

## ACCELERATED PAPER

# Allelic imbalance at intragenic markers of *Tbx18* is a hallmark of murine osteosarcoma

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**We have recently identified a locus exhibiting a high frequency of allelic imbalance (AI) in both spontaneous human (HSA 6q14.1-15) and radiogenic murine (MMU9, 42 cM) osteosarcoma. Here we describe the fine mapping of the locus in osteosarcoma arising in (BALB/c × CBA) F<sub>1</sub> hybrid mice. These studies have allowed us to identify *Tbx18*, a member of the T-box transcriptional regulator gene family, as a candidate gene. Three intragenic *Tbx18* polymorphisms were used to map the region of maximum AI to within the gene itself; 16 of 17 tumours exhibited imbalances of at least one of these markers. The highest frequency was found in exon 1, where 14 of 17 tumours were affected at a single nucleotide polymorphism at 541 nt. Two polymorphic CA repeat markers in intron 2 and intron 5 demonstrated overlapping regions of imbalance in several tumours. Both markers flanking the *Tbx18* gene (D9Osm48 and D9Mit269) revealed significantly lower frequencies of imbalance and confirmed the limitation of the common interval to *Tbx18*. Examination of both the mouse and human annotated genomic sequences indicated *Tbx18* to be the only gene within the interval. Sequence analysis of the *Tbx18* coding region did not reveal any evidence of mutation. Given the haploinsufficiency phenotypes reported for other T-box genes, we speculate that AI may influence the function of *Tbx18* during osteosarcomagenesis.**

## Introduction

Osteosarcomas are characterized by a high degree of chromosomal alteration (1–3), making it difficult to identify those genes specifically involved in the process of osteosarcomagenesis. Nevertheless, a consistent picture of mutations of genes acting as negative regulators of the cell-cycle pathway (Rb1 and p16) has evolved (4–8), highlighting the importance of dysregulated cell-cycle control. Mutation of p53 or amplification of Mdm2 are also frequently associated with osteosarcoma formation, showing requirement for reduced apoptosis or failure to undergo DNA damage-induced cell-cycle arrest (4,6,8–11).

Although frequent mutations or deletions of these genes are

now evident, their importance for bone tumourigenesis could only be established because of prior knowledge of their function, acting either as general tumour suppressor or oncogenes. Genes with a specific function in osteoblast growth regulation or differentiation are not known so far to be mutated in osteosarcoma.

In the case of Paget-associated bone tumours, PDB2 (Rank, OMIM 603499) is a prominent candidate gene at the LOH locus 18q22.1 (12), as it is specifically involved in bone tissue homeostasis. However, Rank mutations have not been reported yet in osteosarcoma. As Rank acts as an osteoclast-activating factor (13,14), one would also expect it to be associated with osteoclastic rather than osteoblastic osteosarcomas, with the latter representing the predominant histological subtype of all bone sarcomas.

There is considerable heterogeneity both in histological appearance and therapeutic response of osteosarcoma, and the possibility of predicting tumour progression from observed genetic alterations is rather poor. This may well be due to the complex pattern of chromosomal and genomic changes in osteosarcomas, which could easily obscure specific gene alterations.

In a recent study we have successfully employed a parallel analysis in human and murine osteosarcoma to map chromosomal loci affected by allelic imbalance (AI) (15). Allelotyping of DNA markers and genes has allowed us to identify a new locus in a region of conserved synteny between mouse chromosome 9 (43 cM) and human chromosome 6 (6q14.1-15), exhibiting a high degree of genetic alterations in osteosarcoma of both species. An interval of ~4 Mbp was identified as the common region of AI and was thus postulated to harbour a yet unidentified tumour-suppressor gene.

The present paper describes further mapping of this gene by allelotyping murine osteosarcoma for a number of strain-specific polymorphism in this interval. As *Tbx18* was found to be the only gene located in the region, we have used intragenic *Tbx18* markers to assay AI. We conclude that *Tbx18* is a strong candidate for the gene affected by AI on mouse chromosome 9.

