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Ladder-Like Amplification of the Type I Interferon Gene Cluster in the Human Osteosarcoma Cell Line MG63

Narasimha Rao V. Marella¹, Michael J. Zeitz¹, Kishore S. Malyavantham¹, Artem Pliss¹, Sei-ichi Matsui², Sandra Goetze³, Juergen Bode³, Ivan Raska⁴, and Ronald Berezney^{1,*}

¹Department of Biological Sciences, University at Buffalo, State University of New York, Buffalo, NY 14260, USA

²SKY Core Resource Facility, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

³HZI, Helmholtz Centre for Infection Research/Epigenetic Regulation, Inhoffenstr. 7, -38124 Braunschweig, Germany

⁴First Faculty of Medicine, Charles University in Prague and Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Albertov 4, 128 00 Prague, Czech Republic

Summary

The organization of the type I interferon (IFN) gene cluster (9p21.3) was studied in a human osteosarcoma cell line (MG63). Array comparative genomic hybridization (aCGH) showed an amplification of ~six-fold which ended at both ends of the gene cluster with a deletion that extended throughout the 9p21.3 band. Spectral karyotyping (SKY) combined with fluorescence in situ hybridization (FISH) identified an arrangement of the gene cluster in a ladder-like array of 5–7 “bands” spanning a single chromosome termed the “*IFN chromosome*”. Chromosome painting revealed that the IFN chromosome is derived from components of chromosomes 4, 8 and 9. Labeling with centromeric probes demonstrated a ladder-like amplification of centromeric 4 and 9 sequences that colocalized with each other and a similar banding pattern of chromosome 4, as well as alternating with the IFN gene clusters. In contrast, centromere 8 was not detected on the IFN chromosome. One of the amplified centromeric 9 bands was identified as the functional centromere based on its location at the chromosome constriction and immunolocalization of the CENP-C protein. A model is presented for the generation of the IFN chromosome that involves breakage- fusion- bridge (BFB) events.

Keywords

human osteosarcoma cells; interferon (IFN) gene cluster; gene amplification; IFN chromosome; fluorescence in situ hybridization (FISH); spectral karyotyping (SKY); array comparative genomic hybridization (aCGH)

Introduction

Osteosarcoma is a highly malignant bone sarcoma seen in children with a peak incidence occurring during adolescence, thus establishing a relationship between bone development

*Correspondence: Email: berezney@buffalo.edu; Tel: 716-645-2363 ext 154; Fax: 716-645-2975.

Present Address of Sandra Goetze³: Center for Model Organism Proteomes, Institute of Molecular Biology, University of Zurich, Winterthurerstrasse 190 8057 Zurich Switzerland

Present Address of Kishore S. Malyavantham¹: IMCO Diagnostics Ltd, 60 Pine View Drive, Buffalo, NY 14228

and tumor formation (Unni, 1998, Stock et al., 2000, Arndt and Crist, 1999, Wang, 2005, Picci, 2007). Various cytogenetic and molecular analysis studies have shown that osteosarcomas are characterized by numerous and complex chromosomal aberrations and ploidy changes ranging from translocations to deletions, duplications, double minutes and homogenously stained regions (hsr's) (Bridge et al., 1997, Ladanyi and Bridge, 2000, Ragland et al., 2002, Lim et al., 2004, Lim et al., 2005). Advances in chromosomal analysis such as spectral karyotyping (SKY) (Schrock et al., 1996, Macville et al., 1997) and comparative genomic hybridization (CGH) techniques (Pinkel et al., 1998, Kallioniemi et al., 1992, Vissers et al., 2003, Inazawa et al., 2004) have led to a further characterization of the complex chromosomal abnormalities and DNA copy number changes in a variety of human cancers.

The type 1 interferon gene cluster spans about 400 Kbp within the chromosome 9p21-p22 region (Diaz et al., 1994, Diaz, 1995). It consists of one beta gene (at the telomeric end), one omega gene, 13 alpha genes and 11 pseudogenes ((Diaz et al., 1994, Diaz, 1995) ;see Figure 1 for gene map). The 9p21-p22 region has been well documented to contain rearrangements in a variety of solid and hematological cancers (Olopade et al., 1992a, Diaz et al., 1990, Fountain et al., 1992, Center et al., 1993, Cheng et al., 1993, Stadler et al., 1994, Cairns et al., 1994, Rakosy et al., 2008, Bode et al., 2000). In osteosarcomas, regions containing tumor suppressor genes are often mutated or deleted (Miller et al., 1996a, Patino-Garcia and Sierrasesumaga, 1997, Tsuchiya et al., 2000, Toguchida et al., 1992, Miller et al., 1996b). Frequent deletions within 9p21-p22 hinted at the possibility that this region harbors tumor suppressor genes (Bohlander et al., 1994, Cairns et al., 1993, Bode et al., 2000). The cell cycle controlling tumor suppressor genes *CDKN2A/MTS-2* (that code for the p15/p16 proteins, respectively) located in this critical region are possible candidates whose deregulation might lead to tumor progression (Zhang et al., 1996, Bonetta, 1994, Southgate et al., 1995, Hartwell and Kastan, 1994, Jagasia et al., 1996, Park et al., 2002, Usvasalo et al., 2008, Okamoto et al., 1994, Nielsen et al., 1998, Kohno and Yokota, 2006a). Consistent with this proposal it was reported that p16 is deleted in MG63 human osteosarcoma cells (Miller et al., 1996a, Park et al., 2002).

