Characterization of a Hotspot Region on Chromosome 12 for Amplification in Ring Chromosomes in Atypical Lipomatous Tumors

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Ring chromosomes are cytogenetic hallmarks of genomic amplification in several bone and soft tissue tumors, in particular atypical lipomatous tumors (ALT). In ALT, the ring chromosomes invariably contain amplified material from the central part of the long arm of chromosome 12, mainly 12q12→15, but often also segments from other chromosomes are involved. Previous studies have shown that one of the recurrent amplicons in ALT, located in 12q13.3-14.1 and harboring the candidate target genes *TSPAN31* and *CDK4*, often has a sharp centromeric border. To characterize this breakpoint region in more detail, 12 cases of ALT with ring chromosomes were analyzed by array comparative genomic hybridization and fluorescence in situ hybridization. In the seven cases showing a sharply delineated amplicon in 12q13.3-14.1, the breakpoint region was further investigated by real time quantitative polymerase chain reaction and Vectorette PCR. The breakpoints clustered to a 146-kb region containing 11 genes. Whereas there was no indication that the breakpoints gave rise to fusion genes, in silico analysis revealed that the breakpoint region was enriched for repeated elements that could be important for ring chromosome formation in ALT.

INTRODUCTION

Although ring chromosomes may occur in a wide variety of human neoplasias, they are especially frequent in mesenchymal tumors of low grade or borderline malignancy, such as atypical lipomatous tumor (ALT), low grade fibromyxoid sarcoma, myxoinflammatory fibroblastic sarcoma, myxofibrosarcoma, parosteal osteosarcoma, and dermatofibrosarcoma protuberans (Heidenblad et al., 2006; Mertens et al., 2009). In ALT, also known as atypical lipoma or well-differentiated liposarcoma, ring chromosomes have been detected in 133 of 156 (85%) reported cases (Rosai et al., 1996; Dei Tos and Pedeutour, 2002; Mitelman Database of Chromosome Aberrations in Cancer, 2009). Molecular genetic, fluorescence in situ hybridization (FISH), and array-based comparative genomic hybridization (CGH) studies of ring chromosomes in ALTs have shown that they consistently contain amplified material from the central part of the long arm of chromosome 12, mainly 12q12→15. In addition, ring chromosomes in ALTs may include sequences from several other chromosomes, in particular chromosomes 1, 3, 6, 12, or 20 (Meza-Zepeda et al., 2001; Nilsson et al., 2004; Heidenblad et al., 2006; Italiano et al., 2008).

Ring chromosomes can have an impact on cancer development through different mechanisms: loss of genetic material distal to the involved chromosomal fragments, rearrangement of one or more genes in the breakpoint regions, such as the formation of the fusion genes *PDGFB/COL1A1* in dermatofibrosarcoma protuberans (Sirvent et al., 2003) and *FGFR1/PLAG1* in salivary gland tumors (Persson et al., 2008), or overexpression of amplified genes (Italiano et al., 2008). As ring chromosomes in ALT are always supernumerary, only the two latter models seem valid in this tumor type.

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TABLE 1. Karyotypes and Array CGH Findings in 12 Atypical Lipomatous Tumors

Case	Karyotype	Chromosomes with amplicons at CGH
1	47∼48,XY,+1∼2r/46∼47,XY,-5,der(20)t(5;20)(q13;p13),+1∼3r	12
2	47∼50,XY,+1∼4r	12
3	47,XX,+r/48∼49,XX,+2∼3r/47,XX,+1∼2r	1,12
4	47,XY,+r	1,12
5	47∼48,XX,+2r/86∼92,XXXX,+3∼4r	1,9,12
6	48,XY,+2r	3,12
7	47,XY,+r	12
8	47,XX,+r/48,idem,+r/49,idem,+r,+mar/50,idem,der(3)t(3;13)(p25;q12),-13,+2r,+mar/90-92,XXXX, +2∼3r	1,10,12
9	47∼49,XY,+1∼3r	9,12
10	44∼46,XX,+2r/90∼94,idemx2	1,12
11	43∼48,XY,+2∼3r/93∼95,idemx2	2,12,18
12	47∼48,XX,+r,+mar/47∼48,idem,+9,-18	1,12

