Imaging, Diagnosis, Prognosis

Genomic Alterations and Allelic Imbalances Are Strong Prognostic Predictors in Osteosarcoma

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Abstract

Purpose: Osteosarcoma, the most common primary malignant tumor of the bone, is characterized by complex karyotypes with numerous structural and numerical alterations. Despite attempts to establish molecular prognostic markers at the time of diagnosis, the most accepted predictive factor remains the histologic evaluation of necrosis after neoadjuvant chemotherapy. The present approach was carried out to search for genome-wide recurrent loss of heterozygosity and copy number variations that could have prognostic and therapeutic impact for osteosarcoma patients.

Experimental Design: Pretherapeutic biopsy samples of 45 osteosarcoma patients were analyzed using Affymetrix 10K2 high-density single nucleotide polymorphism arrays. Numerical aberrations and allelic imbalances were correlated with the histologically assessed response to therapy and clinical follow-up.

Results: The most frequent genomic alterations included amplifications of chromosome 6p21 (15.6%), 8q24 (15.6%, harboring MYC), and 12q14 (11.1%, harboring CDK4), as well as loss of heterozygosity of 10q21.1 (44.4%). All these aberrations and the total degree of heterozygosity of each tumor were significantly associated with an adverse outcome of patients and were used to define a chromosomal alteration staging system with a superior predictive potential compared with the histologic regression grading.

Conclusions: Structural chromosomal alterations detected by single nucleotide polymorphism analysis provide a simple but robust parameter to anticipate response to chemotherapy. The proposed chromosomal alteration staging system might therefore help to better predict the clinical course of osteosarcoma patients at the time of initial diagnosis and to adapt neoadjuvant treatment in patients resistant to the current protocols. Clin Cancer Res; 16(16): 4256–67. ©2010 AACR.

Due to advances in therapy over the last decades, long-term survival in osteosarcoma patients with localized disease has improved and now reaches about 65% with multimodal therapy. Nevertheless, 30% to 40% of patients die because of tumor progression or relapse (1). Individualized intervention schemes based on the biological characteristics of each tumor have been shown to be successful in other pediatric cancer types, e.g., in acute lymphatic leukemia and in neuroblastoma. Also concerning osteosarcomas, several studies have been carried out to combine common histologic parameters with genetic and molecular findings to predict the clinical outcome of patients (2–4). However, the histologic response to neoadjuvant chemotherapy ("regression grade") assessed after definitive surgery is still the gold standard concerning prognostic prediction (5, 6).

Osteosarcomas are characterized by highly complex karyotypes and a high frequency of chromosomal copy number changes (3, 7–12). It has already been shown in other tumors, such as gastrointestinal tract carcinomas, that a high complexity of chromosomal instability is correlated with an unfavorable outcome (13). In this study, we describe the use of Affymetrix single nucleotide polymorphism (SNP) arrays in a genome-wide high-resolution approach. Both loss of heterozygosity (LOH)
and variations in DNA copy numbers (CNV) were assayed to identify possible genomic fingerprints for the prediction of response to chemotherapy and prognosis. Furthermore, the osteosarcoma genomes were investigated for chromosomal gains and losses to identify candidate genes as potential therapeutic targets.

**Materials and Methods**

**Tissue samples and patients characteristics**

Our series of pretherapeutic fresh frozen biopsy samples included 79 osteosarcomas that were numbered consecutively (OS1-OS79). Due to insufficient size or inadequate amount of vital tumor only 45 samples were selected for the present study, including osteosarcoma of the extremities \( (n = 43) \) and the pelvis \( (n = 2) \). In nine cases, tumor and blood samples from the same patient were available. There were 25 males and 20 females with a mean age of 16.5 years (median, 14 years; range, 4-51 years); 10 patients had lung metastases at the time of initial diagnosis. All patients were treated between 1993 and 2007. Preoperative and postoperative chemotherapy was given according to the protocols of the Cooperative German-Austria-Swiss Osteosarcoma Study Group (reviewed and approved by the appropriate ethics committee) after informed consent. Response to chemotherapy was assessed according to the Salzer-Kuntschik (S-K) histologic 6-graded scale. S-K grades 1 to 3 (≤10% viable tumor cells) were classified as good responders and S-K grades 4 to 6 (>10% viable tumor cells) as poor responders. Information on histologic response was available for 44 patients. Follow-up data (0.6-12.7 years; mean, 3.84 years) were available for all patients (Table 1). Additionally, blood samples from five healthy donors were used as hybridization quality controls.