In this study we have deciphered the organization of the type I interferon gene cluster in the human osteosarcoma cell line MG63 utilizing a combination of fluorescence in situ hybridization (FISH), chromosome painting, SKY and CGH techniques. Bacterial artificial clones (BACs) as well as cosmid probes to the individual genes were used to study this entire region. We found that the IFN gene cluster is highly amplified (~six fold). Moreover, this level of amplification is specific for the IFN gene cluster as sequences flanking both ends of the cluster were deleted. FISH analysis revealed that the IFN gene cluster is arranged as a ladder of 5–7 repeating bands that span from one end of the chromosome to the other. This chromosome, termed the “*IFN chromosome*” is composed of elements from chromosomes 4, 8 and 9. Centromere amplification of chromosomes 4 and 9 and chromosome 4 were also observed in a similar ladder of 5–7 repeating bands that alternate with the IFN gene cluster bands. Staining with CENP-C antibodies demonstrated that centromere 9 is the true centromere at the constriction of this chromosome. The ladder-like amplification of the IFN gene cluster and centromeres as well as the presence of the fragile sites FRA9A and FRA9C (Moriarty and Webster, 2003, Buttel et al., 2004) near the gene cluster leads us to propose that the phenomenon of BFB is responsible for the generation of this complex chromosome.

Materials and Methods

Cell culture

The cell lines used in these studies (human osteosarcoma MG63, U-2-OS, Saos-2, SK-ES-1 and normal human diploid fibroblasts, WI38) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Advanced DMEM (Invitrogen, Carlsbad, CA) with 2.5% fetal bovine serum at 37°C in a 5% carbon dioxide (CO₂) substituted incubator.

Metaphase spread preparation

Cells were arrested in metaphase by incubation with the reversible microtubule inhibitor, nocodazole (0.04 µg/ml) for 2–3 h (Zieve et al., 1980). The detached mitotic cells were collected by vigorous shaking and other adherent cells were released by mild trypsinization for 2–3 min. The cells were then pelleted by centrifugation at 800 rpm for 5 min, resuspended in a hypotonic solution and incubated at 37°C for about 10 min to allow swelling of the cells. Following centrifugation, a suspension of ice-cold methanol: acetic acid (3:1) was added drop-by-drop to the cells and subjected to centrifugation at 800 rpm for 5 min. This process was repeated 3 times to quantitatively remove the cell debris. The final cell pellet was resuspended in 2 ml of the fixative and this cell suspension was stored for use at –20°C for up to six months. To prepare metaphase spreads, 20–30 µl of the cell suspension was dropped onto a coverslip and air dried to allow for even spreading of the chromosomes.

Array comparative genomic hybridization (aCGH) analysis

aCGH array of ~6000 RPCI-11 BAC clones from the RP-11 human BAC library, chosen by virtue of their sequence-tagged site (STS) content and association with cancer, was generated as described previously (Cowell and Nowak, 2003, Nowak et al., 2005). Each clone was spotted in triplicate at intervals which corresponds to ~0.5 Mbp genomic resolution. MG63 genomic DNA was prepared from cell pellets using the DNeasy Isolation Kit (Qiagen, Inc.) Two control DNA pools were used for BAC CGH array analysis. The male control and female control pools each contained DNA from 15 cytogenetically normal individuals. For procedural quality control, all analyses were performed as sex-mismatch hybridizations. This allows observation of chromosome X and Y copy number differences. 1 µg of control and MG63 genomic DNA was random primer labeled using a BioPrime DNA labeling kit (Invitrogen, Inc.) for 3h at 37°C with the appropriate Cy dye (Cy3 or Cy5). After ethanol precipitation, the probes were resuspended in H₂O, combined and purified of unincorporated Cy dye by passage over a Qiagen spin column. The labeled probes were dried and stored at –20°C until hybridization. Hybridization to the CGH arrays was conducted for 16 h at 65°C. After hybridization the slide was washed in decreasing concentrations of SSC and SDS, followed by 0.1×SSC, 95% ethanol and centrifugal drying for 3 min. The hybridized slides were scanned using an Affymetrix 428 Scanner to generate high-resolution (10 µm) images for both Cy3 and Cy5 channels. Image analysis was performed on the 16-bit raw image files using ImaGene (V4.1BioDiscovery).

Spectral karyotyping (SKY) analysis

Preparations of metaphase chromosome spreads were subjected to the SKY procedure recommended by Applied Spectral Imaging (ASI, Carlsbad, CA). The images were captured using a combination of Rhodamine, Texas-Red, Cy5, FITC and Cy5.5 filter sets mounted on a Nikon fluorescence microscope equipped with a spectral cube and an interferometer module. Karyotypic analysis of the images was carried out using the SKY View software.

