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 hsrs 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 hsrs 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 (hsrs) and double minute chromosomes (dmins), 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 hsrs 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 (Afffymetrix 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℃, 30 sec at 55℃ and 1 min at 72℃), 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.

Table I. Primers used for PCR.

A, RT-PCR

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

B, Genomic PCR

Primer	Sequence
Forward	
MMRP19-4S-2 (APIP exon 5)	CTCTTTCCAGGACGGGAGTT
Reverse	
FGFR2-1123P6A (exon 6)	ACGTGTGATTGATGGACCCG
FGFR2-8bA (exon 8)	CACGTATATTCCCCAGCGTC
FGFR2-1123P10A (exon 10)	GTTGAAGAGAGGCGTGTTGT

RT, reverse transcription; PCR, polymerase chain reaction.

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℃ for 1 min, followed by 30 cycles of denaturation at 98℃ for 20 sec, annealing and extension at 68℃ for 10 min with 15-sec increments per cycle, and a final extension at 72℃ 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).
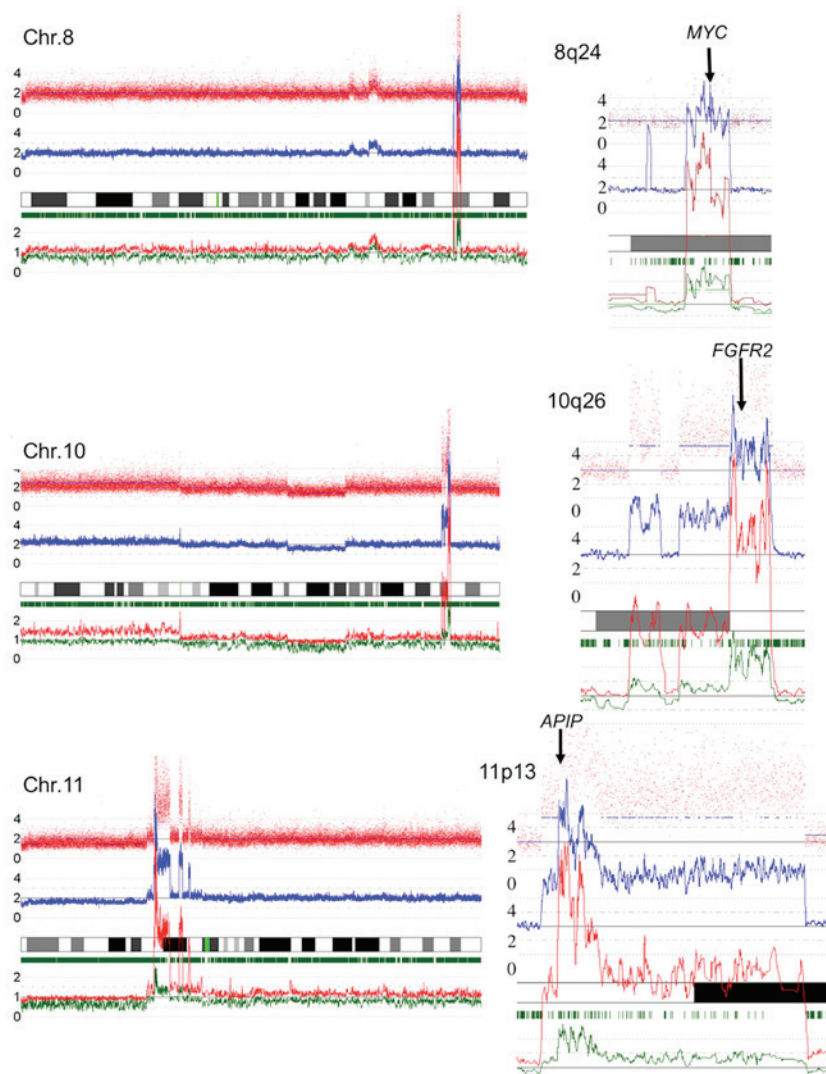
Figure 1. Amplification of 8q24.1, 10q26 and 11p13 in SNU-16 cells, as demonstrated using genome copy number analysis. Genome copy number analysis of the SNU-16 cell line revealed high-level amplifications at Chr. 8q24.1 (0.8 Mb region), 10q26 (1.1 Mb) and 11p13 (1.1 Mb). These amplified regions contained *MYC* and *PVT1* at 8q24.1, *BRWD2*, *FGFR2* and *ATE1* at 10q26, and 24 genes, including *APIP*, *CD44*, *RAG1* and *RAG2*, at 11p13. Chr., chromosome.

