AACAAGAACCCCTCCAGTAC |

Primers are represented as 5’>3’. MMP, matrix metalloproteinase.
of the GNAI1 gene and exons 1, 2, 3, 8 and 9 of the MMP27 gene were as described previously (5,6). For the mutational analysis of the MMP27 gene, in addition to the above primers, additional primers were used to shorten the amplicon size. These primers are shown in Table I. The PCR conditions for the amplification of the MMP27 with the additional primers were as follows: One cycle of 94˚C for 3 min; ten cycles of 94˚C for 30 sec, 67˚C for 30 sec with a 1˚C reduction for each cycle and 72˚C for 30 sec; thirty two cycles of 94˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec; followed by 72˚C for 7 min as a final extension; and 4˚C as the storage temperature. The primer sequences for the amplification of the FGDI gene are shown in Table I. The PCR reaction conditions for the FGDI amplification were as follows: After an initial denaturation at 94˚C for 2 min, the amplification was performed at 94˚C for 1 min, followed by annealing temperatures (exons 11, 14 and 18, 55˚C; exons 1, 2 and 16, 57˚C; exons 5, 6, 7, 8, 13 and 17, 58˚C; exons 3, 12 and 15, 60˚C; and exons 4, 9 and 10, 62˚C) for 1 min for 35 cycles, with a final extension at 72˚C for 7 min. The primer sequences and PCR conditions for the amplification of exon 1 of the TRRAP gene and exons 1, 2, 3, 4 and 5 of the GRM3 gene were as described previously (7,8). The PCR products were directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing ready reaction (Applied Biosystems, Foster City, CA, USA). These exons were investigated as they harbored the majority of the reported mutations in these genes in human cancers. The GenBank accession numbers were NM_002067.2 (GNAI1), NM_022122 (MMP27), NM_004463 (FGDI), NM_003496 (TRRAP) and NM_000840 (GRM3).

Results

The strategy of the present study was to investigate the gene exons that were the most likely to carry mutations. In particular, exons 4 and 5 of the GNAI1 gene were examined for mutations since all of the known GNAI1 mutations have been reported in codons 209 and 183 of these exons. Exons 1, 2, 3, 8 and 9 of the MMP27 gene were selected for sequencing as they have been shown to carry somatic mutations in melanomas. All the exons of the FGDI gene were analyzed for mutation, as mutations in this gene have never been reported in human cancers. Exon 1 of the TRRAP gene and exons 1, 2, 3, 4 and 5 of the GRM3 gene were analyzed as these exons have also been reported to harbor somatic mutations in melanoma.

The sequencing results showed no mutations in and around the hot spot of codons 209 and 183 in the GNAI1 gene in 12 thyroid cancer cell lines and 46 thyroid cancer samples (including 26 FTC and 20 ATC samples). No novel MMP27 somatic mutations were identified in 12 thyroid cancer cell lines and 15 ATC tumor samples. As shown in Fig. 1A and B, an infrequent [1/16 (6.3%)] C>T transition was observed at nucleotide position 351, resulting in a codon change of AAC>ATG and amino acid N117N in exon 3. In exon 8, an infrequent [1/17 (5.8%)] C>T transition was observed at nucleotide position 1089, resulting in a codon change of TCC>TCT and amino acid S363S. In exon 9, a frequent [7/17 (41.2%)] G>A transition at nucleotide position 1227 was also observed, resulting in a codon change of GGG>GGA and amino acid G409G. The two N117N and S363S mutations were rare and novel silent mutations that have not been previously reported in the SNP database.

No FGDI mutations were identified in 12 thyroid cancer cell lines. However, as illustrated in Fig. 1C and D, an infrequent [1/16 (6.3%) ]T>C mutation was observed at nucleotide position 2091, resulting in a codon change of ACT>ACC and amino acid T697T. An infrequent [1/17 (6.3%)] A>G was also observed at nucleotide position 2136, resulting in a codon change of CCA>CGG and amino acid P712P. These silent T697T (rs12011120) and P712P (rs1126744) mutations were rare SNPs that have been reported in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Mutations were not identified in the TRRAP gene in 12 thyroid cancer cell lines and 16 PTC tumor samples. No GRM3 mutations were detected in 12 thyroid cancer cell lines. A G>A mutation was observed at nucleotide position 1323, resulting in a codon change of ACG>ACA and amino acid T441T in all 12 thyroid cancer cell lines and 16 PTC samples. An infrequent [1/16 (6.3%)] G>A mutation was also observed at nucleotide position 1424 resulting in a codon change of GGT>GAT and amino acid G475D (Fig. 1E). The T441T mutation was a novel synonymous SNP that has not previously been reported in the SNP database. G475D (rs17161026) was a non-synonymous SNP that has previously been reported in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Fig. 1 shows the mutations identified in the present study, and a summary of the results is presented in Table II.

