Identification of Novel Candidate Oncogenes in Chromosome Region 17p11.2-p12 in Human Osteosarcoma

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Abstract

Osteosarcoma is the most common primary malignancy of bone. The tumours are characterized by high genomic instability, including the occurrence of multiple regions of amplifications and deletions. Chromosome region 17p11.2–p12 is amplified in about 25% of cases. In previous studies, COPS3 and PMP22 have been identified as candidate oncogenes in this region. Considering the complexity and variation of the amplification profiles for this segment, the involvement of additional causative oncogenes is to be expected. The aim of the present investigation is to identify novel candidate oncogenes in 17p11.2–p12. We selected 26 of 85 osteosarcoma samples (31%) with amplification events in 17p11.2–p12, using quantitative PCR for 8 marker genes. These were subjected to high-resolution SNP array analysis and subsequent GISTIC analysis to identify the most significantly amplified regions. Two major amplification peaks were found in the 17p11.2–p12 region. Overexpression as a consequence of gene amplification is a major mechanism for oncogene activation in tumours. Therefore, to identify the causative oncogenes, we next determined expression levels of all genes within the two segments using expression array data that could be generated for 20 of the selected samples. We identified 11 genes that were overexpressed through amplification in at least 50% of cases. Nine of these, c17orf39, RICH2, c17orf45, TOP3A, COPS3, SHMT1, PRPSAP2, PMP22, and RASD1, demonstrated a significant association between copy number and expression level. We conclude that these genes, including COPS3 and PMP22, are candidate oncogenes in 17p11.2–p12 of importance in osteosarcoma tumourigenesis.

Introduction

Osteosarcoma is the most frequent primary bone tumour in children and adults with a peak incidence in adolescence and a second smaller peak after age 50 [1]. The tumours develop mainly in regions with high osteoblastic activity like the metaphyseal regions of the long bones, including the distal femur, proximal tibia and proximal humerus [2]. Primary osteosarcomas arise from primitive mesenchymal cells producing osteoid [3]. The main site of metastasis is the pulmonary region and the metastasized regions are expected to contain one or more oncogenes of which the amplification-induced overexpression is important for osteosarcoma tumourigenesis. Indeed RUNX2 and CDC5L, both encoding cell cycle regulators, in 6p12-p21, and MIC in 8q24 have already been identified as likely candidate oncogenes for osteosarcoma in these chromosomal segments [11–13]. In an earlier small-scale study, we found genes COPS3 in 17p11.2 and PMP22 in 17p12 to be most consistently overexpressed after amplification in osteosarcomas, making these genes candidate oncogenes in that segment [14]. However, due to technical restrictions, the expression status of only about 60 genes and expressed sequence tags in the amplified region could be analyzed. Moreover, the available (chromosome and array) CGH and microsatellite marker data from us and others did not allow an accurate demarcation of the amplified segments within 17p11.2–p12 in the analyzed tumours [8–10,15–19].

Recent studies in other tumour types suggest that amplicons may contain multiple oncogenes that can act independently or

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cooperatively [20,21]. In order to identify all possible candidate oncogenes in the 17p11.2-p12 region, we performed a detailed amplification and expression profiling of this segment in osteosarcomas. To increase the sensitivity of our assays, we restricted our analyses to tumours that were selected for the presence of amplification events in that region.

Materials and Methods

Osteosarcoma samples and osteoblasts

Eighty-five osteosarcomas were selected form a collection, which was established over a period of 15 years (1993-2008) at the Academic Medical Center in Amsterdam. Age of the patients (53% male/47% female) ranged from 6 to 78 (median 16). For those in the 17p-selected group (40% males/60% females) age ranged from 6 to 58 (median 16). All osteosarcoma samples were checked for high (>90%) tumour cell content by an experienced pathologist (J.B.). Human primary fetal osteoblasts were cultured in osteoblast basal medium with osteoblast growth supplement (Cell Applications, Inc, San Diego, CA USA).

Ethics statement

The research was performed at the Department of Genome Analysis of the Academic Medical Center (AMC), Amsterdam, The Netherlands. Clinical samples were irreversibly anonymised and results of scientific research could not be linked to individual patients. The Committee Medical Ethics of the Academic Medical Center specifically waived approval for this study because it falls under paragraph 7:467 Civil Law Code of The Netherlands.

