Molecular heterogeneity in the novel fusion gene
APIP-FGFR2: Diversity of genomic breakpoints in gastric cancer with high-level amplifications at 11p13 and 10q26

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Abstract. Several novel fusion transcripts were identified by next-generation sequencing in gastric cancer; however, the breakpoint junctions have yet to be characterized. The present study characterized a plethora of APIP-FGFR2 genomic breakpoints in the SNU-16 gastric cancer cell line, which harbored homogeneously staining regions (hsrs) and double minute chromosomes. Oligonucleotide microarrays revealed high-level amplifications at chromosomes 8q24.1 (0.8 Mb region), 10q26 (1.1 Mb) and 11p13 (1.1 Mb). These amplicons contained MYC and PVT1 at chromosome 8q24.1, BRWD2, FGFR2 and ATE1 at chromosome 10q26, and 24 genes, including APIP, CD44, RAG1 and RAG2, at chromosome 11p13. Based on these findings, reverse transcription-polymerase chain reaction (PCR) was performed using various candidate gene primers to detect possible fusion transcripts, and several products using primer sets for the APIP and FGFR2 genes were detected. Eventually, three in-frame and two out-of-frame fusion transcripts were detected. Notably, PCR analysis of the entire genomic DNA detected three distinct genomic junctions. The breakpoints were within intron 5 of APIP, which contained three distinct breakpoints, and introns 5, 7 and 9 of FGFR2. Fluorescence in situ hybridization showed several fusion signals within hsr using two short probes (~10-kb segments of a bacterial artificial chromosome clone) containing exons 2-5 of APIP or exons 11-13 of FGFR2. Although, for any given fusion, a multiplicity of transcripts is thought to be created by alternative splicing of one rearranged allele, the results of the present study suggested that genomic fusions of APIP and FGFR2 are generated in hsr with a diversity of breakpoints that are then faithfully transcribed.

Introduction

Gastric cancer is the second most common cause of cancer-associated mortality worldwide (1,2). Multiple genomic alterations accumulate during the development and progression of gastric cancer. Mutations of tumor suppressor genes and oncogenes, including APC (3), KRAS (4), TP53 (5), RUNX3 (6), E-cadherin (7) and β-catenin (8), have been reported, along with amplifications of MYC (9), FGFR2/KSAM (10), MET (11) and HER2/ERBB2 (12). In addition, previous studies have identified a large number of fusion transcripts, including AGTRAP-BRAF, FPPP1RB-STARD3, DUS4L-BCAP29 and PVT1 fusions with six different partners, using next-generation transcriptome sequencing (13-15).

Characterization of chromosomal translocations and inversions may help to identify genes implicated in the development of epithelial tumors and hematological malignancies. CD44- SLC1A2 was recently identified in a paracentric chromosomal inversion at chromosome 11p13-15 in gastric cancer (16); however, karyotypic analysis of gastric cancer, including spectral karyotyping (SKY), has been precluded by the complicated and cryptic nature of rearrangements (17). Conversely, homogeneously staining regions (hsr) and double minute chromosomes (dmin), which are cytogenetic manifestations of high-level DNA amplifications, are easily characterized using high-resolution oligonucleotide microarrays. Combined with next-generation transcriptome sequencing, oligonucleotide microarrays identified several fusion transcripts associated with genomic amplification in various solid tumors, including lung cancer and medulloblastoma, that harbored hsr and dmins (18-20). The identification

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of fusion transcripts may help researchers to develop novel therapeutic strategies and elucidate the molecular mechanisms underlying carcinogenesis. Furthermore, characterization of genomic fusions and breakpoint junctions may help to elucidate the mechanisms of fusions associated with DNA amplification.

The present study aimed to identify fusion genes associated with genomic amplification in gastric cancer. A comprehensive molecular analysis of high-level DNA amplifications in a gastric cancer cell line harboring hsrs and dmins, SNU-16, was performed. Several APIP-FGFR2 fusion transcripts were identified with diverse genomic breakpoints.