Probes and antibodies

Individual chromosome paints and centromere specific probes were obtained from Vysis (Downers Grove, IL). BAC probes spanning the interferon genes were obtained from the human RP-11 BAC library at Roswell Park. Antibodies to the centromeric proteins were a kind gift from Dr. William Earnshaw, Wellcome Trust for Cell Biology, University of Edinburgh, UK.

Fluorescence In situ Hybridization (FISH)

A map showing the interferon gene cluster and the positions of the DNA probes used in these studies is presented in Figure 1. DNA extracted from the BAC and cosmid probes spanning the interferon gene cluster were labeled with either biotin or digoxigenin using the biotin-16- dUTP or digoxigenin-11-dUTP nick translation kits from Roche Applied Science (Indianapolis, IN) as per manufacturer's protocol. Probe fragments above 4 Kbp were removed by purification through a min-elute column from Qiagen (Valencia, CA) to obtain a size range favorable for hybridization. The probes were then subjected to a standard hybridization procedure (Henegariu et al., 2001). Briefly, the cells were dropped onto coverslips and the metaphase spreads were air dried and dehydrated in 70%, 100% ethanol. Following denaturation with 70% formamide/2×SSC at 75°C for 1 min on a thermocycler, the cells were again dehydrated using 70%, 100% ethanol followed by air drying. The probe was prepared in a hybridization buffer consisting of 50% formamide, 2×SSC and 10 % dextran sulphate. Repetitive sequences were blocked by adding ~1 µg of human Cot-1 DNA (Roche Applied Science, Indianapolis, IN) to the probe mixture. This was then denatured at 75°C on a thermocycler for 10 min, snap cooled at 4°C and placed on a glass slide. Coverslips were incubated overnight at 37°C in a chamber humidified with 50% formamide/2×SSC. Post-hybridization washes with three different solutions of 45 min each: (a) 50% Formamide/2×SSC with 0.05% Tween (b) 2×SSC /0.05% Tween (c) 1×SSC was performed followed by two washes with PBS.

Probes were detected using either anti-biotin rabbit antibody (Roche Applied Science, Indianapolis, IN) or anti-digoxigenin mouse antibody (Roche Applied Science, Indianapolis, IN). Secondary detection of the signal was accomplished by using anti-rabbit or anti-mouse Alexa 488 and Alexa 594 antibodies (Invitrogen, Carlsbad, CA). Directly conjugated fluorescent chromosome and centromere probes were detected as per manufacturer's protocol. After labeling, cells were mounted in Prolong gold antifade (Invitrogen, Carlsbad, CA) and left for overnight curing protected from light. Images were captured using an Olympus BX 51 (Olympus America Inc, Center Valley, PA) microscope equipped with a Sensicam QE digital CCD camera (Cooke Corporation, Romulus, MI), motorized z-axis controller (Prior), Slidebook 4.0 (Intelligent Imaging Innovations, Denver, CO) and Image-Pro plus 4.1 (Media Cybernetics Inc, Bethesda, MD) softwares.

Line scanning profiles to evaluate co-localization were performed by utilizing the line profile tool provided in the Image-Pro plus 4.1 software. To verify the accuracy of our co-localization studies, we imaged 0.5 µm diameter Fluoresbrite beads (Polysciences, Inc. Warrington, PA) in both the red and green channels and examined the merged images. Observation of completely yellow beads confirmed the overall validity of our results. We further determined that the average sub-pixel shift between the red and green channels of the imaged beads was 0.54 pixels (~ 0.03 microns) under the microscopic conditions used in our experiments (Malyavantham et al., 2008)

Results

Identification and amplification of the IFN gene cluster in human osteosarcoma MG63 cells

Spectral karyotyping (SKY) of the human osteosarcoma cell line MG63, confirmed its hypotriploid nature with >50% of the population having a modal chromosome number of 66. Additionally a large number of chromosomal rearrangements were also detected such as: complex translocations, deletions, additions and amplifications which make assignment of absolute chromosome numbers misleading at times (Fig 2A, B and Fig. S1 A–E). For example, the number of chromosome 8 copies designated by SKY varied from 3–6 but typically only two copies appeared at the correct size and color for chromosome 8 (Fig S1 compares MG63 with normal diploid human fibroblasts). The remaining copies were often much larger and clearly derived from components of several other chromosomes in addition to 8 (Fig 2A and Fig. S1 A–E).

To decipher the chromosomal organization of the type I IFN gene cluster in relationship to the overall SKY karyotype, we stripped the chromosome spreads of the SKY probes and performed FISH using various probes that span the gene cluster (Fig 1). Initial results demonstrated a linear, ladder-like distribution of the probe signal in 5–7 bands that transverse the entire length of a chromosome (Fig 2C and Fig S2). This chromosome is termed the “*IFN chromosome*” and is readily identified on the corresponding SKY images as one of the copies of chromosome 9 (see arrows in Fig 2A, B and C) albeit much larger than a normal chromosome 9 (Fig 2A, Fig S1 A–E and Fig S2). Moreover, the IFN chromosome with its characteristic linear repeats of the IFN gene cluster was always detected in the over 300 chromosome spreads that were imaged in this study and that encompassed over 40 passages of the cell line (Fig S2 & Fig S3 for additional examples) and was always identified as an aberrant chromosome 9 by SKY (Fig. 2A and Fig. S1 A–E). Our studies on two MG63 cell lines obtained from ATCC >10 years apart showed identical karyotypes including the chromosome with linear amplification for the IFN gene cluster. These results indicate that the IFN chromosome has been stably inherited across a large number of generations in the MG-63 cell line. In contrast, FISH signals to the IFN probes were found in a more variable fashion as typically 1–2 signals distributed on 3–7 other chromosomes (Fig S2, arrowheads).

