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Short communication

# Combined spectral karyotyping, multicolor banding, and microarray comparative genomic hybridization analysis provides a detailed characterization of complex structural chromosomal rearrangements associated with gene amplification in the osteosarcoma cell line MG-63

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## Abstract

The advancement of fluorescence in situ hybridization–based assays has permitted more refined delineation of chromosomal loci involved in complex chromosomal rearrangements (CCRs) and gene amplification. In this detailed molecular cytogenetic analysis, spectral karyotyping (SKY), multicolor banding (mBAND) analysis, and microarray comparative genomic hybridization (CGH) were used to refine the analysis of chromosomes with amplifications and small intrachromosomal rearrangements such as inverted duplications and interstitial deletions present in the osteosarcoma cell line MG-63. SKY analysis has limited resolving power to delineate cryptic chromosomal rearrangements, so mBAND assays were performed for a subset of chromosomes (i.e., 6, 8, 17, and 20). Of the 10 clonal CCRs analyzed in detail with mBAND, 5 were found to have rearrangements between 8q24 and either 6p23–pter or 6p21, with multiple copies of this translocation inserted at various sites in the different chromosomes. In two CCRs, 6p21 and 8q24 generated an alternating pattern of mBAND probe hybridization, indicating the presence of a large coamplified repeat unit within homogeneously staining regions. Microarray CGH analysis demonstrated focal high-level amplification of 8q23–q24, 6p22–pter, and 6p21, in agreement with the pattern of chromosome subband gains identified with mBAND. Thus, sequential SKY, mBAND, and microarray CGH provided a comprehensive description of some of the intricate chromosomal aberrations present in the complex MG-63 karyotype and permitted reconstruction of the fine structure of the genomic rearrangements, thus providing some important mechanistic clues concerning the details of the amplification process in tumors. © 2004 Elsevier Inc. All rights reserved.

## 1. Introduction

Osteosarcoma (OS) is the most common of primary bone malignancies, with an initial peak incidence in the pediatric and early adult population (age 15–29 years) and a second peak incidence in later adult life. OS tumors and cell lines exhibit karyotypes with a high degree of aneuploidy and unbalanced structural rearrangement that have made it difficult

to identify specific recurrent chromosomal aberrations. Conventional cytogenetic analyses have revealed complex karyotypes with cryptic structural rearrangements and unidentifiable marker chromosomes [1,2]. Spectral karyotyping (SKY) [3] has recently been used for detailed characterization of OS structural chromosomal rearrangements and identified chromosomes 6, 8, 17, and 20 to be most frequently involved in these aberrations [4,5]. One limitation of SKY analysis is that it cannot assign chromosomal subregional identities below the level of 1–2 Mb [6]. Furthermore, because of the high degree of structural complexity of OS and equivocal chromosomal morphology, conventional

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cytogenetic analysis performed with SKY may not provide sufficient banding information for the precise identification of the chromosomal breakpoints involved in the complex chromosome rearrangements of OS karyotypes. This drawback has recently been addressed with high-resolution multicolor banding (mBAND) analysis, which can be used to more accurately identify translocation breakpoints, as well as small insertions, deletions, and inversions associated with more complex aberrations [7–9].

Metaphase comparative genomic hybridization (CGH) studies have identified frequent gains and amplifications at 1p32, 1q21, 5p13, 6p12, 8q24, 8cen–q13, 17p11.2, and Xp21 and frequent losses at 6q16, 10p12pter, and 10q22q26 [1,10]. More specifically, amplifications at 8q, 6p, and 17p have been the focus of more recent work using traditional metaphase-based CGH (reviewed by Sandberg and Bridge [1]) as well as microarray CGH [11]. Advances in mapping resolution using microarray CGH [11–13], have greatly improved resolving power in comparison to metaphase CGH, and provide more details regarding both the complexity and exact location of genomic rearrangements leading to copy number imbalances.

