Alu-*Alu* Fusion Sequences Identified at Junction Sites of Copy Number Amplified Regions in Cancer Cell Lines

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Key Words

Alu elements • BFB cycle model • Cancer cell lines • Gene amplification • Inverted repeats

ments in the process of BFB cycle-mediated amplification, sequence homology of *Alu* elements is frequently used to repair the broken ends.

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Abstract

Alu elements are short, ~300-bp stretches of DNA and are the most abundant repetitive elements in the human genome. A large number of chromosomal rearrangements mediated by *Alu*-*Alu* recombination have been reported in germline cells, but only a few in somatic cells. Cancer development is frequently accompanied by various chromosomal rearrangements including gene amplification. To explore an involvement of *Alu*-*Alu* fusion in gene amplification events, we determined 20 junction site sequences of 5 highly amplified regions in 4 cancer cell lines. The amplified regions exhibited a common copy number profile: a stair-like increase with multiple segments, which is implicated in the breakage-fusion-bridge (BFB) cycle-mediated amplification. All of the sequences determined were characterized as head-to-head or tail-to-tail fusion of sequences separated by 1–5 kb in the genome sequence. Of these, 4 junction site sequences were identified as *Alu*-*Alu* fusions between inverted, paired *Alu* elements with relatively long overlapping sequences of 17, 21, 22, and 24 bp. Together with genome mapping data of *Alu* elements, these findings suggest that when breakages occur at or near inverted, paired *Alu* ele-

Alu elements are short, ~300-bp stretches of DNA and are the most abundant repetitive elements in the human genome, with over 1,000,000 copies [Stenger et al., 2001]. It is well known that homologous recombination between *Alu* elements generates chromosomal rearrangements including duplications, deletions, and translocations, which contribute to evolutionally expanding genetic diversity in the human genome [Cordaux and Batzer, 2009]. A large number of *Alu*-*Alu* recombination-mediated genomic rearrangements in the germline have been identified by comparative genomics [Van de Lagemat et al., 2005; Sen et al., 2006] and reported as inherited disorders including familial cancers [Batzer and Deininger, 2002; Kolomietz et al., 2002; Franke et al., 2009; Belancio et al., 2010; Konkel and Batzer, 2010; Mitsui et al., 2010; Resta et al., 2010; Boone et al., 2011; Cozar et al., 2011; Kuiper et al., 2011; Borck et al., 2012]. On the other hand, only a few rearrangements mediated by *Alu*-*Alu* recombination in somatic cells have been identified. These include deletions in the *CAD* gene in hepatoma [Hsieh et al., 2005], partial tandem duplications in the *MLL* gene in acute myeloid

leukemia [Strout et al., 1998], and tandem duplications in the *MYB* gene in acute lymphoblastic leukemia [O'Neil et al., 2007]. These are created by recombination between 2 direct *Alu* repeats and until now no rearrangements created by recombination between 2 inverted *Alu* repeats have been reported, although such inverted repeats are known to be highly unstable [Gebow et al., 2000].

Gene amplification, defined as an increase in the copy number of specific regions, is commonly observed in human cancer cells and contributes to cancer development by overexpressing oncogenes [Albertson, 2006]. Some amplifications are explained by the breakage-fusion-bridge (BFB) cycle-mediated amplification model [McClintock, 1941; Debatisse and Malfoy, 2005]. In each round of the BFB cycle a broken end of a chromosome is repaired by end-to-end fusion and an inverted duplicated chromosome bearing 2 centromeres is formed. Its junction site sequence exhibits a characteristic structure: a head-to-head or tail-to-tail fusion with a 1–5-kb intervening sequence, as shown in previous studies [Hyrien et al., 1988; Legouy et al., 1989; Okuno et al., 2004; Kitada and Yamasaki, 2007; La Farina et al., 2008]. Such a junction structure is thought to be generated by the non-homologous end joining repair mechanism, which requires no sequence homology. However, it is possible that the structure can also be generated by recombination between inverted, paired *Alu* elements using their long homology. Indeed, the occurrence of such recombination events has been demonstrated in model constructs [Lobachev et al., 2002; Tanaka et al., 2002; Weinstock et al., 2006; VanHulle, 2007]. In this study, we determined 20 junction site sequences of 5 highly amplified genome regions in 4 cancer cell lines. All the junction sites possessed head-to-head or tail-to-tail fusion sequences with 1–5 kb intervening sequences. Of these, 4 junction sites were identified as *Alu-Alu* fusion sequences between inverted, paired *Alu* elements.

