Abstract

**Background:** Osteosarcoma is a highly malignant bone neoplasm of children and young adults. It is characterized by extremely complex karyotypes and high frequency of chromosomal amplifications. Currently, only the histological response (degree of necrosis) to therapy represent gold standard for predicting the outcome in a patient with non-metastatic osteosarcoma at the time of definitive surgery. Patients with lower degree of necrosis have a higher risk of relapse and poor outcome even after chemotherapy and complete resection of the primary tumor. Therefore, a better understanding of the underlying molecular genetic events leading to tumor initiation and progression could result in the identification of potential diagnostic and therapeutic targets.

**Methods:** We used a genome-wide screening method – array based comparative genomic hybridization (array-CGH) to identify DNA copy number changes in 48 patients with osteosarcoma. We applied fluorescence in situ hybridization (FISH) to validate some of amplified clones in this study.

**Results:** Clones showing gains (79%) were more frequent than losses (66%). High-level amplifications and homozygous deletions constitute 28.6% and 3.8% of tumor genome respectively. High-level amplifications were present in 238 clones, of which about 37% of them showed recurrent amplification. Most frequently amplified clones were mapped to 1p36.32 (**PRDM16**), 6p21.1 (**CDC5L**, **HSPCB**, **NFKBIE**), 8q24, 12q14.3 (**IFNG**), 16p13 (**MGRN1**), and 17p11.2 (**PMP22**, **MYCD**, **SOX1**, **ELAC27**). We validated some of the amplified clones by FISH from 6p12-p21, 8q23-q24, and 17p11.2 amplicons. Homozygous deletions were noted for 32 clones and only 7 clones showed in more than one case. These 7 clones were mapped to 1q25.1 (4 cases), 3p14.1 (4 cases), 13q12.2 (2 cases), 4p15.1 (2 cases), 6q12 (2 cases), 6q12 (2 cases) and 6q16.3 (2 cases).

**Conclusions:** This study clearly demonstrates the utility of array CGH in defining high-resolution DNA copy number changes and refining amplifications. The resolution of array CGH technology combined with human genome database suggested the possible target genes present in the gained or lost clones.
Background

Osteosarcoma (OS) is a primary malignant tumor of bone arising from primitive bone-forming mesenchymal cells and it accounts for approximately 60% of malignant bone tumors in the first two decades of life [1]. These tumors typically arise in the metaphyseal regions of long bones, with the distal femur, proximal tibia and proximal humerus. A significant number of osteosarcomas are of conventional type which can be subdivided into three major categories based on their predominant differentiation of tumor cells: osteoblastic, chondroblastic, and fibroblastic. Currently, only the histological response (degree of necrosis) to therapy represent gold standard for predicting the outcome in a patient with non-metastatic osteosarcoma at the time of definitive surgery [2]. Patients with lower degree of necrosis have a higher risk of relapse and poor outcome even after chemotherapy and complete resection of the primary tumor. Therefore, a better understanding of the underlying molecular genetic events leading to tumor initiation and progression could result in the identification of potential diagnostic and therapeutic targets.

Chromosomal aberrations in osteosarcoma are highly complex and characterized by high frequency of amplifications. These amplifications may result in the overexpression of genes and contribute to the genomic instability in osteosarcoma. The identification of genes within the amplified sites is crucial for understanding the biology and clinical behavior of osteosarcoma. Until, recently gene amplification has been detected by PCR, southern blot analysis or FISH-based approach using gene specific probes. These techniques are inherently restricted to the previously known amplified genes in the genome. In contrast, genome-wide screening of amplified chromosomal regions with CGH has become an important tool for the detection of amplified regions in the tumor genome. So far published chromosomal CGH studies in osteosarcoma have identified several high-level chromosomal amplifications at 1p22, 1p31, 1q21, 1q23, 2q24, 3p25, 3q26, 6q24.3, 4q12, 5p14-p15, 5q33, 6p12-p21, 6q24.3, 7p21-p22, 8q12-q23, 10p21, 10q11.1, 10q22, 11q13, 11q23, 12p13, 12q12-q15, 17p11.2, 17q21, 18q22, 19p13.1 and 20p11.2 [3-7]. However, conventional CGH has limited sensitivity and resolution (~10–15 megabases) because of its dependence on the morphology of metaphase chromosomes. In addition, extensive follow-up work is required to identify candidate genes after regions of gain or loss have been identified. Recently, novel method termed as array-based comparative genomic hybridization (array CGH) has been described, which enables high throughput quantitative measurement of high-resolution DNA copy number changes throughout the genome [8]. This method is based on hybridization of differentially labeled test and reference DNAs to an array of mapped human genomic DNA fragments (~100–200 kb) and has been recently applied to human and mouse tumors [9-14]. To identify high-resolution copy number, we used array CGH to the panel of 48 tumors. The resolution of array CGH technology combined with human genome database not only allowed a precise identification of amplicons but also suggested the possible target genes within the amplicons.