DNA and RNA extraction

Tumour tissue samples were cut in 10 μm sections. These were collected in alternate fashion for DNA and RNA isolation. Genomic DNA was isolated with proteinase K digestion and chloroform extraction according to standard methods. For RNA extraction, fetal osteoblasts were cultured to 90% confluence. Total RNA was extracted from tumour tissue sections and cultured osteoblasts using the Trizol method (Invitrogen, Breda, The Netherlands) and additionally purified according to the RNaseasy protocol (Qiagen, Venlo, The Netherlands). RNA quality was assessed using the BioAnalyzer (Agilent Technologies Inc, Palo Alto, CA, USA). Only samples with RNA Integrity Number (RIN) score higher than 7.5 were used for subsequent analyses.

Quantitative Real-Time PCR

For gene dosage measurements, quantitative PCR (qPCR) was performed using the LightCycler 480 Real-Time PCR system (Roche, Almere, The Netherlands) according to the manufacturer's instructions. Target gene dosages in the tumour tissue samples were normalized against reference gene 36B4. Primers and probes for the target genes and reference genes were designed using the UniversalProbe Library Assay Design Center (Roche, http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) and are listed in Supplemental Table S1. Primers were tested in-silico for specificity (http://www.genome.ucsc.edu/cgi-bin/hgPcr) and on agarose gels for single band amplification. All PCR reactions were performed in triplicate. Normalized ratios were calculated using the Relative Quantification software, provided by the manufacturer, and normal blood as reference. A normalized ratio of 1 is equivalent to the presence of two copies of a target gene in the tissue under study. Amplification was defined as target gene copy number >3.5 in the tumour sample.

Quantitative Real-Time RT-PCR

Quantitative reverse transcription (RT) PCR was used for measurement of mRNA expression levels of genes. For this purpose, total RNA (0.5 μg) was reverse transcribed into cDNA by oligo-dt or random priming, according to standard methods. The cDNA content of target genes in the tumour tissue samples was normalized against that of reference gene SDH5. Design of primers and probes, listed in Supplemental Table S2, specificity testing, and PCR reactions were performed as described for the qPCR method. Fold changes in the expression of target genes were calculated as described above, using cultured human fetal osteoblasts as reference. Target genes that showed a 2-fold or more increase in expression in the tumour sample compared to the expression in the osteoblasts sample were considered to be overexpressed.

Single nucleotide polymorphism (SNP) array analysis

DNAs extracted from tumours with significant amplifications in 17p11.2-p12 were analyzed for whole-genome copy number variation by using Illumina HumanCNV370-Quad BeadChips. The arrays contain probes for 373,397 SNPs. Processing of DNA samples, hybridization, staining, and scanning of the BeadChips, and primary data extraction were all performed according to the Illumina Infinium II protocol at the array facility of ServiceXS (Leiden, the Netherlands). Scoring of genotypes was done using the standard cluster file from Illumina. The arrays were analyzed with Illumina GenomeStudio software (version 2009.2). The LogR ratio (LRR) and the B allele frequency (BAF) data were processed into the OverUnder plugin as described by Attiyeh et al [22], and copy numbers were calculated using Illumina BeadStudio (version 3.2.2). The OverUnder algorithm calculates copy numbers of tumours based on the allelic imbalance and signal intensities and corrects for aneuploidy. The resulting copy numbers calculated for each SNP were then used as input for Gain and Loss Analysis of DNA (GLAD) [23]. GLAD detects the altered regions in the genomic pattern and assigns labels of “normal”, “gained” or “lost” to the segmented data. These output data were used as input for Genomic Identification of Significant Targets in Cancer (GISTIC) analysis [24]. This program identifies and analyses significant chromosomal aberrations across a set of tumour samples, based on the amplitude of the aberrations as well as their frequency of occurrence across the samples and calculates a value for this significance, called the G-score. Multiple hypothesis testing was accounted for using False Discovery Rate (FDR) q-value statistics. A cutoff q-value of 0.25 was used to select significant regions. The SNP data are described in accordance with MIAME guidelines and have been deposited in the NCBI's GEO Omnibus database under accession number GSE32964.

