RESEARCH ARTICLE

The breakage–fusion–bridge (BFB) cycle as a mechanism for generating genetic heterogeneity in osteosarcoma

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Abstract Osteosarcoma (OS) is characterized by chromosomal instability and high copy number gene amplification. The breakage–fusion–bridge (BFB) cycle is a well-established mechanism of genome instability in tumors and in vitro models used to study the origins of complex chromosomal rearrangements and cancer genome amplification. To determine whether the BFB cycle could be increasing the de novo rate of formation of cytogenetic aberrations in OS, the frequency of anaphase bridge configurations and dicentric chromosomes in four OS cell lines was quantified. An increased level of anaphase bridges and dicentrics was observed in all the OS cell lines. There was also a strong association between the frequencies of anaphase bridges, dicentrics, centrosomal anomalies, and multipolar mitotic figures in all the OS cell lines, indicating a possible link in the mechanisms that led to the structural and numerical instabilities observed in OS. In summary, this study has provided strong support for the role of the BFB cycle in generating the extensive structural chromosome aberrations, as well as cell-to-cell cytogenetic variation observed in OS, thus conferring the genetic diversity for OS tumor progression.

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Introduction

Genomic instability, reflecting the susceptibility of the genome to acquire multiple genetic alterations, is now known to play a major role in tumorigenesis and tumor progression. Because the rate of random mutational events alone appears to be insufficient to account for the numbers of genetic alterations found in most cancers, Loeb (1991) postulated the need for a mutator phenotype mechanism that destabilizes the genome and drives tumor progress. This phenotype is now known to appear in two main forms: microsatellite instability (MIN) and chromosomal instability (CIN). MIN leads to an increased point mutation rate (approx. a 1,000-fold increase), whereas CIN refers to an enhanced rate of accumulating gross chromosomal aberrations (Nowak et al. 2002). While MIN arises primarily from changes at the nucleotide level, the molecular basis of CIN seems far more complex in nature. This instability is generally defined as an excess of chromosome alterations, at the structural and/or numerical level, occurring at each

cell generation and these changes may not necessarily be transmitted through cell generations. It appears to be an important feature of tumor cells since propagation of such a diverse cell population may facilitate evasion of standard therapies.

Most members of the sarcoma family have well-defined recurrent chromosomal translocations that result in fusion genes with oncogenic fusion protein products (Tomescu and Barr 2001). However, osteosarcoma presents a unique challenge as it represents the extreme member of a group of sarcomas in which multiple chromosomal abnormalities occur on many of the chromosomes. We, along with several others (Akerman et al. 1996; Al-Romaih et al. 2003; Atiye et al. 2005; Bayani et al. 2003; Lim et al. 2004, 2005; Mertens et al. 1993; Squire et al. 2003; Stock et al. 2000; Tarkkanen et al. 1995; Zielenska et al. 2001, 2004), have identified complex karyotypes with multiple numerical and structural chromosome aberrations and gene amplification through the cytogenetic analyses of osteosarcoma (OS) tumors and cell lines. There are deletions as well as duplications of part or whole chromosomes and multiple areas of recombination within and between chromosomes. Multiple marker chromosomes, double minutes, and/or homogenously staining regions are also frequently exhibited (Stock et al. 2000). Also, a common finding in this tumor is extensive intra- and intertumor heterogeneity in the pattern of structural and numerical chromosome aberrations.

In other tumors exhibiting a similarly complex karyotype, such as oral squamous cell carcinomas, it has been proposed that such cytogenetic patterns could be the result of an inherent genomic instability in the neoplastic cells brought about by disruption of systems that maintain genetic fidelity. Some of these processes include defects in chromosomal segregation, telomere stability, cell-cycle checkpoint regulation, and the repair of DNA damage, all of which could play a role in generating the genomic imbalances observed in these complex karyotypes (Gollin 2004; Michor et al. 2005). A mechanism that could generate the intratumor heterogeneity seen in OS maybe the breakage–fusion–bridge cycle (BFB), originally described using corn half a century ago (McClintock 1941). In the past few years, Gisselsson has evoked this same mechanism to explain the extensive intratumor heterogeneity in tumors exhibiting chromosomal instability (Gisselsson 2003; Gisselsson et al. 2000). This model has also been used to explain the generation of a variety of gene amplifications or genomic instabilities in human cancer cells such as the amplification of the *DHFR* or *AMPD2* genes in CHO cells (Kaufman et al. 1983; Masuda and Takahashi 2002; Shimizu et al. 2005; Toledo et al. 1992; Trask and Hamlin 1989). Structures indicative of the BFB phenomena has

been observed in OS tumors and cell lines (Lim et al. 2004, 2005) and include ladderlike fluorescence staining patterns of amplicons, inverted duplications, anaphase bridges and dicentrics. However, a direct quantitative study was lacking to determine if these mutational events occurred at a frequency high enough to explain the heterogeneity observed in OS. Thus, the current study was designed to investigate the hypothesis that the BFB cycle is involved in perpetuating some of the de novo chromosomal instability observed in OS cell lines.