Materials and methods

Gastric cancer cell lines. Nine gastric cancer cell lines, including SNU-16, MKN-1, MKN-45, SNU-5, KATO-III, HGC-27, NUGC-4, SH-10 and H-111, were analyzed. SNU-16 and SNU-5 cell lines were obtained from the Korean Research Institute of Bioscience and Biotechnology (Taejon, South Korea). MKN-1, MKN-45, KATO-III, HGC-27, NUGC-4, SH-10 and H-111 cell lines were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The culture conditions were described previously (17).

Chromosome preparation and DNA/RNA extraction. Metaphase spreads of tumor cells were prepared from a short-term culture of SNU-16 cells, which were derived from a poorly-differentiated adenocarcinoma. Genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). Total RNA was extracted using the Isogen-LS kit (Nippon Gene, Co., Ltd., Tokyo, Japan). Total RNA (4 µg) was reverse transcribed into cDNA in a total volume of 33 µl with random hexamers using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, Chalfont, UK).

Genome copy number analysis. Genome copy number analysis was performed using the Genome-Wide Human single nucleotide polymorphism (SNP) Array 6.0 (Affymetrix Inc., Santa Clara, CA), according to the manufacturer’s protocol. The copy numbers and chromosomal regions with gains or losses were individually evaluated using the Copy Number Analyzer for Affymetrix GeneChip (CNAG) 3.3.0.0 program (21). The genomic breakpoint was defined as lying within the boundaries marked by copy number changes. This region was then mapped on the National Center for Biotechnology Information MapViewer platform (http://www.ncbi.nlm.nih.gov/mapview/) and the precise breakpoint region was determined on the physical map.

Reverse transcription (RT)-polymerase chain reaction (PCR), genomic PCR and sequencing analyses. RT-PCR and genomic PCR analyses were performed using the AmpliTaq Gold 360 Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), as described previously (22). Briefly, after 35 rounds of PCR (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C), 5 µl of PCR product was separated by 3% agarose gel electrophoresis. The PCR primers used for detecting the APIP-FGFR2 fusions are shown in Table I.

| Primer | Sequence |
|--------|----------|
| Forward APIP-1-2S (exon 1) | TCGGGAGGGAGACTGTTGTT |
| Reverse FGFR2-11A (exon 11) | TGCTTCCGCCATGACCACCTT |

B. Genomic PCR

| Primer | Sequence |
|--------|----------|
| Forward MMRP19-4S-2 (APIP exon 5) | CTCTTTCAGGACGGAGGT |
| Reverse FGFR2-1123P6A (exon 6) | ACGGTGTATTGATGGACCCG |
| FGFR2-8bA (exon 8) | CACGTATATTTCCAGCGTC |
| FGFR2-1123P10A (exon 10) | GTTGAAGAGAGCGGTGTTG |

The nucleotide sequences of the PCR products and, if necessary, those of subcloned PCR products were analyzed as described previously (22). RT-PCR for detecting PVT1-PDHX, PVT1-ATE1, CLN6-CALML4, APIP-PVT1, CD44-FGFR2, PVT1-PPAPDC1A and CD44-SLC1A was performed as described previously (14,16).

Fluorescence in situ hybridization (FISH) analysis. Double-color FISH (DC-FISH) analysis was performed as described previously (17,23). Briefly, the bacterial artificial chromosome (BAC) clones RP11-412L22, RP11-62L18 and CTD-3056O22 (Advanced GenoTechs Co., Tsukuba, Japan) were used as probes to assess APIP, FGFR2 and MYC rearrangements in tumor cells, respectively. To detect the chromosomal fusion of APIP and FGFR2, DC-FISH analysis using two different probes, L4 and L1, which were prepared from ~10-kb long-distance PCR products obtained from RP11-412L22 and RP11-62L18 templates, respectively, was performed. For the long-distance PCR, each reaction mixture (50 µl) contained 1 ng BAC DNA, 10 pmol of each primer, 8 µl of dNTP mixture (2.5 mM each), 5 µl LA PCR Buffer II and 2.5 U of Takara LA Taq HS (Takara Bio, Inc., Otsu, Japan). Reaction conditions were as follows: denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 98°C for 20 sec, annealing and extension at 68°C for 10 min with 15-sec increments per cycle, and a final extension at 72°C for 10 min. L4 contained exons 2-5 of APIP (nucleotides 118, 330-128, 595 in RP11-412L22), and L1 contained exons 11-13 of FGFR2 (nucleotides 27, 769-38, 427 in RP11-62L18).
RT-quantitative (q)PCR. The mRNA expression levels of FGFR2 in the gastric cancer cell lines were determined using the TaqMan Universal Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with specific primer probe sets from the ABI Assays-on-Demand™ (Applied Biosystems; Thermo Fisher Scientific, Inc.) and on the ABI Prism 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. After an incubation at 95°C for 10 min, the cDNA was amplified for 40 cycles of denaturation at 95°C for 15 sec and combined annealing/extension at 60°C for 1 min. Each sample was analyzed in duplicate. The primer set used was the Assays-on-Demand™ FGFR2 15-16 (Hs01552919_m1). Data were calculated using the relative standard curve method, and the mRNA levels were normalized to that of β-actin.

