ASSOCIATION OF A FUNCTIONAL RB1 PROMOTER POLYMORPHISM WITH SUSCEPTIBILITY FOR RADIATION-INDUCED OSTEOSARCOMA IN MICE

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Various high–penetrance cancer syndromes such as Li-Fraumeni and familial retinoblastoma are associated with an elevated risk for osteosarcoma, but they account for less than 10% of all cases. Cancer predisposition may also be influenced by low penetrance alleles of susceptibility genes, but their identification in human osteosarcoma is hampered by limited number of cases and genetic heterogeneity. Using a mouse model of alpha-radiation induced osteosarcoma, we recently found suggestive linkage in testcrossed Tx(C3Hx102) mice to loci on chromosome 7 and 14. In an extended linkage analysis using BALBx(BALBxCBA) backcrossed mice we now confirmed the chromosome 14 locus and identified the *Rb1* tumor suppressor as the most likely candidate gene. This finding is supported by frequent allelic loss of the BALB/c allele as detected in F1 tumors. The BALB/c allele of the *Rb1* promoter was found to carry a hexanucleotide duplication, which is predicted to generate two additional binding sites for WT1 and SP1. Using CAT reporter assay and direct *Rb1* mRNA quantification we found that this insertion causes an approximately 1.8fold increase in expression. An enhanced *Rb1* expression by this promoter variant is consistent with the BALB/c allele being associated with reduced osteosarcoma susceptibility as found in the linkage analysis.

Although bone tumors have a much lower incidence than many other neoplasia, several observations suggest a significant contribution of genetic predisposition in modifying the individual risk (1,2). A number of high–penetrance cancer syndromes such as Li-Fraumeni, familial retinoblastoma as well as Werner- and Rothmund-Thompson syndrome are associated with an elevated risk for osteosarcoma (3), but they account for less than 10% of all diagnosed cases. Familial cancer syndroms with osteosarcoma as the primary tumor are unknown. There is only anecdotal evidence for increased prevalence of osteosarcoma in close relatives, respectively, who don't show signs of a generalized tumor-prone syndromes (4,5,6). In the case of Li-Fraumeni syndrome and familial retinoblastoma, respectively, evidence for germline mutations in p53 or Rb1 are found only in a subset of affected families (7). It is possible that phenocopies of these conditions are caused by alterations in genes that are part of the p53 or Rb1 pathways. To explain incomplete penetrance of the tumor-propensity in a Li-Fraumeni family interaction of multiple modifier genes was already suggested in the late 70s (8). Recently it was proposed that a congenital cancer predisposition caused by multiple low penetrance alleles would result in a larger fraction of tumors having a genetic etiology than currently estimated (9,10). Since familial clustering would not be detected in those cases (11), population based association studies using SNPs are now expected to discover some of these more complex cancer-prone syndromes. In the case of human osteosarcoma, however, the small number of patients together with their genetic and phenotypic heterogeneity renders gene mapping by such a strategy difficult. For other tumors the use of mouse models as an alternative has been suggested (12,13). Since differences in cancer predisposition are well documented among inbred strains, linkage studies can be performed in a directed and reproducible way. We thus used a mouse model of alpha-radiation induced osteosarcoma, for which we have recently shown suggestive linkage in testcrossed T-stock x (C3H x 102) mice to loci on chromosomes 7 and 14 (14). To extend and verify these results in other mouse strains we performed another linkage analysis using backcrosses of the strains BALB/c and CBA/CA, which are known to differ in their predisposition to radiogenic osteosarcoma (15).

Methods

Mice breeding and tumor induction. 169 female BALB/cHe x (BALB/cHe x CBA/Ca) mice (obtained from GSF breeding stocks) were treated with a single i.p. injection of 185Bq/g ^{227}Th (as Thorium Citrate) at the age of 100 days (14). Mice were housed 5 to a cage and examined 5 days a week for the development of tumors or other life-threatining conditions. Bone tumors were diagnosed radiologically, and confirmed by histological examination after EDTA decalcification. Typical histologies and X-ray images of tumors were deposited at the Pathbase database (http://www.pathbase.net Acc.No. 3410, 3411, 3412, 3414). All animal procedures were approved by the state gouvernment (file no. RegOB 211-2531-49/00) and carried out in accordance with the national animal welfare guidelines.

DNA Extraction and Microsatellite Genotyping. Genomic DNA was extracted from 5mm tail tips as described before (14). Genotyping for 58 polymorphic microsatellite markers distributed evenly over the entire genome was carried out by PCR amplification in 96well plates using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, Ca, USA) followed by 90min electrophoresis on 3% agarose / TBE gels. Primer sequences and reaction conditions were as recommended by Dietrich et al (16).

Mapping of susceptibility loci. For an initial exclusion linkage analysis, pointwise LOD scores were calculated for linkage of marker genotypes with tumors development. For QTL intervall mapping, only markers within the region showing significant linkage on chromosome 14 were analysed using MapManagerQTX (17). For this purpose , a normalized censored tumor latency time was calculated for each mouse as follows:

$t(L)_C = (t_{max} - t(L)) / t_{max}$ (mice with osteosarcoma)
$t(L)_C = - t(L) / t_{max}$ (mice without osteosarcoma),

with t(L) beeing the intervall between treatment and death and t_{max} being the maximal lifetime observed. $t(L)_C$ thus runs from –1 to 0 for osteosarcoma free mice and from 0 to +1 for mice developing osteosarcoma, with –1 reflecting the longest tumor-free survival and +1 early arising tumors.

Sequencing. The *Rb1* coding sequence was determined from RT-PCR amplified liver or spleen cDNA obtained from untreated BALB/c and CBA/CA mice. The *Rb1* promoter sequence was determined from genomic DNA derived from tail tips. Primer sequences and PCR conditions are in table 1 (supplemental material). Cycle sequencing was carried out using the AB BigDye system with the reaction conditions as recommended by the manufacturer. Sequences were analysed on an ABI 377 sequencer (Applied Biosystems, Foster City, Ca, USA).

Analysis of allelic imbalance in osteosarcoma. Genotyping was performed in 17 osteosarcomas induced in BALB/c x CBA/CA hybrid mice for a set of polymorphic microsatellite markers on chromosome 14 and for a SNP in the *Rb1* 3' UTR (3090nt). For details of the method see Nathrath et al 2002 (18).