Materials and methods

Cell lines, culture, and cytogenetic preparation

The OS and the Ewing's sarcoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD); these included SAOS-2 (HTB-85), HOS (CRL-1543), U-2 OS (HTB-96), MG-63 (CRL-1427), and the Ewing's sarcoma (CRL-7598). All of the OS lines were derived from pediatric OS patients. Cytogenetic analysis performed on the control Ewing's sarcoma cell line identified the t(11:22) translocation. No additional cytogenetic changes were observed. All the cell lines were cultured according to the supplier's protocols. Cytogenetic slides were prepared as previously described (Al-Romaih et al. 2003). Briefly, the cell lines were treated with 0.1 μg/ml Colcemid (Invitrogen, Canada) for 2 h, hypotonically treated with 0.075 M KCl, fixed in 3:1 methanol:acetic acid, and then prepared for slides. Slides were aged for 3–5 days before hybridization with fluorescence in situ hybridization (FISH) probes.

Analysis of anaphase cells and anaphase bridge formation

For analysis of anaphase preparations, cells were cultured on slides to approximately 80% confluence and harvested without Colcemid by fixation in methanol:acetic acid (3:1). The slides were counterstained with DAPI in Antifade (Vector Laboratories, Burlingame, CA) and viewed with an epifluorescence microscope. Each cell line was cultured on four slides. Criteria for anaphase bridge configurations required a well-separated parallel anaphase plate displaying at least one anaphase bridge. The number of anaphase bridges per total anaphase figures was then evaluated. To determine baseline values, a Ewing's sarcoma cell line was used as a disease control. Ewing's is a pediatric bone tumor with a well-defined translocation (Tomescu and Barr 2001).

Fluorescence in situ hybridization with peptide nucleic acid probes and multicolor banding analysis

FISH for the alpha-satellite repeats of centromeric regions was carried out with pan-centromeric fluorescein isothiocyanate-labeled peptide nucleic acid (PNA) probes (Applied Biosystems, Foster City, CA). Slide pretreatment consisted of a 5-min incubation in a pepsin/0.01 N HCL solution at 37°C, washes in a 1× phosphate-buffered saline (PBS)/$MgCl_2$ solution, 10-min postfixation in 1% formaldehyde in 1× PBS/$MgCl_2$, an additional wash in 1× PBS, and dehydration through an ethanol series. The probe and chromosome preparation were co-denatured on the HYBriteTM system (Vysis, Downers Grove, IL) for 1.5 min at 80°C. Hybridization was carried out in a humidified chamber for 1 h at room temperature (RT), followed by two 15-min washes at RT in 70% formamide/10 mM Tris/0.1% bovine serum albumin (pH 7.0–7.5), three 5-min washes at RT in 0.1 M Tris/0.1 5M NaCl/0.08% Tween™ 20 (pH 7.0–7.5), and dehydration through an ethanol series at RT. Chromosomes were counterstained with DAPI, mounted and viewed with an epifluorescence microscope. Since OS cell lines are characterized by having additional levels of ploidy, the number of dicentrics/multicentrics was determined based on an analysis of 40 metaphase cells. Multicolor banding (mBAND) analysis was done as previously described (Lim et al. 2004). Chromosomes 1, 6, 8, and 9 were analyzed because they were already shown to be most frequently involved in rearrangements by spectral karyotyping (Bayani et al. 2003). Images of at least 20 metaphases were captured for each experiment using a Zeiss Axioskop Plus microscope (Zeiss, Toronto, ON, Canada) equipped with the appropriate filter sets, and analyzed with MetaSystems ISIS imaging software.