Methods

Patient samples

A total of 48 tumors from 42 patients (20 males and 22 females) were collected from the Texas Children's Cancer Center, Houston, TX (tumors 193, 196, 204, 209, 221, 226, 248, 274, 295, 311, 326, 341, 345, 360, 400, 464, 481, 501, 527, 591 and 606) and Memorial Sloan Kettering Cancer Center, New York (tumors 06, 15, 24, 25, 27, 29, 32, 34, 40, 48, 68, 76, 78, 79, 80, 82, 83, 85, 88, 95, 98, 99, 102, 123, 423, 425, and 474). All tissues in this study were obtained after IRB approved informed consents were signed. The age at diagnosis ranged from 5 years to 71 years at diagnosis. The histological information of 42 patients is presented in Table 1.

Array CGH

The array used in this study consists of 967 human BACs, which were spaced approximately 3 megabase across the whole genome. These arrays were obtained from Spectral Genomics, Houston, TX. The experiments were performed according to the manufacturer's protocol. Arrays were pre-hybridized with human Cot-I DNA (GIBCO Invitrogen, Carlsbad, CA) and salmon testes DNA to block the repetitive sequences on BACs. One microgram of normal DNA (reference) and tumor DNA (test) was labeled with cy5-dUTP and cy3-dUTP respectively, by random priming. To avoid dye bias, we performed dye swap experiments for each sample. The probe mixture is dissolved in hybridization mixture, denatured, cooled, and mounted with 22 x 60 mm coverslip. Hybridizations were performed in sealed chambers for 16–20 hours at 60°C. After post hybridization washes, arrays were rinsed, dried with compressed air, and scanned into two 16-bit TIFF image files using Gene Pix 4000A two-color fluorescent scanner (Axon Instruments, Inc., Union City, CA) and quantitated using GenePix software (Axon Instruments, Union City, CA).