The amplifications on chromosome 12, involving a large number of genes such as *MDM2*, *CDK4*, *DYRK2*, *TSPAN31* (formerly known as *SAS*), and *HMGA2*, are usually discontinuous and of varying size (Heidenblad et al., 2006; Italiano et al., 2008). Several of the amplified genes could play a key role in cancer development. For instance, both *MDM2* and *CDK4* are involved in cell cycle regulation by inhibiting TP53 and promoting RB1 phosphorylation, respectively. *DYRK2* encodes a protein kinase involved in cellular growth, presumably by phosphorylation of histones H3 and H2B, the TSPAN31 protein is recruited in the regulation of cell development, activation, growth and motility, and the HMGA2 protein contains structural DNA-binding domains and may act as a transcriptional regulator.

Previous studies of the amplicons in 12q in ALT and other soft tissue tumors with supernumerary ring chromosomes have identified a distinct recurrent, approximately 0.4 Mb, amplicon in 12q13.3-14.1, containing the *TSPAN31* and *CDK4* genes (Heidenblad et al., 2006; Italiano et al., 2008). Interestingly, this amplicon seems to have a sharp proximal (centromeric) border, suggesting that this region may be prone to breakage and amplification. In the present study, we used array CGH, FISH, quantitative PCR and Vectorette PCR to investigate in more detail the location of the breakpoints.

MATERIALS AND METHODS

Tumors

Frozen samples from 12 ALT with supernumerary ring chromosomes at G-banding analysis were selected for array CGH analysis (Table 1).

Array CGH

Each sample was analyzed using microarrays containing 32,433 partly overlapping, individual BAC clones, generating a whole coverage of the human genome. The arrays were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University (http://swegene.onk.lu.se), as previously described (Jönsson et al., 2007), using the BACPAC May 2004 data (hg 17). Extraction, labeling, hybridization of genomic DNA from freshly frozen tumor biopsies, and pretreatment and washing of slides were performed as previously described (Heidenblad et al., 2006; Hallor et al., 2008). A DNA pool derived from multiple healthy male donors (Promega, Madison, WI) was used as control for normal copy number.

Image and Data Analysis

Primary data were collected using the GenePix Pro 4.0 software (Axon Instruments inc., Foster City, CA), and the quantified data matrix was deposited into the web-based database BioArray Software Environment (BASE) (Saal et al., 2002). The following correction, normalization and copy number detection were performed as previously described (Heidenblad et al., 2006).

FISH

Each sample was tested by FISH to validate the array CGH results. BAC and fosmid clones covering the centromeric border of the recurrent amplicon in 12q13.3-14.1 were selected according to the latest release of the UCSC Genome Browser (March 2006). The probes used, obtained from the BACPAC Resource Center