Materials and Methods

Cell Lines

Two breast cancer cell lines, COLO824 and BT474, a pancreas cancer cell line, AsPC1, and a skin cancer cell line, A431, were used. COLO824 was purchased from DSMZ and the other 3 cell lines were purchased from ATCC.

Array-CGH

Genomic DNA was extracted from culture cells with a GenomicTip 100 (Qiagen, Valencia, Calif., USA). The procedure for labeling DNA with Cy5-dUTP or Cy3-dUTP and hybridization to microarrays was described in the manufacturer's instructions (Agilent Technologies, Santa Clara, Calif., USA). Human genome

244K standard microarrays and high density custom microarrays with a mean resolution of 125 bp covering the amplified genome regions were used. Fold copy number changes of sample DNA relative to the normal genomic DNA (Promega, Madison, Wisc., USA) were visualized by plotting them in log₂ scale against the human genome coordinate using SignalMap software (Nimble-Gen Systems, Madison, Wisc., USA).

Quantitative PCR

The quantification of DNA copy numbers was performed using the SYBR green PCR kit in an ABI 7900 system (Applied Biosystems, Foster City, Calif., USA). Primer pairs were designed using Primer3Plus software [Untergasser et al., 2007]. Primer sequences are listed in online suppl. table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000342885). Real-time qPCR was done twice in 30- μ l reaction mixtures containing 3 different amounts of genomic DNA (25, 50, and 100 ng). Calculated copy numbers are represented as fold change values relative to the normal genomic DNA.

FISH Analysis

FISH analysis was performed as previously described [Kitada and Yamasaki, 2008]. BAC clones (Invitrogen, Carlsbad, Calif., USA), corresponding to the chromosomal locations of the most amplified segments (between a and b in fig. 1), were labeled with biotin-16-dUTP and used for hybridization. Hybridization signals were detected by avidin-fluorescein (Roche Applied Science, Mannheim, Germany). The BAC clones used were: RP11-795C4 and RP11-458P19 for 6p22.3 of COLO824; RP11-62N3 and CTD-3244J20 for 7q22.1 of AsPC1; RP11-116H11 and CTD-2257H21 for 7p11.2 of A431; RP11-395N21 and RP11-201P13 for 9p13.3 of BT474; RP11-1146L22 and RP11-361G24 for 11q13.3 of A431.

Sequence Analysis of Junction Sites

Based on high density array-CGH data, PCR primer pairs were designed using Primer3Plus software: one at the end of inverted duplicated regions and another in intervening sequence regions. When array-CGH data did not give information on where intervening regions in the genome sequence were located, primers were designed every 1 kb (or 0.5 kb) to cover the whole sequence region between 2 neighboring segments. Primer3Plus software was used for primer design and LA-PCR (TaKaRa Bio, Otsu, Japan) was used for PCR reactions. The resulting PCR products were directly sequenced with a BigDye Terminator Cycle Sequencing method (Applied Biosystems).

Genome Sequence

The human genome sequence of NCBI Build 36.1 (<http://genome.UCSC.edu/>) was used. *Alu* elements with sequence sizes >150 bp were used for the genome mapping.

Results

Characteristics of Copy Number Amplified Regions in Cancer Cell Lines

We examined 5 amplified regions in 4 cancer cell lines: 6p22.3 in COLO824, 7q22.1 in AsPC1, 7p11.2 in A431,

9p13.3 in BT474, and 11q13.3 in A431. All amplified regions show a characteristic pattern in the copy number profile: a stair-like increase with multiple segments on one side and a single sharp drop on the other side (fig. 1). Copy number increases with this pattern can be explained by the BFB cycle-mediated gene amplification (online suppl. fig. 1). Junction a, located where the copy number drops sharply in figure 1, would have been generated by repair of the double-strand breakage which initiates the BFB cycle. The amplified regions consisted of 3–5 segments and the most amplified segment (between a and b in fig. 1) has a size of 1,245, 634, 264, 397, and 638 kb for 6p22.3 (COLO824), 7q22.1 (AsPC1), 7p11.2 (A431), 9p13.3 (BT474), and 11q13.3 (A431), respectively. Fold copy number change of this segment measured by quantitative PCR was 3.7, 14.1, 12.9, 6.4 and 10.7, respectively. Some of these regions have been previously reported as amplified regions [Lin et al., 1984; Merlino et al., 1984; Ullrich et al., 1984; Yoshida et al., 1988; Heidenblad et al., 2004; Shadeo and Lam, 2006].