**Translational Relevance**

Osteosarcoma is a highly aggressive neoplasm, with 30% to 40% of patients dying due to progressive disease despite intense multidisciplinary treatment protocols. Up to now, the clinical course of patients cannot be anticipated reliably at the time of initial diagnosis and generally the histologically assessed response to neoadjuvant chemotherapy after definitive surgery has to be awaited. Because up to 50% of osteosarcoma patients only poorly respond to neoadjuvant treatment, molecular predictors might help to stratify patients more accurately in distinct prognostic and therapeutic subgroups. In the present study, we propose that genomic alterations, determined by using a chromosomal alteration staging system, are strong prognostic predictors in osteosarcoma that can be verified at initial biopsy and are superior to the histologic regression grading.

**Affymetrix 10K2 high-density SNP arrays**

Genomic DNA was extracted using the QIAamp-Mini DNA extraction kit (Quiagen) from frozen tissue and blood samples.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients ( (N = 45) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4-51</td>
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<tr>
<td>Mean</td>
<td>16.5</td>
</tr>
<tr>
<td>Median</td>
<td>14</td>
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<tr>
<td>Gender ( (n) )</td>
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<tr>
<td>Male</td>
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</tr>
<tr>
<td>Female</td>
<td>20</td>
</tr>
<tr>
<td>Blood samples available</td>
<td>9</td>
</tr>
<tr>
<td>Site of primary tumor ( (n) )</td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>27</td>
</tr>
<tr>
<td>Tibia</td>
<td>8</td>
</tr>
<tr>
<td>Humerus</td>
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</tr>
<tr>
<td>Pelvis</td>
<td>2</td>
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<tr>
<td>Fibula</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>Histologic differentiation ( (n) )</td>
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<tr>
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<tr>
<td>Chondroblastic</td>
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</tr>
<tr>
<td>Fibroblastic</td>
<td>4</td>
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<tr>
<td>Teleangiectatic</td>
<td>2</td>
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<tr>
<td>Metastasis ( (n) )</td>
<td></td>
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<tr>
<td>Lung</td>
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<td>Follow-up (years)</td>
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<tr>
<td>Range</td>
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<tr>
<td>Mean</td>
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<tr>
<td>Clinical outcome ( (n) )</td>
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<tr>
<td>AWD-lost</td>
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<tr>
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<td>Response to chemotherapy ( (n) )</td>
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<tr>
<td>S-K grade I</td>
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<td>S-K grade II</td>
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<td>S-K grade III</td>
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<tr>
<td>Good</td>
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<td>S-K grade IV</td>
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<td>S-K grade V</td>
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<td>S-K grade VI</td>
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<td>Poor</td>
<td>21</td>
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<tr>
<td>No S-K available</td>
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</tbody>
</table>

**NOTE:** S-K grade I, no residual viable tumor; grade II, solitary viable tumor cells; grade III, ≤10% viable tumor, grade IV, 10-50% viable tumor; grade V, 50-80% viable tumor; grade VI, >80% viable tumor. Abbreviations: CR, complete remission; lost, lost to follow-up; AWD, alive with disease; DOD, dead of disease.
DNA samples were processed according to the standard GeneChip Mapping 10K (V2.0) Xba Assay protocol (Affymetrix Inc.) as suggested by the manufacturer [http://www.affymetrix.com/products/arrays/index.affx]. In brief, 250 ng of DNA were digested with XbaI and ligated to the Xbal adaptor prior to PCR amplification using AmpliTaq Gold (Applied Biosystems) and primers that recognize the adapter sequence. PCR-amplified DNA was fragmented using DNase I, end-labeled with a fluorescent tag, and hybridized to the array. Hybridized arrays were processed with an Affymetrix Fluidics Station 450, and fluorescence signals were detected using the Affymetrix GeneChip Scanner 3000.

The primary hybridization data were processed using Affymetrix GCOS and GTYPE software, yielding the genotype and hybridization intensity for each individual SNP marker. In a second step the Affymetrix GeneChip Chromosome Copy Number Analysis Tool (CNAT and GTYPE Version 4.1) was applied to visualize the data corresponding to their chromosomal location and to convert the hybridization intensities into DNA copy numbers. CNV with a score <0.75 were classified as losses and with a score >3 as gains.

CNAT also detects genomic regions with unusual or long stretches of contiguous homozygote SNPs and assigns them a score for the likelihood of being caused by a tumor-specific LOH. To distinguish tumor-specific somatic LOH from potential germline homozygosity, a cutoff LOH value of 5 was used. The value varies between 0.01 (no LOH = heterozygous SNP) and 75 (no heterozygous SNP on the entire chromosome arm) and has been explicitly discussed in Affymetrix White Papers. We validated this threshold by comparing regions that showed allelic losses in a small subset of tumor and blood samples. The cutoff LOH value of 5 had the best fit with the LOH data of matched samples and is equivalent to stretches of homozygosity >4 Mb. A homozygous interval of this length was not observed either in the investigated blood samples or in the genome of more than 100 healthy donors (control data provided by Affymetrix). All physical positions were determined using the NetAffx Analysis Center’s UCSC Genomic Query Tool provided by the manufacturer and correspond to the National Center for Biotechnology Information sequencing database (version GRCh37, hg19).