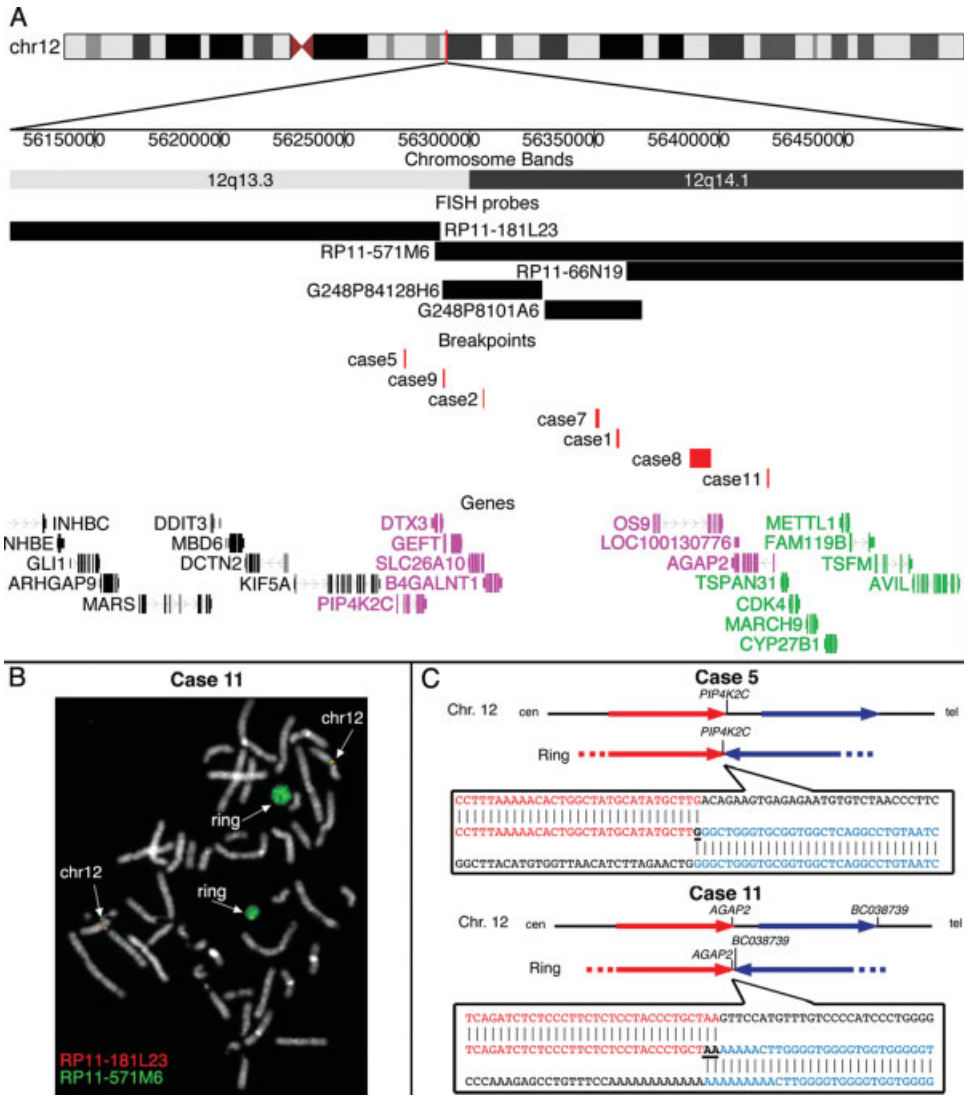

Figure 1. (A) Schematic representation of the overall results obtained by FISH and RQ-PCR, showing the map position of the breakpoints for each of the seven cases with 12q13.3-14.1 amplification in rings. The breakpoints are indicated by red blocks. Genes mapping within the investigated genomic region are indicated in black (if not amplified), violet (if interrupted or amplified in some cases) or green (amplified in all cases) at the bottom of the figure. (B) FISH analysis (Case 11) using the probes RP11-181L23 (red, not amplified in rings) and RP11-571M6 (green, amplified in rings) illustrating the sharp border of the amplicon in 12q13.3-14.1. (C) Schematic representation of the arrangement and structure of amplicon fusion junctions at 12q13.3-14.1 in ring chromosomes in cases 5 and 11. The arrows indicate the orientation of the fragments with respect to the orientation on normal chromosomes 12. The genes interrupted by the breakpoints are also indicated.