Data processing and analysis

After scanning of the slide, the fluorescent intensities of the green and red channels were background subtracted. The resulting values were normalized by intensity based local weighted regression method (Lowess) to correct for systematic bias in dye labeling and fluorescent intensity [15]. Then the ratio of the red/green channel of each clone was calculated and log base 2 transformed (log ratios).
| Tumor No. | Sex | Age-Dx | Site          | Histological Subtype                              | Type       | Metastatic Disease | Huvos Grade | Response |
|----------|-----|--------|---------------|--------------------------------------------------|------------|--------------------|-------------|----------|
| 06       | Female | 7     | Distal Femur  | NA                                                | Biopsy    | L                  | II          | PR       |
| 15       | Male   | 35    | Distal Femur  | Osteoblastic+MFH-Like                             | Biopsy    | L                  |             |          |
| 24       | Female | 16    | Clavicle      | Chondroblastic                                    | Biopsy    | L                  | Minimal     |          |
| 25       | Male   | 8     | Skull         | Osteoblastic                                      | Mets      | L                  |             |          |
| 27       | Female | 34    | Ischium       | Mixed                                             | Mets      | L                  |             |          |
| 29       | Male   | 19    | Ilium         | NA                                                | Biopsy    | M                  | II          | PR       |
| 32       | Female | 25    | Ilium         | Chondroblastic                                    | Biopsy    | L                  | Minimal     |          |
| 34       | 15 Femur |  | Chondroblastic And Osteoblastic                | Biopsy    | L                  | II          | PR       |
| 40       | Female | 11    | Left Distal Femur | Giant Cell/Osteoblastic/Fibroblastic | Biopsy    | None               | IV          | GR       |
| 48       | Female | 32    | Proximal Tibia | Osteoblastic/Fibroblastic                         | Biopsy    | None               | I           | PR       |
| 68       | Male   | 18    | Femur         | Telangectatic                                     | Mets      | L                  |             |          |
| 76       | Female | 20    | Thigh         | N/A                                               | Biopsy    | L                  |             |          |
| 78       | Male   | 5     | Humerus       | N/A                                               | Biopsy    | L                  | III         | GR       |
| 79       | Male   | 9     | Tibia         | Telangectatic                                     | Mets      | L                  | III         | GR       |
| 80       | Male   | 46    | Tibia         | Osteoblastic                                      | Biopsy    | L                  | I           | PR       |
| 82       | Male   | 23    | Humerus       | Osteoblastic                                      | Mets      | L                  | I           | GR       |
| 83       | Female | 12    | Femur         | Telangectatic                                     | Biopsy    | L                  | IV          | GR       |
| 85       | Male   | 34    | Femur         | Fibroblastic                                      | Biopsy    | L                  | I           | PR       |
| 88       | Male   | 17    | Humerus       | Chondroblastic                                    | Biopsy    | L                  | IV          | GR       |
| 95       | Male   | 71    | Femur         | Giant Cell Rich                                   | Biopsy    | L                  |             |          |
| 98       | Female | 31    | Ilium         | Epitheloid                                        | Biopsy    | L                  |             |          |
| 99       | Female | 22    | Humerus       | N/A                                               | Biopsy    | L                  |             |          |
| 102      | Male   | 70    | Humerus       | Fibrohistiocytic                                  | Mets      | L                  |             |          |
| 123      | Female | 16    | Femur         | N/A                                               | Biopsy    | L                  | I           | PR       |
| 209      | Female | 17    | Distal Femur  | Osteoblastic                                      | Biopsy    | No                 | II          | GR       |
| 221      | Female | 17    | Femur         | Osteoblastic                                      | Biopsy    | No                 | IV          | GR       |
| 248      | Female | 13    | Tibia         | Pleiomorphic                                      | Biopsy    | No                 | ?           |          |
| 311      | Female | 13    | Distal Femur  | Osteoblastic                                      | Biopsy    | No                 | III         | GR       |
| 326      | Female | 20    | Femur         | Osteoblastic                                      | Biopsy    | No                 | IV          | GR       |
| 341      | Male   | 12    | Femur         | Fibroblastic                                      | Biopsy    | N                  | II          | PR       |
| 345      | Male   | 10    | Distal Femur  | Osteosarcoma                                      | Biopsy    | No                 | IV          | GR       |
| 360      | Female | 18    | Distal Femur  | Osteoblastic                                      | Biopsy    | Yes                | IV          | GR       |
| 400      | Female | 15    | Distal Femur  | Chondroblastic                                    | Yes       |                   |             |          |
| 423      | Male   | 30    | Proximal Humerus | Giant Cell                                           | Biopsy    | None               | I           | PR       |
| 425      | Male   | 24    | Tibia         | N/A                                               | Biopsy    | Proximal Femur     | I           | PR       |
| 474      | Female | 17    | Pelvis        | Chondroblastic                                    | Biopsy    | None               | II          | PR       |
| 591      | Male   | 15    | Proximal L Tibia | Telangectatic                                   | Biopsy    | None               | II          | PR       |
| 193a     | Female | 17    | Distal Femur  | Osteoblastic                                      | Biopsy    | No                 |           |          |
| 196a     | Female | 15    | Distal Femur  | Osteoblastic                                      | Pul Met   | Yes                |           |          |
| 274b     | Male   | 13    | Distal Femur  | Osteoblastic/Chondroblastic                       | Biopsy    | No                 | II          | PR       |
| 295b     | Male   | 13    | Femur         | Osteoblastic/Chondroblastic                       | Yes       | II                 | PR         |          |
| 464c     | Female | 15    | Distal Femoral Head | Osteoblastic/Fibroblastic                          | Biopsy    | No                 | II          | PR       |
| 501c     | Female | 15    | Femur         | Osteoblastic                                      | Biopsy    | None               | II          | PR       |
| 606c     | Female | 14    | Distal Femur  | Osteoblastic                                      | Biopsy    | Yes                | II          | PR       |
| 481d     | Male   | 10    | Distal Femur  | Osteoblastic                                      | Biopsy    | No                 | II          | PR       |
| 527d     | Male   | 10    | Proximal Tibia | Osteoblastic                                      | Biopsy    | Yes                | III         | GR       |
| 204e     | Female | 18    | Distal Femur  | Osteoblastic                                      | Biopsy    | No                 | I           | PR       |
| 226e     | Female | 18    | Distal Femur  | Telangectatic                                     | Biopsy    | Definitive         | No          | I        |

a, b, c, d, e - Tumor samples obtained from the same patient.
Each experiment was repeated once with dye reversal to addressing the confounding effect of the dye and experiment. The average of the dye-reversal experiment pair was calculated by reversing the sign of one experiment so that the log ratio reflects the tumor versus normal ratio.