As a step towards determining what portion of the total IFN gene cluster is represented in the repeating bands, we performed FISH experiments with both BAC (Fig 2D, E, F) and cosmid (Fig 2G, H, I) probes that spanned the entire IFN gene cluster. A similar ladder-like array was found in all cases. This is consistent with the amplified regions containing the entire IFN gene. As expected, the probes used in this study did not show any amplification when investigated in the normal diploid human fibroblast primary cell line, WI38 (Fig 2 K–O). Moreover, the ladder-like amplification of the IFN gene cluster was specific to the MG63 cell line since it was not detected in several other osteosarcoma cell lines (Saos-2, U-2 OS and SK-ES-1; Fig S4).

To further study the degree of amplification of the IFN gene cluster and to what extent the entire cluster is represented in the ladder-like array of bands on the IFN chromosome, we performed aCGH analysis using a library of human genomic BAC probes. As previously reported for MG63 (Tarkkanen et al., 1995, Bridge et al., 1997, Zielenska et al., 2001, Ozaki et al., 2003, Atiye et al., 2005, Lim et al., 2005, Ohata et al., 2006, dos Santos Aguiar et al., 2007, Lu et al., 2008), significant levels of gains and losses in DNA sequences were detected in virtually all the chromosomes (Fig S5). A 5.6 fold amplification was estimated across the entire IFN gene cluster in 9p21.3 (Table 1, Table S1 and Fig 3). Based on the average hypotriploid overall genomic content of MG63, this would correspond to ~16 copies of the IFN gene cluster across the genome. Consistent with this level of amplification, an

average of 15 spots or bands of IFN gene sites was estimated from chromosome spreads of MG63 using IFN gene probes (Fig S2 and Fig. S3). In contrast, BAC probes that were immediately flanking both ends of the cluster were depleted as was the great majority of the remaining ~13 Mbp 9p21 band with the exception of the IFN gene cluster (Table 1, Fig 3 and Table S1). The adjacent and much smaller ~5.4 Mbp 9p22 band near the IFN cluster showed preferential gains including one region that was amplified ~10 fold beginning at the end of 9p22 (9p22.3) and into 9p23 (Fig 3, Table S1). Taken together these results indicate that the ladder-like amplification on the IFN chromosome contains the IFN gene cluster and is depleted of neighboring sequences in 9p21.

Translocation of chromosomes 4, 8, 9 and the interferon gene cluster distribution

While the initial SKY analysis pointed to the IFN chromosome as being a chromosome 9 type, the often multiple chromosomal rearrangements in osteosarcoma cell lines such as MG63 (Fig 2A, B and Fig S1 A–E), make assignment of an absolute chromosome number of limited value (Lim et al., 2004, Lim et al., 2005, Lau et al., 2004). More detailed analysis of our SKY data hinted at possible complex translocations among chromosomes 4, 8 and 9 on the IFN chromosome. To investigate this further, metaphase FISH analysis was performed in MG63 cells by double labeling of chromosomes 4, 8 and 9 with probes to the IFN gene cluster. All three chromosome paints decorated the IFN chromosome, albeit more weakly for chromosomes 8 and 9 (Fig 4A–L). The chromosome 4 decoration showed a banding pattern (Fig 4A arrow and 4B) that appeared to alternate with the IFN gene cluster probes (Fig 4C). Line profile analysis confirmed the alternating bands of chromosome 4 and the IFN gene cluster (Fig 4J). In contrast, the extremely weak staining for chromosomes 8 and 9 over the IFN chromosome was diffuse with no indication of banding (Figs 4D, G, arrow and 4E, H). This was confirmed by the corresponding line profile analysis (Fig 4K and L). In addition to decoration of the IFN chromosomes, all three chromosomes also stained several other chromosomes or portions of chromosomes in the MG63 metaphase spreads (Fig 4 A, D, G, arrowheads). As a control, painting of chromosomes 4, 8 and 9 revealed a normal complement of two chromosomes in metaphase spreads of WI38 normal diploid human fibroblast cells. Labeling of chromosomes 4 and 9 in the WI38 cell line is presented in Fig 2N & O, respectively.

Amplification of centromeric regions and their relationships to the chromosomal and IFN gene cluster organization

Results from both SKY analysis and chromosome painting experiments (Fig 2A–C, Fig 4A–L and Fig S1A–E) indicate that the IFN chromosome is composed of complex rearrangements involving chromosomes 4, 8 and 9. Since the centromere is a characteristic structural and functional feature of every chromosome, we have investigated the localization of centromere 4, 8 and 9 sequences on the IFN chromosome using centromere specific FISH probes. Both centromeric 4 and 9 probes showed a ladder-like banding pattern similar to that of the IFN gene cluster (Fig 5A, C arrows). In contrast, the chromosome 8 centromeric probe did not stain the IFN chromosome (Fig 5B).