All statistical analyses were done with STATISTICA 4.5 (StatSoft). The correlation analysis carried out in this study used Spearman's rank order correlation coefficient. Differences between good and poor responders to chemotherapy were considered significant at \( P < 0.05 \) (\( \alpha \)-2 sides) using the Wilcoxon-Mann-Whitney rank test.

Results

We collected frozen pretherapeutic biopsy specimens of osteosarcomas from 45 patients, all but one with a record of the subsequent response to chemotherapy. We used Affymetrix 10K SNP microarrays to assay DNA copy-number changes and LOH. A mean call rate of 88.5% (range, 82.71-96.2%) resulted in a minimum of SNP genotypes per case.

Heterozygosity and LOH

In all osteosarcoma samples we observed multiple regions of contiguous SNP homozygosity, ranging in length from 4.7 Mb to 156.6 Mb. These regions were classified by Affymetrix analysis software as areas with LOH (LOH-likelihood score 5 to 70, determined by Affymetrix CNT tool).

An appropriate method to estimate the whole-genome LOH frequency is to compare the frequency of SNP genotypes in a tumor with the population average. All SNPs used in the array are known to be heterozygous in at least 38% of the reference population (data by Affymetrix). The effective heterozygosity in the investigated blood samples as well as in control DNA provided by Affymetrix was on average 34.4%. The percentage of heterozygote markers in the osteosarcoma samples, however, decreased to an average of 27% in good responders and even to 24% in poor responders (Fig. 1A). Applying the LOH scoring algorithm as described above this converts into approximately 100, 1,200, and 2,000 of 9,769 SNPs being homozygote due to a putative allelic loss in blood samples, good-responding tumors, and poor-responding tumors, respectively (Fig. 1B).

Any LOH in a tumor sample is evident from a reduction of the average heterozygosity over contiguous stretches of a chromosome. LOH accompanied by a copy number loss along an entire chromosome was found in 22 of 45 tumors. In all investigated osteosarcomas LOH affected at least 6 chromosomes with a nonrandom distribution throughout the genome. Preferentially affected were chromosomes 2, 3, 5, 6, 10, and 13, which together harbored 51% of all SNPs scored as LOH.

LOH score and LOH profile as putative prediction factors

Response to chemotherapy and metastatic disease are well-known prognostic factors that were also shown in our cohort of patients: the response to chemotherapy significantly reflected a favorable outcome (event-free survival in 73% of the good responders compared with 38% of the poor responders; \( P = 0.005 \), data not shown), whereas the presence of metastases represented the most decisively negative prognostic factor (Fig. 1C and D). However, the frequency of LOH was also strongly associated with the patients’ outcome (Fig. 1E). The most striking finding was the unequal distribution of LOH with respect to the histologically evaluated response to chemotherapy: patients with a high LOH score (more SNPs scored as LOH than the average of 1,500) significantly more often had a poor response to chemotherapy than had patients with a low LOH-score (Wilcoxon-Mann-Whitney rank test, data not shown). The prognostic value of heterozygosity depended on the chromosomal region of the affected SNP. The percent distribution of the LOH values along the
The genome of all investigated osteosarcomas, stratified by their response to chemotherapy, is shown for each autosomal chromosome in Fig. 2A.

**LOH regions in relation to response to therapy and relapse**

The most common LOH locus (in 22 of 45 cases; 48.9%) was found on a 4.2 Mb region of chromosome 13 (13q14.13-13q14.2; minimal deleted region in positions 45.553.450-49.731.408; 24 SNPs) harboring among other genes the osteosarcoma associated tumor suppressor gene **RB1**. LOH on 13q did not show any correlation to the response to chemotherapy or event-free survival (Fig. 2B and E).

The region 11p15.1-11p15.4 on chromosome 11 exhibited LOH in 5 separate SNP stretches (in 12 of 44 cases; 27%), the most prominent measuring 3.6 Mb (positions 8.460.319-11.973.737; 16 SNPs) and harboring among others, the genes **WEE1, ST5**, and **LMO1**. LOH of 11p was found significantly more often in patients with poor response to chemotherapy (P < 0.005). LOH in this region was significantly associated with event-free survival, independent of the absence (P = 0.0108) or presence (P = 0.0089) of primary lung metastases (Fig. 2D and G).