Using these complementary techniques, we refined breakpoint locations for chromosomes 6, 8, 17, and 20 in the OS cell line MG-63 and revealed the origin of inserted material from some of these chromosomes, which contributed to the amplification of regions delineated with microarray CGH. Furthermore, the patterns of amplification and relative genomic loss or deletion in flanking regions show that high-resolution microarray CGH can provide important clues concerning the nature of gene amplification at the genomic level.

## 2. Materials and methods

### 2.1. Cell line and normal reference specimens

The cell line MG-63 (CRL-1427), established originally from a 14-year-old male pediatric OS tumor, obtained from American Type Culture Collection (Rockville, MD) and cultured according to the supplier's instructions, was consistent with previous cytogenetic findings concerning this cell line [4,5]. Metaphase spreads were prepared from passage 19 for the current study, with standard Colcemid treatment and methanol–acetic acid fixation. Metaphase spreads from a normal peripheral blood culture were also prepared. High molecular weight DNA was also extracted from both sources by means of standard phenol–chloroform extraction.

### 2.2. mBAND

Metaphase slides from MG-63 and normal lymphocytes were hybridized according to the manufacturer's instructions, with the mBAND paints (MetaSystems, Altlußheim, Germany). Each chromosome paint is derived of differentially labeled DNA from a specific chromosomal band, thus

revealing a specific hybridization pattern along the length of the chromosome [7–9,14]. Because chromosomes 6, 8, 17, and 20 were shown with SKY to be frequently rearranged in this cell line [5], these were the chromosomes we analyzed. The use of normal chromosomes allowed optimal false-color classifiers to be generated (Fig. 1A), as required by the ISIS imaging software (MetaSystems). For each experiment, images of at least 20 metaphases were captured using a Zeiss Axioskop 2 Plus microscope (Zeiss Canada, Ontario, Canada) equipped with the appropriate filter sets and were analyzed with MetaSystems ISIS imaging software.

### 2.3. Microarray CGH analysis

Microarray-based comparative genomic hybridization was performed according to the manufacturer's protocols on human genomic bacterial artificial chromosome (BAC) microarrays obtained from Spectral Genomics (catalog no. KTH3-1400; Spectral Genomics, Houston, TX). The microarrays consisted of 1400 clones spotted in duplicate from the RPCI-1, RPCI-3 P1-derived artificial chromosome (PAC), and the RPCI-11 bacterial artificial chromosome (BAC) libraries at a resolution of 2–4 Mb. Briefly, equal amounts of normal female reference DNA and DNA from MG-63 were differentially labeled and hybridized to the arrays. A reverse experiment was also performed, as required by the manufacturer and as indicated for the accompanying software. The arrays were washed and scanned using the Axon GenePix 4000A confocal scanner. Generation of final microarray CGH profiles was based on the Spectral Genomics Array Software; however, more stringent normalization parameters were applied. Normalization scripts were based on previous cytogenetic data [5]. Chromosome 2 was not found to be altered, and so the mean intensity ratios of spots mapping to chromosome 2 were used to determine baseline levels; thresholds of gain and loss were determined to be at two standard deviations (0.14) from the mean. Regions of high-copy amplification typically exhibited ratios of above 2.5; areas of chromosomal imbalance due to lower copy number gains exhibited ratios between 1.14 and 2.5. Regions of loss were identified as ratios less than 0.86. Areas of equal and opposite ratio imbalance indicate copy number change. Final microarray CGH profiles reflect the change in dye ratios for both experiments.

## 3. Results

Ten derivative chromosomes were identified with mBAND analysis to be clonal rearrangements between at least three different chromosomes, involving at least three distinct breakpoints; these were thus classified as complex chromosome rearrangements (CCRs) [15]. Additional changes observed were not included in this report due to their nonclonal nature. All 10 of the clonal CCRs had been