Using FISH probes prepared from the most amplified segments, localization of amplified regions in metaphase chromosomes was examined. One strong fluorescent signal was observed in COLO824 cells, while 2 strong signals were observed in the other cell lines (fig. 2). In most of the cells of AsPC1, BT474, and A431 (11q13.2), 2 signals with similar intensities were located at the ends of 2 chromosomes, which appeared identical in shape. These 2 chromosomes are presumed to have been generated by duplication of 1 chromosome carrying the regional amplification. Near-tetraploidy of BT474 and near-triploidy of A431 would be related to the duplication. In A431 (7p11.2), 2 signals with different intensities appeared in different-shaped chromosomes, suggesting that these 2 regional amplifications occurred independently. Further study, especially individual analysis of individually isolated chromosomes, as described before [Kitada et al., 2011], is needed for detailed characterization.

Determination of Junction Site Sequences

To determine junction site sequences, DNA fragments containing junction sites were obtained by PCR-based amplification and sequenced. In total, 20 junction site sequences were determined and the results are summarized in table 1. Junction sites a, b, c, d, and e correspond to junctions a, b, c, d, and e in figure 1.

The sequence structure of junction site A431-7-b is complicated. An inverted duplication was formed by head-to-head fusion at sequence positions 55,204,390 and 55,208,110 and 3 short sequences were sandwiched:

Fig. 1. Copy number amplifications in cancer cell lines. Five amplified genomic regions in 4 cancer cell lines were selected: **A** 6p22.3 in COLO824; **B** 7q22.1 in AsPC1; **C** 7p11.2 in A431; **D** 9p13.3 in BT474; **E** 11q13.3 in A431. Copy number profiles analyzed by array-CGH. Fold change values of genomic DNA of cell lines relative to the normal genomic DNA are visualized by plotting them in log₂ scale (vertical axis) against the sequence coordinate in Mb scale (horizontal axis). Each amplified segment is marked by a red bar with junction IDs (a, b, c, d, and e).

Fig. 2. Chromosomal locations of amplified regions. FISH probes, which were generated by fluorescently labeling BAC clone DNA derived from genomic regions of the most amplified segments (between a and b in fig. 1), were hybridized to spreads of metaphase chromosomes. Hybridized signals were visualized as green dots in the DAPI-stained chromosomes. **A** 6p22.3 in COLO824; **B** 7q22.1 in AsPC1; **C** 7p11.2 in A431; **D** 9p13.3 in BT474; **E** 11q13.3 in A431.

775 bp from chromosome 4 and 79 and 345 bp from chromosome 7 (online suppl. fig. 2A). Except for A431-7-b, all 19 junction sites showed a common structure: head-to-head or tail-to-tail fusions with intervening sequences with a mean size of 2.4 kb, ranging from 0.2 to 8.8 kb. Short overlapping sequences of 1–4 bp were found in 14 junction site sequences and an insertion of 10 bp was found in 1 junction site, BT474-9-c. For the remaining 4 junction sites, relatively longer overlapping sequences were identified: 24, 22, 17, and 21 bp, for AsPC1-7-c, AsPC1-7-e, BT474-9-d, and A431-11-b, respectively. Sequence alignment revealed that these overlapping sequences came from identical sequences between paired, inverted *Alu* elements (fig. 3). Various *Alu* subfamilies were combined: *AluSc-AluY* (separated by 2.9 kb) for AsPC1-7-c; *AluSg-AluSx* (2.5 kb) for AsPC1-7-e; *AluJo-AluSx* (1.6 kb) for BT474-9-d; *AluSx-AluSx* (3.4 kb) for A431-11-b.