Double labeling with the centromeric 4 and 9 probes, revealed colocalization of the banded patterns for these two centromeric sequences as indicated by the yellow color (Fig 5D). Colocalization of the centromeric 4 and 9 signals was also observed on another chromosome in ~70% of the examined metaphase spreads (Fig 5D, arrowhead). Further investigation of this chromosome was not a part of this study. Line profile analysis of the banded chromosome showed overlapping peaks of centromere 9 (red) and centromere 4 (green) with the exception of a single large red peak in the center that corresponds to the position of the centromeric constriction for the IFN chromosome (Fig 5E).

We then investigated the relationship of centromeric 9 repeats to labeling of chromosomes 4, 8 and 9 on the IFN chromosome. Chromosome 4 labeling (green) was distributed in a banding pattern that colocalized with the ladder-like centromeric 9 (red) amplification at those centromeric repeats that were not found at the chromosomal constriction for the centromere (Fig 5F). This was verified by line profile analysis which demonstrated overlapping peaks for both the centromere 9 probe and the chromosome 4 signals except at the intense centromere 9 signal in the middle of the scan which corresponds to the location of the true centromere for the IFN chromosome (Fig 5F). The chromosome 8 and 9 paint signals (green) were very faintly distributed with respect to the centromere 9 signals (red) (Figs 5G, H) and line profile analysis of double labeling of chromosome 8 and 9 (green) and centromere 9 (red) showed strong red peaks for the centromere 9 signals over faint green backgrounds (Fig 5G, H). As anticipated, the repeating bands of centromere 4, co-localized within the wider bands of chromosome 4 on the IFN chromosome (Fig 5I). Moreover, the ladder arrays for centromeres 4 and 9 alternated with the corresponding ladder patterns for the IFN gene cluster (Fig 5J, K).

Our findings suggested that centromere 9 is the true functional centromere for the IFN chromosome based on its unique position at the chromosome constriction and the absence of centromere 4 at this position. To further test this possibility, experiments were performed with the centromeric probes of chromosomes 4, 9 and antibodies to the CENP-C protein which is a marker protein for the true functional centromeres of chromosomes (Politi et al., 2002, Earnshaw et al., 1989, Fukagawa et al., 1999, Page et al., 1995, Saitoh et al., 1992). Double labeling of centromeric 4 and CENP-C showed a linear banding pattern of the centromeric 4 along the entire length of the IFN chromosome while CENP-C was exclusively localized at the chromosomal constriction (Fig 5L, arrow). Line profile analysis of this chromosome did not show colocalization of CENP-C and the centromeric 4 signals on the IFN chromosome (Fig 5N), while CENP-C colocalized with the centromeric 4 signal on other chromosomes (Fig 5L, arrowheads). Corresponding double labeling experiments with centromeric 9 probes and CENP-C showed a similar linear repeat of the centromeric 9 signals along the length of the IFN chromosome (Fig 5M, arrow). In this case, however, the signal from the centromeric 9 probe colocalized with the CENP-C protein labeling as demonstrated with line profile analysis (Fig 5O). We thus, conclude that the functional centromere of the IFN chromosome is derived from chromosome 9. Other chromosomes in the metaphase spread also showed colocalization of centromeric 9 sequences and CENP-C (Fig 5M, arrowheads).

Discussion

Osteosarcomas are highly malignant tumors of the bone that are characterized by very complex, mostly imbalanced karyotypic changes (Mertens et al., 1993). Many human osteosarcoma cell lines mimic these features. For example, complex chromosome translocations (CCR's) and gains and losses in certain genomic sequences are common in the MG63 cell line (Lim et al., 2004, Tarkkanen et al., 1995, Tarkkanen et al., 1999, Ozaki et al., 2003, Zielenska et al., 2001). High levels of amplification was especially reported in regions of 6p12-21, 8q23-24, 17p11-13 and 20q (Bayani et al., 2003, Lau et al., 2004, Forus et al., 1995, Lu et al., 2008).

In this study, we have performed a detailed analysis in MG63 osteosarcoma cells of the ~400 Kbp human type I interferon gene cluster located on the short arm of chromosome 9 (9p21-p22). aCGH analysis revealed that the entire gene cluster was amplified ~5.6 fold. Based on the average hypotriploid content of the MG63 cells, this corresponds to ~16 copies of the gene cluster per genome. Combined SKY and FISH investigations led to the visualization of the IFN gene cluster as a ladder-like array of 5–7 bands that spanned the

length of a highly enlarged and rearranged chromosome “9” as depicted by SKY. This chromosome was termed the “*IFN chromosome*” and was consistently observed in over 300 chromosome spreads from MG63 cells.