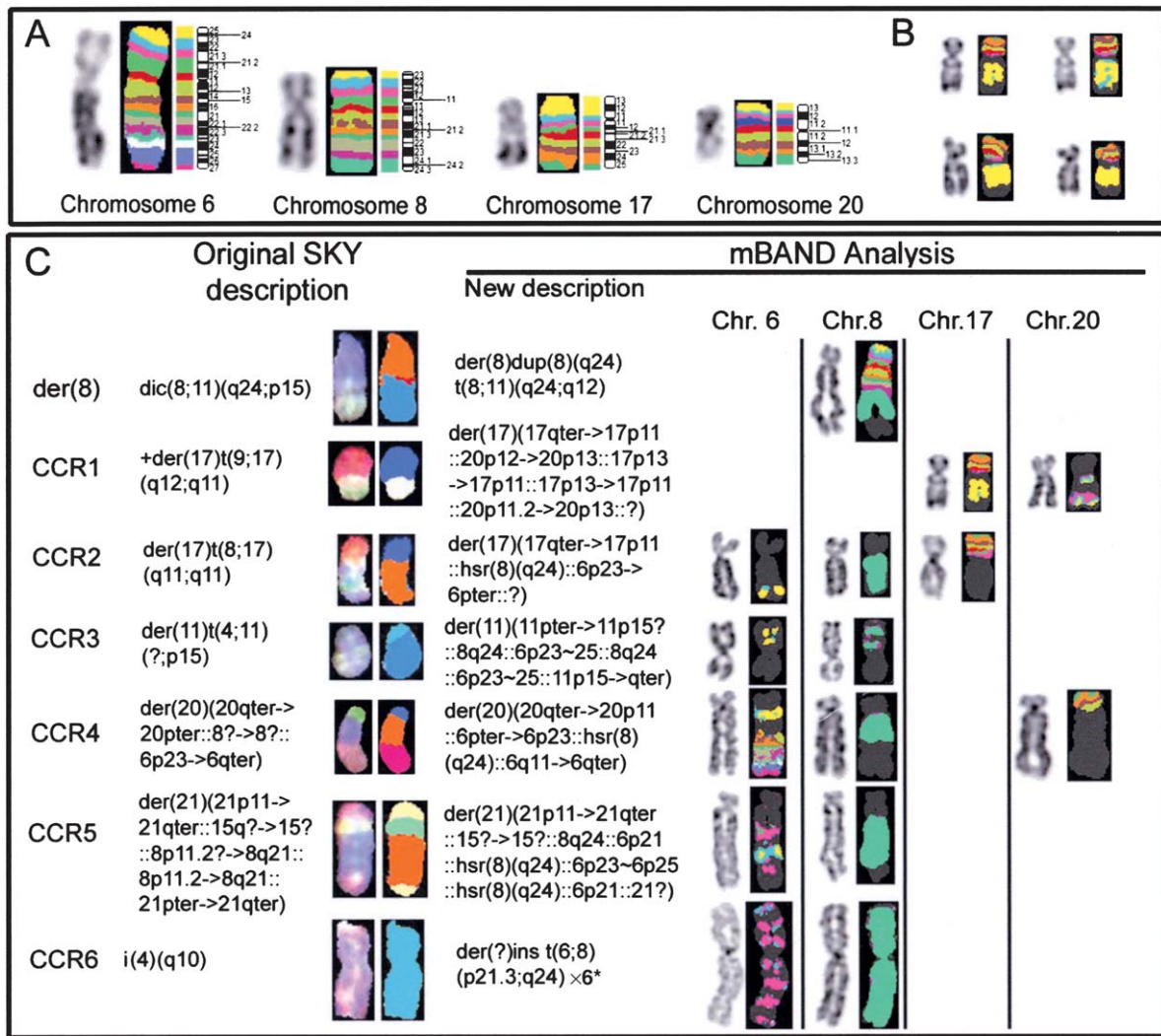


Fig. 1. (A) Normal chromosomes 6, 8, 17, and 20 showing, for each chromosome (left to right) the inverted 4',6-diamidino-2-phenylindole (DAPI) image, the false-color banded image, the false-color classifier used for analysis, and the ideogram showing cytogenetic band assignments at the 550-band level. For clarity, not all subband assignments are labeled. (B) Consistent mBAND results for CCR1: inverted DAPI and false-color banded images of CCR1, captured from four different metaphases, demonstrated little variability in the banding pattern. (C) Molecular cytogenetic analysis with mBAND6, 8, 17, and 20 of complex chromosome rearrangements found in cell line MG-63. Cytogenetic descriptions with SKY and with mBAND are compared for seven of the 10 chromosomes found to stain positively with at least one of the five probe kits. For each chromosome, the SKY RGB (red-green-blue) and false-color images are shown, as well as the inverted DAPI and mBAND false-color banding pattern images. If orientation of a chromosomal segment was not clear, the region is identified by the breakpoints and the approximate symbol (~). der(8): mBAND8 revealed a duplication of 8q24 and revised the breakpoint on chromosome 11. CCR1: Analysis with mBAND17 shows that the short arm comprises material from 17q rather than 17p. Chromosome 9 does not appear to be involved, but instead, small insertions of 17pter and 20pter are each present as duplications. Note the inverted orientation of 17q and 20p11.2~p13. CCR2-CCR4: Chromosomes show complex rearrangements involving regions 6p23~pter and 8q24. In CCR2, there is a whole-arm gain of 17q. CCR5-CCR6: Multiple insertions of regions 6p21 and 8q24 on the same translocation products. In CCR6, six insertions of the (6;8) translocation are found (asterisk). (D) mBAND6 and mBAND8 hybridization in CCR5 and CCR6 (left to right): mBAND6 false-color banding, inverted DAPI, SpectrumOrange-labeled probe for 6p21; diethylaluminum chloride (DEAC)-labeled probe for 6p23~pter (CCR5) or 6p21.3 (CCR6); and mBAND8 false-color banding, inverted DAPI, and TexasRed-labeled probe for 8q24. The DEAC signals in the two CCRs are differentially classified due to differences in the fluorescence intensity ratio relative to Cy3. Analysis