We developed a new analytical method called invariant analysis to define the significant copy number changes. This method is designed to: i) increase the power of the analysis by combining all the cases in our dataset to define an invariant population (unchanged population); and, ii) to address the signal to noise differences among individual cases due to sample and hybridization variability. Our goal is to define a set of unchanged clones that can be used to calculate the upper and lower bound thresholds of the log ratios for the unchanged population in each experiment. First, we calculated the variance of each clone from all the experiments. We computed the \( p \)-values of the each clone by comparing to the clone with median variance using chi-square distribution [http://linus.nci.nih.gov/~brb/TechReport.htm](http://linus.nci.nih.gov/~brb/TechReport.htm). The clones that have \( p \)-value greater than preset cutoff 0.9 were considered as invariant clone set, i.e. clones that do not vary significantly in all experiments. Then the mean and standard deviation of the log ratios of these invariant clones in each experiment were calculated. The clones with log ratios that exceed mean +/- 2 × SD of the invariant set were considered gains and losses, respectively. For amplification and homozygous deletions, clones were defined to have at least 2 fold of the upper bound threshold and 4-fold of lower bound threshold, respectively. The gene(s) present in the clones were identified using UCSC browser [http://genome.ucsc.edu/cgi-bin/hgGateway?org=human](http://genome.ucsc.edu/cgi-bin/hgGateway?org=human) by downloading gene table (refFlat) from human gene assembly, July 2003. We search the candidate genes based on linear mapping position, which include 100 kb up and downstream from the clone center position. The supplemental data for this article is available at: [https://bigfile.bcm.tmc.edu/outbound/prao/supplemental%20data%20Man%20et%20al.xls](https://bigfile.bcm.tmc.edu/outbound/prao/supplemental%20data%20Man%20et%20al.xls)

**Statistical analysis**

Significant clones in 6p, 8q, 12q and 17p amplicons were calculated using 2-sample t-test with randomized variance model [http://linus.nci.nih.gov/~brb/TechReport.htm](http://linus.nci.nih.gov/~brb/TechReport.htm). The experiments in each of the two groups, amplification and normal, used for comparison were defined based on the invariant analysis (see above). The clones that have \( p < 0.001 \) were considered as significant. We chose a stringent cutoff to minimize the multiple testing problem.

**FISH**

FISH was performed to validate and quantify chromosomal amplicons using clones from 6p12-p21 (RP11-91E11, AL391415, RP11-81F7, RP11-79I2, RP11-90H17 and RP11-79F13), 8q24.3 (RP11-89K10), and 17p11.2-p12 (RP11-64B12, RP11-89K6 and RP11-189D22) on tumors metaphase/interphase cells from cases 274, 364, 425, 426, 527 and 628. We confirmed the map positions of all clones on normal human metaphase cells by FISH. The bacterial artificial chromosome (BAC) clones, and centromeric clone from 6 (pEDZ6) were labeled with Spectrum Red or Spectrum Green (Vysis, Downers Grove, IL) by nick translation. Hybridization and FISH analysis was performed as described previously [16].

**Results**

To define the gains and losses in our experiments, we used invariant analysis for the first time to describe genomic changes by array CGH. In this method, we defined an invariant clone set that has low variance of log ratios among all the array experiments. After the mean and standard deviation of the log ratios in the invariant set of each experiment were calculated, clones that have higher or lower log ratios than the mean +/- 2SD of the invariant set (upper bound and lower bound) were used to define gains and losses. We chose to use this method because it addresses some of the shortcomings of the modeling method, such as using all information provided in a set of experiment to determine the unchanged population instead of using one experiment at a time. However, the variation of each experiment is accounted for because the thresholds are calculated using the invariant set from each experiment. It also does not require a separate reference set for comparison. Finally, it provides an adjustable cut-
off to optimize the thresholds to the training data, if provided.

The amplified and homozygously deleted clones were defined to have at least 2 fold of the upper bound and 4-fold of lower bound, respectively. Figure 1 summarizes the high-resolution DNA copy number changes identified by array CGH in 48 osteosarcomas derived from 42 patients. Copy number changes were detected involving small genomic regions, whole chromosomes, and chromosomal arms showing homozygous deletions and high-level amplifications.

**Overview of genomic profiles**

Copy number changes excluding clones from sex chromosomes were involved in a significant fraction of most tumor genome. The estimated average genomic distance between clones was ~3–4 Mb. The frequency of clones showing gains (79%) was greater than losses (66%). High-level amplifications and homozygous deletions constitute 28.6% and 3.8% of tumor genome respectively. The most frequently deleted clones were identified from the chromosomal regions 2q31.1, 3p14.1, 4p16.2, 6q12, 6q21, 6q27, 7q35, 10p15.1, 10q22-q23, 10q25-q26, 11q25, 13q12.2, 13q14.3, 13q22.1, 17p13.3 and 17q12 (Table 2). Most frequently gained clones were mapped to chromosome 1p36, 4p16, 6p12-p21, 8q21, 8q23-q24, 12q14.3, 16p13.3, 16q24.3, 17p11-p12, 19p13.3 and 21q22.3 (Table 3). We explored the possible statistical relationship between copy number alterations and histological and clinical parameters. We found no significant relationship between copy number changes and primary/metastatic disease, or histological type or histological response. This may be due to the involvement of large number of genomic loci and insufficient sample size.