## Materials and methods

### *Tumour induction and diagnosis*

The mouse osteosarcoma model used in these studies has been described elsewhere (15–17). Briefly, female BALB/c×CBA F<sub>1</sub> hybrid mice were injected at the age of 100 days with a single i.p. dose of 185 Bq/g body wt Th227 (as thorium citrate), which induces osteosarcoma in ~35% of animals. Osteosarcoma was confirmed by radiological and histological examination.

### *DNA–RNA extraction and reverse transcription*

DNA and RNA from tail tips and fresh-frozen osteosarcoma was extracted using the DNA–RNA midi kit (Qiagen GmbH, Hilden, Germany). Prior to the lysis, the tissue was homogenized for 10 s using an Ultra-Turrax T8 (IKA Labortechnik Staufen, Germany).

For tumours <2 mm in diameter, DNA and RNA was extracted from 10 µm tissue sections after microdissection of tumour cells and surrounding normal tissue cells. DNA purification from these samples was carried out using the QIAamp DNA kit including proteinase K lysis (Qiagen GmbH),

**Abbreviations:** AI, allelic imbalance; PCR, polymerase chain reaction.

whereas RNA was extracted using 24 h SDS-Proteinase K lysis followed by classical phenol (pH 4)-chloroform purification.

Reverse transcription using 1 µg total RNA was carried out at 42°C with Superscript II polymerase (Gibco BRL Life Technologies, Karlsruhe, Germany) primed with random hexamers according to manufacturers instructions.

#### Radiation hybrid mapping

For radiation, hybrid mapping 4 µl of each of the T31 panel DNA templates (Research Genetics, Huntsville, AL) was probed by PCR amplification for a 3' UTR genomic fragment of the murine *Tbx18* gene using the following primers: 5'-GGTACACTTGAAAGTTGGTAAGATG-3' (*Tbx18RHf*); 5'-CA-TTCTCACATCAAGTCCAAGG-3' (*Tbx18RHr*).

Reaction mixtures contained 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 1 U *rTaq* polymerase, 0.3 µmol of each dNTP (all Amersham Pharmacia, Freiburg, Germany) and 5 pmol of each primer in a final volume of 20 µl. PCR conditions were as follows: 4' initial denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final 7 min incubation at 72°C on a GeneAmp9700 (Perkin Elmer Applied Biosystems, Foster City, CA). The panel was scored for presence or absence of the amplicon in 3% agarose-TBE gels containing 30 µg/l ethidium bromide after 1 h electrophoresis at 4 V/cm. The raw data vector for *D9Mit9*, *Mod1*, *D9mit269* and *Nt5* were derived from the MGD database (<http://www.jax.org/resources/documents/cmdata/rhmap>) and loaded into MapManager QT (version b29) for mapping (18).

#### Sequencing of *Tbx18*

*Tbx18* cDNA was amplified in overlapping fragments covering the entire coding sequence using the following primer-pairs: 5'-CATCGGGAG-CTGCGGAGC-3' (*Tbx18/245f*); 5'-GAGATCTTCACCCGCATTGCTG-3' (*Tbx18/900r*); 5'-GTCTCCCGTACCTGGCTG-3' (*Tbx18/729f*); 5'-CTCAC-TGCTCTGGTTGGTAAG-3, (*Tbx18/1710r*); 5'-CACATCCTCACCTGTTG-TCTG-3' (*Tbx18/1523f*); 5'-CATTCTCACATCAAGTCCAAGG-3' (*Tbx18/2671r*).

For the reaction mixture see above. PCR conditions were as follows: 4' denaturation at 94°C, 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min 30 s at 72°C, followed by a final 7 min incubation at 72°C.

PCR amplification of *Tbx18* exon 1 was carried out in 20 µl using 20 ng genomic DNA, 1 U Pfu polymerase, 1× PCR buffer (both Stratagene, La Jolla), 4% DMSO, 0.3 µmol of each dNTP (Amersham Pharmacia) and 5 pmol of each primer. Primer sequence were as follows: 5'-CTTCTCT-GTGAGGCACTGATCG-3' (*Tbx18/408f*); 5'-GCAGCCGCTTCTTCGT-CG-3' (*Tbx18/575r*).