with mBAND6 shows small insertions in CCR5 and a distinct ladder amplification pattern in CCR6. mBAND8 revealed more homogeneous staining, but gaps can be seen between signals on CCR5 and, although less obvious, the ladder effect in CCR6 is also seen with mBAND8; however, the overrepresentation of 8q24 amplification resulted in classification saturation of this region (green). In previous studies [5] this aberration was assigned as i(4)(q10) with inconclusive classification. (E) Microarray CGH profiles for chromosomes 6 and 8. Centromeres and amplified regions are marked. CGH ratio data from two independent microarrays are shown. Red and blue traces represent normalized data from the microarray in which the MG-63 DNA sample was labeled with Cy3 or Cy5, respectively. Each data point along the ratio plot represents the normalized ratio of individual clones linearly ordered such that the leftmost clone is consistent with the p-arm terminus and the rightmost clone is consistent with the q-arm terminus. Because the normalized Cy5: Cy3 ratio is computed for both microarrays, loss of a particular clone in MG-63 is manifested as the simultaneous deviation of the ratio plots from a modal value of 1.0, with the red ratio plot showing a positive deviation (upward) and the blue ratio plot showing a negative deviation at the same locus (downward). Conversely, DNA copy number gains show the opposite pattern. Areas of equal and opposite ratio imbalance indicate copy number change without any indication of experimental error due to hybridization inequalities. Where MG-63 DNA was labeled with Cy5, regions of high-copy amplification typically exhibit ratios above 2.5. Areas of chromosomal imbalance due to lower copy number gains exhibit ratios between 1.14 and 2.5, and regions of loss would exhibit ratios below 0.86. Chromosome band locations correspond to those of the mapped location of BAC clones as assigned by the U.S. National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Chromosome 6 profile shows distinct regions of focal amplification, corresponding to the gains of 6pter and 6p21 seen with mBAND6 (false-color classification bars shown below). Chromosome 8 profile shows high-level amplification of 8q23~q24, correlating with mBAND8 analysis of chromosomes CCR2-CCR6.