Homomygous deletions were noted for 32 clones (3.8%). Recurrent homozygous deletions were noted for 7 clones that are were mapped to 1q25.1 (4 cases), 3p14.1 (4 cases), 13q12.2 (2 cases), 4p15.1 (2 cases), 6q12 (2 cases), 6q12 (2 cases) and 6q16.3 (2 cases). Figure 2A is showing a homozygous deletion at 3p14.1 in tumor 06. Loss of 6q12 region was noted in 35% of the osteosarcomas. This region was covered with four clones spanning ~4.2 Mb. Two tumors (tumor 27 and 345) showed low intensity ratios indicting homozygous deletions in this region, one tumor (tumor 345) showed all 4 deleted clones spanning ~4.2 Mb with RP1-129L7 having the lowest ratio intensity decrease. In another case (tumor 27), two clones (RP1-46B1 and RP1-129L7) showed decreased intensity ratios indicating homozygous deletions. Both these clones spanning approximately 2.6 Mb of 6q12 region.

**Table 2: Most frequently lost clones.**

| Clone       | Map Position (Mb) | Cyto Position | Frequency (%) | Genes     |
|-------------|-------------------|---------------|---------------|-----------|
| RP11-79K15  | 31.9              | 17q12         | 27            | PSMB1     |
| RP1-140C12  | 170.4             | 6q27          | 22.9          |           |
| RP1-90M15   | 24.8              | 13q12.2       | 20.8          |           |
| RP1-79H4    | 73.4              | 13q22.1       | 20.8          | KLF12     |
| RP1-79K22   | 101.7             | 6q16.3        | 20.8          | MTMR6, NUPL1 |
| AC004B89    | 148.401           | 7q35          | 20.8          | OR2A4     |
| RP11-89H7   | 116.5             | 10q25.3       | 18.8          |           |
| AL359B36    | 128.22            | 10q26.11      | 18.8          |           |
| RP11-80L16  | 67                | 6q12          | 18.8          |           |
| RP11-80D10  | 2.8               | 10p15.1       | 16.7          |           |
| AC021027    | 84.029            | 10q22.3       | 16.7          |           |
| RP11-79E24  | 88                | 10q23.2       | 16.7          |           |
| RP11-90B19  | 131.5             | 10q26.3       | 16.7          |           |
| RP11-835G21 | 143.17            | 11q25         | 16.7          |           |
| RP11-80H2   | 50                | 13q14.3       | 16.7          |           |
| RP11-81D9   | 72.8              | 13q22.1       | 16.7          |           |
| RP5-1029F21 | 1.21              | 17p13.3       | 16.7          |           |
| AC0206B1    | 175.95            | 2q31.1        | 16.7          | PDK1      |
| RP11-89A12  | 68                | 3p14.1        | 16.7          |           |
| RP11-9A1    | 71.28             | 3p14.1        | 16.7          |           |
| RP11-492I23 | 3.64              | 4p16.2        | 16.7          |           |
| RP1-46B1    | 69.4              | 6q12          | 16.7          | BAII      |
| RP3-454N4   | 106               | 6q21          | 16.7          |           |
Table 3: Most frequently gained clones.