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 hsrs and dmins 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 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, 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).
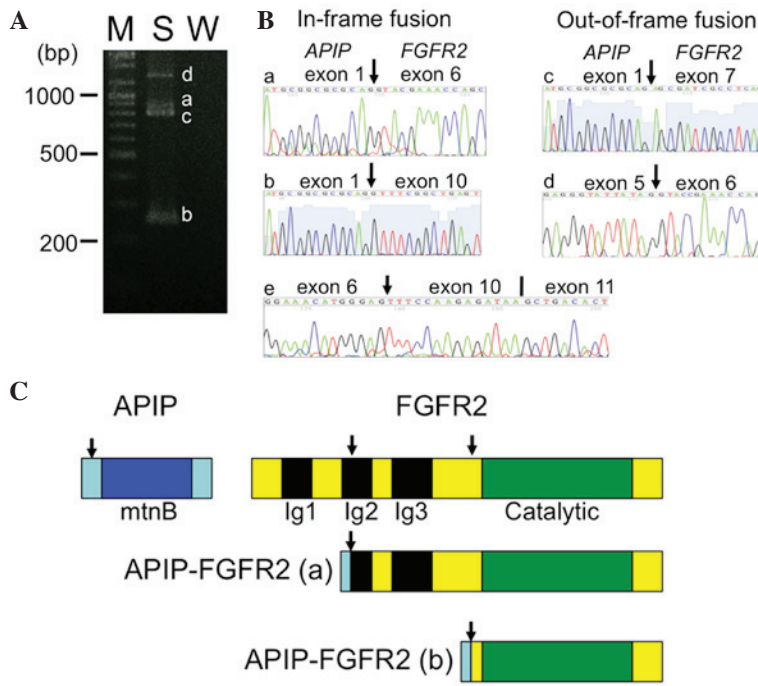
Figure 2. Cloning of the *APIP-FGFR2* fusion gene. (A) Detection of *APIP-FGFR2* chimeric transcripts using reverse transcription-polymerase chain reaction. Primers were APIP-1-2S and FGFR2-2A. (B) Sequencing of three in-frame (a, b and e) and two out-of-frame (c and d) *APIP-FGFR2* fusion transcripts. 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.
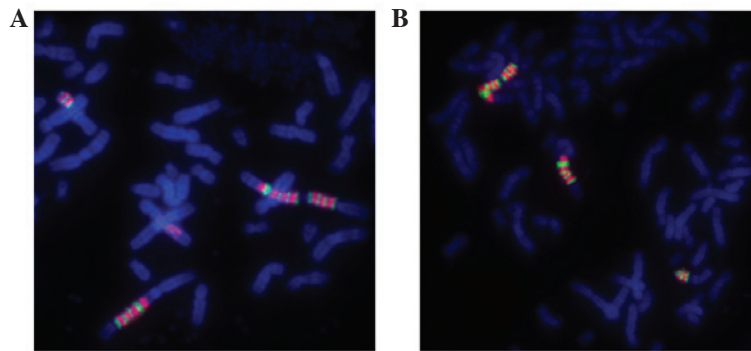

Figure 3. Dual color-fluorescence *in situ* hybridization analysis using BAC clones targeting *APIP, FGFR2* and *MYC*. Tandemly repeated amplifications of (A) *APIP* (green) and *FGFR2* (red) and (B) *MYC* (green) and *FGFR2* (red) were observed in homogeneously staining regions. BAC clones targeting *APIP* (RP11-412L22), *FGFR2* (RP11-62L18) and *MYC* (CTD-3056O22) were used as probes. BAC, bacterial artificial chromosome.
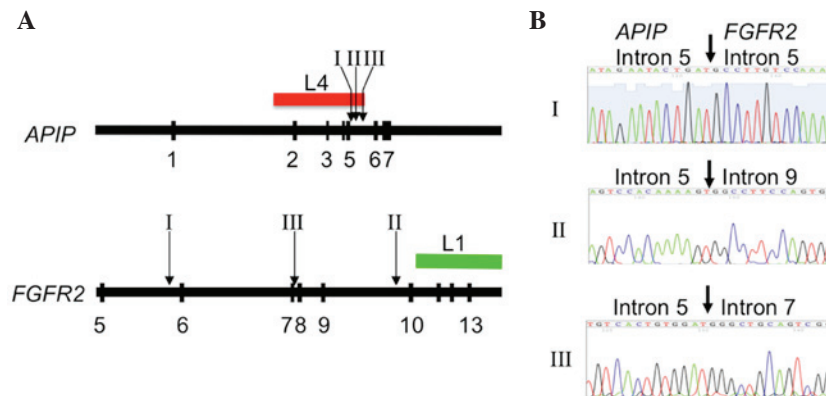