PCR conditions were as follows: 4' denaturation at 94°C, 35 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C, followed by a final 7 min incubation at 72°C. The PCR products were size fractionated by gel electrophoresis in 1.5% agarose-TAE with ethidium bromide staining and purified for sequencing using the Qiagen gel extraction kit (Qiagen GmbH).

For cycle sequencing, 100 ng of the purified PCR products were mixed with 3 µl Big Dye terminator kit (Applied Biosystems, Weiterstadt, Germany), 2.5 pmol primer and water to a final volume of 15 µl. Sequencing primers were identical to those used in the preceding PCR reaction. Sequencing conditions were as follows: 10 s initial denaturation at 94°C followed by 25 cycles of 10 s at 96°C, 5 s at 50°C, 4 min at 60°C. Reaction products were purified according to manufacturers recommendations and analysed on an ABI377 sequencer using 5% long ranger polyacrylamide gel (FMC Bio-products, Rockland, ME). All PCR and sequencing reactions were carried out on a GeneAmp9700 cyclor (Perkin Elmer Applied Biosystems).

#### Restriction fragment analysis

For restriction fragment length polymorphism (RFLP) detection of the *Tbx18* exon 1 single nucleotide polymorphism (SNP), PCR was performed from normal and tumour DNA using primers: 5'-GCAGCATGCTAAGCCTC-AAGG-3' (*Tbx18\_gen380f*); 5'-TAGAGAAGTGGAAAAGCAGCGGCAG-3' (*Tbx18\_gen929r*).

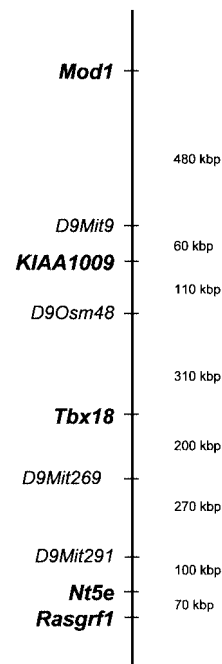
The resulting 549 bp long PCR products were purified using Qiagen PCR purification kit and digested using *BmyI* (Roche Diagnostics GmbH, Penzberg, Germany). Fragments were analysed on 2% agarose-TBE-ethidium bromide gels.

#### Analysis of STS markers in *Tbx18* intron 2 and intron 5 and at an extragenic locus

From the genomic *Tbx18* sequences available at [http://www.ensembl.org/Mus\\_musculus/](http://www.ensembl.org/Mus_musculus/) (chromosome 9, 88 650 000–88 690 000 bp) two polymorphic CA repeat markers in *Tbx18* intron 2 and intron 5 were found. Another polymorphic CA repeat marker (*D9Osm48*) was found ~270 kb proximal to *Tbx18* (ensembl 88 399 172–88 399 223 bp).

To allelotyping tumour and normal tissue DNA for these markers, the following PCR primers were used: 5'-ATGTTCCAGGCCTCAGTTTT-3'

Mmu9



**Fig. 1.** RH mapping using T31 panel places *Tbx18* between markers *D9Mit9* and *D9Mit269* and between the genes *Mod1* and *Nt5*, respectively. This interval also includes the osteosarcoma LOH locus as described by Nathrath *et al.* Markers and genes used for RH mapping are underlined. All other genes and markers together with the distances in kilobase pairs are derived from the ensemble mouse genome database ([http://www.ensembl.org/Mus\\_musculus/](http://www.ensembl.org/Mus_musculus/)).

(*D9Osm48f*); 5'-GCTCTGCATCTCTAGCCTGT-3' (*D9Osm48r*); 5'-CCTTC-AAAAGGTTACTTGCA-3' (*Tbx18i5f*); 5'-GGACAGCGACTCAACACT-CT-3' (*Tbx18i5r*); 5'-GTTCCAACGTTTTTGCATTC-3' (*Tbx18i2f*); 5'-CCC-AGTCCATGGTGTATTGT-3' (*Tbx18i2r*).