Prediction of promoter function. A 352bp fragment of the *Rb1* promoter from BALB/c and CBA/CA mice, including all upstream regulatory sequences, the 5'-UTR and the initiation codon ATG was analyzed for potential TF binding sites using MatInspector software (Genomatix Software GmbH, Munich, Germany) (19,20). Predefined transcription factor weight matrix descriptions are based on the TRANSFAC-database (all-vertebrates library) with the following default parameter: core similarity=0.75 and matrix similarity=calculated optimized.

Cloning of a *Rb1* promoter fragment into CAT reporter vector. A 451 or 457 bp fragment of the *Rb1* promoter was PCR amplified from CBA/CA and BALB/c genomic DNA, respectively, using primers 6F and 8.2B, Pwo-polymerase (Peqlab, Erlangen, Germany) and reaction conditions as given in table 1 (supplemental material). The fragment was cloned into TOPO TA vector (Invitrogen BV, Groningen, The Netherlands) and directionally subcloned into the pCAT3 basic vector (Promega Corp. Madison WI, USA) using SacI and XhoI restriction sites. Size, sequence, and orientation of the resulting insert was confirmed by restriction digestion using ScaI and ClaI (all enzymes from Roche Diagnostics, Mannheim, Germany) and sequencing. Clones with the *Rb1* promoter inserts (Rb1pCAT) in positive orientation relative to the CAT reporter gene were designated BALB 36 and CBA 11, whereas clone CBA 14-5 contains the promoter fragment in reverse orientation relative to the CAT reporter gene.

CAT reporter assay. Plasmids BALB 36, CBA 11 and CBA 14-5 were purified using Quiagen plasmid MaxiPrep kit (Quiagen GmbH, Minden, Germany) yielding a final concentration of 2 µg/µl and OD260/OD280 absorption ratios between 1.68 and 1.73. A luciferase expression vector pGL3 (Promega Corp., Madison WI, USA) under control of the RFB LTR was kindly provided by M.Neumann (GSF Institute for Molecular Virology) and served as an internal control for transfection efficiency. For reporter analysis, ROS 17/2.8 rat osteoblasts were plated at a density of $5*10^5$ cells per 60mm plate (Nunc , Denmark) in DMEM containing 1g glucose/l and 10% FCS (Gibco Life Technologies, Eggenstein, Germany). Two days after plating, cells were transiently transfected with 2µg Rb1pCAT and 0.2µg pGL3 using FuGene lipofection (Roche Diagnostics GmbH, Mannheim, Germany). 48h after transfection, cells were harvested using reporter lysis buffer (Promega Corp, Madison WI, USA) and 20µg protein extracts were assayed for CAT activity using the FastCAT yellow assay (Molecular Probes Europe, Leiden, The Netherlands). After thin-layer chromatography, TLC plates (Sigma Aldrich, Germany) were visualized on a Storm laser scanner (Amersham Pharmacia,Uppsala, Sweden) and quantified using Image Quant software (Molecular Dynamics, Sunnyvale CA, USA). Transfection efficiency was controlled for by cotransfection with pGL3 (see above) and quantification using the luciferase assay (Promega Corp. Madison WI, USA) on an Auto-Lumat LB953 luminometer (EGG Berthold, Pforzheim, Germany). Promoter activity was expressed in arbitrary units as AU = 1000*acetyl-CA / (CA + acetyl-CA)*luciferase, wherein CA is the integral density of the chloramphenicol signal and acetyl-CA the related value of the acetylated chloramphenicol.

***Rb1* mRNA quantification.** Whole embryo RNA was extracted from day 16 p.c. BALB/c and CBA/CA embryos by homogenisation in liquid N_2 followed by GTC extraction and phenol/chloroform purification. For each strain, 6 embryos from 3 pregnant mice were used. Following northern blot of 10µg of each

RNA sample, membranes (Roche Diagnostics, Germany) were probed with a 630 bp ^{32}P labelled mouse *Rb1* cDNA fragment spanning exon 10 to 13. Signals were quantified using a Storm scanner (Amersham Pharmacia,Uppsala, Sweden) and Image Quant software (Molecular Dynamics, Sunnyvale CA, USA). Relative expression levels (AU) are given as the integrated optical densities of the *Rb1* signal normalized to the sum of the 28S and 18S RNA signals.

Results

Mapping of susceptibility loci

Osteosarcoma susceptibility loci were mapped in 169 female mice from a BALB/c x (BALB/c x CBA/CA) backcross following treatment with the bone-seeking alpha-emitter ^{227}Th. During the entire follow-up period 44 mice were diagnosed with radiologically and histologically confirmed osteosarcoma, appearing with latency times between 182 and 645 days after injection (Fig. 1a). For genome-wide mapping of susceptibility loci a selective genotyping approach was used. Mice that died tumor-free before the age of 600 days and those which developed tumor with latency periods of more than 400 days were excluded from the analysis, thus concentrating on the "extreme" ends of the phenotype. This helps to minimize interference of phenocopies or reduced penetrance. On chromosome 14 a locus was found between markers D14Mit234 and D14Mit97 with the BALB/c allele being significantly linked with reduced tumor susceptibility (p=0.0002). BALB/c homozygosity at D14Mit225 was found in 26 of 36 long-term tumor-free mice compared to only 5 of 24 mice which developed a tumor early in life , yielding a LOD-score of 3.4. Additional regions with suggestive linkage were found on chromosome 4, 5, 12 (susceptibility associated with BALB/c homozygosity) and on chromosome 18 (susceptibility associated with CBA/BALB heterozygosity, Fig. 2a). For further QTL mapping on the main locus on chromosome 14, a normalized, censored latency time $t(L)_C$ was calculated to serve as a trait parameter both for tumor-bearing and for tumor-free mice (Fig. 1b). Likelihood ratio statistics for a QTL in this intervall exceeds 20, equivalent to LOD = 4.39 (Fig. 2b). This region on chromosome 14 overlaps with a osteosarcoma susceptibility locus we previously found in other mouse strains (14) and was thus used to fine map a putative modifier gene. For this purpose haplotype analysis was carried out for all 169 mice using additional polymorphic markers between D14Mit234 and D14Mit97 (Fig. 2c). The lowest number of recombinants was found for D14Mit192 and D14Mit225, which span a 3 Mbp intervall according to the published mouse genome sequence. D14Mit125, which maps approximately 13 Mbp distally and is clearly separated from D14Mit225 by marker D14Mit91, shows a second local minimum in the number of recombinants. Although it is indeed possible that two closely linked genes modify tumor predisposition, we restricted our further analysis to the intervall between D14Mit192 and D14Mit225 due to the higher number of informative animals. Somewhat unexpectedly we found the BALB/c allele to be associated with tumor resistance, although the BALB/c inbred strain itself is more susceptible than the strain CBA/CA (15). We assume that this is due to the influence of additional susceptibility loci mentioned above, three of which show susceptibility associated with the Balb/c allele but only one with the CBA-allele (Fig. 2a).