Figure 4. (A) Schematic illustration of genomic breakpoint regions in *APIP* and *FGFR2* in the SNU-16 cell line. Vertical arrows indicate the fusion points of three clones (I, II and III) detected in the same SNU-16 cell line. L1 (green) and L4 (red) are probes used for the fluorescence *in situ* hybridization analysis. (B) Sequencing of three *APIP-FGFR2* genomic junctions. Vertical arrows indicate the fusion points.
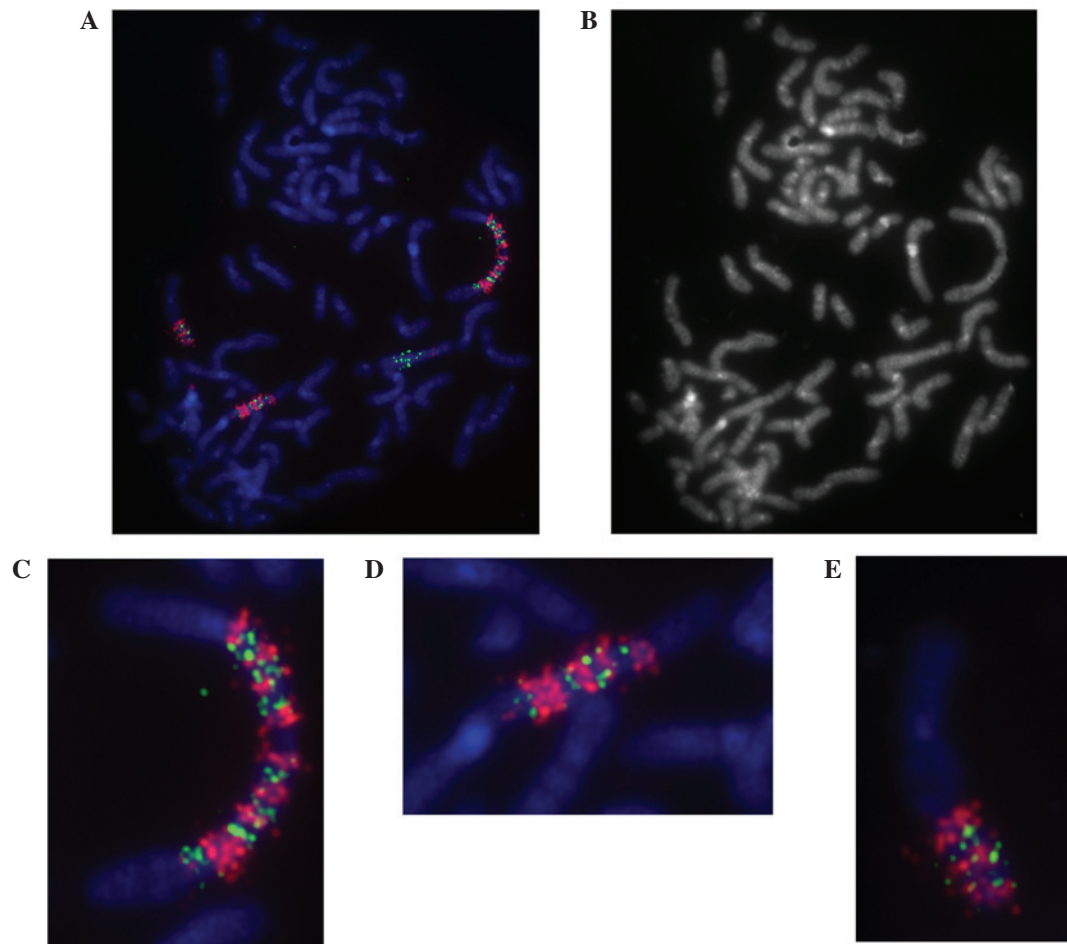
Figure 5. DC-FISH analysis using a probe set consisting of two long-distance polymerase chain reaction products (~10 kb in size). (A) DC-FISH analysis was performed using L4 (red), containing exons 2-5 of *APIP*, and L1 (green), containing exon 10 of *FGFR2* (magnification, x1,000). (B) DAPI image corresponding to the metaphase of (A), which was captured in conjunction with spectral classifications as inverted Q-bands by fluorescence using Hoechst 33258 for the identification of chromosomal breakpoints. (C-E) Enlarged views of tandemly repeated amplifications of *APIP* and *FGFR2* in three homogeneously staining regions within (C). DC-FISH, dual color-fluorescence *in situ* hybridization.

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). The *MYC* FISH signals were observed in regions assigned to *APIP*. 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 PCR 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
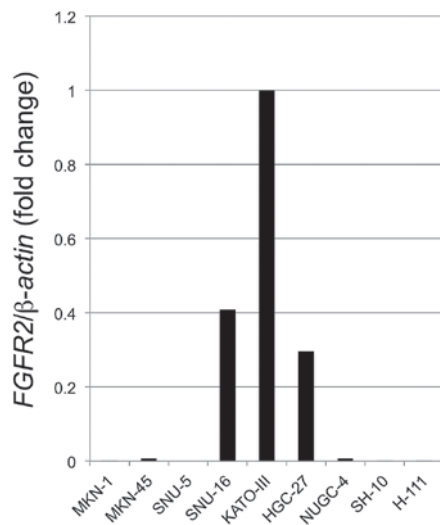
Figure 6. Relative mRNA expression levels of *FGFR2* in gastric cancer cell lines, as compared with that of the KATO-III cell line, which was set to an expression level of 1.

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 hsrs, 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/hsrs (18-20). Further studies are required to elucidate the exact relationship between the gene fusion, hsrs 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 hsrs 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 hsrs resulting in several transcripts from rearranged alleles with either normal or alternative splicing.

Acknowledgements

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