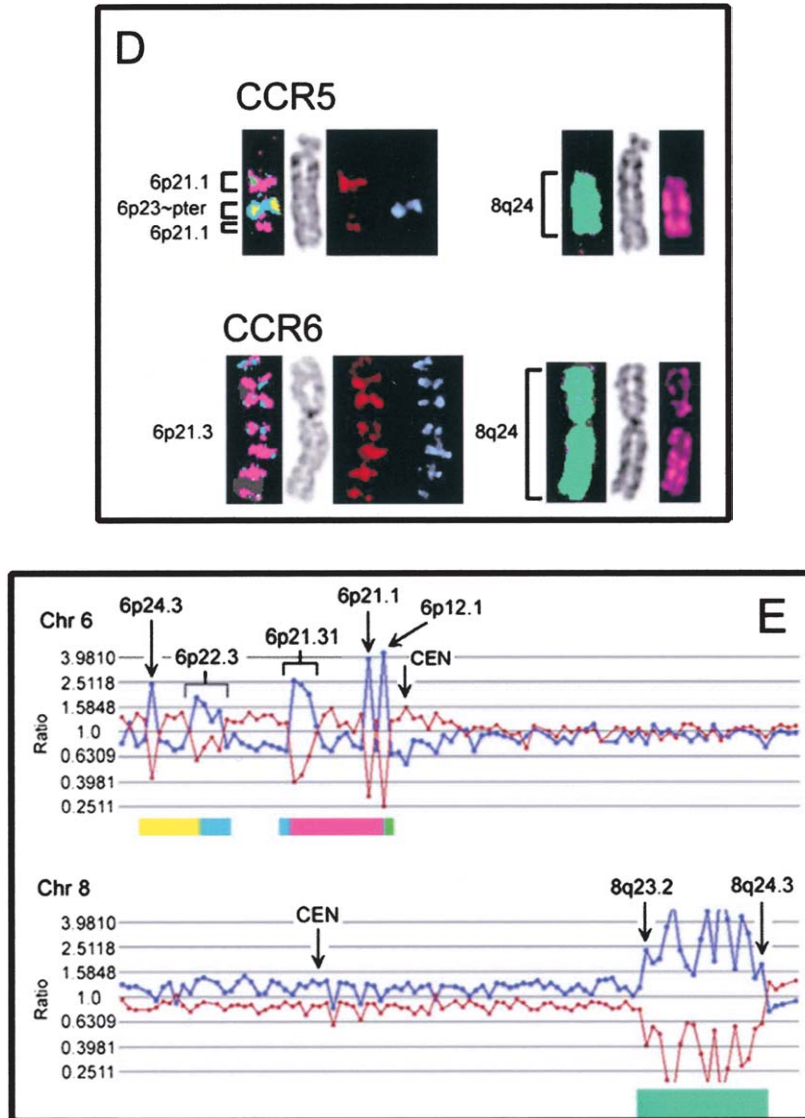


Fig. 1. Continued

previously detected with SKY [5], thus indicating that the clonal markers in the MG-63 cell line are stable, despite the fact that different passages of the cell line were used for SKY and for mBAND. Cell-to-cell variability of CCR banding patterns was minimal, as shown for CCR1 in Fig. 1B. Seven of the 10 clonal CCRs had refined cytogenetic descriptions, which are summarized in Fig. 1C. Using the mBAND17 probe, small insertions of 17p11~p13 and 20p12~p13, not previously identified, could now be detected in CCR1, and chromosome 9 was excluded from involvement in this translocation. The small insertions were also identified with mBAND to be in inverted orientation, which was not apparent with SKY and G-banding. In CCR1 and CCR2, the pericentromeric breakpoint was localized with mBAND17 to occur at 17p11, resulting in whole-arm gain of 17q. Although there was no net copy number change of 17p11~p13, the region was rearranged and inserted at two positions on CCR1; mBAND17 did not detect any normal

chromosome 17, and two copies of chromosome 20 were identified with mBAND20.