Rb1 strain polymorphisms

Searching the MGD Database for potential modifier genes between D14Mit192 and D14Mit225 we considered the *Rb1* tumor suppressor gene as the most promising candidate. Sequencing the entire coding region of *Rb1* in BALB/c and CBA/CA mice we found only minor strain differences in the 3' UTR (supplemental material, Table 2). These were not considered to be functionally relevant since they do not alter sequence motifs involved in mRNA stability (21). When we analyzed the *Rb1* promoter region up to 1190bp upstream of the first exon, however, a TCGCCC hexanucleotide duplication in the BALB/c allele was revealed between nt1053 and nt1084 (Fig. 3a, base numbering according Genebank M86180) . This duplication is located 177 bp upstream of the first exon, 5' to the main binding sites for Sp1, ATF and E2 (Fig.3b).

Rb1 promoter variant in germline and tumors

The genotype at the *Rb1* promoter was determined for each mouse by PCR-amplification using primers 1005f and 1128r (Fig. 4a) and tumor incidences were plotted separately for $Rb1p^{B/B}$ and $Rb1p^{B/C}$ mice, respectively (Fig. 4b). This confirmes that mice inheriting the B/C genotype have a significantly shorter latency time and higher overall osteosarcoma incidence than mice with the B/B genotype (p=0.0007, Log-Rank Test). Interestingly, in an accompanied search for allelic imballance in 17 osteosarcoma arising in BALB/c x CBA hybrid mice we found preferential loss of the BALB-allele (10 of 17 cases, 58.8 %) as compared to the CBA-allele (3 of 17 cases, 17.6%) at microsatellite markers flanking the Rb1 locus (Fig. 5). According to Knudson's hypothesis this non-random loss of the Balb/c allele is a further hint for it carrying a germline-polymorphism with a higher efficiancy in tumor-suppression.

Prediction of altered TF binding sites

To investigate if the detected *Rb1* promoter polymorphism has the potential to change binding of transcription factors (TF), the computer algorithm MatInspector was used that predicts TF binding sites based on the TRANSFAC-database (see methods section). The BALB/c specific TCGCCC insertion was predicted to generate two additional binding sites, one for each of the two transcription factors WT1 and SP1 (Supplemental material , Fig. 7). Moreover, the additional SP1 site in conjunction with a preexisting SP1 site forms a potential new SP1-SP1 promoter module, as has been previously described as an active promoter module in the human HCK gene (22).

Effect of *Rb1* promoter variant onto transcription

To test the impact of the BALB/c specific *Rb1* promoter insertion on gene expression, a CAT reporter assay was performed. For this purpose 451bp or 457bp long fragments (primer f6 and r8.2) from the CBA/CA and BALB/c allele of the *Rb1* promoter (encompassing the ubiquious SP1, ATF and E2 binding sites together with the site of the TCGCCC insertion) were directionally cloned into pCAT vectors (supplemental material , Fig 8). Following transient transfection of ROS17/2.8 rat osteoblasts, the *Rb1* promoter fragment activated the CAT reporter gene in an orientation-dependent and reproducible manner (figure 6a). Relative to the value of the CBA variant (2.42 AU, CI 2.19 – 2.65), the BALB/c allele has 1.8 fold higher activity (4.38 AU, CI 4.19 – 4.57). This difference is highly significant (p=0.0003, t-test). Inverse orientation of the *Rb1* promoter fragment in clone CBA14-5 shows only 38% activity and extracts from mock-transfected cells have less than 15% basal activity, relative to the value of the positively orientated CBA/CA construct (Fig. 6b).

To verify the effect of the promoter variant in-vivo effect, levels of *Rb1* transcript were quantified in RNA extracts from BALB/c and CBA/CA embryos. As shown by northern blots (Fig. 6c), expression of *Rb1* mRNA is indeed higher in embryos of the BALB/c strain (232 AU, CI 165 – 299) than in the CBA/CA strain (183 AU, CI 138 – 228) (p=0.038, t-test). This difference in *Rb1* expression, which we attribute to the promoter variation, is consistent with the *Rb1* locus beeing both genetically linked to tumor susceptibility and showing preferential loss of the BALB/c allele in F1 tumors.

Discussion

Together with results from a recently studied T-stock x (C3H x 102) cross (14), we now have evidence for linkage of osteosarcoma predisposition to the *Rb1* locus (compound LOD score 5.44). In the BALB/c x CBA/CA backcross analysed in the present paper tumor susceptibility is asscociated with a BALB/c specific insertion in the *Rb1* promoter region. Mice with the B/C heterozygote genotype at the *Rb1* gene show a significantly shorter latency time and higher tumor incidence than B/B homozygote mice. 500 days after tumor induction, more than 40% of the B/C mice developed osteosarcoma as compared with just 12% tumors in mice of B/B genotype. The strains C3H and 102, which also exhibit genetic linkage to this Rb1 locus (14), both harbour the CBA promoter variant. In the 3' UTR of Rb1 3 single-base polymorphisms were found between these two strains, which may causes differences in the mRNA stability, although they do not affect conserved stem-loop structures (21). Alternatively, the C3H and 102 strains might carry allelic differences in cis-acting regulatory sequences residing 5' of *Rb1* promoter.