Immunostaining of human centromere protein A and the kinetochore complex

To discriminate between functional and nonfunctional dicentrics, the OS cell lines were cultured on slides to approximately 80% confluence. Specific detection of human centromere protein A (CENP-A) and the kinetochore complex was carried out to discern functional from nonfunctional (constitutional) centromeres in the dicentrics. Briefly, slides were washed in 1× PBS, fixed with cold methanol:acetone (1:1) for 10 min on ice, then washed and permeabilized with 0.1% Tween-20 in PBS for 30 min at 37°C. The cells were incubated for 1 h at 37°C with mouse monoclonal anti-CENP-A (product KAM-CC006; Stressgen Bioreagents, Ann Arbor, MI, USA), followed by three washes with 1× PBS/0.1% Tween-20. The cells were then incubated for in the dark 1 h at 37°C with rhodamine-conjugated goat anti-mouse IgG (product 115-297-003;

Jackson ImmunoResearch Laboratories, PA, USA). For kinetochore identification on the metaphase chromosomes, autoimmune serum from patients with the CREST variant of scleroderma was used as a primary, followed by rhodamine-conjugated goat anti-mouse IgG (product 109-297-003; Jackson ImmunoResearch Laboratories). The protocol was otherwise identical to that employed for the detection of CENP-A. Studies of dicentrics, as well as marker chromosomes containing neocentromeres, have shown that CENP-A is present on active, but not inactive, centromeres, suggesting that CENP-A has an essential role in centromere function (Van Hooser et al. 2001). Additional alpha-satellite repeats may be found at constitutional centromeres in dicentric/multicentric chromosomes, but these will not necessarily be active, and thus will not have CENP-A co-localization (Warburton et al. 1997). As such, immunocytochemistry should produce positive signals from both centromeres of dicentrics if the BFB cycle is still occurring in the cell line. Alternatively, positive signals from centromeres involved in anaphase bridges will also provide additional support for BFB events.

Results

Analysis of anaphase bridge formation and presence of dicentric chromosomes

Anaphase bridges were quantified by identifying mitotic figures in anaphase with a filamentous connection linking two perpendicularly aligned plates (Fig. 1a, 1). Anaphase bridge configurations (ABC) were observed at a frequency of 1.7% in the Ewing's cell line (Fig. 2). Comparable low frequencies were reported in other neoplastic cell lines with simple karyotypes (Gisselsson et al. 2004). All the OS cell lines showed varying degrees of bridge formation frequency with SAOS-2 exhibiting the most and U-2 OS the least. SAOS-2 had an ABC frequency of 35.7%, followed by MG-63 at 32.0%, HOS at 17.0%, and U-2 OS at 10.3% (Fig. 3). An elevated number of micronuclei (Fig. 1b, 6–7), chromatin strings, and nuclear blebs (Fig. 1c, 2–3) were also observed in the OS cell lines compared to the control, representing bridges that had broken after anaphase. Micronuclei are defined as small fragments of chromatin that remain physically distinct from the main nucleus, whereas for nuclear blebs, the interior volume appears to remain connected to the nucleus (Hoffelder et al. 2004).

Frequency of mitotically unstable chromosomes (dicentrics)

To visualize the alphoid repeats that are found in the centromeric region, metaphase PNA FISH was performed using a pan-centromeric probe. To analyze the level of