| Clone      | Map Position (Mb) | Cyto Position | Frequency (%) | Genes            |
|------------|-------------------|---------------|---------------|-----------------|
| RP11-81F7  | 43.8              | 6p21.1        | 45            | HGNC, MRLP14    |
| RP1-163G9  | 2.6               | 1p36.32       | 43            | PRDM16          |
| RP11-79F13 | 44.6              | 6p21.1        | 37.5          | CDC5L, HSPCB, NFKBIE |
| RP11-90H17 | 65.3              | 6p21.3        | 37.5          | UCP4            |
| RP11-64L12 | 0.68              | 16p13.3       | 37.5          | MSLN, SOX8      |
| RP11-637F16| 144.6             | 8q24.3        | 35.4          |                |
| RP3-447E11 | 46                | 6p21.1        | 35.4          | CLIC5           |
| RP11-79I2  | 6p21.1            | 31.2          |               | EGFL            |
| RP11-80F24 | 78.3              | 8q21.13       | 29            |                |
| RP4-753D5  | 50.9              | 6p12.3        | 29            |                |
| AC005263   | 0.95              | 1p31.3        | 29            | AMH, GNRPX, DIT1L |
| RP1-189D22 | 19.56             | 17p11.2       | 29            | TPP3A, SMCR5, DRG2, FLI |
| RP11-89P19 | 119.5             | 8q23.3        | 27            |                |
| RP11-89H1  | 77.4              | 8q21.13       | 25            |                |
| RP1-283E3  | 43.7              | 21q22.3       | 25            |                |
| RP11-89P9  | 125.4             | 8q24.13       | 22.9          | MTSS1           |
| AL391415   | 43.06             | 6p21.2        | 22.9          | GLO1, DNAH8     |
| RP11-492I23| 3.64              | 4p16.2        | 22.9          |                |
| RP1-163M9  | 16.2              | 1p36.13       | 22.9          |                |
| RP1-383B15 | 2.89              | 19p13.3       | 22.9          |                |
| RP11-89K6  | 13.1              | 17p12         | 22.9          |                |
| RP11-89K10 | 127.3             | 8q24.13       | 20.8          | NSE2            |
| RP1-90D11  | 98.8              | 8q22.1        | 20.8          |                |
| RP11-91E11 | 37.4              | 6p21.2        | 20.8          | PIM1            |
| RP11-91K23 | 37.4              | 6p21.2        | 20.8          |                |
| RP3-417II  | 63.04             | 6p11.2        | 20.8          | BAG2, RAB23     |
| RP11-79O4  | 19.9              | 17p11.2       | 20.8          | ULK2, AKAP10    |
| RP11-46C24 | 101.27            | 16q24.3       | 20.8          |                |
| RP11-89P19 | 67                | 12q14.3       | 20.8          |                |

Amplification is a frequent phenomenon in osteosarcoma

Previous studies using CGH have identified several chromosomal amplification sites in osteosarcoma. Because of the limitation of the method, it fails to pinpoint the precise site of amplicon. However, the present study by array CGH has identified 238 clones (28.6%) with high-level amplifications. Recurrent amplifications were noted in ~37% of the total amplified clones (Figure 3). These amplified clones were mapped to 1p22, 1p31.1 (ROR1), 1p36.1 (PRDM16), 1q21, 1q23 (TNFF6), 2q24, 3p25, 3q26.1, 4p16.3, 5p14, 5q33, 6p11.2-p21, 7p21, 8q12.1, 8q24.13, 10p21, 10q11.1, 10q22 (KCNA1), 11q13, 11q23 (GRK4), 12q12, 12q13-q15, 12q21-q21.33, 17p11.2-p12, 17q21 (NGFR), 18q22, and 19p13.1 (NFAT). Of these amplified sites, 6p11.2-p21, 8q12.1, 8q24.13, 12q12, 12q13-q15, 12q21-q21.33, 16p13 and 17p11.2-p12 were frequent.

Gain of clones from 6p12-p21 regions was noted in 33/48 (~65%) cases analyzed. High-level amplification of the clones from same region was noted in 25% of the cases by array CGH. We found that most of the cases with amplification of 6p12-p21 displayed either increased or slightly varying degree of copy number increase across the 6p12-p21 region. The combined log ratios from all the cases defined the boundaries of amplification between RP3-329A5 and RP11-79F13. The amplicon spans approximately 9.4 Mb with amplification peak for clone RP11-81F7. Further, we used FISH to validate 6p amplicon on tumor metaphase and interphase cells from cases 274, 364, 426 and 527. Increased copy numbers for clones RP11-91E11, AL391415, RP11-81F7, RP11-79I2, RP11-90H17 and RP11-79F13 were noted in interphase cells with maximum copy number increase for clone RP11-81F7 (Figure 4A). This was consistent with amplification peak for clone RP11-81F7 in the tumors profiled by array CGH (Figure 2B). In addition, we used 2-sample t-test with randomized variance model to define significant clones from 6p12-p21 amplicon. By this method, we identified RP11-79F13 (p = 0.00000007), RP11-79I2 (p = 0.00000007) and RP11-81F7 (p = 0.00000007) as statistically significant clones.

Most cases with 8q gain, displayed varying degree of copy number increase predominantly from 8q12.1 (16.9%), 8q21.13 (29%), and 8q24.3 (35%). High-level amplification...
tions were also noted from 8q12.1 (RP11-550I15 – 6.3%; Figure 2C), 8q21.13 (RP11-89H1 – 6.3%), 8q24.3 (RP11-89K10 – 6.3%) and RP11-637F16 (12.5%). FISH using clone RP11-89K10 (p = 0.00049) on interphase cells from case 527 confirmed the amplification (10–12 copies) (Figure 4B).