At *Tbx18i2* (intron 2, ensembl 88 682 000–88 682 032 bp) the fragment sizes are ~210 bp for the BALB/c allele and 188 bp for the CBA allele. At *Tbx18i5* (intron 5, ensembl 88 667 050–88 667 066 bp) the fragment sizes are ~180 bp for the BALB/c allele and 150 bp for the CBA allele. The primer pair for *D9Mit269* was purchased from Research Genetics. PCR conditions for all CA repeat markers were according to Dietrich *et al.* (19). Quantification of BALB and CBA alleles and evaluation of AI was done as described earlier (15). Briefly, following gel-electrophoresis on 3% agarose or 12% polyacrylamide (for marker *D9Osm48*), gel-images were shot on a digital gel documentation device (DokuGelV, Scanalytics, Fairfax, VA) and quantified using IQ software (BioImageSystems, Ann Arbor, MI). Allele ratio of tumour samples was compared with allele ratio of normal tissue. If the allele ratio in a tumour sample differed by >50% from the related value in normal tissue, the case was classified as showing AI.

## Results

### Radiation hybrid mapping of *Tbx18*

PCR amplification of the 3' UTR of *Tbx18* from genomic DNA using the primer *Tbx18RHf* and *Tbx18RHr* yields a mouse-specific 202 bp long fragment. Probing the T31 radiation hybrid panel we found *Tbx18* amplicons in 34% of all clones. The raw data vector has been deposited at [www.jax.org/resources/documents/cmdata/rhmap/9data.html](http://www.jax.org/resources/documents/cmdata/rhmap/9data.html). According to the RH mapping algorithm ([www.jax.org/resources/documents/cmdata/rhmap/rhsubmit.html](http://www.jax.org/resources/documents/cmdata/rhmap/rhsubmit.html)) the maximal LOD score was found when *Tbx18* was placed distally to marker *D9Mit9* and gene *Mod1* and proximally to marker *D9Mit269* and gene *Nt5* (Figure 1). Thus, *Tbx18* maps at the lower part of the osteosarcoma AI locus identified previously as lying between *D9Mit9* and *D9Mit269* (15).

*Tbx18* sequence in osteosarcoma

*Tbx18* coding sequences (nucleotides 729–2671) were determined from 12 osteosarcoma. In none of the cases, alterations in the cDNA fragment sizes or losses of an entire fragment were detected, thereby excluding the possibility of large intragenic deletions or defects in transcription of the *Tbx18* gene (data not shown). Two base alterations were found in all tumours compared with the published *Tbx18* wt sequence (20; accession number AF306666). Two transitions (A to G or Thr to Ala at nucleotide 1606 and C to T at nucleotide 2339 of

the 3' UTR) were not tumour specific, as they were both present in the normal tissue of BALB/c and CBA/CA mice.