Fig. 1 Chromosomal instability in U-2 OS, HOS, MG-63, and SAOS-2 cell lines. **a***1* Chromatid bridge showing a perpendicular filamentous connection linking two aligned plates, *2* multipolar mitotic figure. **b** Alpha-satellite staining. *1–4* Dicentric chromosomes visualized by PNA FISH for alpha-satellite repeats in all the OS cell lines (*white arrows*); *5* dual PNA FISH signals for the alphoid repeats in the chromatin strings of anaphase bridges (*white arrows*); *6* micronuclei with staining for alphoid repeats; *7* micronuclei negative for the alphoid repeats (acentric). **c** Active centromere staining. *1* Multiple signals for CENP-A were visualized along the chromatin string (*white arrows*). *2* Internuclear string at telophase with a signal for CENP-A at one end, while *3* shows protrusions exhibiting signals in these blebs. *4* Enlargement of a functional dicentric with two signals for the kintetochore complex (*white arrow*; CREST antibodies)
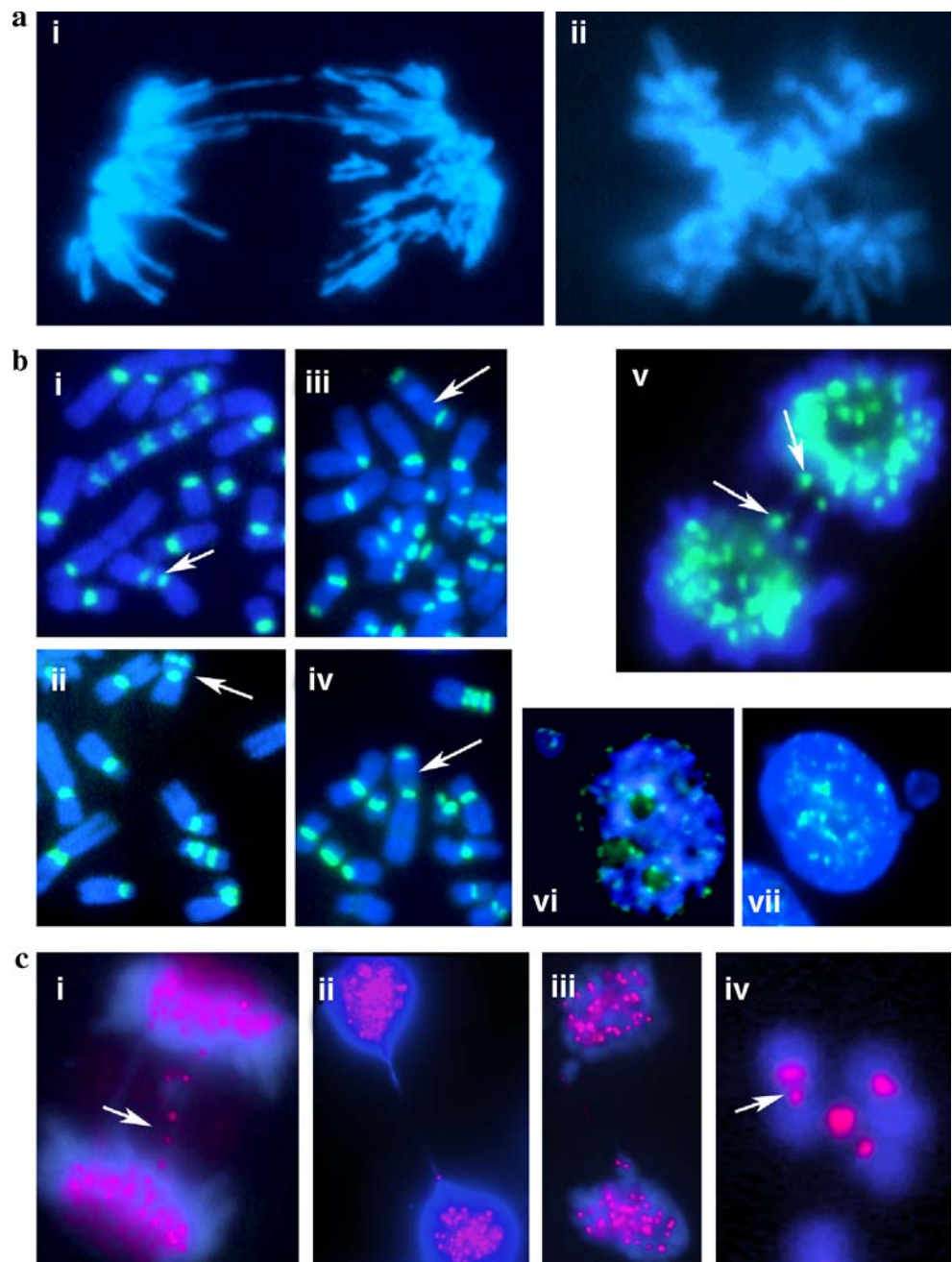
mitotically unstable chromosomes in the OS metaphases relative to that of a normal complement, the frequency of dicentrics were normalized to the diploid level. No dicentrics were found in the Ewing's sarcoma cell line. However, in all the OS cell lines, the presence of several clonal and non-clonal dicentrics/multicentrics strongly suggests the presence of BFB events (Fig. 1b, 1–4, *white arrows* and Fig. 2). SAOS-2 had the greatest frequency of dicentrics with 3.5 dicentrics per 46 chromosomes, followed by MG-63 at 2.7, HOS at 2.2, and U-2 OS at 1.8. Multiple clonal dicentrics were observed in all the cell lines (Fig. 1b, 1–4, *white arrows*). MG-63 also had a