Six different chromosomes showed gain of 8q24 [der(8) and CCR2–CCR6] and five of these (CCR2–CCR6) involved additional focal gains of 6p21 or 6p23ter~pter (or both). Insertions of 8q24 were detected at two positions in CCR3, and insertions of 6p23~pter were also found at these locations. In chromosomes CCR2 and CCR4, the 8q24 probe exhibited extensive hybridization over a much larger region than signals observed from control metaphases. Thus, these aberrations were described as hsr(8)(q24). Note that, in both of these chromosomes, the region 6p23~pter was found to be adjacent to 8q24. Further complexity in translocation between chromosomes 6 and 8 can be seen in CCR5 and CCR6, in which 6p21 and 8q24 appear to be translocating concurrently. Analysis of CCR6 with mBAND6 shows a distinct ladder amplification pattern. Hybridization of mBAND8 probes showed a similar staining pattern for

CCR6 (Fig. 1D); however, the overrepresentation of 8q24 amplification resulted in classification saturation of this region (green). The same is true for CCR5: the false-color bands corresponding to region 6p23~pter appear to overlap with the 8q24 staining, but hybridization images (Fig. 1C) show distinct gaps between 8q24 signals, likely corresponding to the position of 6p23~pter material. The colocalization of chromosome 6 and 8 material on the same CCRs was confirmed with fluorescence in situ hybridization (FISH) using the respective whole-chromosome paints (Vysis, Downers Grove, IL) (data not shown).

Array CGH of chromosome 6 (Fig. 1E) showed several distinct regions of focal amplification, occurring at 6p24.3, 6p22.3, 6p21, and 6p12.1. The resolution of the BAC microarray is superior to that of mBAND, so it is evident that additional gains were detected with microarray CGH. Notably, many of these regions of focal amplification on 6p were closely flanked by regions of deletion. The pattern in the region 6pter~q14 is consistent with an area of gross loss interspersed with focal amplification at the several distinct loci, which may have occurred through subsequent events. This observation of focal 6p amplification alternating with interstitial deletion was not evident in the mBAND data, which showed extensive gains of the 6pter region and 6p21. The loss, observed with CGH to be between the gains at 6p24.3 and 6p22.3, corresponds to a subregion of 6p24.3. It therefore would not be classified differentially from the 6p24.3 gain in mBAND analysis. The loss identified with CGH at 6p21.1~p21.2 was also not detectable with mBAND, because this region is not differentially labeled from 6p21.31, a region found to be amplified.

The high-level amplification of 8q24 seen with mBAND8 was observed in the microarray CGH data (Fig. 1D). The profile for chromosome 8 showed that at least five hybridization spots, mapped within the 8q24.12~q24.3 region, each exhibited a ratio greater than 4.0. The overall gain of chromosome 8 is evident from the normalized microarray data output, and corroborated with the mBAND data, which showed at least four copies of 8pter~qter, two being normal and two involving a terminal translocation from 11q. Much like the focal gains on 6p, the additional high-level amplification at 8q24 may also reflect later events, thus exhibiting focal amplifications superimposed upon the background of whole chromosome gain.

#### 4. Discussion

Our findings show that mBAND analysis greatly improves the cytogenetic description of complex chromosomal rearrangements when used in conjunction with 24-color FISH assays such as SKY. These multicolor analytical techniques rely on a chromosome-specific labeling strategy, analysis of digitized images, and conventional banding information to analyze structural changes. When complex rearrangements take place involving small genomic regions,

combinatorial FISH assays, particularly 24-color painting probes, have inherent limitations and drawbacks stemming from fluorescence flaring [16,17] and the size of the hybridization target. Misclassification of chromosomal identities due to fluorescence flaring effects has been seen with multicolor karyotyping [16,17] and may occur between non-homologous chromosomes participating in a translocation. The contribution of fluorescence, at the translocation site, from nonhomologous chromosomes can either obscure a true insertion or create a false insertion. Furthermore, small insertions may not be detected due to fluorescent flaring, the size of the insertion and chromosome condensation, or a combination of some or all of these factors. Alternatively, if the labeling scheme of the larger fusion partner is partially shared by a small insertion, then flaring effects of the larger segment at the breakpoint region may saturate the weaker spectra of an insertion. This effect was observed with the SKY classification of CCR2 and CCR4, in which the signal from 6pter was likely overwhelmed by saturation with high-level amplification of flanking 8q24 material. Because the SKY dye combinations of chromosomes 6 and 8 are similar, misclassification can occur for either chromosome or for other chromosomes that share those similar dye combinations (including chromosome 4, as in the case of the SKY description of CCR6). Thus, mBAND detection of these 6p insertions indicates the greater resolving power of this technique in comparison to SKY.