Table II. Mutations of the MMP27, FGDI and GRM3 genes in thyroid cancers.

| S. No. | Gene | Exon | Nucleotide | Codon | Amino acid | Type of mutation |
|-------|------|------|------------|-------|------------|-----------------|
| ATC# 12 | MMP27 | 3 | C351T | AAC-AAT | N117N | Silent |
| ATC# 19 | MMP27 | 8 | C1089T | TCC-TCT | S363S | Silent |
| ATC# N3 | MMP27 | 9 | G1227A | GGG-GGA | G409G | Silent |
| PTC# 4 | FGDI | 14 | T2091C | ACT-ACC | T697T | Silent |
| PTC# 52 | FGDI | 14 | A2136G | CCA-CCG | P712P | Silent |
| PTC# 6 | GRM3 | 3 | G1323A | ACG-ACA | T441T | Silent |
| PTC# 46 | GRM3 | 3 | G1424A | GGT-GAT | G475D | Missense |

MMP, matrix metalloproteinase.
Discussion

In the present study, several genes were analyzed for the first time for possible mutations in thyroid cancer. GNA11 mutations were analyzed in all types of thyroid cancer (PTC, FTC and ATC) as they have been frequently identified in uveal melanoma and are known to activate the MAPK signaling pathway (5), which is one of the most deregulated signaling pathways in thyroid cancer (3). However, no mutations were detected in and around codons 209 and 183. These two hot spot codons were selectively analyzed as GNA11 mutations have consistently been identified only in these two residues (5). The MMPs are proteolytic enzymes that degrade the components of the extracellular matrix and basement membranes, which are associated with cancer metastasis (6,10-12). ATC is the most aggressive type of thyroid cancer that is often associated with deadly metastasis (17). Therefore ATC was particularly analyzed for the mutation of the MMP27 gene. Two MMPs have been reported to occasionally be mutated in melamnas. The MMP27 gene was analyzed for mutation in ATC in the present study as we had already previously analyzed the second gene, MMP8, in thyroid cancer (18). Three uncommon
mutations were identified; C351T resulting in N117N, C1089T resulting in S363S and G1227A resulting in G475D silent mutations. The silent mutations are unlikely to be involved in thyroid carcinogenesis as these mutations do not change the basic amino acids.

We previously revealed that FGD1 was normally maintained, hypomethylated and overexpressed by BRAF (V600E) in thyroid cancer cells and in turn observed that it was hypermethylated after ShRNA-mediated knockdown of BRAF (V600E) in thyroid cancer cell lines (9). Based on these findings and the high transforming and invasive potential of the FGD1 gene (4), we considered there to be a high possibility of identifying oncogenic mutations in FGD1. All 18 exons of the gene were sequenced to be analyzed for mutations, but only two silent mutations (T697T and P712P) were detected. These mutations are unlikely to have a significant role in PTC. No somatic missense mutations were identified in the FGD1 gene.

The TRRAP gene has been reported to be mutated in a particular codon, S722F (7). As GRM3 activates the MAPK signaling pathway (8), the present study investigated whether GRM3 is mutated in PTC samples, since the majority of PTCs harbor genetic deregulation in the MAPK signaling pathway. No mutations were detected in TRRAP, while two SNPs (T441T and G475D) were identified in the GRM3 gene. This suggests that TRRAP and GRM3 may not have important roles in the pathogenesis of this type of thyroid cancer.

In conclusion, the present findings suggested that genetic alterations in the GNA11, MMP27, FGD1, TRRAP and GRM3 genes may not be significant in the tumorigenesis of thyroid cancer. It is not surprising that mutations in these genes are not common in thyroid cancer since a number of the upstream effectors involved in cellular transformation, growth and metastasis, including EGFR, RET/PTC, ALK, RAS, BRAF, PTEN, PIK3CA, PIK3CB and PDK1, are commonly genetically altered via mutations or genetic amplifications that are able to independently activate the MAPK or PI3K/Akt pathways in thyroid cancer (3,4,16,18,19).

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