Results

Identification of APIP-FGFR2 fusion transcripts. In our previous study, SKY and FISH identified hsr and dmin in the SNU-16 cell line (17). Therefore, in the present study, genome copy number analysis using SNP arrays was performed to identify precise genomic changes in the amplicons. High-level amplifications at chromosomes 8q24.1 (0.8 Mb region), 10q26 (1.1 Mb) and 11p13 (1.1 Mb) were observed (Fig. 1). These amplified regions contained MYC and PVT1 at 8q24.1, BRWD2, FGFR2 and ATE1 at 10q26, and 24 genes, including APIP, CD44, RAG1 and RAG2, at 11p13. Based on these findings, RT-PCR using various candidate gene primer sets was performed to detect fusion transcripts. Using combination sets of primers for APIP and FGFR2, several PCR products, markedly different in size, were successfully amplified (Fig. 2A). Sequence analysis of these PCR products detected three in-frame and two out-of-frame fusion transcripts (Fig. 2B). For one of the in-frame fusion transcripts, the fusion point was within exon 6 of APIP and exon 10 of FGFR2; however, the corresponding band to this transcript was not found on the gel of separated RT-PCR products (Fig. 2A). Putative predominant APIP-FGFR2 fusion proteins commonly retained the N-terminal 18 amino acids of APIP and the catalytic domain of FGFR2 (Fig. 2C).
Figure 2. Cloning of the APIP-FGFR2 fusion gene. (A) Detection of APIP-FGFR2 chimeric transcripts using reverse transcription-polymerase chain reaction. Primers were APIP-1-2S and FGFR2-2A. (B) Sequencing of three in-frame (a, b and c) and two out-of-frame (c and d) APIP-FGFR2 fusion transcripts. Vertical arrows indicate the fusion points. Transcripts a, b, c and d correspond to those in (A), while transcript e was not found. (C) Schematic representation of putative APIP, FGFR2 and APIP-FGFR2 proteins. Vertical arrows indicate breakpoints or fusion points. M, size marker; S, SNU-16 cells; W, water; mtnB, methylthioribulose-1-phosphate dehydratase domain; Ig1-3, immunoglobulin-like domain 1-3.

Figure 3. Dual color-fluorescence in situ hybridization analysis using BAC clones targeting APIP, FGFR2 and MYC. Tandemly repeated amplifications of (A) APIP (green) and FGFR2 (red) and (B) MYC (green) and FGFR2 (red) were observed in homogeneously staining regions. BAC clones targeting APIP (RP11-412L22), FGFR2 (RP11-62L18) and MYC (CTD-3056022) were used as probes. BAC, bacterial artificial chromosome.

Figure 4. (A) Schematic illustration of genomic breakpoint regions in APIP and FGFR2 in the SNU-16 cell line. Vertical arrows indicate the fusion points of three clones (I, II and III) detected in the same SNU-16 cell line. L1 (green) and L4 (red) are probes used for the fluorescence in situ hybridization analysis. (B) Sequencing of three APIP-FGFR2 genomic junctions. Vertical arrows indicate the fusion points.
Detection of tandemly repeated amplification of APIP, FGFR2 and MYC in hsrs.