multicentric marker chromosome, which hybridized with the alphoid sequences over its entire length in the pattern of "ladder-rungs." However, in the cell line U-2 OS, we also found a non-clonal tricentric marker (Fig. 1b, 1), which was observed in only one of the 40 metaphases used for dicentric enumeration. The hybridization intensity at the primary constriction in the dicentrics was always brighter than for any of the secondary centromeric regions. PNA FISH on ABC exhibited dual signals on the resolving bridge, representing centromeres migrating to opposite poles of the mitotic spindle (Fig. 1b, 5, *white arrows*).
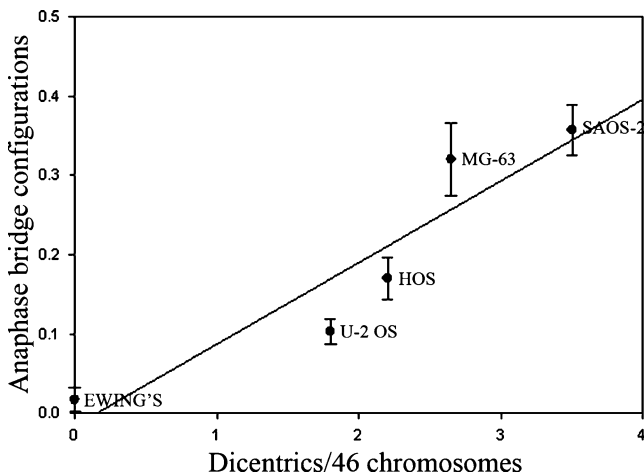
Fig. 2 Correlation between dicentric/multicentric chromosomes and anaphase bridge configurations. A positive correlation was found between the number of dicentrics/multicentrics per metaphase and ABC among the OS cell lines. ABC frequencies are displayed on the *y-axis*, while the number of dicentric/multicentric chromosomes is shown on the *x-axis*. The number of dicentrics was expressed as a ratio where the total number of chromosomes within each of the 40 metaphases counted was used as a denominator. This ratio was then multiplied by 46 to derive a representative measure of the degree of mitotic instability for each cell line relative to the normal chromosomal complement. Correlation is shown with regression analysis using Sigmaplot v9.0. The Pearson correlation coefficient is 0.929. The *p* value is 0.0222

A significant correlation was observed between the cell lines exhibiting the highest number of dicentrics with those that had the highest number of ABC (Fig. 2), highlighting the important role of mitotically unstable chromosomes in generating structural variability ($r=0.929$; $P=0.022$, Pearson
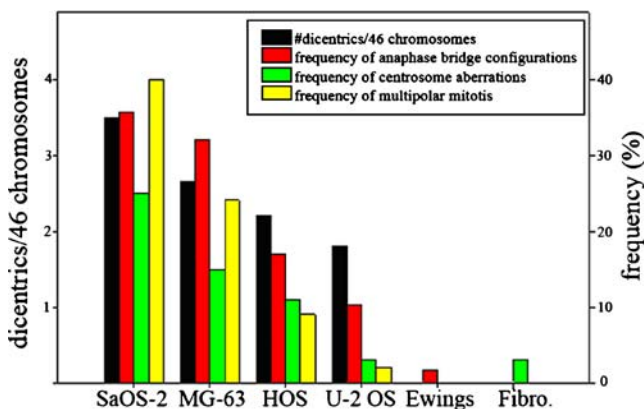

Fig. 3 Frequency of various segregational anomalies in HOS, MG-63, U-2 OS, and SAOS-2 cell lines. Frequencies of ABC, centrosomal anomalies (CEN) and multipolar mitoses (MM) are represented in percentages on the *left y-axis*. The numbers of dicentrics/multicentrics are tabulated on the *right y-axis* per 46 chromosomes. Normal primary fibroblasts and Ewing's sarcoma cell lines were used as negative controls. All of the OS cell lines exhibit a higher percentage of ABCs, CEN, MM, and dicentric/multicentric chromosomes. Regardless of the segregational parameters or the number of dicentrics/multicentrics, SAOS-2 is the most abnormal cell line followed by MG-63, HOS, and then U-2 OS

correlation). SAOS-2, which had the highest number of dicentrics, also exhibited the highest number of ABC. This was followed closely by MG-63 and HOS, and lastly by U-2 OS, which had the lowest number of dicentrics and the lowest frequency of ABC.

mBAND analysis of the four OS cell lines identified inverted duplications in several complex chromosomal rearrangements involving at least three different chromosomes. These observations were found to be in agreement with a previous mBAND study by our group (Lim et al. 2005).