The region 10q21.1 on chromosome 10 (2.5 Mb; positions 55.666.767-58.131.081; 17 SNPs), harboring **PCDH15** and **ZWINT1**, among others, showed a very high LOH frequency (21 of 45 cases; 46.7%) and was significantly more often found in patients with poor response to chemotherapy (P < 0.005). LOH in this region was significantly associated with event-free survival, independent of the absence (P = 0.0108) or presence (P = 0.0089) of primary lung metastases (Fig. 2D and G).

**Clusters of CNV**

LOH can be associated with DNA CNV. Signal intensity data for the quantification of allelic copy numbers changes indicated that 41% of the LOHs found in osteosarcoma genotypes were due to allelic losses and gains, including events of duplication or chromosomal loss. The remaining 59% of SNPs showed LOH without CNV.

Long stretches of homozygosity were frequently associated with CNVs. To predefine groups, the osteosarcomas were clustered according to their CNV similarity using the processed CNV score-profiles of all SNPs. It was found that there were three different groups of CNV similarity on individual SNPs (Fig. 3A; squares A, B, and C).
A, the chromosomal pattern of genome-wide LOH probability derived by SNP arrays in 44 osteosarcomas (proportional-sized probe sets for each chromosome). Each chromosome illustration shows the average percentage of LOH-affected SNPs, calculated for the good (left greenish column) and the poor (right reddish column) response to chemotherapy patient group, respectively. In the heatmap diagram, cold colors represent low, hot colors high frequencies of LOH. B to D, LOH distribution in osteosarcomas on chromosomal regions. Average percentage of LOH affected SNPs was calculated separately for patients with poor (black) and good (grey) response to chemotherapy. B, chromosome 13 contained a region on 13q14.2, harboring the RB1 gene, of high LOH frequency that affected 22 of 44 osteosarcomas (50%). C, five distinct regions (red colored bars) on chromosome 11p15.1-4 (red dots) found in 12 of 44 osteosarcomas (27%) strongly discriminated between poor and good response to chemotherapy. D, chromosome 10q21.1 contained a region with LOH in 21 of 44 osteosarcomas (47%) that also decisively distinguished between good and poor responders. E to G, Kaplan-Meier analysis for event-free survival (EFS; patients with ppm were excluded in dotted and included in solid lines). No significant correlation was found between LOH on 13q14.2 and relapse (E; \( P = 0.3 \)) or between LOH on 11p15.1-4 and relapse (F; \( P = 0.4 \)); between LOH on 10q21.1 and relapse a highly significant correlation was detected (G), independent of including \( (P = 0.0089) \) or excluding \( (P = 0.0108) \) ppm patients.
The three defined clusters illustrate the expected heterogeneity of osteosarcomas. Medial cluster B conjoins osteosarcomas (22 of 45) of heterogenic and variable characteristics (short branches indicate low distinguishing potential) and with no obvious relation to response and prognosis; Kaplan-Meier analysis of event-free survival reveals no significant correlation with the clustering. However, clusters A and C, which represent the both extreme characters of the dendrogram (longest branches indicate the highest dissimilarity with other subclusters), include those osteosarcomas that exhibit in a great majority either uniformity in response to chemotherapy (Fig. 3A, cluster C: poor response) or in frequency of LOH per SNP (Fig. 3B, cluster A: on average 10% of SNPs affected).

**Regions of high amplification**

CNV analysis enabled a construction of profiles (including aneuploidy and common variations on chromosome arms) and the detection of amplified “hot spot” regions. In our cohort, 27 of 45 osteosarcomas exhibited gains of >2-fold that affected at least one chromosomal region (>5 contiguous SNPs), but only 13 of these osteosarcomas showed high-level amplifications (>3-fold copy number gains). Amplifications were found to be most frequent (10 of 45 osteosarcomas) on 6p12-p21 (Fig. 4A), where three separate hot spot regions could be identified. In one case, an additional amplified region of 0.4 Mb was identified at 6p22 harboring the E2F3 gene. Six SNPs in a row displayed a signal that...
Fig. 4. A, chromosomal region 6p12-p21 spans a stretch of nearly 25 Mb between the positions 54741994 (6p12) and 30200689 (6p21). High amplification patterns in osteosarcomas found on chromosome 6 showed three distinct loci of amplification with a mean peak of frequency in a 5 Mb stretch between positions 40910958 and 45607978. The additional single event of high amplification found on 6p22 (at positions 19921598-20345022) harbors the gene **E2F3**. B to D, high-amplification regions in osteosarcomas. Kaplan-Meier analysis for event-free survival (the patients with ppm were excluded in dotted and included in solid lines). B, 6p12-p21. Amplification was significantly associated with poor event-free survival (**P** = 0.018). C, 8q24.21. All patients affected by amplification suffered from relapse within 2 years after initial diagnosis, irrespective of including (**P** = 0.004) or excluding (**P** = 0.0003) ppm patients. D, 12q14. All five patients affected by amplification showed primary metastases; there was a trend towards event-free survival (**P** = 0.07). E to G, Kaplan-Meier analysis for event-free survival comparing the S-K regression grading (dotted lines) and the proposed CAS systems (solid lines). E, CAS1. F, CAS2. G, CAS3. Kaplan-Meier analysis for event-free survival comparing the S-K regression grading (dotted lines) and the proposed CAS systems (solid lines) only for patients without primary metastases. H, CAS1. I, CAS2. K, CAS3.
was on average 16 times higher than the surrounding signals (Fig. 4A).