Surprisingly, backcrossed mice homozygote for the BALB/c allele at the *Rb1* locus are more resistant than B/C heterozygote ones, although the BALB/c inbred strain is generally more sensitive than CBA/CA (15). It is possible that additional modifier loci on chromosome 4, 5 and 12, at which the BALB/c allele is associated with susceptibility, confer in total a stronger effect than the Rb1 locus alone, although each of these minor loci exhibits only suggestive linkage. Detection of resistance alleles derived from a sensitive inbred strain is not an uncommon phenomenon in whole-genome linkage studies in mice (23,24) . This is perhaps best explained by epistatic interaction between multiple genes which can even cause counteracting effects (25). A better understanding of such complex genetic mechanisms requires insight into the function of the underlying genes and the consequence of strain-specific polymorphisms. For the *Rb1* gene, which was the best candidate within the haplotype-defined intervall in the present study, no polymorphisms in the coding sequence were detected between the BALB/c or the CBA-allele. Instead, we found a 6bp TCGCCC insertion in the promoter region of the BALB/c strain. From a teleologic point of view it is impossible to tell whether the CBA- or the BALB/c promoter represents the wildtype sequence. Testing different mouse strains, however, we found the insertion in only 3 strains (A, C57B6 and BALB/c), compared to 8 strains (AKR, SWR, C3H, 102, 129Sv, CBA/CA, CBA/H, DBA) showing the sequence originally published by Zacksenhaus et al (1993) (data not shown).

Comparison of the potential TF binding sites in *Rb1*-promoter of strain BALB/c and CBA/CA reveals, that the 6bp insertion potentially creates additional binding sites for the transcription factors Wt1 and SP1. The potential of the BALB/c specific promoter insertion to modify transcription was experimentally verified using in-vitro CAT-reporter analysis and direct quantification of *Rb1* mRNA from BALB/c and CBA/CA embryos, showing 1.8 fold and 1.27 folder higher expression from the BALB/c promoter variant, respectively.

Rb1 mutations in man predispose not only for retinoblastoma, but also for osteosarcoma (26,27,28) . The Rb1 protein acts as a tumor suppressor by its ability to regulate the G1/S cell cycle

transition (29) and might thus shift the balance between proliferation and osteogenic differentiation (30). The overproportionally high risk of Rb1 mutation carriers to develop secondary tumors following radiotherapy (31,32,33) and the importance of the cdk2-Rb1-pathway for cell-cycle arrest after radiation exposure (34), suggest that a sufficient level of functional Rb1 protein is crucial to protect cells from entering S-phase with unrepaired DNA lesions. The general importance of this pathway for cancer predisposition was recently shown in a mouse model of pristane-induced plamacytoma, where a p16 promoter variant in BALB/c causes down-regulation of this Cdk-inhibitor and is linked with tumor susceptibility (35). The majority of Rb1 mutations detected in retinoblastoma-families cause partial or complete losses of the gene (41%) and frameshift or nonsense mutations (42%) (36,37,38), resulting in Rb1 haploinsufficiency and consequently give rise to highly penetrant phenotypes. A small fraction of retinoblastoma families, however, exhibit incomplete penetrance of tumor predisposition, which can be caused by mutations or hypermethylations in the Rb1 promoter region (39,40,41,42). Osteosarcoma are frequently diagnosed in pedigrees segregating the severe type of Rb1 mutations, whereas patients inheriting the low penetrance alterations in the Rb1 promoter are not known yet to carry an increased bone tumor risk. From the available data, however, it can not be ruled out that an existing osteosarcoma predisposition in these low-penetrance families remains undetected because of the general low osteosarcoma incidence and the small number of such families.
It would thus be of interest to test if among “sporadic” osteosarcoma patients, which make up about 90% of all diagnosed cases, a preponderance of low-penetrance Rb1 polymorphisms such as the one detected in the present study can be found. In the promoter sequence of the human Rb1 gene an insertion similar to the 6nt BALB/c specific TCGCCC insertion has not been identified. It is of interest to note that several potential TF binding sites for WT1, SP1, E2F, and ATF seem to be conserved in both promoters, indicating a common regulatory organization.

Germline mutations in the coding sequence of the tumor suppressor genes Rb1 and P53 are responsible for about 8% of all human osteosarcoma. A minor fraction of cases are associated with germline mutations in Rank (OMIM 602080), Wrn (OMIM 277700) and Recq4 genes (OMIM 268400), but these mutant alleles have a low population frequency and tumors are of late-onset and exhibit atypical histologies.
In general, an inherited predisposition to cancer is most frequently observed in patients harbouring hemizygote germline mutations in tumor suppressor genes (P53, Rb1, Pten, APC, Ptch). This causes haploinsufficiency and, consequently increases the risk for complete functional losses by somatic deletions of the remaining wildtype allele. Probably due to their low fidelity such mutations are relatively rare within the population and thus can account for a small portion of all diagnosed tumors only. There is growing interest, however, in a possible congenital tumor predisposition caused by less severe mutations, which might have a reduced penetrance or exert their phenotypic effect only by interaction with other genes in a polygenetic manner, thus masking the inherited causality (12,13,43,44). Epidemiological studies in cancer patients show that subtle alterations in modifier genes such glutathion-S-transferases, cytochrome p450 or N-acetyltransferase, although not associated with any obvious phenotype, can alter tumor predisposition in a quantitative manner. For the APC tumor-suppressor gene it was recently shown, that inheritance of an allele with just 50% reduced expression can confer a cancer-prone phenotype (45). Interestingly, an association between inherited allele variants and altered expression level was recently found for a large portion of randomly selected human genes (46). Tumor suppression by Rb1 in mice seems to rely on a sufficient expression of both parental alleles, rather than on the total *Rb1* expression level (47).
For osteosarcoma, the identification of low penetrance modifier alleles by population based studies is hampered by the low incidence of this tumor type. Nevertheless, involvement of as yet unidentified genetic factors causing bone tumor predisposition is indicated by the preferential appearance of this cancer in adolescence and its association with a preceeding discordant tumor (1). We have shown here that mapping of modifier genes in model organisms such as mice can be a valueable tool to identify new genetic variations which have the potential to modify tumor predisposition.

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Fig. 1
Bone tumor induction in 169 female BALB x (BALBxCBA) backcrossed mice by ^{227}Th. **a,** Kaplan-Meier curve of osteosarcoma development following injection with 185 Bq/g ^{227}Th at the age of 100 days. **b,** Distribution of the censored tumor latency time t(L)c for all mice. Calculation of t(L)c is described in materials and methods. Values between –1 and 0 represent tumor-free mice (open bars, t(L)c = -1 are the longest tumor-free survivors), whereas values between 0 and 1 represent mice which developed osteosarcoma (filled bars, t(L)c =1 represent mice with shortest observed latency time).