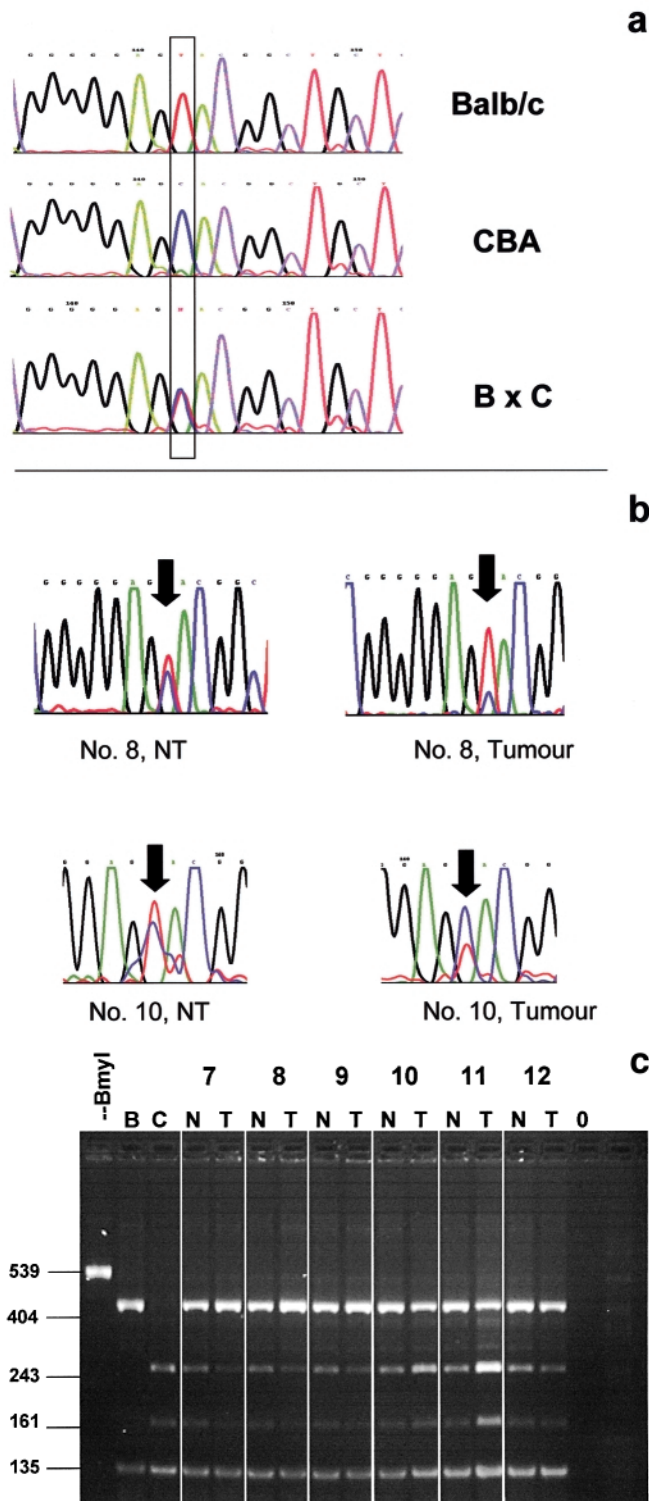
In only two of the tumour cases a detectable RT-PCR product for the most 5' part of the *Tbx18* coding sequence (245f/900r) was found. We speculate that this is due to partial RNA fragmentation during decalcification of the tumour, as RT-PCR for shorter fragments in this region worked well for all tumours (see below). Within the 5' region of *Tbx18* we found an additional heterozygotic C/T transition at nucleotide 541, which is associated with an amino acid change from histidine (codon CAC) to tyrosine (codon TAC). This alteration affects the region of the *Tbx18* protein proximal to the T-box domain. Testing embryonic cDNA from BALB/c and CBA/CA mice confirmed that this is a variation between the two strains, with the CBA allele coding for tyrosine and the BALB/c allele coding for histidine (Figure 2a).

*Allelotyping osteosarcoma for Tbx18<sup>nt541</sup>*

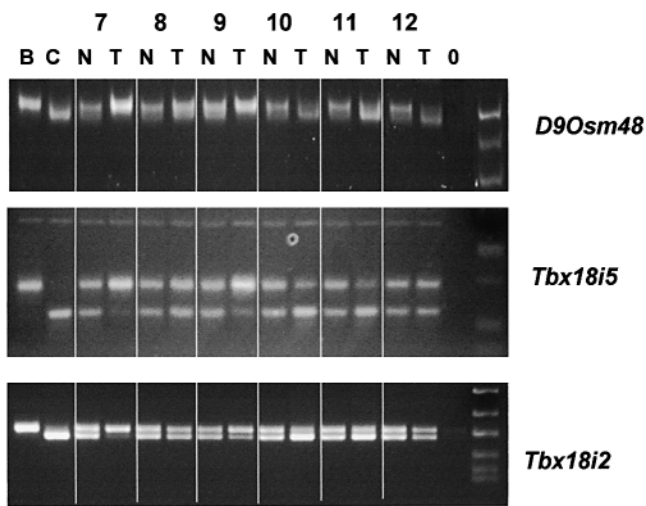
The C/T polymorphism at *Tbx18<sup>nt541</sup>* was used as a SNP to allelotype all tumours using cDNA sequencing (Figure 2b) and RFLP analysis from a PCR amplified genomic fragment (Figure 2c). From 17 tumours analysed for this locus, 15 exhibit relative reduction of one of the two alleles. The paternal CBA allele was lost in eight cases (47%) and the maternal BALB/c allele in seven cases (41%). Figure 4 shows that three osteosarcoma cases (nos 2, 4 and 8), which were initially used to define the LOH1 locus as they retained heterozygosity at either of the flanking markers D9Mit9 or D9Mit269, were all affected by LOH at this *Tbx18* marker. Three cases (nos 3, 16 and 17), which were not affected by AI at either of the flanking markers show signs of AI at the *Tbx18* locus.