(http:/bacpac.chori.org), were: BACs RP11-181L23, RP11-571M6 and RP11-66N19 and fosmids G248P84128H6 and G248P8101A6 (Fig. 1A, Table 2). Metaphase spreads were hybridized in situ with probes directly and indirectly labeled by nick translation as previously described (Surace et al., 2008). Digital images were obtained using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, Boston, MA). Cy3 (red; New England Nuclear, Boston, MA), fluorescein (green; Fermentas Life Sciences, Milan, Italy), Cy5 (IR; New England Nuclear), and DAPI (blue) fluorescence signals, which were detected using specific filters, were recorded separately as gray-scale images. Pseudocoloring and merging of images were performed with Adobe Photoshop software (Adobe Systems, Seattle, WA). Initial FISH experiments were carried out co-hybridizing BACs RP11-181L23, RP11-571M6 and RP11-66N19, followed by cohybridization of fosmid clones G248P84128H6 and G248P8101A6.

TABLE 2. FISH Results in 12 Atypical Lipomatous Tumors

		Case no.											
Clone	Position	1	2	3	4	5	6	7	8	9	10	11	12
RP11-181L23	chr12:56,116,000-56,288,135	−	−	−	+	−	−	−	−	−	−	−	−
G248P84128H6	chr12:56,288,430-56,328,414	−	+			+		−	−	+		−	
G248P8101A6	chr12:56,333,753-56,374,072	+	+			+		+	−	+		−	
RP11-571M6	chr12:56,286,137-56,497,675	+	+	−	+	+	−	+	+	+	−	+	−
RP11-66N19	chr12:56,362,976-56,513,586	+	+	−	+	+	−	+	+	+	−	+	−

+, Amplified in rings; −, not amplified in rings.

Real-Time Quantitative Polymerase Chain Reaction

To map the amplicon breakpoints in more detail, genomic real-time quantitative PCR (RQ-PCR) experiments were performed. To identify regions of switch between amplified and non-amplified sequences, multiple primer pairs were designed, by means of the Primer3 software v.0.4 (http://frodo.wi.mit.edu/), for the genomic sequence overlapping the fosmids G248P84128H6 and G248P8101A6. In three tumors (cases 5, 8, and 11), the results obtained led us to select additional primer pairs proximally and/or telomerically to this region. The segment encompassing the breakpoint was further narrowed down using the same approach, i.e., by selecting additional primer pairs within that region. Genomic DNA was extracted from archived freshly frozen tumor biopsies using DNeasy Tissue Kit (Qiagen, Valencia, CA), including the optional RNaseH treatment, and DNA from a healthy individual (Human Control Genomic DNA, Applied Biosystems) was used as a control. The DNA was amplified by means of Applied Biosystems Real-Time PCR System 7300 using the SYBR Green PCR kit (Applied Biosystems, Monza, Italy). The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C for all of the primer pairs used. All measurements were performed at least in triplicate.

Vectorette-PCR

To clone and sequence the amplicon junctions at 12q13.3-14.1, Vectorette PCR experiments were performed according to handbook protocol (Vectorette™ Genomic Systems, Universal Vectorette™ System, Sigma-Aldrich®). PCR conditions were as follows: 3 min at 93°C followed by five cycles of 20 s at 93°C, 20 s at 65°C, and 3 min at 68°C; redenaturation for 20 s at 93°C followed by 35 cycles of 20 s at 93°C, 20 s at 60°C and 3 min at 68°C followed by 7 min at 68°C. PCR products were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen) and sequenced. Reference genome sequence data were obtained from the UCSC browser (http://genome.ucsc.edu/cgi-bin/hgGateway?db = hg18; March 2006 release), and sequence comparison was performed using the BLAST software tool (www.ncbi.nlm. nih.gov/BLAST/).

Bioinformatic Analysis

To disclose the genomic architecture of the 12q region frequently affected by breakage in the analyzed tumors, the mapped breakpoint regions in cases 5 and 11, as well as the overall "hotspot" breakpoint region, were analyzed in silico.

The 300-kb chromosomal segments surrounding the breakpoints in cases 5 and 11 (chr12:56,200,001-56,500,000; chr12:115,300,001-115,600,000; chr12:67,200,001-67,500,000), the overall "hotspot" breakpoint region (chr12:56,273,902-56,419,899), the whole chromosome 12 (chr12:1-132,349,534), and the whole human genome were analyzed for the presence of segmental duplications and interspersed repeated elements. The latter were investigated together, as well as by repeat type.