Amplification in one or more of the three 6p-regions was found in 10 of 45 osteosarcomas (22%); the most frequently amplified region (in 6 of 45 osteosarcomas; 14%) was approximately 4.7 Mb in size, localized between physical positions 40,910,958 and 45,607,978, and included the potential candidate genes CCND3, PTK7, and RUNX2. Amplifications on 6p12-p21 were found in both response groups, namely in 4 of 23 patients with good and 6 of 22 patients with poor response. However, event-free survival was significantly ($P = 0.018$) less common in patients whose osteosarcomas showed high amplifications in this region (Fig. 4B).

The MYC-containing region 8q24.21, which is 0.8 Mbp in size, was found to be amplified in 7 cases, including 5 highly amplified cases. Amplifications were significantly associated with a worse overall survival ($P = 0.01$) and event-free survival ($P = 0.0003$). Three of seven patients exhibiting an 8q-amplification had primary pulmonary metastases (ppm). However, even after excluding these three patients with ppm from the collective, the remaining four had an invariable significant association with event-free survival ($P = 0.004$) and overall survival ($P = 0.01$; Fig. 4C).

The CDK4-harboring region 12q14, which is 4 Mbp in size, was found to be amplified in 5 cases, including 3 highly amplified cases. All 5 amplifications were associated with primary lung metastases (Fig. 4D) and with relapse ($P = 0.07$). Additionally, 10 cases had LOH stretches at the same region that were significantly associated with poor event-free survival in the whole collective ($P = 0.002$) and in patients without primary metastases ($P = 0.011$).

### Chromosomal alteration staging as predictive factor

The association between chromosomal alterations and clinical outcome of patients was investigated using Fisher’s exact test. Therefore, three distinct chromosomal alteration staging (CAS) constellations of genomic events were defined and subsequently compared with the S-K regression grading system concerning their prognostic impact: CAS1 included tumors with either an above average LOH score (more SNPs scored as LOH than the average of 1,500) or amplification of 6p, 8p, or 12q; CAS2 comprised osteosarcomas with either LOH on 10q or amplification of 6p, 8p, or 12q; and CAS3 comprehended cases with a combination of two of the following four events: LOH on 10q, amplification of 6p, 8q, or 12q.

Table 2 compares the S-K regression grading and the proposed CAS constellations regarding the number of correctly predicted cases ($nC$). The occurrence of relapse was used to differentiate between cases with good and poor prognosis. No difference was found between the group of good responders (S-K I-III) and CAS3 ($18$ correctly predicted cases with no relapse in both groups), but in all other subgroups CAS1 to CAS3 were superior in predicting

### Table 2. Correlation between the Salzer-Kuntschik regression grading and the proposed chromosomal alteration staging systems

<table>
<thead>
<tr>
<th></th>
<th>nC</th>
<th>CAS1</th>
<th>CAS2</th>
<th>CAS3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nC</td>
<td>Above average LOH score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cases ($n = 45$)</td>
<td>nR = 18 (40%)</td>
<td>28 (62.2%) ns</td>
<td>30 (66.6%) $P = 0.0053$</td>
<td>31 (68.8%) $P = 0.0049$</td>
</tr>
<tr>
<td>S-K I-III ($n = 23$)</td>
<td>nR = 5 (21.7%) nC = 18 (78.3%)</td>
<td>11 (47.8%) ns</td>
<td>11 (47.8%) ns</td>
<td>13 (56.5%) ns</td>
</tr>
<tr>
<td>S-K III-IV ($n = 26$)</td>
<td>nR = 9 (34.6%) nC = 17 (65.4%)</td>
<td>15 (57.7%) ns</td>
<td>18 (69.2%) $P = 0.0243$</td>
<td>20 (76.9%) $P = 0.0045$</td>
</tr>
<tr>
<td>S-K IV-VI ($n = 21$)</td>
<td>nR = nC = 13 (61.9%)</td>
<td>16 (76.2%) $P = 0.0408$</td>
<td>18 (85.7%) $P = 0.0028$</td>
<td>17 (81.0%) $P = 0.0117$</td>
</tr>
<tr>
<td>S-K V-VI ($n = 7$)</td>
<td>nR = nC = 5 (71.4%)</td>
<td>6 (85.7%)*</td>
<td>6 (85.7%)*</td>
<td>6 (85.7%)*</td>
</tr>
</tbody>
</table>

Abbreviations: nC, number of cases with correct prediction of the clinical course; nR, number of cases with relapse, ns, not significant.