Expression microarray analysis

RNAs extracted from tumours with significant amplifications in 17p11.2-p12 and from human fetal osteoblasts were analyzed for whole genome gene expression analysis using Illumina HumanHT-12 v3 Expression Beadchips. Each array contained 48,804 probes, spanning the entire human transcriptome. Labeling of RNA samples, hybridization, staining, and scanning of the Beadchips, and primary data extraction were all performed according to the Illumina Infinium II protocol at the array facility of ServiceXS (Leiden, the Netherlands). Fold changes in expression of a probe were determined by normalizing the intensity of the average signal in the tumour sample against the average signal in the osteoblasts sample. The expression microarray data are described in accordance with MIAME guidelines.
and have been deposited in the NCBI’s GEO Omnibus database under accession number GSE32964.

Results

SNP array analysis identifies two significant amplicons in 17p11.2-p12

To select for osteosarcoma samples with amplification events in the 17p11.2-p12 region, we performed qPCR for test genes SCO1 at 10.6 Mb (p12), MAP2K4 at 11.9 Mb (p12), MYOCD at 12.6 Mb (p12), COX10 at 14.0 Mb (p12), PMP22 at 15.1 Mb (p12), NCOR1 at 16.1 Mb (p11.2-p12), COPS3 at 17.2 Mb (p11.2), and TOM1L2 at 17.8 Mb (p11.2) on 85 tumours. Twenty-six osteosarcomas displayed amplification events (normalized copy number >3.5) for one or more of the test genes. These samples were subjected to whole genome high-resolution SNP array analysis using Illumina HumanCNV370-Quad BeadChips. The arrays were analyzed with Illumina GenomeStudio software. The resulting LogR ratio (LRR) and the B allele frequency (BAF) data for all SNPs were processed into the OverUnder plugin [22], and absolute copy numbers were calculated using Illumina BeadStudio (see Materials and Methods for details). Amplified regions were found on chromosomes 1, 5–8, 11, 14, 16, 17, 19, and 20 (sex chromosomes excluded). Large deletions were limited to chromosomes 3, 5, 6, 9, 10, and 13. A comparison of the genome-wide copy number profile of these selected tumours with that of unslected osteosarcomas will be presented elsewhere (Both et al., in preparation). Representative chromosome 17 copy number profiles of three osteosarcoma samples (OS2, OS11, OS20) are shown in Figure 1. OS2 exhibits multiple amplification peaks in 17p, superimposed on copy number increases for whole chromosome 17. Figures 1B and 1C show localized amplification events on 17p and diploid copy numbers for the other parts of chromosome 17. The 17p copy number profiles in the other selected osteosarcomas often proved to be complex, as in sample OS2, but always included 17p11.2 and/or 17p12. To determine the most significantly amplified segments in these profiles, we subjected the whole genome copy number data to Genomic Identification of Significant Targets in Cancer (GISTIC) analysis. This method identifies regions that are aberrant (amplified or deleted) more often than would be expected by chance, and gives greater weight to high-amplitude events [24]. The whole genome GISTIC amplification analysis is shown in Figure 2A, a detailed view for chromosome arm 17p is presented in Figure 2B. As expected, the amplification event on chromosome arm 17p (marked by 17p11.2) proved to be most significant, as this was the condition of inclusion. The GISTIC amplification profile also demonstrates significant peaks for chromosomal regions 6p12.3, 8q24.21, and 19q12, containing the recently identified osteosarcoma candidate oncogenes RUNX and CDCL5, MYC, and CCNE1, respectively [11–13,25]. The detailed GISTIC analysis for 17p shows a minimal significant amplified segment in the 17p11.2-p12 region, with two major peaks centered on 13.5 and 17.2 Mb, respectively. The positions of PMP22 and COPS3, which we previously suggested as candidate oncogenes in this region, are indicated.

Integration of copy number and expression data for genes in 17p11.2-p12

Overexpression as a consequence of gene amplification is a major mechanism for oncogene activation in tumours [26,27]. Thus, the candidate oncogenes in the 17p11.2-p12 region should be found to be overexpressed in a significant fraction of the tumours in which they were amplified. In addition, the

Figure 1. Representative examples of copy number (CN) changes along chromosome 17 in osteosarcomas OS2, OS11, and OS20. SNP-array data were processed using the OverUnder algorithm described by Attiyeh et al [22], and copy numbers were calculated using Illumina BeadStudio.