The UCSC Table Browser (http://genome.ucsc.edu/cgi-bin/hgTables) was queried for summary analysis about the items belonging to the tracks "Self Chain" and "Repeat Masker." Bioinformatic analysis, by means of the Fuzznuc software (http://bioweb2.pasteur.fr/docs/EMBOSS/fuzznuc.html), was locally performed on the whole chromosome 12 sequence and on the three chromosome 12 breakpoint regions in cases 5 and 11, to disclose consensus sequences (motifs) known to be involved in chromosome instability. To test the statistical significance of the repeated element frequencies observed in the breakpoint regions, we randomly selected 24 intervals of 300 kb from the human chromosome 12 and measured the repeat rates for each of these. Starting from these

values, we computed the SD. We assumed the repeat rate on the whole chromosome 12 (w12) as the mean value, and the computed SD as the SD of a Gaussian distribution of repeat rates on chromosome 12. We considered repeat rates larger than w12 + 1.96 × SD ($P = 95\%$) as statistically significant, and rates larger than w12 + 2.58 × SD ($P = 99\%$) as highly significant.

RESULTS

The arrayCGH analysis disclosed DNA copy number alterations in all 12 tumors (Table 1). Apart from chromosome 12, which had amplifications in all cases, also chromosomes 1 (six cases) and 9 (two cases) were repeatedly involved (Supporting Information Table 1 and Supporting Information Fig. 1). The amplicons on chromosome 12 varied in size from regions covered by a single BAC to 5.3 Mb. All informative cases showed gain/amplification of the genomic regions 64,486,879-64,635,771 (harboring the *HMGA2* gene), 67,447,777-67,816,916 (harboring the *MDM2* and *CPM* genes), and 68,369,508-68,556,720 (harboring the *BEST3* and *RAB3IP* genes). A narrow peak of amplification at 12q13.3-14.1 was seen in six of nine informative tumors, in accordance with the results reported by Heidenblad et al., (2006) (Supporting Information Table 1). The proximal border of the amplicon was delineated by the BAC RP11-571M6 covering the *CDK4* and *TSPAN31* genes (Supporting Information Table 1).

As some cases were not informative for RP11-571M6 or the nearest, overlapping BAC on the centromeric side, and to study the centromeric border of the 12q13.3-14.1 amplicon in more detail, FISH cohybridization experiments were carried out in all cases, using the BAC clones RP11-181L23, RP11-571M6, and RP11-66N19 (Fig. 1B). In seven tumors (Cases 1, 2, 5, 7, 8, 9, and 11), the breakpoints were delineated by clones RP11-181L23, showing signals on both normal chromosomes 12, and RP11-571M6, with additional multiple spots on ring chromosomes (Fig. 1B), as well as RP11-66N19. In Cases 3, 6, 10, and 12, none of the three clones was amplified, while Case 4 showed amplification of all three probes. Apart from Case 8, which did not show gain of BAC RP11-571M6 at array CGH analysis, the FISH results were in agreement with the array-based results.

To characterize further the breakpoint region delineated by RP11-571M6, fosmid clones G248P84128H6 and G248P8101A6, overlapping with the centromeric portion of RP11-571M6, were used. In Cases 1 and 7, the fosmid G248P84128H6 gave only normal signals on the two chromosomes 12, while G248P8101A6 was shown to be amplified in ring chromosomes. In Cases 2, 5, and 9, both fosmids were amplified in ring chromosomes, clearly indicating the location of the breakpoint region between the BAC clone RP11-181L23 and the fosmid G248P84128H6. Moreover, in Cases 8 and 11, both fosmids gave signals only on the two normal chromosomes 12, mapping the breakpoint position distally to G248P8101A6. The overall FISH results are listed in Table 2.