Immunostaining of CENP-A and kinetochore complexes in ABC and dicentrics

Immunostaining for the detection of functional dicentrics, using anti-CENP-A antibody specific for the kinetochore complex, allowed visualization of dicentrics. This analysis suggested that both the regions that exhibit staining for alphoid sequences with PNA FISH were functional and likely to contribute to BFB cycles. Due to the special fixation techniques required to preserve kinetochore protein structure, the morphology of the chromosomes was reduced but permitted two signals to be clearly visualized on the dicentric chromosome (Fig. 1c, 4, white arrow). All of the OS cell lines were tested for functional dicentrics. Only MG-63 and SAOS-2 yielded functional dicentrics for visualization. Immunocytogenetic analysis of cells grown on slides with anti-CENP-A antibody showed evidence of active kinetochores distributed along an anaphase bridge (Fig. 1c, 1, white arrow). Such multiple signals for CENP-A that could be visualized along the resolving bridge showed delayed segregation during mitosis. Internuclear strings between telophase cells were seen with a signal for CENP-A at one end (Fig. 1c, 2); protrusions exhibiting signals in blebs were also seen (Fig. 1c, 3), indicating involvement of dicentrics in bridges that have fragmented after bridge resolution.

Discussion

The structural and numerical chromosomal alterations that characterize OS are important indications of CIN in tumor cells, manifesting as complex rearrangements, amplifications, and ploidy changes, leading to the inter- and intratumoral heterogeneity (Gisselsson 2003). BFB is one mechanism that could drive the patterns of structural aberration and amplification observed in OS. Aberrant double-strand break repair processes can precipitate the formation of structural changes indicative of the BFB cycle. Aberrations within DNA repair pathways such as nonhomologous end-joining may explain the involvement of non-

syntenic chromosomal regions in several OS derivative chromosomes (Lim et al. 2004). These mechanisms were associated with genomic instability and gene amplification in tumors arising in DNA repair knockout mice (Wang et al. 2005). Mutations of genes involved in homology-dependent repair pathways were also shown to be involved in human chromosomal aberration syndromes (Pfeiffer et al. 2004). Formation of dicentric chromosomes resulting from improper repair would then lead to subsequent BFB cycles and the evolution of complex chromosomal rearrangements, as has been observed in the murine model for the development of pro-B lymphoma (Zhu et al. 2002). It was postulated that BFB cycles are responsible for perpetuating the complex chromosomal abnormalities shown to occur in many malignant solid tumor, including head and neck, pancreatic, ovarian carcinomas, as well as leiomyosarcoma, malignant fibrous histiocytoma, and atypical lipomatous tumor (Gisselsson et al. 2000).

Several recent studies have reported the occurrence of BFB intermediates in OS cell lines (Gisselsson et al. 2004; Lim et al. 2004, 2005; Scheel et al. 2001). However, there was no previous study done to determine whether these events occurred at a frequency high enough to explain the role of this process in generating genomic heterogeneity in OS. To determine if BFB is the mechanism underlying the genomic instability in OS, we quantified the frequencies of several parameters such as anaphase bridge configurations, metaphase cells containing mitotically unstable chromosome aberrations such as dicentrics, and the presence of inverted duplications by mBAND analysis, in cell lines with well-documented karyotypes.

All the OS cell lines showed a high frequency of ABC. Moreover, there was a strong association between ABC frequency and the number of dicentrics in the OS cell lines. The finding that the ABC correlated to the dicentrics is a novel observation in OS cell lines, which suggests that dicentric chromosomes may be unstable and more prone to loss or rearrangements through involvement in BFB cycles. Furthermore, it was shown that one functional dicentric was confirmed by the presence of additional kinetochore staining regions in addition to the primary constriction. It is worth noting, however, that not all of the dicentrics are active in the generation of BFBs. Relative to the number of dicentrics and multicentrics in all the OS cell lines, chromosomes exhibiting more than one functional kinetochore complex were found at lower numbers, indicating that some of the dicentrics only harbor a single functional kinetochore. The data indicates that a minimal number of functional dicentrics could be crucial for BFB cycles in moderating the rate of chromosomal evolution, thus allowing the perpetuation of a low-level of de novo genomic changes. The data also indicates that this would enhance phenotypic diversity for further tumor evolution

and adaptation to selective pressures in the microenvironment (Cahill et al. 1999). An elevated number of micronuclei, chromatin strings, and nuclear blebs were also observed in the OS cell lines compared to the control, representing bridges that had broken after anaphase. In a study examining the fate of anaphase bridges in cultured oral squamous cell carcinoma cells in real-time, these structures frequently broke into multiple fragments that became incorporated into micronuclei in the following interphase (Hoffelder et al. 2004). This exclusion of material from fragment bridges is interesting in light of two observations made during the quantification of ABC and dicentrics in the OS cell lines. Both centric and acentric micronuclei were observed in all the OS cell lines. Also, cell lines exhibiting the highest frequency of ABC seemed to have the greatest number of micronuclei, positive for pan-centromeric signals in some cases. These observations can be explained by the results of Hoffelder's study showing that upon fragmentation of an ABC, acentric fragments and centric chromosomes would be produced. In the current OS study, a number of internuclear strings between anaphase bridges and telophase cells were flanked by centromeric signals. This finding was consistent with these strings originating from fragmented anaphase bridges and protrusions originating from bridges that had undergone fragmentation and complete breakage.