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amplification-induced overexpression of these genes should result in a positive correlation between copy number and expression level in the respective tumours. To identify these candidate genes, we determined expression levels for all genes in the two established amplicons. Expression data for the genes in this region could be generated for 20 of the 26 osteosarcoma samples that were subjected to SNP array analysis. We extracted the expression data from a whole genome expression profiling analysis of 35 osteosarcoma samples with 17p11.2-p12 amplifications and of human osteoblasts, using Illumina HT12 expression array BeadChips. A comparison of the genome wide expression profile of these selected tumours with that of unselected osteosarcomas will be presented elsewhere (Both et al., in preparation).

To establish the correlation between overexpression and amplification, we first calculated for each gene in the 17p11.2-p12 segment in each of the 20 tumours the fold-change in expression relative to the expression in normal osteoblasts. Genes with a two-fold or more increase in normalized expression were considered to be overexpressed. In this way, we identified 53 genes that proved to be overexpressed in at least one of the 20 osteosarcoma samples. We then determined copy numbers for each of these genes in each tumour using the absolute copy numbers of SNPs within or nearest to their coding sequence. The latter were extracted from the preceding SNP array analyses. Genes with copy number $>3.5$ were considered to be amplified. The amplification and overexpression status of each of the 53 genes in the 20 tumours with 17p11.2-p12 amplification is given in Supplemental Figure S1.

Based on this correlative analysis, we identified the 11 genes listed in Table 1, which proved to be overexpressed as a consequence of amplification in more than 50% of cases.

To assess the significance of the association between copy number and expression for these top ranking genes, we performed qPCR and qRT-PCR for each of the genes in each tumour and established the strength of the relationship between these two variables by calculating Pearson’s correlation coefficient $R$. Using a positive $R$ of approximately 0.5 or more and a 2-sided $P$ of 0.05 or less as cut off values for a statistically significant association between copy number and expression, we discarded two of the eleven top genes listed in Table 1, i.e. $GRAP$ and $ALKBH5$, as candidate oncogenes in 17p11.2-p12 (Table 2).

Discussion

Osteosarcomas are characterized by the presence of highly complex genomic aberrations. The p11.2-p12 region on chromosome 17 has already been known for some time to be amplified in
a substantial fraction (25%) of these tumours [9,10,15–19]. However, the great variation and complexity of the amplification profiles in this region, exemplified in Figure 1, have hampered the identification of the driver oncogenes for these amplifications. In this study, we performed a systematic search for these genes based on the principle that a causative gene should exert its oncogenic action via amplification-induced overexpression. We first selected tumours with 17p11.2-p12 amplifications. This enabled us to enrich the dataset for amplification events in our area of interest. Secondly, we applied the GISTIC procedure, which incorporates enriching the dataset for amplification events in our area of interest. 

## Table 1. Top 11 genes most frequently overexpressed through amplification in 17p11.2-p12.

| Gene   | Location | Band Mb | A  | O  | O/A | %  |
|--------|----------|---------|----|----|-----|----|
| SHMT1  | p11.2    | 18.25   | 17 | 15 | 13  | 76.5|
| PMP22  | p12      | 15.15   | 16 | 14 | 12  | 75  |
| RASD1  | p11.2    | 17.39   | 18 | 13 | 12  | 66.7|
| TOP3A  | p11.2    | 19.5    | 17 | 12 | 11  | 64.7|
| PRPSAP2| p11.2    | 17.9    | 16 | 10 | 10  | 62.5|
| COP53  | p11.2    | 17.17   | 18 | 12 | 11  | 61.1|
| GRAP   | p11.2    | 18.94   | 17 | 12 | 10  | 58.8|
| C17orf39| p11.2    | 17.95   | 18 | 10 | 10  | 55.6|
| RICH2  | p12      | 12.75   | 17 | 11 | 9   | 52.9|
| ALXH5  | p12      | 18.1    | 17 | 10 | 9   | 52.9|
| C17orf45| p11.2    | 16.34   | 18 | 10 | 9   | 50  |

Genes were scored for the number of times a feature was found in the tumour (data taken from Supplemental Figure S1). A: number of tumours with amplification of a gene; O: number of tumours with overexpression of that gene; O/A: number of tumours with overexpression and amplification of that gene; %: percentage of tumours in which the amplified gene is overexpressed.