Further studies demonstrated that the IFN chromosomes contained components of chromosomes 4, 8 9, and centromeres 4, 9. The centromeric 4 and 9 sequences were also amplified in repeating bands that co-localized with each other and alternated with the IFN gene clusters. Chromosome 4 was also detected as bands that overlapped with the centromeric 4/9 sites. The one exception was the single intense signal for centromere 9 near the constriction characteristic of the functional centromere of the chromosome. Co-localization of a centromere 9 sequence at this site with the integral centromere protein CENP-C confirmed its role as the functional centromere and established the IFN chromosome as a highly rearranged derivative of chromosome 9 that also contains components of chromosomes 4, 8 including centromeric repeats of chromosomes 4 and 9. In regard to this latter point, amplifications in the alpha centromeric regions and centrosomes are a common feature among tumors thus promoting instability and rearrangement of the chromosomes (Lengauer et al., 1998, Ghadimi et al., 2000, Al-Romaih et al., 2003). A model illustrating the banding arrangement of the IFN chromosome is presented in Figure 6.

Previous investigations have demonstrated ladder-like amplification of chromosomal regions in MG63 including the 9p21-p22 bands (Lim et al., 2004, Lim et al., 2005). Our analysis demonstrates that the amplification within 9p21 is specific for the relatively small IFN gene cluster (~400 Kbp) located in 9p21.3, since immediately flanking the cluster on both sides are sequence losses that extend throughout the large (~13 Mbp) 9p21 region (Fig 3, Table 1 and Table S1). In contrast, the relatively small 9p22 band (~ 5.4 Mbp) shows high levels of amplification near its telomeric end (9p22.3) that continues into 9p23 and could potentially contribute to the ladder-like banding pattern of the IFN chromosome (Fig 3, Table S1). Our findings of large gains in 9p22-p23 and corresponding losses in 9p21 could also provide the basis for previous reports of losses in osteosarcoma cells of chromosome 9 (Bridge et al., 1997, Stock et al., 2000, Zielenska et al., 2001) or 9p (Tarkkanen et al., 1995, Ozaki et al., 2003) as well as a previous report of gains in 9p22 (Ozaki et al., 2003).

Investigations into gliomas, bladder cancers and melanomas suggest that deletions within the 9p21-22 region might have a potential tumor suppressor activity thus aiding in tumor progression (Stadler et al., 1994, Southgate et al., 1995, Olopade et al., 1992b). Recent data indicates that the interstitial deletion of *CDKN2A* tumor suppressor gene on 9p21.3 (also called P16), that encodes the CDKN2A/p16protein, is characteristic of many human cancers including osteosarcoma (Packenham et al., 1995, Miller et al., 1997, Miller et al., 1996a, Park et al., 2002, Kohno and Yokota, 2006b, Miller et al., 1996b). Our aCGH analysis confirmed that the sequence region which contains the P16 gene and flanks the IFN gene cluster on the centromeric end is deleted in MG63 (Table 1, Table S1).

Previous studies have proposed the BFB mechanism as the basis for ladder-like amplification of genes across chromosomes (Coquelle et al., 1997, Hellman et al., 2002, Richards, 2001, Chernova et al., 1998, Lim et al., 2004, Lim et al., 2005). This phenomenon was first described in maize (McClintock, 1941) and later associated with structural abnormalities in a variety of cancer types (Gisselsson et al., 2000, Ciullo et al., 2002, Masuda and Takahashi, 2002) including osteosarcoma (Selvarajah et al., 2006, Lim et al., 2004). The mechanism of BFB leads to anaphase bridges formation, dicentrics and centrosomal anomalies. BFB cycles are highly enhanced by the presence of fragile sites and double strand mismatch repair mechanisms. Fragile sites are described as those regions of the genome that are highly susceptible to DNA instability in cancer cells. In most cases they are located in close proximity to oncogenes and tumor suppressors (Richards, 2001,

Hellman et al., 2002, Popescu, 2003, Pichiorri et al., 2008, Smith et al., 2007). The ladder-like amplification of the IFN gene cluster, the presence of known fragile sites (FRA9A, C) just upstream of the interferon beta gene (Moriarty and Webster, 2003, Buttel et al., 2004, Bode et al., 2000), CGH analysis demonstrating deletion of the sequences flanking the IFN gene cluster and the overall losses of sequences throughout the 9p21 band, all lead us to propose the BFB mechanism for the generation of the IFN chromosome.

Further supporting the BFB model is the co-amplification of centromere 4 and 9 signals that colocalize with each other but alternate with the interferon gene cluster. Various studies on BFB cycles in osteosarcoma have shown that certain centromeric regions can facilitate translocations and recombinations among chromosomes (Al-Romaih et al., 2003, Gisselsson et al., 2000, Lim et al., 2004, Lim et al., 2005). A number of rearrangements especially inversions involving chromosome 1, 9, 13 have been reported (Selvarajah et al., 2006). The presence of highly repetitive DNA in centromeric regions (chAB4 and Neurofibromatose1-related) of chromosome 9 facilitates “jumping translocations” (Berger and Bernard, 2007) and gives it the ability to recombine easily with other chromosomes. The break points for jumping translocations have been reported to be at fragile sites and other viral DNA integration sites (Berger and Bernard, 2007, Padilla-Nash et al., 2001).