TABLE 3. Mapping of Genomic Breakpoints in Chromosome 12 by Real-Time Quantitative PCR

Case	Location of breakpoints	Range (bp)	Interrupted gene
1	chr12:56,358,765-56,360,012	1,248	
2	chr12:56,305,320-56,305,756	437	*SLC26A10*
5	chr12:56,273,902-56,274,309	408	*PIP4K2C*
7	chr12:56,350,587-56,351,872	1,286	
8	chr12:56,388,184-56,396,527	8,344	*OS9*
9	chr12:56,289,499-56,290,017	519	*DTX3*
11	chr12:56,419,308-56,419,899	592	*AGAP2*

To map the breakpoints at higher resolution, we performed genomic RQ-PCR assays. PCR products corresponding to amplified sequences on ring chromosomes displayed, on average, a 17-fold copy number increase, yielding a clear distinction between amplified and non-amplified sequences. This approach allowed a more precise definition of the boundaries of the breakpoint regions to within sequence chr12:56,273,902-56,419,899. This 145,998 bp region encompasses 11 genes, including *DTX3*, *PIP4K2C*, *SLC26A10*, *OS9*, and *AGAP2*. The overall RQ-PCR results are listed in Table 3.

After the detailed mapping of the breakpoint region in each case, Vectorette PCR experiments were performed to identify the genomic segments fused to the "hotspot" breakpoint region in 12q13.3-14.1. In Cases 5 and 11, additional chromosome 12 sequences were found at the breakpoint region (Fig. 1C). Sequencing of the obtained PCR products in Case 5 revealed the junction of the 12q13.3-14.1 amplicon at nucleotide (nt) 56,273,939 [within the phosphatidylinositol-5-phosphate 4-kinase (*PIP4K2C*) gene] with an amplified 12q24.22 sequence at nt 115,452,133, in opposite sequence orientation, where no known gene is annotated. In Case 11, a

junction occurred at nt 56,419,527 (within the *AGAP2* gene) and nt 67,363,345 (within the *BC038739* gene) in 12q15, in opposite sequence and transcriptional orientation. Both joined sequences disclosed nucleotide microhomologies at the fusion junctions: one (G) and two (AA) nucleotides, respectively, in cases 5 and 11 (Fig. 1C). Unfortunately, all of the reiterative Vectorette PCR experiments performed in the remaining ALT cases failed.

To investigate the genomic architecture of regions involved in the chromosomal rearrangements occurring in the ring chromosomes, bioinformatic analysis was performed. The chromosomal regions surrounding the breakpoints in Cases 5 and 11 were checked for the presence of segmental duplications, repeats, and consensus motifs of genomic fragility. The results obtained showed no intrachromosomal segmental duplication within the mapped breakpoint regions in Cases 5 and 11. Furthermore, there were no significant differences in Cases 5 and 11 between the three mapped chromosome 12 breakpoint regions and the entire chromosome 12 with regard to the distribution of known sequence motifs (data not shown). The repeatmasker analysis revealed that the investigated breakpoint regions are enriched for specific classes of repeated elements in the genomic regions containing breakpoints, as well as in the "core" (chr12:56,310,001-56,400,000) of the overall "hotspot" breakpoint region (chr12: 56,273,902-56,419,899), and in the regions surrounding the breakpoints of the sequences attached to 12q13.3-14.1 in Cases 5 and 11 (chr12:115,300,001-115,600,000 and chr12:67,200, 001-67,500,000) (data not shown). More specifically, in the two regions joined to the 12q13.3-14.1 amplicon in Cases 5 and 11, we found a SINE content of 31.9% and 30.3% respectively, which is higher than in the whole chromosome 12 (15.0%). However, the SINE content of overall hotspot breakpoint region was only 23.1%, not significantly higher than in the whole chromosome 12. Whereas there was no enrichment of SINEs (23.1% vs. expected 15.0%) in the core of the hotspot breakpoint region, LINEs and LTRs were more commonly represented than in the whole chromosome 12 (33.9% vs. 20.5% and 18.5% vs. 8.6%, respectively).