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## Table 2. Pearson’s correlation coefficient \( R \) estimating the relationship between gene copy number and expression level for the top 11 genes most frequently overexpressed through amplification in 17p11.2-p12.

| Gene            | Function of gene product                                                                 | \( R \)  | 2-sided \( P \) |
|-----------------|------------------------------------------------------------------------------------------|----------|----------------|
| C17orf39        | Subunit of complex that co-activates transcription from RNA pol II promoters               | 0.77     | 0.00003        |
| RICH2           | GTPase activator for Rho-type GTPases                                                    | 0.75     | 0.00009        |
| C17orf45        | Non-coding RNA with unknown function                                                     | 0.74     | 0.0001         |
| TOP3A           | Alteration of DNA topologic state during transcription                                   | 0.73     | 0.0001         |
| COP53           | Embryonic development/signal transduction                                                | 0.72     | 0.0002         |
| SHMT1           | One-carbon compound metabolism                                                          | 0.67     | 0.0007         |
| PRPSAP2         | Nucleic acid metabolism                                                                  | 0.64     | 0.001          |
| PMP22           | Component of myelin/cell growth control                                                  | 0.62     | 0.002          |
| RASD1           | Activator of G-protein signaling                                                        | 0.49     | 0.02           |
| GRAP            | Cytoplasmic signaling                                                                    | –0.13    | 0.6            |
| ALXH5           | Alkylation repair homolog                                                                | –0.16    | 0.5            |

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Rab proteins also belong to this family and TBC1D16, encoding Rab3-GAP, has recently been identified as a driver oncogene in melanoma [40]. In analogy, RICH2 might have a comparable function in osteosarcoma tumourigenesis.

The gene product of RASD1 is another member of the RAS superfamily of small GTPases and, as such, presumed to have an oncogenic function. However, Vaidyanathan et al [41] found that expression of RASD1 in a number of cell lines suppressed cell growth, contrary to what would be expected of RAS family members. This would make RASD1 a poor candidate oncogene for osteosarcoma tumourigenesis. However, in accordance with an oncogenic function, knockdown of the expression of RAB27A, encoding another member of the Ras superfamily, was shown to inhibit the growth of melanoma cells [40]. In analogy, RASD1 might have such an oncogenic aspect as well.

Two types of topoisomerases are found in mammals, type I which alters topology by a single strand break and type II which inhibits the growth of melanoma cells [40]. In analogy, RASD1 might have a comparable function.

SHMT1 functions as a regulator in the de novo synthesis of thymidine nucleotides. Several polymorphisms in the SHMT1 gene have been linked to a higher chance of cancer, like acute lymphoblastic leukaemia [49,50], ovarian cancer [51] and prostate cancer [52]. PRPSAP2 is another protein involved in nucleic acid metabolism. The PRPSAP2 gene encodes part of the enzyme PRPP synthetase, which catalyzes the formation of phosphoribosylpyrophosphate, being a primary substrate for newly formed pyrimidine nucleotides. Depletion of the PRPP synthetase causes growth arrest [53], the reverse has not been shown. Overexpression of SHMT1 and PRPSAP2 (and also COP5) has been reported to occur in multiple myelomas [54].

Conclusion

Our systematic screening of genes in the 17p11.2-p12 region has yielded nine candidate oncogenes associated with osteosarcoma tumourigenesis. Functional studies are underway to test these candidate genes for their oncogenic potential. To test their prognostic potential, we are currently investigating the expression of our candidate genes at the RNA and protein level in an extended series of osteosarcomas with or without metastasis or local recurrence. It has already been demonstrated that there is a significant relationship between strong COP5 staining and patients that develop metastasis [30]. However, prognostic studies for the other candidate genes require the development of new antibodies (CI1af59, RICH2, RASD1) or testing of available antibodies on osteosarcoma tissues (TOP3A, COP5, SMH1, PRPSAP2, PMP22), which is presently underway. The candidate oncogenes could be useful as biomarkers for improvement of diagnosis and as possible targets for therapeutic intervention in osteosarcoma and in other tumour types as well.

Supporting Information

Figure S1 Amplification and overexpression status of genes in chromosome region 17p11.2-p12 in osteosarcoma. Cells are colored as follows. Blue: gene amplified, yellow: gene overexpressed, green: gene amplified and overexpressed, blank: gene without amplification and overexpression.

Table S1 qPCR primers.

Table S2 qRT-PCR Primers.

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Author Contributions

Conceived and designed the experiments: J. Both TJMH. Performed the experiments: J. Both TW J. Bras GRS. Analyzed the data: J. Both FB TJMH. Contributed reagents/materials/analysis tools: J. Bras GRS FB. Wrote the paper: J. Both TJMH.

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