We, therefore, predict that the fragile sites in the pericentromeric regions of 9p had undergone the phenomenon of jumping translocations with chromosome 4 resulting in a complex phenotypic chromosome. Breakage at the fragile sites located near the interferon gene cluster then occurred followed by illegitimate DNA repair mechanisms leading to fusion of the chromosome segments with centromeric regions of chromosome 4 and 9. Thus the phenomena of jumping translocations along with the fragile sites resulted in a highly rearranged chromosome showing linear amplification of the interferon region on chromosome band 9p21.3 that alternated with bands containing centromeric 4, 9 and chromosome 4 regions.

The complex chromosomal rearrangements that resulted in an amplification of the type I interferon gene cluster into a precise ladder-like array spanning a single chromosome (see Fig 6) will likely have important implications for our understanding of gene regulation in relationship to chromosomal organization and nuclear architecture. One important issue is: *“How is the ladder-like linear array of interferon gene clusters seen in the mitotic chromosome arranged within the three-dimensional context of the interphase cell nucleus?”* Since normal chromosomes are arranged into specific chromosome territories in the cell nucleus (Cremer and Cremer, 2001, Cremer et al., 2001, Cremer et al., 2006, Gilbert et al., 2005), it will be of further significance to determine if the territorial organization is maintained in this highly rearranged and aberrant IFN chromosome and how the different chromosomal regions might fit together into the overall territorial arrangements.

Another fundamental question is: *“What effect(s) does this amplification have on the replication timing of this gene and its transcriptional regulation?”* It is well known that the position of a gene in the chromosome is one of the factors that determine its replication timing (Aladjem, 2007, Zink, 2006, Grasser et al., 2008). Study of the replication timing of the ladder-like array of IFN gene clusters that span from one end to the other of the IFN chromosome in MG63, offers an intriguing model to study the replication timing of amplified genes in relationship to their chromosomal positions. Since the MG63 cell line was previously shown to mass produce the interferon beta protein after induction with polyinosinic : polycytidic acid (Billiau et al., 1977) and gene amplification in human cancers is often associated with over expression of the genes (Albertson, 2006), analysis of the transcriptional activity and regulation of the amplified IFN gene cluster in the MG63 cell

nucleus could provide new insight into the relationships between gene amplification, chromosomal positioning and transcriptional regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of abbreviations

IFN	Interferon
FISH	Fluorescence in situ hybridization
SKY	Spectral karyotyping
aCGH	array Comparative genomic hybridization
BFB	Breakage Fusion Bridge
CENP	Centromeric protein
BAC	Bacterial artificial chromosome
CCR	Complex chromosome translocations
FRA	Fragile sites
CCD	Charge Coupled Device

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Figure 1. DNA Probes Covering the Type I Interferon Gene Cluster
The ~ 400 kbp human type I interferon gene cluster resides on the short arm of chromosome 9 (band 9p21.3) and consists of 13 α , 1 β , and 1 ω genes interspersed with multiple pseudogenes (not shown). **Red bars:** Three bacterial artificial chromosome (BAC) probes abbreviated P8, G20, and I7 which partially overlap and together span the entire gene cluster were used for FISH studies. **Blue Bars:** Five cosmid probes abbreviated C9, 133D4, 49D4, F5 and C12 that are positioned across the entire gene cluster were also used in FISH experiments. The full names of the abbreviated probes are shown below the map.

Figure 2. Spectral karyotyping (SKY) and IFN gene cluster FISH analysis of the MG63 cell line (A), SKY karyotype of MG63 cells; (B), SKY of the corresponding chromosome spread that led to (A); (C), Magnified image of SKY treated chromosome spread shown in (B) that has been stripped of the SKY probes and re-hybridized with cosmid probe 133D4 that spans across the interferon ω gene; stained with DAPI to visualize the chromosomes. As indicated by the arrows in (B) and (C), a chromosome tentatively identified as a chromosome “9” by SKY in (A, arrow) shows amplification of the IFN gene signal in 6–7 bands that span the chromosome in a ladder-like array (C); (D–I) Representative images of chromosome showing amplification of various probes that span across the IFN gene cluster (left panels): BAC probes P8 (D), G20 (E), I7 (F) and cosmid probes C9(G), F5(H), C12 (I). Right panels show the corresponding IFN gene probe signal and DAPI. (K–M) Representative images of IFN gene cluster probes on WI38 metaphase spreads: (K), P8 (red) and chromosome 9 (green) ; (L), G20 (red) and chromosome 9 (green) ; (M), C9 (red) and DAPI (blue); (N), Triple labeling of centromeric 9 (red), chromosome 9 (green) and DAPI (blue) in WI38; (O), Triple labeling of centromeric 4 (red), chromosome 4 (green) and DAPI (blue) in WI38.