Fig. 2
Mapping of osteosarcoma susceptibility genes. **a,** result of whole genome linkage analysis of the osteosarcoma censored latency time t(L)c using MapManager QTX and microsatellite-based genotyping. Position of markers is given by small diamonds and the sweep-radius by red lines right to the chromosomes. For chromosomal regions filled in red the Balb/c allele conferes susceptibility, whereas at regions filled in blue susceptibility is associated with the CBA-allele. LOD Scores for pointwise linkage are given at the bottom of chromosomes. Green bars on the left of chromosomes show regions exhibiting linkage in T-stock x (C3Hx102) mice (14). A selective genotyping approach was used for the linkage analysis based upon 35 mice with the shortest tumor latency time and 35 tumor-free mice with the longest survival time. **b,** Likelihood ratio statistics of markers along chromosome 14 using QTL intervall mapping with t(L)c as trait parameter. **c,** haplotype analysis for additional markers at the chromosome 14 region using all mice with and without tumor. BALB/BALB homozygosity is shown in red and BALB/CBA heterozygosity in blue.
Numbers of animals with each haplotype are shown at the bottom.

Fig. 3
Rb1 Promoter polymorphism between Balb/c and CBA/CA mice. **a,** Sequence of the *Rb1* promoter region between nt1053 and nt1084 determined for strains BALB/c and CBA/CA , showing structure of the BALB/c specific TCGCCC insertion. Base numbering according to Zacksenhaus et al (48)(Genebank Acc.-No. M86180). **b,** Position of the BALB/c specific hexanucleotide insertion relative to the SP1, ATF and E2 core binding sites according to Zacksenhaus et al (1993)**.**

Fig. 4
Association of the *Rb1* promoter genotype with tumor induction**. a,** PCR products following amplification of an 123 / 129 bp fragment (primer insF /insR) encompassing the site of the BALB/c specific insertion. The insertion specific fragment was used for genotyping all mice of the backcrossed cohort**. b,** Kaplan-Meier plot of osteosarcoma appearance in BALB x CBA backcrossed mice grouped according to their genotypes at the *Rb1* promoter. Difference between the B/B and the B/C genotypes is highly significant (p=0.0007, Log Rank test).

Fig. 5
Analysis of allelic imbalance in 17 osteosarcomas induced in Balb/C x CBA hybrid mice**. a,** strain polymorphism in the 3' UTR used for genotyping tumor vs. normal tissue. In the lower panel two representative cases with loss of the Balb/c- and CBA-alleles are shown, resp**. b,** pattern of allelic imbalances at the *Rb1* locus and at flanking microsatellite markers. Black areas indicate retention of heterozygosity, whereas reduction or loss of the Balb/c allele is depicted in red colour and of the CBA-allele in blue colour.

Fig. 6
Analysis of transcriptional activity of the variant allele of the *Rb1* promoter. **a,** CAT activity as measured by TL chromatography of ROS17/2.8 rat osteoblast protein extracts following transient transfection with the Rb1promoter/CAT constructs. **b,** Quantification of the CAT reporter signal driven by BALB/c and CBA/CA promoter variants (positive orientation), BALB/c promoter (inverse orientation) and mock-transfected cells. Transfection efficiency was normalized by simultaneous measurment of luciferase expression from cotransfected RFBpGL3 constructs. **c,** *Rb1*-Expression in BALB/c vs. CBA/CA mouse embryos 16 days p.c.

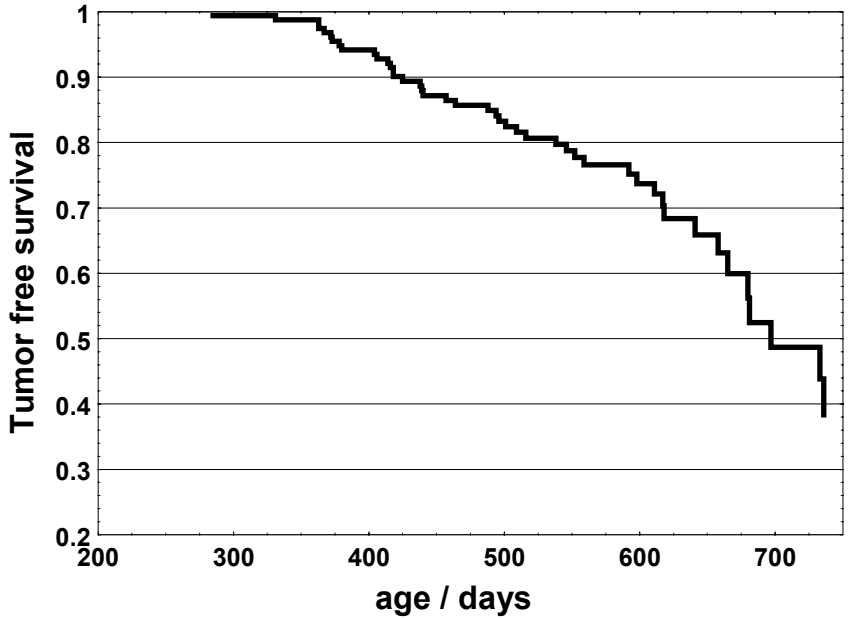
Tumor free survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
200
300
400
500
600
700
age / days

Fig 1a

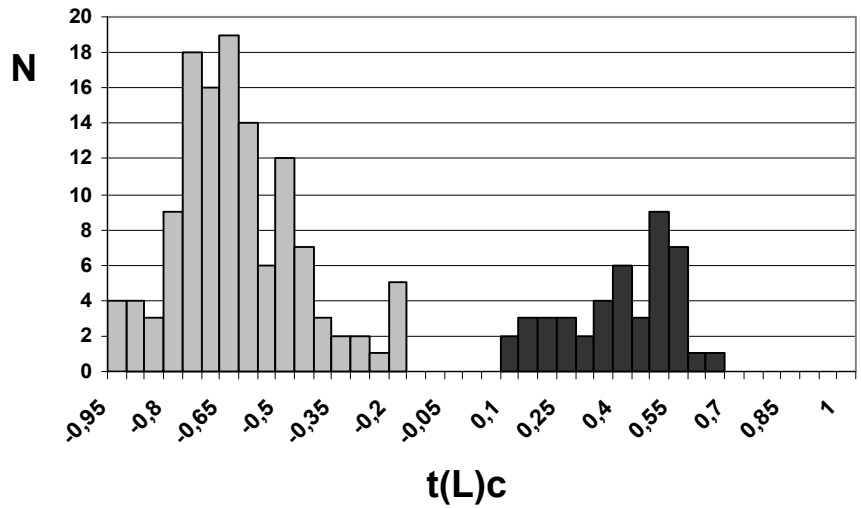
N
20
18
16
14
12
10
8
6
4
2
0
-0,95
-0,8
-0,65
-0,5
-0,35
-0,2
-0,05
0,1
0,25
0,4
0,55
0,7
0,85
1
t(L)c