*Insufficient case numbers for statistics.
the correct clinical course of patients compared with the S-K score. Because the cutoff of 10% viable tumor cells defines good and poor responders we firstly focused on all cases showing S-K grade III or IV, comprising exactly the cases that were close to that cutoff score. Among these cases, CAS3 predicted the clinical course accurately in 80.8% of patients compared with only 65.4% using the S-K regression grading. Also in the group of S-K poor responders (IV-VI), CAS3 predicted >25% more patients correctly than the S-K score (i.e., nC = 61.9% in S-K IV-VI versus nC = 90.5% in CAS3). In Fig. 4E to G the proposed CAS categories were evaluated concerning event-free survival and compared with the S-K regression grading (Kaplan-Meier analysis). CAS3 unequivocally displays a better prediction of the clinical outcome of patients compared with the histologically assessed response to therapy.

Because metastases at the time of initial diagnosis are the most decisive prognostic factor in osteosarcoma patients (Fig. 1C and D) we omitted these cases (n = 10) in a separate calculation. In Fig. 4H to K the proposed CAS categories were again compared with the S-K score and still show a highly significant predictive impact. Nevertheless, there were two cases with primary metastases that were CAS1-3 negative and clinically showed a complete remission following therapy. One case has been free of disease for 4.77 years, the other case only for 0.76 years. Although nine month of follow-up is definitely not long enough to consider a patient cured, at least one patient with metastatic disease at the time of diagnosis was correctly classified as CAS negative. The remaining eight cases with primary metastases were all CAS positive and relapsed (n = 1) or died of disease (n = 7) subsequently. Chromosomal alteration staging therefore seems to be meaningful also in patients with primary metastases.

We did not find statistically significant correlations between osteosarcoma subtypes and genomic alterations, but we found at least one interesting trend: 11 of 45 osteosarcomas showed at least partial chondroblastic differentiation, and 8 of 11 were classified as poor responders according to S-K grade. However, 4 of 8 poor responders are currently free of disease (range of follow-up, 1.56-5.47 years, mean 3.20 years) and, thus, were prognostically misclassified. Interestingly, all these cases were CAS negative contrary to the remaining 4 of 8 poor responders that indeed revealed a poor clinical outcome and all turned out to be CAS positive.

Discussion

Although the understanding of the molecular pathogenesis of osteosarcoma has advanced in the last two decades, risk assessment continues to be based mainly on clinical and histopathologic parameters (14–16). Many studies have been conducted in recent years to establish molecular markers that might help to identify patients for whom maximal therapy is necessary and others for whom therapy of reduced intensity is sufficient to achieve long-term survival. So far, such molecular markers have not been identified. The present study was undertaken to search for genes or chromosomal locations with potential patho-
genetic, prognostic, and therapeutic impact in a genome-

LOH patterns in osteosarcoma

In the vast majority of the genomes of the investigated osteosarcomas we observed LOHs and CNVs indicating a high degree of chromosomal aberrations and allelic imbalances. Significantly more chromosomal variations, especially LOHs, were observed in tumors with a poor response compared with tumors with a good response to preoperative chemotherapy. Furthermore, patients with tumors showing above average LOH scores developed recurrent disease more often than did patients with tumors showing low LOH scores. A correlation between chromosomal imbalances and the prognosis of patients has recently been shown not only in osteosarcomas, but also in other tumors, including gastric carcinomas, but the biological basis of these observations is poorly understood (13, 17, 18). The high LOH scores in osteosarcomas with a poor response to chemotherapy found in our study might represent an imbalanced loss of various genes involved in cell cycle and apoptosis regulation and therefore might reflect a more aggressive phenotype with a potential survival advantage against chemotherapy.

The locus most frequently affected by LOH in our study was found on a 4.2 Mb region of chromosome 13, harboring the tumor suppressor gene RB1. The RB1-LOH rate of 43% in our study was comparable with the rate of 37.2% to 39% published in the literature (19, 20). Allelic imbalances of the RB1 locus have previously been reported in osteosarcoma, but their predictive impact remains controversial. Earlier studies revealed high LOH rates for osteosarcoma at the 13q14 locus, and the authors found them to be associated with a poor prognosis (20, 21). More recently, Heinsohn et al. were not able to confirm this prognostic impact, which is in accordance with our findings (19). Remarkably, the LOH region on chromosome 10q21.1, harboring PCDH15 and ZWINT1, was more important in respect of prognostic discrimination (Fig. 2D and G). The observed LOH frequency was equivalent to those of the RB1 locus, but occurrence of LOH in this region was adversely correlated with event-free and overall survival. Another region with a recurring LOH pattern in 22% of the investigated cases was found on chromosome 12q13-14 harboring CDK4, which was described in other studies before (16, 22). In our study, we remarkably did not only find amplifications of this region but also high-score LOH stretches that were significantly correlated with relapse.