A significant finding in this study was the occurrence of interstitial alpha-satellite repeats in dicentrics preferentially located near the ends of the chromosomes. It was previously shown by our group that there was an increased propensity for rearrangements to occur in the pericentromeric region in OS tumors and cell lines (Bayani et al. 2003). It could be postulated that, given the increased frequency of pericentromeric breaks, the resultant sticky ends could become stabilized with processes such as telomere capture (Bosco and Haber 1998), thus resolving some of the BFB cycles. Studies have shown that the alpha-satellite repeats found in the centromeric regions of human chromosomes may facilitate translocations in some solid tumors (Jin et al. 2001) and have been specifically observed as highly amplified sequences in OS (Gisselsson et al. 1999). The pericentromeric regions of certain chromosomes are known to contain duplications and unique low copy repeat sequences, which have been implicated in generating hot spots for recombination and translocations (Eichler 1998).

One of the characteristics of the BFB cycle is the presence of inverted duplications (Lo et al. 2002). Using mBAND analysis, further support for the role of BFB by the presence of inverted duplications in OS was previously shown by our group. In these studies, rearrangements between at least three different chromosomes involving at least three distinct breakpoints were classified as complex

chromosome rearrangements (Lim et al. 2004, 2005). In this study, chromosome 1 analysis of SAOS-2 cell line exhibited the most number of complex rearrangements showing inverted duplications (Fig. 4). In one rearrangement (Fig. 4b), material from 1p35–p36 was duplicated and then inverted in a der(9)t(1;9). In the other rearrangement (Fig. 4c), material from bands 1p21 and 1p32 were duplicated and inverted in a der(13)t(1;13). Based on these aberrations, the repeated breaks and fusions in a BFB cycle could eventually lead to intratumor heterogeneity characterized by amplifications and deletions. Using the findings of SAOS-2, it is possible that a double-strand break telomeric to 1p36, followed by repair using the sister chromatid as the template, could result in the structure of an inverted duplication (Fig. 4b). Together with ongoing impaired DNA repair functions, these regions of the genome may facilitate the appearance of focal regions of amplification early on in the instability process.

Centrosomal defects have been documented in several types of carcinomas including breast, gall bladder, lung, bone, pancreas, colorectal, prostate, head and neck cancers, and have been linked to aneuploidy and CIN in a variety of studies (Saunders 2005). Additional segregational anomalies such as multipolar mitoses were frequently observed in OS. A previous study by our group suggested that an intrinsic disturbance of the chromosomal segregation mechanism is likely associated with centrosome aberrations, thus leading to the numerical instability frequently observed (Al-Romaih et al. 2003). Since spindle multipolarity was shown to be associated with anaphase bridge occurrence in several types of neoplastic cell lines, it has been suggested that this linkage could be due to bridges blocking cytokinesis and leading to aneuploidy (Gisselsson et al. 2002, 2004; Reshmi et al. 2004; Saunders 2005; Stewenius et al. 2005). Both the numerical and structural instabilities observed in OS may be associated with similar molecular events. This possible mechanistic link is the observation that some of the key features of genomic instability occurred together.