Fig 1b

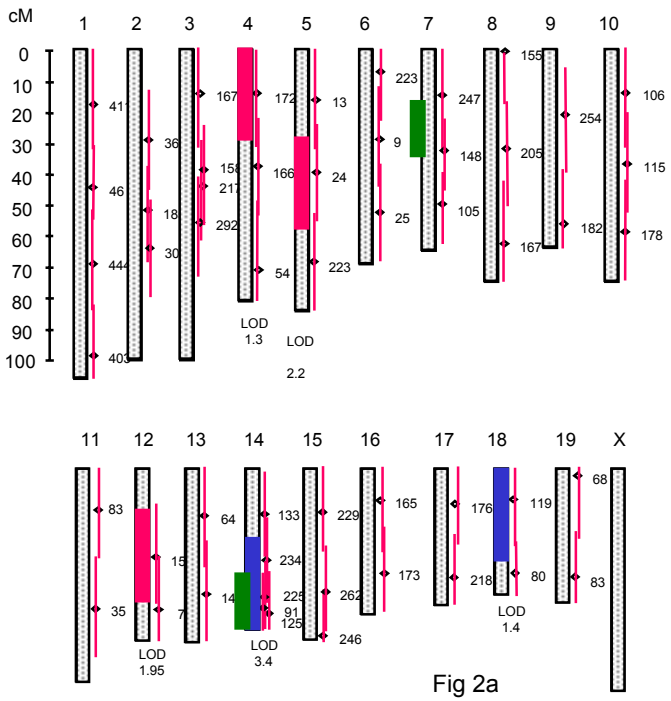

Fig 2a

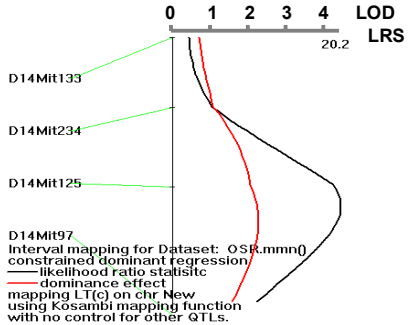

Fig 2b

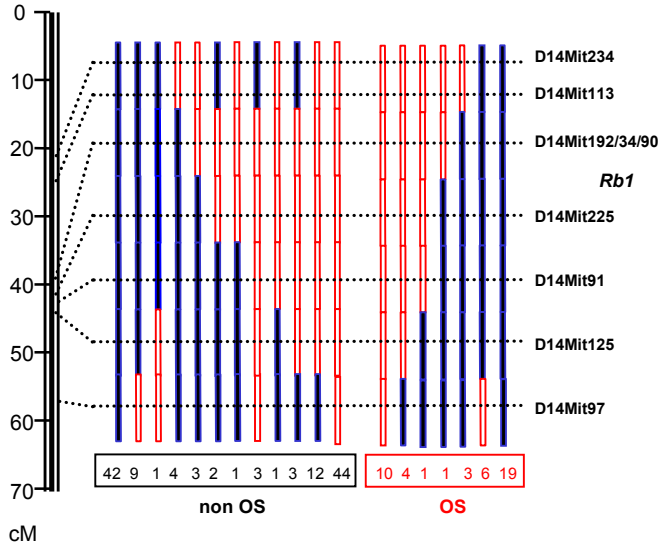

Fig 2c

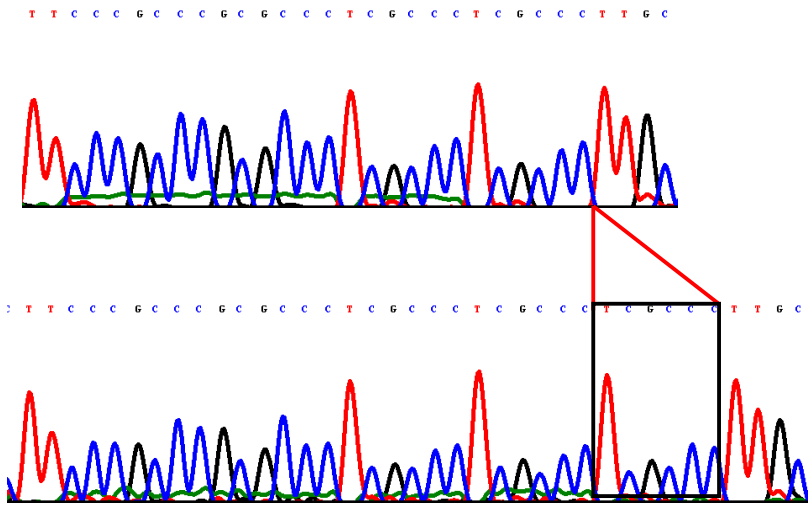

Fig 3a

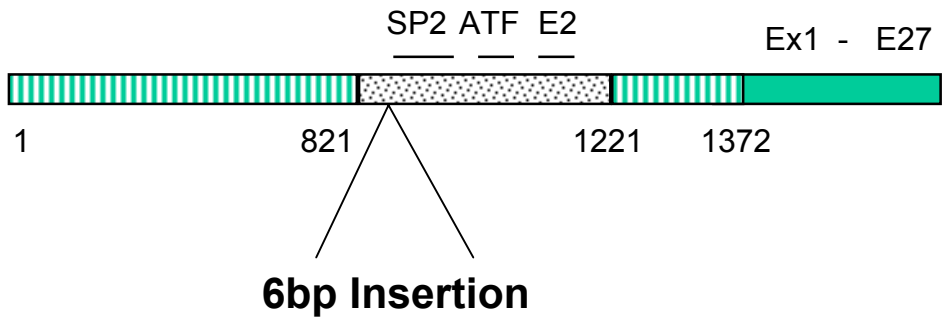

Fig 3b

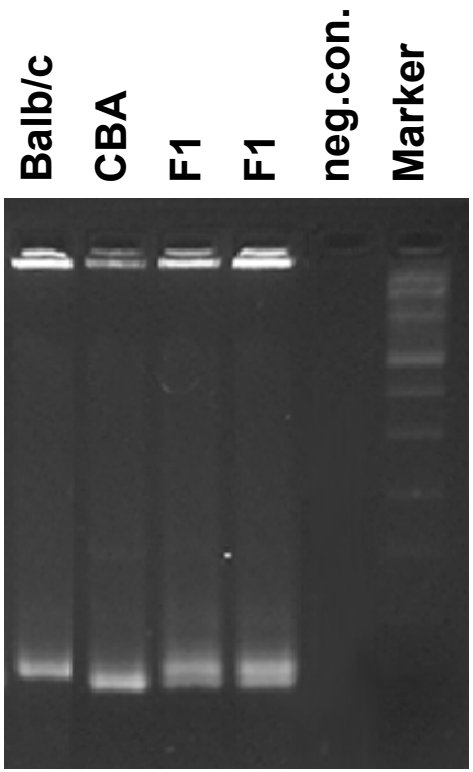
Balb/c
CBA
F1
F1
neg.con.
Marker

Fig 4a