Only recently, the deletion of the 9p region containing the CDKN2A gene has been reported to represent an early event in mouse models for osteosarcoma development and to constitute an independent factor for adverse clinical outcome of osteosarcoma patients (23, 24). Due to the limited resolution of the 10K2 high-density SNP arrays...
used, our data did not allow determining deletions of the CDKN2A gene but detected LOHs of the respective locus in 5 of 45 cases (11%), which is in agreement with the literature. However, we found no correlation to clinicopathologic parameters, including the clinical outcome of patients. Freeman and colleagues reported the common event of copy number gains in the EGFR and copy number losses in the PTEN gene and therefore suggested a dysregulated PI3K-AKT/mTOR pathway to play a role in the development of osteosarcoma (25). In our study we identified LOHs in the region of the EGFR gene in 13 of 45 cases (29%) and of the PTEN gene in 6 of 45 cases (13%). In agreement with the study of Freeman et al. we did not find correlations to clinicopathologic parameters. The region 3q13.31, harboring the LSAMP gene, has recently been reported to be commonly affected by chromosomal alterations in osteosarcoma. These findings, predominantly representing deletions, were correlated with disease progression and poor survival (26, 27). Due to sufficient resolution of the respective region, we were able to detect deletions in 15 of 45 cases (33%) in our series, 5 of which additionally showed LOHs in the flanking DNA. Additionally, we found four cases with LOH that did not show coincidental deletion of 3q13.31. However, although not statistically significant, the deletion of 3q13.31 showed a trend towards adverse outcome in our series, which is in agreement with the literature (26, 27).

**CNV and aneuploidy**

To estimate the differentiation power of CNV values determined in our study we constructed clusters of similarity (Fig. 3). The resulting tree revealed three major similarity groups and one outlier (OS49). Two groups (denoted A and C) covered osteosarcomas that exhibited a good correlation between LOH and the response to chemotherapy. The remaining cases (group B, harboring the majority of patients with primary metastases) represented another subgroup of osteosarcomas that lacked this concordance. Thus, a definite classification of osteosarcomas is not feasible on the basis of the CNV pattern, most probably due to the high heterogeneity of these tumors.

Suggested proclivity for genomic instability in cancer cells is reflected by the degree of aneuploidy (28). It has been suggested that imbalances affecting thousands of genes as caused by long-range chromosomal gains or losses may be an independent contributor to carcinogenesis (29, 30). Frequent amplification of centromeres or abnormalities in spindle apparatus development leading to missegregation of chromosomes has been observed in numerous types of malignant tumors and is considered as the major contributing factors for chromosome instability in cancer cells (31). Interpretation and combination of our LOH and CNV data allow an overview of frequencies of which chromosomes or chromosomal parts are affected by allelic duplications or losses (virtual karyotyping). Aneuploidy is very common in osteosarcomas; only two samples within our collective showed a normal karyotype without aneuploidy. On average 5.5 chromosomes were affected in every sample (median, 6), with a maximum of 16 chromosomes in the karyotype of one patient. Aneuploidy is a common characteristic of tumors and has been proposed to drive tumor progression (32–34). Our results show that high-level aneuploidy in osteosarcomas is related to poor response to chemotherapy, probably reflecting the aggressiveness of these tumors.

**Amplification hotspots**

Chromosome 6p is often affected by copy number gains in several types of cancer, as revealed by numerous CGH studies (35, 36). The most commonly amplified genomic interval of 6p21-p23 harbors, among others, several candidate genes: angiogenesis-associated VEGFA, cell cycle-associated E2F3, CCND3, and RUNX2, and carcinoma-associated PTK7. These genes are directly or indirectly involved in the pathogenesis and pathways of malignant tumors, and amplifications have also been described in a high percentage of osteosarcomas (4, 11, 12, 17, 37–39). In the present study, we found high amplification spots in 24% of the investigated tumors (Fig. 4B) that were significantly correlated with poor event-free survival. Our data imply a complex pattern of imbalances. There is not a single amplicon covering the whole central region of 6p; instead we found a few smaller hot spot regions harboring CCND3, RUNX2, and VEGFA. These genes have already been found to be overexpressed in osteosarcomas (38), and high expression of VEGFA has been correlated with a poor prognosis (40, 41). Interestingly, the cell-cycle-associated E2F3, a downstream target of RB1, which has been found amplified and overexpressed in other tumors, e.g., in urothelial cancer (42), was the only gene located within a small amplified spot suggesting an alternative way to interfere with the RB1 pathway.