Alu elements around breakpoints of junction sites were mapped on the genome sequence (online suppl.

fig. 3). The numbers of *Alu* elements which resided between 2 breakpoints joined at each junction site are summarized in table 1. Out of all 19 junction sites (after removal of junction site A431-7-b due to the complicated structure), 9 contained no *Alu* elements and 4 contained 1 or 2 *Alu* elements with the same orientation. The remaining 6 junction sites contained more than 1 pair of inverted *Alu* elements. Of these, 4 junction sites were found as fusions of inverted, paired *Alu* elements (fig. 3).

Discussion

We have determined 20 junction site sequences of 5 amplified regions in 4 cancer cell lines. We believe these amplified regions are likely to have been amplified by the repetition of BFB cycles from the following findings. First, in metaphase FISH analysis, amplified regions appeared as homogeneously staining regions. Second, in array-CGH analysis, the amplified regions displayed a

Table 1. Junction site structures

Cell line	Region	Junction site	Junction structure ^a	Intervening sequence (bp)	No. of <i>Alu</i> ^b (+, -)	<i>Alu-Alu</i> fusion
COLO824	(6p22.3)	a	← 18703449(TCT)18704971 →	1,522	0	
		b	→ 19948891(TCTT)19951075 ←	2,184	0	
		c	→ 23167971(TGA)23171431 ←	3,460	0	
AsPC1	(7q22.1)	a	→ 98834927(CTTC)98836175 ←	1,248	0	
		b	← 98200770(CTG)98209612 →	8,842	14 (6, 8)	
		c	← 97434987(CCTCAGCCTCCCAAAGTGCTGGGA)97437875 →	2,888	4 (1, 3)	<i>Alu-Alu</i>
		d	← 93723026(AATA)93723819 →	793	0	
		e	← 92019728(GCTCACTGCAACCTCTGCCTCC)92022398 →	2,670	4 (2, 2)	<i>Alu-Alu</i>
		bridge ^c	→ 92068958(A)91940103==(248)==>91940350()92942884 →	nd	nd	
A431	(7p11.2)	a	← 54940436(CT)54940993 →	557	0	
		b	→ 55204390(CTCC)chr4:23858928==(775)= ==>chr4:23859702(AC)55274732<==(79)= ==55274654(CTT)55291783<==(345)= ==55291439[tcca]55208110 ←	3,720	nd	
		c	→ 55598588(GT)55600738 ←	2,150	0	
		d	→ 56613309(TGCA)56614792 ←	1,483	1 (0, 1)	
BT474	(9p13.3)	a	→ 35433279(TG)35435139 ←	1,860	1 (1, 0)	
		b	← 35036330(A)35041583 →	5,253	8 (4, 4)	
		c	← chr9:34383241[tatttaatct]34386096 →	2,855	2 (0, 2)	
		d	← 33988358(GCCCAGGCTGGAGTGCA)33989985 →	1,627	2 (1, 1)	<i>Alu-Alu</i>
		e	← 32547748(TGTA)32549640 →	1,892	2 (0, 2)	
A431	(11q13.3)	a	→ 70315128(TCTT)70315340 ←	212	0	
		b	← 69677606(AATTGCTTGAACCCGGGAGGC)69681032 →	3,426	3 (1, 2)	<i>Alu-Alu</i>
		c	← 68739149(CAGT)68740738 →	1,589	0	

nd = No data.

^a Junction structures are represented as sequence positions and directions (numbers and arrows) with overlapping nucleotides (upper case letters), inserted nucleotides [lower case letters], or no insertions ().

^b The numbers of *Alu* elements between 2 breakpoints which are joined at each junction site are indicated. *Alu* elements on the plus (+) or minus (-) strand are shown in parentheses.

^c Two separated sequence positions are bridged with a small sequence stretch (248 bp), resulting in a deletion of ~900 kb. (See online suppl. fig. 2B for the structure).

characteristic copy number profile; that is, a stair-like increase with multiple segments on one side and a single sharp drop on the other side. Third, in sequence analysis, junction sites of the amplified regions exhibited a characteristic structure such as head-to-head or tail-to-tail fusion with a 1–5 kb intervening sequence at the center, accompanied by overlapping sequences. These junction structures are known to be generated by end-to-end fusion of broken chromosomes using a non-homologous end joining repair mechanism [Okuno et al., 2004; Hefner and Tomkinson, 2005].