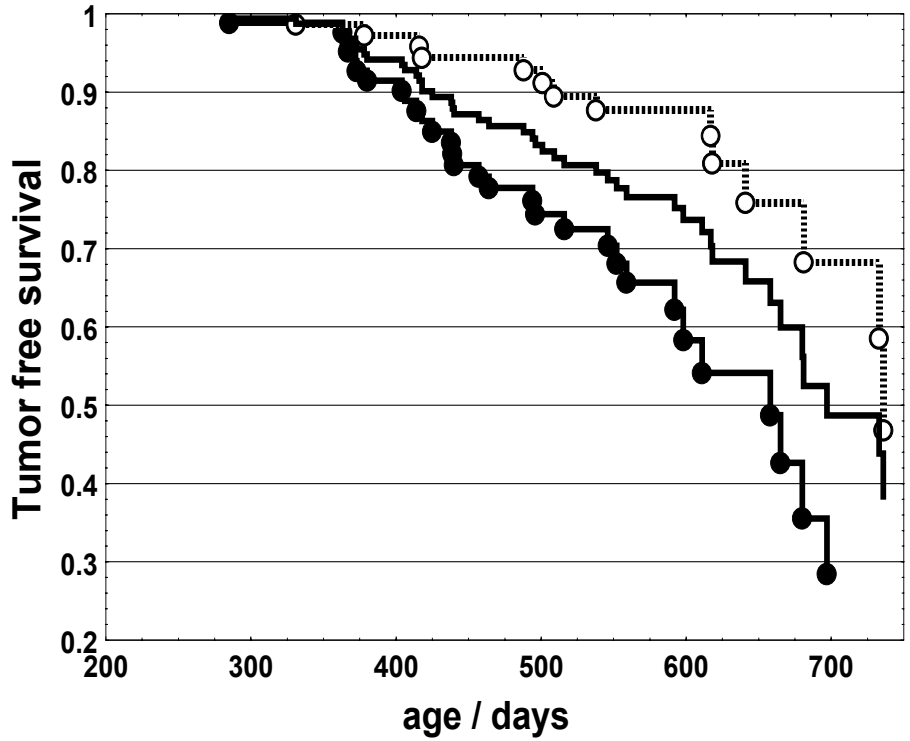
Tumor free survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
200
300
400
500
600
700
age / days

Fig 4b

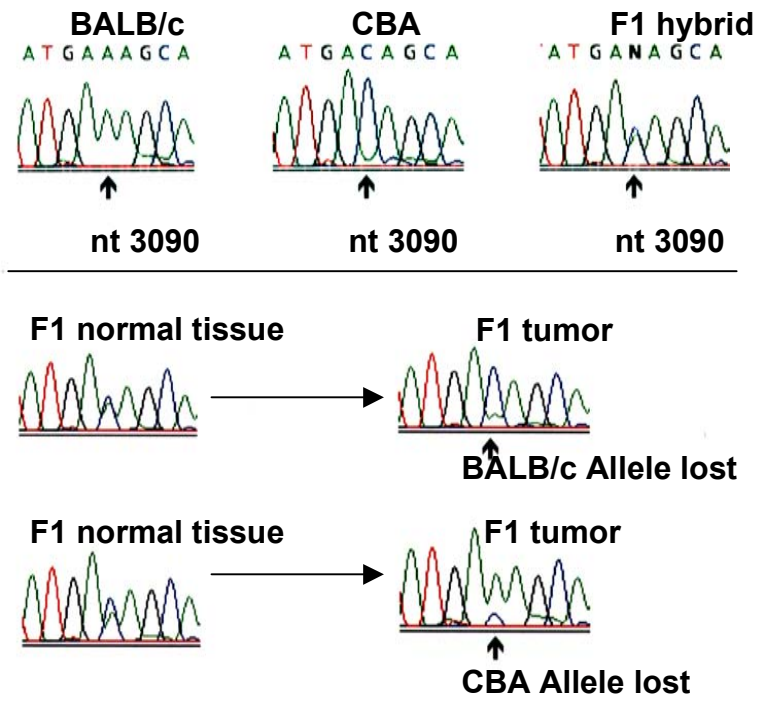
BALB/c
ATGAAAGCA
CBA
ATGACAGCA
F1 hybrid
ATGANAGCA
nt 3090
nt 3090
nt 3090
F1 normal tissue
F1 tumor
BALB/c Allele lost
F1 normal tissue
F1 tumor
CBA Allele lost