Amplifications found on 8q24.21 (MYC) were significantly correlated with poor event-free survival, independent of the presence of primary metastases. Only one patient of our study harboring this amplification is still event-free (short follow-up of only 1.2 years up to now). Prior investigations on mRNA expression of c-MYC in osteosarcomas showed a correlation between c-myc overexpression and event-free survival (43), supporting the suggested unfavorable prognostic value of the MYC gene overexpression. The authors found c-MYC overexpression in 42% of osteosarcomas, more than two times more frequent than in our group of tumors (15.6%). These findings implicate that DNA copy-number gains are not the only possible mechanisms to regulate c-MYC expression. MYC amplification is already known as a poor prognostic marker in other tumors, e.g., neuroblastomas, and leads to therapy stratification. The distinct prognostic value of MYC amplification in our study should be verified prospectively.

Amplification of 12q14 has already been shown in osteosarcomas (44). This region harbors CDK4, an important cofactor in mechanisms regulating cell-cycle progression, and MDM2, often reported to be coamplified with CDK4. Amplification of the genomic interval containing
MDM2 was not found in our study, a strong hint in favor of two independent amplification events (45, 46). Amplifications on 12q14 were found in 11% of the investigated tumors, but exclusively in patients with primary pulmonary metastases, suggesting a potential role of CDK4 in developing a metastatic phenotype at least in a subset of osteosarcomas.

Chromosomal alteration staging system as predictive factors

Our study reports one of the first uses of a SNP array for genome-wide screening in osteosarcoma, a tumor type that is known for its complex genomic alterations. This complexity hampers the identification of genes in all genome-wide approaches, but confers a clear advantage for studies that combine LOH and CNV data concurrently.

With the powerful method of SNP array analysis, we identified a robust and simple marker, the overall genomic instability (the overall LOH score), to predict response to chemotherapy at the time of diagnosis. Additionally, we have shown that this tool is able to find relevant genes for prognosis (MYC, CDK4) and to identify new potential target genes (E2F3, ZWINT-1), which will be subject to ongoing studies.

Based on the detailed analysis of chromosomal alterations we were able to define a staging system (CAS) that suggests high superiority in predicting the clinical outcome of patients compared with the histologically assessed regression grading. Especially the combination of two different events of chromosomal instability (LOH on 10q, amplification of 6p, 8q or 12q), designated CAS3, was significantly more reliable in differentiating patients with good and poor prognosis, even in the S-K groups near the cutoff of 10% viable tumor cells (S-K groups III and IV, defining good and poor responders). Notably, our proposed CAS system is able to predict the prognosis of osteosarcoma patients at the time of initial diagnosis, whereas the histologic assessment is possible not before the end of neoadjuvant treatment. Because the currently used regimens include high-dose chemother-

apy for 10 weeks prior to definitive surgery, knowledge of the achievable effect of this treatment at initial diagnosis might lead to refinement and modification of the therapy schedule. Furthermore, patients who do not benefit from the current chemotherapy protocols could be spared its associated short-term and long-term side effects. Importantly, the proposed CAS system is applicable for both osteosarcoma patients with and without primary metastases, as shown by omitting patients with metastatic disease at the time of diagnosis (Fig. 4 H-K). Even in the subset of cases with initial metastases the CAS system was able to detect CAS-negative cases that are currently free of disease.

The practicability of CAS in the routine diagnostic procedure has to be proven. At present, we are developing a simplified test based on PCR-SNP assays, whose analysis is technologically feasible in a modern diagnostic laboratory. It will be important to validate the findings reported here in prospective clinical trials and to evaluate the usefulness of the chromosomal markers identified here for strategic treatment decisions in osteosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Nina Weber for excellent technical assistance.

Grant Support

J. Smida, E. Korschning, and M. Nathrath are members of the Translational Sarcoma Research Network supported by the BMBF. M. Nathrath is supported by the Wilhelm Sander-Stiftung and the Elterninitiative krebskranke Kinder München e.V. D. Baumhoer was supported by the Novartis Foundation, formerly Ciba Geigy-Jubilee-Foundation.

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Received 02/02/2010; revised 05/30/2010; accepted 06/25/2010; published OnlineFirst 07/07/2010.

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Published OnlineFirst July 7, 2010; DOI: 10.1158/1078-0432.CCR-10-0284

www.aacrjournals.org  Clin Cancer Res; 16(16) August 15, 2010 4267
Clinical Cancer Research

Genomic Alterations and Allelic Imbalances Are Strong Prognostic Predictors in Osteosarcoma

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Clin Cancer Res  Published OnlineFirst July 7, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-0284

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