DISCUSSION

Overexpression of oncogenes through amplification at the chromosome level plays an important role in the development of many malignant tumor types. Cytogenetically, gene amplification can be observed in three principally and mechanistically different forms: double minutes (dmin), homogeneously staining regions (hsr), and ring chromosomes/giant marker chromosomes (hereafter referred to as rings) (Cox et al., 1965; Kopnin, 1981; Barker, 1982; Cowell, 1982; Gebhart et al., 1984; Schwab, 1998; Gisselsson et al., 1999; Pedeutour et al., 1999; Menghi-Sartorio et al., 2001; Storlazzi et al., 2006; Hattinger et al., 2009). Depending on tumor type, the genomic contents of the amplicons vary, with different target genes in different tumors. Although each type of amplification has been observed in most malignant tumor types, rings are especially frequent among bone and soft tissue tumors.

There are several hypotheses to explain the origin of hsr and dmin, all of which rely on the assumption that the initial event is a DNA double strand-break (DSB) (Coquelle et al., 1997; Paulson et al., 1998). An hsr is commonly believed to arise through breakage-fusions-bridge (BFB) cycles, generating "head to head" amplifications, as proposed by McClintock (1951) almost 60 years ago, whereas dmin are thought to originate through the "loop-formation-excision-amplification" process, also known as the "episome model" (Carroll et al., 1988), leading to "head to tail" amplicons (Vogt et al., 2004; Storlazzi et al., 2006). In contrast to the quite extensive literature on the formation of hsr and dmin, little is known about the origin of rings.

In bone and soft tissue tumors, rings are typically found as supernumerary chromosomes, i.e., they occur in addition to a more or less normal diploid set of chromosomes. As they are often quite large and contain material from more than one chromosome, the episome model cannot reasonably be applied to rings. Nor is there deletions of the loci involved in the amplicons from any of the normal homologues, which is a typical feature of tumor cells with dmin, or loss of the chromosomal segment located distal to the amplified regions, which is to be expected in cells with hsr arising through BFB cycles (Pedeutour et al., 1994; Gisselsson et al., 1999; Micci et al., 2002). Furthermore, ring chromosomes show, in the majority of the cases, at least one centromere or neocentromere (Gisselsson et al., 1999; Pedeutour et al., 1999; Italiano et al., 2009), which is not observed in dmin. We have previously shown that there is retained heterozygosity for regions on the two normal-appearing homologues of

chromosome 12 in ALTs that are not present in the rings, indicating that the initial event, i.e., the acquisition of an extra copy of the entire or parts of chromosome 12, occurs in a post replication phase (Mertens et al., 2004).

Bearing in mind the inherent mitotic instability of neoplasia-associated rings, it might be difficult to elucidate their origin by studying tumor samples. The rings that are present in tumors at the time of diagnosis are likely to have been extensively modified due to selective pressure. Nevertheless, we and others have observed that one of the commonly observed amplicons in sub-band 12q13.3-14.1, including the *CDK4* and *TSPAN31* genes, has a very sharp centromeric border (Heidenblad et al., 2006; Italiano et al., 2008). Such a sharp border could be explained by the presence of breakprone sequences, fusion genes, or one or more genes with a strong negative impact on tumor growth just outside the breakpoint region.

In seven cases we found an amplicon in 12q13.3-14.1, starting with BAC clone RP11-571M6, adding further support to the notion that this is a hot spot for breakage and amplification in ALTs. The identified 146 Kb region contains 11 annotated genes and, in five of the seven cases, one of them was interrupted by the breakpoint: *SLC26A10* in Case 2, *PIP4K2C* in Case 5, *OS9* in Case 8, *DTX3* in Case 9 and *AGAP2* in Case 11.