Amplification of 12q was noted in 14/51 (~27%) tumors analyzed by array CGH. Three distinct amplicons – AMP1 (12q12), AMP2 (12q14.1) and AMP3 (12q21.33) were noted across the entire long arm of chromosome 12 (Figure 2D). Of these 14 cases, four of them (80, 123, 248, 341) displayed all three amplicons. The AMP1 was noted in 10 cases covering 1.8 Mb region between RP11-91K15 and RP11-90I21 with peak amplification for clone RP11-

Figure 2
A representative chromosome profiles showing homozygous deletions (A, E) and high-level amplifications (B- E). The clones showing homozygous deletions from 3p14.1 (RP11-89A12) and 17q12 (RP11-79O9) in tumor 06 (A, E) and high-level amplifications from 6p12-p21 in tumor 248(B), 8q12 in tumors 06 and 341 and 8q23-q24 in tumor 06, (C), 12q13-q15 in tumor 48, (D) and 17p11.2 in tumor 06(E), are shown as log ratios (Y-axis). The clones on each chromosome are arranged (pter to qter) on the basis of UCSC mapping positions.
Another amplicon (AMP2) was noted 24.48 Mb distal to AMP1 between RP11-91K23 and RP11-89P15. The AMP3, which was 23.3 Mb distal to AMP2 containing RP11-89F6.

Amplification of 17p11.2 was noted in 27% of the cases analyzed by array CGH. The amplicon was composed of three clones RP11-64B12 (p = 0.0000014), RP11-89K6 (p = 0.0000005) and RP11-189D22 (p = 0.0000001) and covering 3.7 Mb region on the short arm of chromosome 17 (Figure 2E). We used these three clones as FISH probes to validate 17p amplicon in tumors 274, 364, 425 and 628 on interphase/metaphase cells. The distribution of copy number for this amplicon in all the cases ranged from 4–14 copies with peak amplification for clone RP11-189D22 (10–14 copies), followed by and RP11-89K6 (8–10 copies) RP11-64B12 (6–8 copies) (Figure 4C).

**Discussion**

This study represents the first application of genome-wide copy number changes by array CGH in osteosarcoma. Recent studies in breast, renal and bladder cancer showed the potential assessment of this technology in detecting high-resolution copy number changes [9,11,14]. This approach will augment the identification of cancer causing genes by relating the clone information directly with sequence information from human genome database. In this study, we used array CGH to screen for high-resolution DNA copy number changes and precise identification of amplifications in a panel of 48 osteosarcomas.

Gene amplification is an important genetic mechanism in human cancers, as it clearly associated with tumor progression and has a prognostic significance and has even provided a target for therapeutics [17,18]. These amplifi-
cations are often seen at the cytogenetic level as homozygously staining regions (hsrs) or double minute chromosomes (dms). However, cytogenetic recognition of amplifications doesn't contribute to the mapping and identification of amplified DNA sequences. The advent of CGH points an ever-increasing number of chromosomal amplifications in various tumors. These amplifications contribute to the genomic instability in tumors. We have recently shown that the mutation of \( p53 \) significantly correlates with genome-wide DNA instability and seems to represent a major genetic factor contributing to the extremely high levels of genomic instability found in high-grade osteosarcomas [19].

Our analysis have identified frequently amplified clones from 6p12.1-p21, 8q24.13, 12q12, 12q13-q15, 12q21-q21.33, 16p13 and 17p11.2-p12. Amplification of clones from 6p12-p21 region was noted in 25% of the cases analyzed. This was consistent with the previously published results by CGH. By array CGH, we refined the 6p amplicon to 9.4 Mb with amplification peak for clone RP11-81F7. We recently demonstrated the origin of 6p amplicon as consequence of tandem duplication of clones RP11-81F7 and RP11-79F13 [7]. Based on combined array CGH and FISH analysis suggest \( CDC5L \), \( HSPCB \), and \( NFKBIE \), and \( HGNC \) and \( MRPL14 \) are the target genes from 6p12-p21 amplicon. Of these genes, \( CDC5L \) may be an important gene in cancer because of its role as a positive cell cycle regulator for G2/M transition[20]. Consistent with our analysis, overexpression of \( HSPCB \) was shown recently by cDNA microarray studies on osteosarcoma [21]. This protein was shown to play an important role in assemble/disassembly of tubulin by inhibiting tubulin polymerization.