*Allelotyping osteosarcoma for Tbx18 intronic and extragenic STS markers*

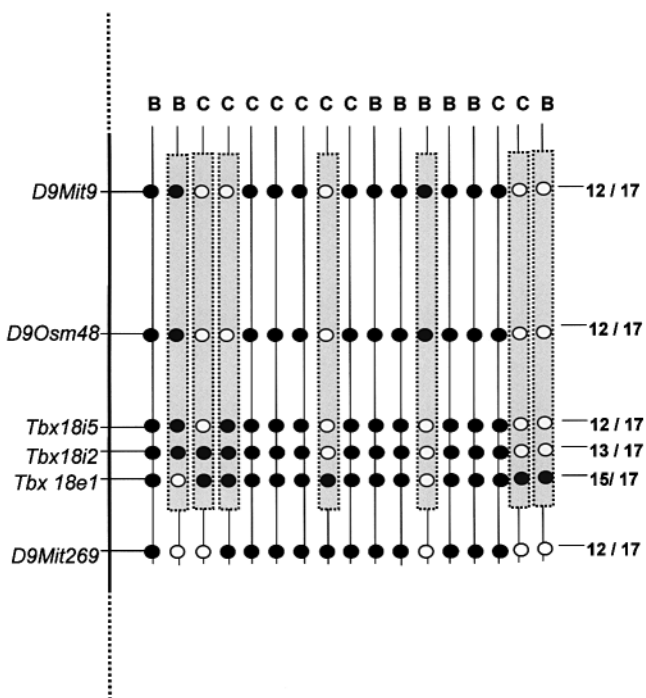
Additional polymorphic STS markers were used to determine the pattern of AI at intron 2 and intron 5 of *Tbx18* (*Tbx18i2* and *Tbx18i5*) (Figure 3) and at a locus 270 kb proximal to the gene (*D9Osm48*). Except for tumour no. 3, the allelotypes at the two intronic markers were found to be identical: AI was obvious at intron 2 in 13 of 17 tumours and at intron 5 in 12 of 17 tumours (Figure 4). Although the portion of tumours exhibiting AI is slightly lower compared with the 541 nt polymorphism in the first exon, one tumour (no. 2), which retained heterozygosity at exon 1 is affected by AI in the second and fifth intron. At marker *D9Osm48*, which resides 270 kb distally to the *Tbx18* 3' UTR, the pattern of AI (12



**Fig. 2.** (a) Electropherogram of *Tbx18* sequence surrounding nucleotide 541 in normal tissue of BALB/c, CBA/CA and BxC mice. Note the heterozygous T/C pattern in BxC hybrid mice. (b) Pattern of AI at the 541 nt locus of *Tbx18* as detected by cDNA sequencing. Note loss of the cytidine, which represents the CBA-allele in tumour no. 8 and loss of the thymidine, representing the BALB/c-allele in tumour no. 10. (c) Pattern of AI at the 541 nt locus of *Tbx18* for a subset of osteosarcoma as detected by genomic PCR and *BmyI* digestion. In the BALB/c allele, the 539 bp long PCR fragment carries one restriction site, leading to two fragments of 135 and 404 bp length. In the CBA-allele of the 404 bp long fragment, the 541 nt polymorphism results in an additional *BmyI* site, thus producing two CBA-specific fragments of 161 and 243 bp. The 135 bp fragment is common for both alleles and serves as an internal control for *BmyI* digestion. The ratio of the 404 bp fragments to the 161/243 bp fragments is a measure of the ratio between the BALB/c and CBA alleles. Note retention of heterozygosity in tumours no. 12, loss of the CBA-specific fragments in tumours 7, 8 and 9 and loss of the BALB-specific fragment in tumours 10 and 11.