Since supernumerary centrosomes and multipolar cell divisions have been demonstrated in more than 90% of sarcomas with complex karyotypes (Gisselsson 2003) and have often been observed in cell lines exhibiting increased ploidy (Ghadimi et al. 2000), we compared the frequencies of ABC observed in this study, with that of centrosomal anomalies and multipolar mitoses previously published by our group (Al-Romaih et al. 2003). In our previous study, the baseline frequencies of these mitotic aberrations were established using normal human fibroblasts. Compared to this control, the OS cell lines HOS, MG-63, and SAOS-2 demonstrated higher frequencies of centrosomal anomalies and multipolar mitoses. It is interesting to note that U-2 OS, the only OS cell line with a functional wild-type p53, exhibited the lowest frequency of centrosomal anomalies and no multipolar mitoses. It was shown that ~50% of OS tumors have mutated p53 (Tsuchiya et al. 2000) and that defects of this checkpoint gene are strongly associated with centrosomal anomalies (Mussman et al. 2000). Thus, it could be reasoned that the functional p53 in U-2 OS probably activates cell-cycle checkpoints. In turn, this would prevent the further accumulation of additional segregational anomalies that is otherwise observed in OS
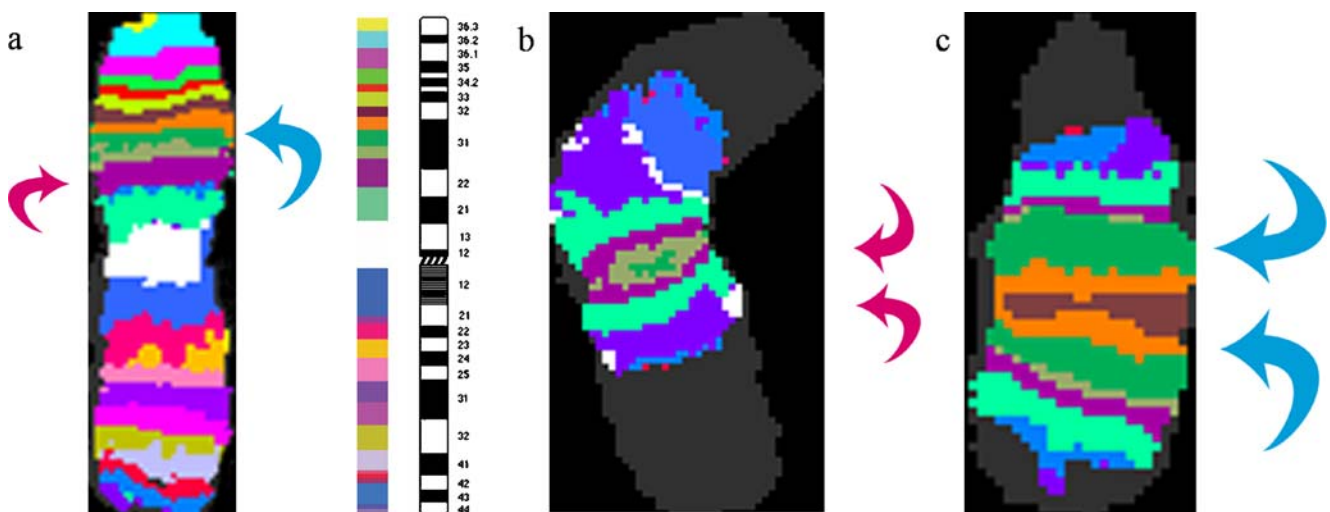

Fig. 4 Inverted duplication of chromosome 1 regions in SAOS-2 OS cell line. Chromosome 1 mBAND probe was hybridized to SAOS-2 and to normal lymphocyte metaphases. a Chromosome 1 false-color banding pattern, false-color ideogram, and corresponding ISCN cytogenetic band assignments (Mitelman 1995) at the 550-band level is shown. The *pink* and *blue arrows* shown by the false-color banding pattern represents the corresponding rearranged regions on the markers. b–c The *pink* and *blue arrows* indicate the orientation of the duplicated region, with the telomeric ends being represented by *arrowheads*. b Material from 1p35–p36 is duplicated and then inverted in a der(9)t(1;9). c Material from chromosome bands 1p21 and 1p32 is duplicated and inverted in a der(13)t(1;13)

cell lines with inactivated p53. Furthermore, the extent of the centrosomal anomalies and multipolar mitoses among the cell lines showed a positive association with each of the parameters of BFB examined in this study. The rank order of the OS cell lines for all the four segregational anomalies was consistent. This association suggests a possible association between the mechanisms that lead to the structural and numerical heterogeneity observed in OS. Double-strand breaks occurring in a cell with dysfunctional repair processes may trigger BFB instability leading to gene deletions, amplifications, and translocations. This in turn can lead to alterations of genes involved in mitotic and cell cycle checkpoints, resulting in segregational errors. This finding can thus explain the structural and numerical heterogeneity observed between individual cells exhibiting complex karyotypes like OS.

The results of this study suggest that the BFB cycle is mechanistically responsible for perpetuation of the CIN phenotype in OS, by facilitating gene amplification and rearrangements conferring a selective advantage. Such genomic instability may drive tumor evolution and serve as a molecular basis of cancer progression.

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