Figure 3. Array Comparative Genomic Hybridization (aCGH) analysis of chromosome 9p
 The results of aCGH for the chromosome 9p short arm in MG63 are displayed graphically. Normal genomic content is represented by a ratio of 1. Upward peaks from this ratio indicate gains in genomic material while downward peaks indicate a loss in the genome. Bands 9p21, 9p22 and 9p23 are represented in various colored boxes as indicated by the legend. The beginning of the centromere region is represented by a single black vertical line. A few points on the 9q arm are also shown. Note that the sequences analyzed across the very large 9p21 band predominantly show losses (ratio <0.85) with the exception of the high degree of amplification of the IFN gene cluster in 9p21.3 denoted with an asterisk. In contrast, the much smaller 9p22 band is highly amplified (see e.g., double asterisk). The complete data for each BAC tested is shown in Table S-1 and the aCGH for the entire genome of MG63 is displayed in Figure S-1.

Figure 4. IFN gene cluster and chromosome painting of the IFN chromosome

Representative images of metaphase spreads of MG63 labeled for Chromosomes 4 (A–C), 8 (D–F) and 9 (G–I) with the G20 BAC probe and their corresponding line profiles (J, K, L). The green and the red peaks in the line profiles correspond to the distribution of the chromosome and gene probe signals, respectively. Chromosomes have been labeled with DAPI (blue) and the chromosomes showing amplification of the IFN gene cluster are indicated by arrows. Arrow-heads, point to other chromosomes or portions of chromosomes in the spread that are stained with chromosome paint.

Figure 5. Centromeres 4, 8, 9 and their relationship to the amplified IFN gene cluster

A–D: Representative images of MG63 showing: (A), Centromere 4 (red); (B), Centromere 8 (green); (C), Centromere 9 (red); (D), overlay of centromeres 4 (green) and centromere 9 (red); (E), corresponding line profile (see below image) for (D). In all the above images the chromosomes are stained with DAPI and those showing amplification for centromeres are indicated by arrows while an arrowhead points to another chromosome in the metaphase spread that show colocalization. F–H: Distribution of centromere 9 signal in relation to: (F), Chr 4; (G), Chr 8; (H), Chr 9 and their corresponding line profiles. Centromere 9 (red) and chromosomes (green) with the line profiles keeping the same color combinations (I), centromere 4 (red) and Chr 4 (green) with the corresponding line profile in the same color

combination. J–K: Magnified images of chromosomes simultaneously labeled for: (J) centromere 4 (red) and IFN gene probe (green); (K) centromere 9 (red) and IFN gene probe (green). Triple labeling with DAPI (blue) are shown on the right panels. L–M: Distribution of centromeric protein (CENP-C) with respect to centromeres 4 and 9. Representative metaphase spreads of double labeling: (L), CENP-C (red) and centromere 4 (green); (M), CENP-C (red) and centromere 9 (green). Chromosomes showing amplification of centromeric signals are indicated by arrows while arrowheads point to other chromosomes in the metaphase spread that show colocalization of both signals. (N) and (O), corresponding line profile analysis for (L) and (M), respectively, keeping the same color combinations. Chromosomes are labeled with DAPI (blue).

Figure 6. A Model for the organization of the amplified IFN gene cluster, centromeres 4, 9 and chromosomes 4, 8 and 9 on the IFN chromosome of MG 63 cells

Based on our results, we propose a model for the amplification patterns characterized on the IFN chromosome of the MG63 human osteosarcoma cell line. Signals from human whole chromosome 8 and 9 paints are very faintly distributed on this chromosome (grey). The interferon gene cluster (blue) is amplified about 6 times in a banding pattern along the length of the chromosome. Both centromeres 4 (green) and 9 (dark red) are also amplified about 6 times in banding patterns that co-localize along the length of the chromosome and alternate with the IFN gene cluster repeats. Chromosome 4 labeling (yellow) is amplified in a banding pattern on this chromosome that colocalizes with the centromere 4 and 9 repeats and also alternates with the IFN gene cluster ladder-like array. We, therefore, illustrate the co-localizing bands containing centromere 4 (green), centromere 9 (dark red) and chromosome 4 (yellow) as a series of orange bands that alternate with the IFN gene cluster bands in blue. The true centromere of the chromosome is labeled with centromere 9 (dark red) that excludes both centromere 4 and chromosome 4 and colocalizes with the centromeric CENP-C protein. A proposal involving *breakage-fusion-bridge* events for the generation of this complex chromosome is presented in the Discussion section.

Table 1

CGH analysis of various BAC probes spanning the IFN gene cluster

BAC probes on Chr 9p21-22	Location on chromosome 9 (Mbp)		Genes covered by individual BAC probe	Linear Ratio
	Start	End		
RP11- 1040L18	20.73	20.91	Outside gene cluster	0.4
RP11-767D19	20.90	21.07	<i>IFNa1</i>	4.6
RP11-1P8	21.06	21.21	<i>IFNβ1, IFNω1, IFNa21, IFN α4, IFNa7, IFNa10, IFNa16, IFNa14</i>	5.1
RP11- 380P16	21.21	21.33	<i>IFNa17, IFNa14, IFNa5</i>	5.7
RP11- 956G20	21.19	21.40	<i>IFNa16, IFNa17, IFNa5, IFNa6, IFNa13, IFNa2, IFNa8</i>	6.2
RP11-344A7	21.50	21.67	<i>IFNa8, IFNa1</i>	6.5
RP11-149I2	21.8	22.0	Outside gene cluster <i>P16</i>	0.4