To confirm the fusion of APIP and FGFR2, DC-FISH analysis was performed using two BAC clones, RP11-412L22 (161 kb), encompassing the entire APIP gene, and RP11-62L18 (174 kb), encompassing the entire FGFR2 gene. No fusion was detected, but alternative and intense signals of APIP and FGFR2 were detected in three hsrs in SNU-16 cells, indicating that both genes were amplified and tandemly repeated in hsrs (Fig. 3A). Subsequently, DC-FISH was repeated using probes for FGFR2 and MYC, which demonstrated an identical alternative pattern of amplified signals in three hsrs (Fig. 3B). These results suggested that the APIP, FGFR2 and MYC loci were amplified and tandemly repeated in the same hsrs.

Identification of genomic fusions between APIP and FGFR2.

As no fusion of APIP and FGFR2 was detected by FISH using BAC clones, the genomic fusion points of APIP and FGFR2 were cloned. Long-distance PCR detected three genomic junctions. All three breakpoints detected in APIP were within intron 5; however, those in FGFR2 were within three different introns, including introns 5, 7 and 9 (Fig. 4A and B). Further DC-FISH analysis was performed using a probe set consisting of two long-distance polymerase chain reaction products (~10 kb in size) (Fig. 4A). These smaller probes detected not only tandemly repeated amplifications of APIP and FGFR2, but also several fusion signals (yellow signals) in hsrs, although these fusion signals could not be fixed definitely because of yellow signals among the too many number of APIP and FGFR2 signals in the hsrs (Fig. 5A-E). These results suggest that several APIP-FGFR2 fusion genes may be generated with different fusion breakpoints within the same hsrs.

Expression of FGFR2.

The expression level of FGFR2 in SNU-16 cells was analyzed and compared with that of several other gastric cancer cell lines using RT-qPCR (Fig. 6). FGFR2 was highly expressed in SNU-16, KATO-III and HGC-27 cells. Furthermore, the FGFR2 region was highly amplified in KATO-III cells, but not in HGC-27 cells (data not shown).

Discussion

The present study identified the APIP-FGFR2 fusion gene in the SNU-16 cell line, which shows a high level of genomic amplification at chromosomes 8q24.1, 10q26 and 11p13. The APIP and
FGFR2 genes were in amplicons from chromosomes 11p13 and 10q26, respectively. The SNU-16 cell line has also been reported to have many fusions, including CD44-SLC1A2 (16), PVT1-PDHX, CLN6-CALML4, APIP-PVT1, PVT1-ATE1, PVT1-PPAPDC1A and CD44-FGFR2 (14). Of these, PPAPDC1A, FGFR2 and ATE1 are within ~1.5 Mb at 10q26, and the CD44, SLC1A2, PDHX and APIP genes are within ~500 kb at 11p13. Notably, in the present study, all these genes, with the exception of PPAPDC1A, were within the same amplicons in SNU-16 cells. Kim et al (14) performed next-generation transcriptome sequencing analysis and detected six types of fusion transcripts in the SNU-16 cell line; however, APIP-FGFR2 and CD44-SLC1A2 were not observed. Of these fusion transcripts, the present study confirmed the presence of PVT1-PDHX, PVT1-ATE1, CLN6-CALML4, APIP-PVT1 and CD44-FGFR2, but not PVT1-PPAPDC1A or CD44-SLC1A, in the SNU-16 cell line using RT-PCR. One possible explanation for this discrepancy is that the expression levels of APIP-FGFR2 and CD44-SLC1A2 were too low to be detected by next-generation transcriptome sequencing. Of the six fusion genes identified by next-generation transcriptome sequencing, only 2-10 junction reads of a total of 72,641,230 reads were sequenced for APIP-PVT1, PVT1-ATE1, PVT1-PPAPDC1A and CD44-FGFR2 (14). Therefore, abundance may be a limiting factor in the detection of fusion transcripts using next-generation transcriptome sequencing.