**Fig. 3.** Allele-pattern of CA repeat markers D9Osm48, *Tbx18i2* and *Tbx18i5* for a subset of osteosarcoma. In all cases the BALB-allele is the longer, the CBA allele the shorter fragment. Note dysbalance in favour of CBA allele in tumour nos 7 and 9 for all three markers, retention of heterozygosity in tumour no. 8 for all markers, dysbalance in favour of the BALB-allele in tumour nos 10 and 11 for all markers. In tumour no. 12, markers *Tbx18i5* and *Tbx18i2* show heterozygosity, whereas D9Osm48 shows loss of the BALB-allele.



**Fig. 4.** Summary of AI pattern in 17 murine osteosarcoma cases over the interval analyzed. Filled circles stand for loss of either of the parental alleles, whereas open circles stand for retention of heterozygosity. Letters above the LOH pattern specify the origin of the reduced allele, where C stands for relative reduction of CBA-allele; and B for relative reduction of BALB-allele. Cases with grey rectangles denote those, which show retention of heterozygosity at either of the two flanking markers D9Mit9 or D9Mit269.

out of 17) resembles that of *D9Mit9*, which defines the proximal boundary of the locus in the original paper (15).

*AI pattern at adjacent markers within Tbx18*

The analysis of AI at three different intragenic markers within the *Tbx18* gene (541 nt, *Tbx18i2*, *Tbx18i5*) shows that only

one tumour (no. 3) is unaffected by AI (Figure 4). The highest frequency appears at the 541 nt position in the first exon, with tumours 8, 16 and 17 showing AI only at this site. Tumour no. 2 exhibit AI in intron 2 or intron 5, but not in the first exon.

**Discussion**

We have mapped previously a locus in a region of conserved synteny between mouse chromosome 9 and human chromosome 6q14.1-15, exhibiting a high frequency of AI in spontaneous and radiation-induced osteosarcoma (15). Using radiation hybrid mapping we have now placed *Tbx18* between the markers *D9Mit9* and *D9Mit269*, an interval that is flanked by the genes *Mod1* and *Nt5* and lies entirely within the region described earlier as showing the highest frequency of AI in murine osteosarcoma (15). In order to fine-map potential osteosarcoma-associated genes in this region we have conducted an allelotyping analysis of 17 murine tumours for polymorphic intragenic and extragenic markers in and around the *Tbx18* gene.

We show here that the genomic regions affected in several tumours overlap at the *Tbx18* gene: six tumours which retain heterozygosity at flanking STS markers exhibit AI for at least one of the six intragenic *Tbx18* markers (Figure 4). Only one of the tumours analysed did not show AI at any of the intragenic *Tbx18* markers. We cannot exclude the formal possibility, however, that parts of the gene or of regulatory elements not analysed here are affected by as yet undetected AI. The high frequency of AI affecting the *Tbx18* gene suggests that it might be closely linked to a gene that is frequently affected by large-scale deletions or amplifications during osteosarcomagenesis, hence leading to AI at flanking genes. Radiation is known to exert its mutagenic effect mainly through large deletions (21,22), which can affect multiple colinear genes (23). Radiation-induced tumours in particular seem to be characterized by deletions/translocations affecting large chromosomal regions (24,25). In this case, *Tbx18* would represent merely a marker for another gene that resides nearby and which has to be altered as an essential step during osteosarcomagenesis.

However, according to the annotated mouse and human genome sequences, the region surrounding *Tbx18* is not a very gene-rich one. The annotated human genome sequence ~700 kb proximal to *Tbx18* shows homology to genes of the SWI/SNF family. We believe this to be a pseudogene, as ORF analysis revealed several stop-codons, which would interrupt translation of any functional protein. This is further supported by the sequence of the mouse genome, which does not show any homology to SWI/SNF genes in this region. About 400 kb proximally to *Tbx18* the mouse homologue of KIAA1009 is annotated. We did not find evidence for transcripts of this gene in normal bone or tumour (data not shown), thus making it unlikely that it has anything to do with suppression of tumour growth. Furthermore, both of these postulated genes map proximal to marker *D9Osm48* and are thus outside the interval of maximal AI frequency. Distally to *Tbx18* we found *Nt5* to be the nearest flanking gene (~570 kb distant from *Tbx18*). This gene, however, already maps distally to markers *D9Mit269* and *D9Mit291*, which represents the lower boundary of the AI interval (15). Thus, if we consider the mapped mRNAs and EST cluster in the annotated human and murine genome sequence to be a true image of genes residing in this interval, *Tbx18* itself remains the only candidate gene.