Several genes, i.e., *MARS*, *DDIT3*, *MBD6*, *DCTN2*, *KIF5A*, and *PIP4K2C*, are located within 100 kb from the centromeric border of the 12q13.3-14.1 amplicon (Fig. 1A). The information on the normal function of most of these genes is limited, but it is of interest to note that forced overexpression of *DDIT3* induces apoptosis in a variety of cell types (Maytin et al., 2001). Thus, it is not unreasonable to speculate that inclusion of *DDIT3* in the amplicon could have a strong negative impact on the fitness of the tumor cells. Nevertheless, Italiano et al., (2008) recently showed that *DDIT3* was co-amplified with *CDK4* and *TSPAN31* in 2/38 (8%) ALTs, and that these two cases also showed strong overexpression of *DDIT3*. In agreement with these data, one of the 12 cases (Case 4) also showed amplification of the *DDIT3* locus (Supporting Information Table 1). Hence, available data do not indicate that the centromeric extension of the 12q13.3-14.1 amplicon is limited by genes that, if included in the ring chromosomes, would negatively affect cell growth.

A number of neoplasia-associated chromosomal breakpoints have been associated with various consensus sequences (motifs) of fragility, such as topoisomerase II binding sites, translin binding sites (Kanoe et al., 1999; Wei et al., 2003; Bystritskiy and Razin, 2004), Chi-like sequences (Wyatt et al., 1992), PurI binding sites, pyrimidine traits (Hirai et al., 1999), eukaryotic replication origin sequences (Bystritskiy and Razin, 2004), nuclear matrix attachment regions (Umanskaia et al., 2005), putative triple helices (Missailidis et al., 2005; Jain et al., 2008), with repeated elements, such as short interspersed elements (SINEs) (Kolomietz et al., 2002; Chisholm et al., 2008; Xiang et al., 2008), long interspersed elements (LINEs) (Fischer et al., 2008), long terminal repeats (LTRs) (Mimori et al., 1999), microsatellites (Uryu et al., 2005; Kobayashi et al., 2006), and with intrachromosomal low copy repeats (Saglio et al., 2002; Stankiewicz and Lupski, 2006; Bea et al., 2009; Thomas et al., 2009). The breakpoint region in 12q13.3-14.1 did not differ from the whole chromosome 12 with respect to distribution of motifs of fragility. Nor was any intrachromosomal segmental duplication detected within the breakpoint region. However, the repeatmasker analysis revealed a significant enrichment of specific repeats. The BAC clone RP11-571M6 maps approximately 113 Kbp proximally to a region (RP11-58A17, chr12:56,610,787-56,772,798) that has been reported to be recurrently involved in amplification in glioblastomas with hsr in 12q14-15 (Fischer et al., 2008). Interestingly, also RP11-58A17 shows a higher content of LINEs than the flanking regions, suggesting that these structures might facilitate the origin of the amplicons. Further support for this hypothesis might be derived from the fact that an elevated content of LINEs has been shown for several human fragile sites (Sawinska et al., 2007). Another aspect to be considered is that BAC clone RP11-571M6 is located at the border of two cytogenetic bands, 12q13 and 12q14, which are light- and dark-staining bands, respectively. It has previously been shown that, in general, light-staining bands are gene-rich and early replicating, whereas dark-staining bands are gene-poor and late replicating (Holmquist, 1992). Many recurrent neoplasia-associated breakpoints described in the literature have been found to map to the interface of light- and dark-staining G-bands, i.e., at the border of chromosomal domains replicating at different times during S-phase (El Achkar et al., 2005; Debatisse et al., 2006).

In summary, our results confirm that chromosome segment 12q13.3-14.1 is a hot spot for

amplicon formation in ALTs with rings, and reveal that different regions become fused to the amplicon, without giving rise to a proper fusion gene. The results also indicate that at least some of the breaks necessary for ring formation occur in unstable regions of chromosome 12, showing enrichment of LINEs and LTRs, followed by complex rearrangements mediated by NHEJ.

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