It is uncertain whether the fusion transcripts detected in gastric cancer cell lines are recurrent or not in clinical samples. In a previous study, CD44-SLC1A2 was detected in only 3 of 149 clinical samples (16), while other fusion transcripts have never been analyzed in clinical samples (14). Unfortunately, the present study did not have enough clinical samples to analyze these fusion transcripts and, thus, SNP array analysis was performed using array data deposited in the Gene Expression Omnibus (GEO) database. CEL files of 243 clinical samples, including 193 from Singapore (GEO accession: GSE31168) (4) and 50 from Russia or Vietnam (GEO accession: GSE29996) (24), were analyzed using Genotyping Console (Affymetrix, Inc.) and CNAG3.3.0.0 or CNAG3.5.1. A total of 15 cases (6%) were found to have copy number gains at the 10q26 region involving FGFR2, including 12 cases from Singapore (4) and 3 cases from Russia/Vietnam (24). Of these, 4 cases (011LGE, 75554796T, 980417T and 990172T) had gains of the 11p13 region involving APIP. These results suggested that FGFR2 rearrangement is a recurrent abnormality in gastric cancer, and that APIP rearrangement is an abnormality related to FGFR2 rearrangement.

Five APIP-FGFR2 fusion transcripts were identified in the present study. These transcripts appeared to originate from variants of APIP and FGFR2 genomic fusions with distinct breakpoint junctions, although some splicing variants may have been included. High-resolution FISH mapping using short probes (~10-kb long-distance PCR products, L1 and L4) supported this notion by demonstrating possible fusion between APIP and FGFR2 in hrs, while FISH using BAC probes (161 and 174 kb) showed only an alternative pattern of amplified signals. A diversity of genomic breakpoints in fusion genes among cases is commonly observed for various fusions caused by chromosome abnormalities; however, the diversity of genomic breakpoints in a fusion gene identified in a single case showing high-level amplifications has not previously been well analyzed. Functional analysis of the molecular diversity exhibited by these fusion transcripts is required to reveal their biological significance and tumorigenic potential in gastric cancer.

In a previous study, next-generation transcriptome sequencing revealed that APIP and FGFR2 were fused to PVT1 and CD44 in SNU-16 cells, respectively (14). PVT1 has also been shown to be involved in three other fusions with PDHX, ATE1 and PPAPDC1A (14). In addition, SLC1A2, another fusion partner of CD44, maps to chromosome 11 at p13-p12 (16). In the present study, the breakpoint of APIP at the fusion junction was in exon 5 of APIP-FGFR2, while exon 6 in PVT1-APIP was reported in a previous transcriptome study (14). These findings, together with our FISH data, suggested that APIP and FGFR2 fuse with each other during the formation of PVT1-APIP and FGFR2-CD44 fusions, a process that is followed by their amplification. Chromothripsis, which is defined as a single catastrophic genetic event (25-27), is the most likely mechanism underlying the formation of these fusion genes with high-level genomic amplification, as suggested in similar reports detecting PVT1 fusion genes in other tumors harboring dmins/hrs (18-20). Further studies are required to elucidate the exact relationship between the gene fusion, hrs and chromothripsis.

APIP encodes the APAF1-interacting protein, which has methylthioribulose 1-phosphate dehydratase activity and is involved in the methionine salvage pathway (28). APIP deficiency is associated with cell death and cancer (29,30). By contrast, FGFR2, which is a member of the fibroblast growth factor receptor family, was detected in ~16% of diffuse gastric cancers (10,31). SNU-16 cells have two FGFR2 fusion genes, APIP-FGFR2 and CD44-FGFR2 (14), and exhibit overexpression of FGFR2. Although it remains unclear how APIP-FGFR2 and CD44-FGFR2 are implicated in the tumorigenesis of gastric cancer, SNU-16 cells have been shown to be sensitive to FGFR inhibition; thus FGFR2 may be an important therapeutic target in gastric cancer (32).
In conclusion, the present study described the APIP-FGFR2 fusion gene in gastric cancer with high-level genomic amplification, and demonstrated fusion signals in hrs by FISH using probes for 10-kb long-distance PCR products. The results of the present study indicated that genomic fusions of APIP and FGFR2 with a diversity of breakpoints are generated in hrs resulting in several transcripts from rearranged alleles with either normal or alternative splicing.

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