High-level amplifications were also noted from 8q12.1 (RP11-550I15 – 6.3%), 8q21.13 (RP11-89H1 – 6.3%), 8q24.3 (RP11-89K10 – 6.3%) and RP11-637F16 (12.5%). There were no candidate genes present in clones RP11-550I15, RP11-89H1 and RP11-637F16, but clone RP11-89K10 contained \( NSE2 \) (breast cancer membrane protein 101 kDa) gene.

High-level amplification of clones on 12q revealed three distinct sites of amplifications – AMP1 (12q12), AMP2 (12q14.1) and AMP3 (12q21.33). Pervious studies have shown the amplification \( GLI \), \( CHOP \), \( SAS \), \( HMGIC-C \), \( CDK4 \), \( HDM2 \), and \( PRIM1 \) from 12q13-q15 region in osteosarcoma [22,23]. The present array CGH analysis identified a possible target gene \( IFNG \) from AMP2 (RP11-298M11; \( p = 0.0000001 \)), which is physically mapped close to the \( HDM2 \) oncogene locus[24]. Previous studies demonstrated that T-cell production of \( IFNG \) strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway. \( IFNG \) induces rapid degradation of the RANK adaptor protein, TRAF6, result-
ing in strong inhibition of the RANKL-induced activation of the transcription factor NFKB and JNK [25]. The AMP3, which was 23.3 Mb distal to AMP2 containing RP11-89F6. Our analysis from AMP3 revealed two interesting candidate genes: transcription factor ELK3 and PCTAIRE protein kinase 2 (PCTK2). ELK3 is a member of the ETS-domain transcription factor family and the protein is activated by signal-induced phosphorylation [26]. The protein encoded by PCTK2 belongs to the cdck2/cdks subfamily of the ser/thr family of protein kinases and play an important role in the regulation of the mammalian cell cycle [27]. High-level amplification of three clones from 12p13 was noted in case 27 and the amplicon span 4.6 Mb with peak amplification for clone RP11-89D16. No candidate genes contained with in this BAC. Amplification 12p has been reported previously in 9/19 high-grade osteosarcomas by CGH. Recent FISH analysis has identified the amplification of CCND2, ETV6, and KRAS2 from 12p region [28].

Amplification of 17p11.2 was noted in 27% of the cases analyzed by array CGH. Our array CGH analysis has identified three clones with high-level amplifications that spans ~3.7 Mb region on 17p11.2. Several candidate genes were identified within these clones (TPP3A, SMCR5, DRG2, FL11, MYCD, SOX 17, ELAC2, and PMP22). Recent studies have shown the amplification of some of the genes identified in the present study (PMP22, and TOP3A) from 17p11.2-p12 in high-grade OS by semi-quantitative PCR and cDNA microarrays [29,30].

The present array CGH analysis has identified seven recurrent clones exhibiting homozgyous deletions from 1q25.1, 3p14.1, 13q12.2, 4p15.1, 6q12, 6q12 and 6q16.3. These chromosomal regions were consistent with previously reported studies by loss of heterozygosity (LOH) and CGH [3-7,31]. The clone, RP11-90M15 (13q12.2) contain possible candidate gene MTMR6, a protein-tyrosine phosphatase gene and shown to be present within a cloned region that encompasses a translocation breakpoint (t(8;13) in an atypical myeloproliferative disorder [32]. Homozygous deletions of two clones spanning approximately 2.6 Mb of 6q12 region containing candidate genes – nuclear fragile X mental retardation protein interacting protein 1 pseudogene (NUFIP1P) and BAI3 gene (brain-specific angiogenesis inhibitor gene), which is to homologous to BAI1 and shown to suppress glioblastoma [33].

Conclusions

In summary, high resolution array-based CGH revealed large number of chromosomal aberrations previously identified in osteosarcoma by chromosomal CGH and conventional cytogenetic methods. The present study allowed precise identification of smaller DNA copy number alterations, which suggest the presence of specific target genes in osteosarcoma. Although this study suggested several possible target genes from amplified regions from 6p, 8q, 1q2 and 17p, but these genes should be validated by other molecular and immunohistochemical approaches on well-defined large patient samples. Further, interaction or association studies between small genomic losses and gains will facilitate the identification of new genetic pathways in the pathogenesis of osteosarcoma.

Competing interests

None declared.

Authors contributions

TKM and KJ have contributed towards the data analysis. LP, ML, RG, and CL were assisted in sample collection and clinical information of the patients. X-YL has involved in array CGH experiments and data collection. CPH has involved in extracting the gene information from BAC clones. SS has provided the arrays used in this study. PHR was involved in the planning, and organization of the project.

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