Fig 5a

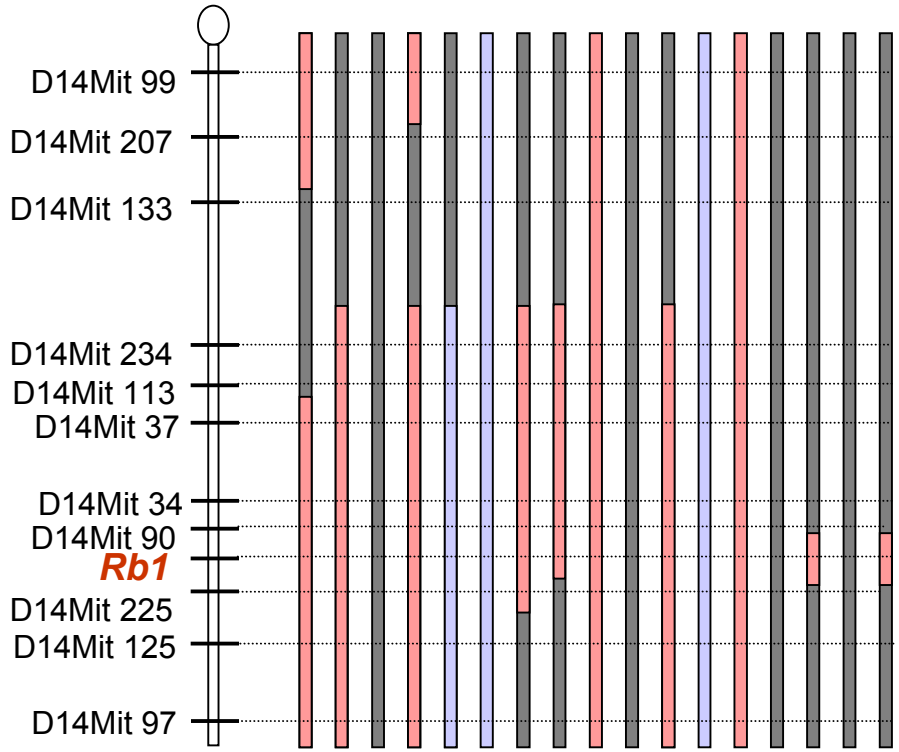
D14Mit 99
D14Mit 207
D14Mit 133
D14Mit 234
D14Mit 113
D14Mit 37
D14Mit 34
D14Mit 90
Rb1
D14Mit 225
D14Mit 125
D14Mit 97

Fig 5b

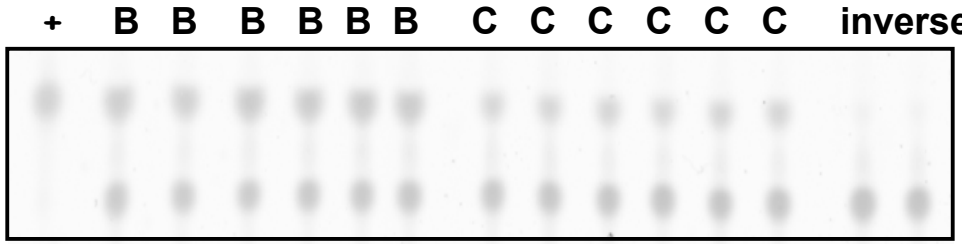

Fig 6a

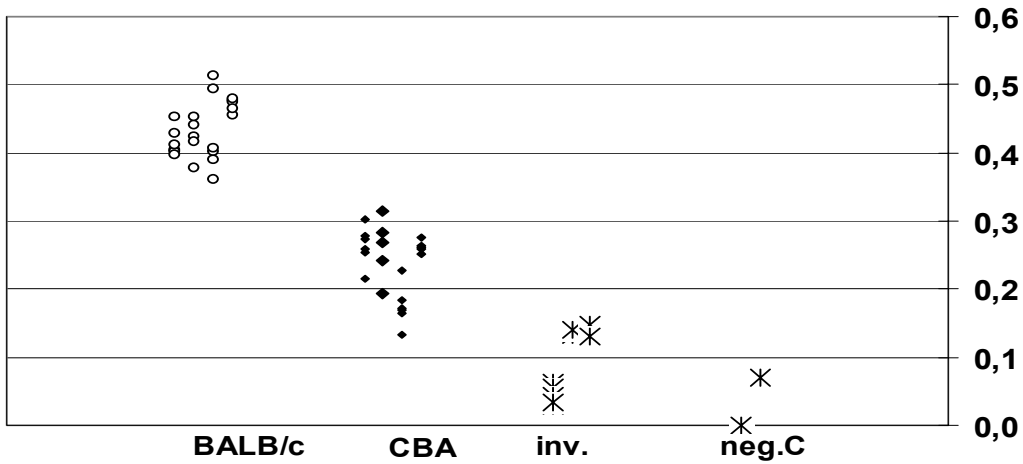

Fig 6b

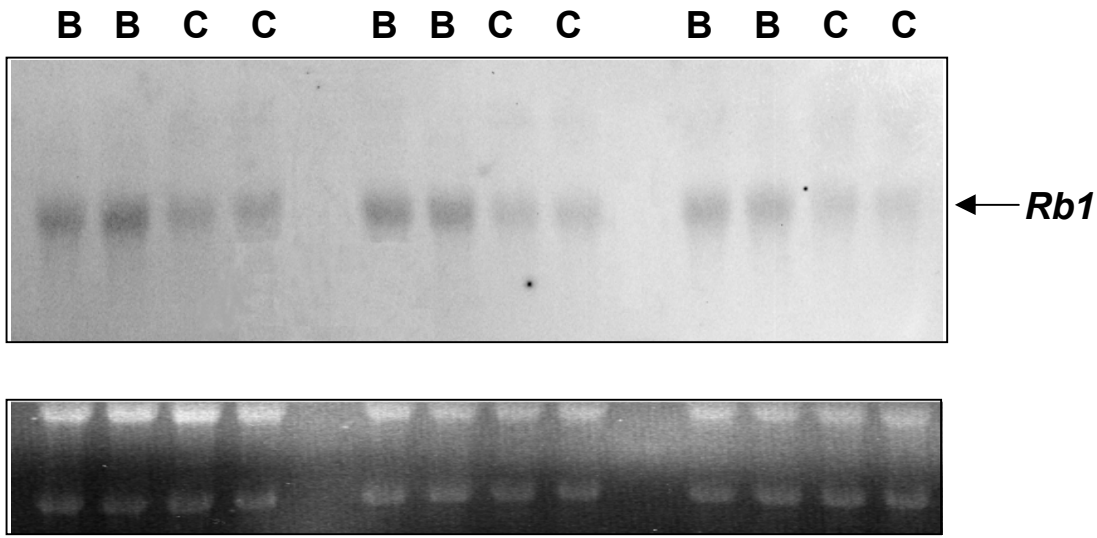

Fig 6c