Analysis obtained from chromosomes 6 and 8 revealed complex and recurrent translocations, specifically between 8q24 and either 6p23~pter or 6p21. The accompanying microarray CGH profiles confirmed these findings, with the pattern of interspersal gain indicating coamplification of subchromosomal regions of 6p and 8q24. Analysis of the microarray CGH data indicates that the two most prominent 6p amplicons detected with mBAND were 6p22.3 and 6p21.31. These regions each comprise ~6 Mb of genomic DNA, and will therefore contribute significantly less hybridization signal than the larger ~32-Mb amplicon arising from the 8q23~q24 region. This inequality may have led to the masking of the 6p signal in SKY, and the subsequent homogeneous false-color classification of 8q24, observed with both SKY and mBAND analysis. Of the nine rearranged chromosomes analyzed with mBAND in this study, five involved a translocation event between 8q24 and either 6p23~pter or 6p21, and multiple copies of this translocation were inserted at various sites along the derivative chromosomes (CCR2–CCR6).

The integrated data from SKY, mBAND, and microarray CGH analysis suggest that the observed ladder-like fluorescence staining pattern (CCR5 and CCR6) may be indicative of a breakage–fusion–bridge (BFB) mechanism of coamplification of the nonhomologous chromosome regions. Similar ladder-like structures have been observed to be associated with gene amplification via a BFB mechanism, in human and murine tumors [18–20]. If the breakpoints marking the amplicon boundaries fall outside of the region for which a

FISH probe is specific, the resulting pattern of hybridization appears as periodic signals. It is conceivable that, if a translocation of 6p22.3 or 6p21.31 with 8q23~q24 were being coamplified via a BFB mechanism, it would be much easier to resolve the ladder pattern in the amplification of 6p, in that these are much smaller regions. The intervening gaps would correspond to the ~32-Mb region from 8q, which results in greater separation between 6p signals. Conversely, with the 8q ladder amplification, the intervening gaps correspond to the much smaller 6p regions, causing the 8q signals to be much closer together, and the flaring effects become more significant. This results in a more homogeneous fluorescence staining and false-color classification.

Gains of 6p have also been reported in OS by our group [11] and others [4,21–24]; however, the high resolution of the Spectral Genomics microarray allowed more detailed analysis of these gains in MG-63 and revealed multiple regions of focal amplification of 6p, not previously reported. These were resolved to occur at 6p24.3, 6p22.3, 6p21.31, 6p21.1, and 6p12.1. The broader regions of gain seen at 6p22.3 and 6p21.31 are each represented by at least three different BAC clones, thus likely representing the same regions of gain seen with mBAND6. These distinct amplicons have not been extensively described in OS; however, a 6p21.3 amplicon has been reported in high-grade ductal carcinoma in situ [25], as well as diffuse histiocytic lymphoma [26], and high-level amplification within 6p21 has been shown in glioblastoma [27] and gastric cancer [28,29]. This amplicon complexity on chromosome 6p is a novel finding in the OS cell line MG-63; in addition, our laboratory has performed microarray CGH on a cohort of primary OS tumors, and a minimal region of gain was identified at 6p21 [11].

Our analysis with mBAND probes for chromosomes 6, 8, 17, and 20 provided an improved karyotype description and a more accurate assignment of some of the small insertions, inversions, and interstitial alterations that were not detected with SKY analysis. Furthermore, we showed with CGH using genomic BAC microarrays that focal amplifications of 2–4 Mb can now be readily resolved. This analysis shows that microarray CGH and mBAND applied in parallel provide a comprehensive description of the complex chromosomal aberrations that characterize the OS cell line MG-63 and can provide some important mechanistic clues concerning the details of the amplification process in general.

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