Analysing *Tbx18* expression in various human and murine tissues, we detected mRNA transcripts in primary osteoblasts, osteosarcoma tissue and cell lines derived from osteosarcoma (data not shown). Although there are no reports in the literature showing *Tbx18* expression other than during embryogenesis, this finding is not completely unexpected. The continuous bone remodelling process involves differentiation of mesenchymal stem cells via preosteoblasts towards differentiating osteoblasts and osteocytes. T-box genes are known to play an important role in determining mesodermal cell commitment at the time point of gastrulation of the early embryo (26,27). *Tbx18* itself is involved in the development of early limb buds, where at day 12.5 its expression concentrates in the mesenchyme surrounding the precartilagenous structures (20). Although initially identified as regulator of embryonal development, some T-box genes appear to control general mechanisms of growth regulation. Examples are *Tbx2*, which can bypass cellular senescence by down-regulating the tumour-suppressor gene *p19<sup>ARF</sup>* (28) and *Tbx5*, which has been shown to exert an antiproliferative effect when overexpressed in canine osteosarcoma cells (29). Both of these cellular mechanisms, control of senescence and proliferation, are important factors in tumour suppression. *Tbx2* was also shown to transactivate pleiotrophin and *osf-2*, both involved in osteogenic differentiation (30).

We did not find any specific mutations in *Tbx18*, which according to Knudson's 'two-hit-hypothesis' (31) of tumour-suppressor gene inactivation should mark the undeleted allele. The His/Tyr amino acid substitution in BALB/c mice due to the nucleotide 541 polymorphism cannot severely impair *Tbx18* function, as the homozygotic Tyr/Tyr BALB/c mouse strain does not exhibit any developmental defects which would be expected to result from altered *Tbx18* function. We also found AI at the *Tbx18* gene to affect both allelic variants with equal frequency, thus making it unlikely that tumour cells gain a growth advantage from the resulting His/Tyr amino acid exchange. There are recent reports in the literature suggesting that both copies of tumour-suppressor genes do not necessarily have to be inactivated to promote tumorigenesis. The so-called class 2 tumour-suppressor genes are assumed to contribute to inappropriate tumour growth if their expression is reduced, perhaps due to haploinsufficiency. Other TSGs show evidence of epigenetic silencing of one allele (32), leading to severe reduction of expression if the active allele is deleted. In this context it is interesting to note that hypermethylation of a CpG island residing ~5 kb upstream of the *Tbx18* promoter was recently reported in ~30% of lung adenocarcinomas (33). Such observations challenge the general validity of the two-hit inactivation mechanism as proposed for tumour-suppressor genes by Knudson (31). For several of the so-called 'caretaker TSGs' a reduction of gene dosage not only increases the risk for a complete functional loss after a second mutagenic event, but also might have a more direct consequence for a cell. It is not unreasonable to imagine that a lower expression level of the tumor-suppressor proteins could result in an impaired function of the pathways, which maintain genomic stability or cellular integrity after stress response. There is a plethora of observations suggesting that carcinogenesis already starts if one copy of a tumour-suppressor gene is lost (34).

The absence of tumour-specific mutations in *Tbx18*, together with the AIs observed in almost all osteosarcoma analysed, lead us to assume, that loss of one functional allele of *Tbx18* contributes to the process of osteosarcomagenesis. Although we found expression of this gene both in normal as well as in

cancerous bone, a direct comparison of transcript or protein levels is hampered by the poor definition of malignant and normal precursor cells in the context of bone architecture. It is thus too early to speculate about a possible molecular function of *Tbx18* in bone homeostasis and transformation.

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