Out of the 20 junction sites, sequences of 4 junction sites, AsPC-7-c, AsPC-7-e, BT474-9-d, and A431-11-b, were found as fusion sequences between inverted, paired *Alu* elements, which are separated by 1–3 kb in the ge-

nome sequence. These fusion sequences contain relatively long overlapping sequences of 17, 21, 22, and 24 bp, identical in both *Alu* elements, when compared with other junction site sequences, which contain 1–8 bp overlapping sequences. This information on the sequences suggests that the junctions may be generated by the single strand annealing pathway of recombination, an alternative repair mechanism using sequence homology between inverted, paired *Alu* elements. The occurrence of such events has been previously demonstrated in model constructs [Lobachev et al., 2002; Tanaka et al., 2002; VanHulle et al., 2007]. With a triggering double strand break at or near inverted, paired *Alu* elements, one *Alu* sequence can anneal to the other inverted *Alu* sequence on the same strand (before DNA replication) or a differ-

Fig. 3. *Alu-Alu* fusions at junction sites. Four junction sites in 3 amplified regions are shown. **A** AsPC1-7-c; **B** AsPC1-7-e; **C** BT474-9-d; **D** A431-11-b. Upper panels: Genome map of *Alu* elements and break-points. *Alu* elements in the plus orientation (solid boxes above the line) and in the minus orientation (solid boxes below the line) are mapped on the genome sequence. The 2 breakpoints, which are joined at each junction site, are mapped on the genome sequence using red and blue colored bars. The sequence between red and blue bars indicates an intervening sequence and the sequence at the side of a red-marked breakpoint is over hanging. Lower panels: Sequence alignment of 2 *Alu* elements fused at the junction site. Underlined sequences represent junction site sequences and sequences in red represent identical sequences in both *Alu* elements, within which the exact fusion point resides. Numbering indicates the sequence positions on the genome sequence.

ent strand (after DNA replication) and an inverted duplicated chromosome with a head-to-head or tail-to-tail fusion junction is formed. In addition, the *Alu* mapping data revealed that 6 junctions, containing at least 1 pair of inverted elements, have the potential to make an in-

verted *Alu-Alu* fusion. Out of these junctions, 4 were found as inverted *Alu-Alu* fusions. The high occurrence rate (4/6) of such a fusion event suggests the possibility that, if the breaks occur at or near inverted, paired *Alu* elements, broken ends induced by the process of BFB cy-

cle-mediated amplification could be mainly repaired by single strand annealing using sequence homology of *Alu* elements. On the other hand, in this study no *Alu-Alu* fusion was found at the junction sites created by the first round of BFB cycles (junctions a in fig. 1 and junction sites a in table 1). This suggests that *Alu*-mediated repair does not participate in the initiation of the BFB cycle. However, the lack of *Alu-Alu* fusion could also be attributed to the absence of *Alu* elements near the breakpoint sites and, therefore, further study is required before conclusions as to relationship between the *Alu*-mediated repair and the initiation of the BFB cycle can be drawn.

So far, *Alu-Alu* fusion-mediated chromosomal rearrangements reported in somatic cells are very few compared with those in human germline cells [Kolomietz et al., 2002; Belancio et al., 2010; Konkel and Batzer, 2010; Deininger, 2011], and those that have been reported are tandem duplications of *MLL* [Strout et al., 1998] and *MYB* [O'Neil et al., 2007] and deletions of *CAD* [Hsieh et al., 2005]. However, the current study shows the possibility that even in somatic cells the *Alu-Alu* fusion occurs in a type of chromosomal rearrangement. Recently, by us-

ing a genome-wide searching assay, DNA fragments with palindromic structures have been isolated from cancer genomes and then mapped to the genome sequence. The mapping shows that such fragments are not randomly distributed in the cancer genomes and are often located in clusters, especially at the boundaries of amplified regions [Guenthoer et al., 2012]. It would be interesting to examine whether some of these fragments contain inverted *Alu-Alu* fusion sequences. In addition, by genome-wide sequence analysis using the next generation sequencing technology, 3 inverted *Alu-Alu* fusion sequences have been identified in a breast cancer cell line [Bignell et al., 2007]. Thus, further studies, especially genome-wide sequence analysis, will identify novel *Alu-Alu* fusions occurring in somatic cells and will further our understanding of the relationship between such fusions and somatic rearrangements.

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