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SHORT COMMUNICATION

Jdp2 downregulates Trp53 transcription to promote leukaemogenesis in the context of Trp53 heterozygosity

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We performed a genetic screen in mice to identify candidate genes that are associated with leukaemogenesis in the context of Trp53 heterozygosity. To do this we generated Trp53 heterozygous mice carrying the T2/Onc transposon and SB11 transposase alleles to allow transposon-mediated insertional mutagenesis to occur. From the resulting leukaemias/lymphomas that developed in these mice, we identified nine loci that are potentially associated with tumour formation in the context of Trp53 heterozygosity, including AB041803 and the Jun dimerization protein 2 (Jdp2). We show that Jdp2 transcriptionally regulates the Trp53 promoter, via an atypical AP-1 site, and that Jdp2 expression negatively regulates Trp53 expression levels. This study is the first to identify a genetic mechanism for tumour formation in the context of Trp53 heterozygosity.

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INTRODUCTION

Genetic alterations of TP53 are frequent events in tumourigenesis and promote genomic instability, impair apoptosis, and contribute to aberrant self-renewal.1-4 The spectrum of mutations that occur in TP53 in human cancers is diverse. Missense mutations that deregulate the DNA-binding domain are common, and prevent or impair the transcriptional regulatory activity of TP53.3 Cytogenetic alterations that delete or disrupt TP53 have also been reported, as has epigenetic silencing due to methylation of the Trp53 promoter.3 In both carcinomas and haematopoietic malignancies, TP53 mutation status has been shown to correlate with prognosis.5,6 TP53 is generally considered a tumour-suppressor gene,7 with inactivation of both copies of the gene seen in many tumours. Paradoxically, several studies have observed accelerated tumourigenesis in Trp53+/− mice that develop tumours despite retaining a wild-type copy of Trp53.7-10 Furthermore the analysis of tumours from Li-Fraumeni patients with germline alterations of TP53, suggest that a significant proportion may retain a wild-type TP53 allele.11-13 Threshold levels of P53 are required for processes such as suppression of apoptosis or induction of cell-cycle arrest.14 In the context of TP53 heterozygosity it is possible that transcriptional silencing of the wild-type TP53 allele by mechanisms such as promoter methylation, altered cis-regulation of the gene that decreases transcription from the wild-type TP53 allele, or post-translational modification of P53, decreases TP53 function to a level such that tumourigenesis can occur. In this paper we set out to identify, which somatically mutated genes can contribute to tumour formation in the context of TP53 heterozygosity. To do this we used mice heterozygous for Trp53 (as well as Trp53 wild type and null controls) to genetically dissect this phenomenon focusing on leukaemogenesis as a model system. This analysis allowed us to identify nine loci that are potentially associated with tumour formation in the context of Trp53 heterozygosity. We show that the Jun dimerization protein 2 (Jdp2) is a site frequently targeted by transposon insertion events leading to upregulated Jdp2 expression and a decrease in Trp53 expression levels. Further we illustrate that Jdp2 regulates the Trp53 promoter via an atypical AP-1 binding site. This study is the first to identify a genetic mechanism for tumour formation in the context of Trp53 heterozygosity.

RESULTS AND DISCUSSION

Mouse lines carrying the mutant Trp53 allele, Trp53+/−, which are null for Trp53,15-16 the SB (Sleeping Beauty) transposon array, T2/Onc,16 and the SB transposase allele, Rosa26SB11, (see Dupuy et al.17) were intercrossed to generate mice that were homozygous, heterozygous or wild type for the Trp53+/− allele (hereafter referred to as Trp53−/−, Trp53−/+ or Trp53+/+ mice, respectively) with or without SB transposition occurring (that is, on a T2/Onc+/−/−, Rosa26−/−/− or T2/Onc+/−/−, Rosa26−/−/− background, respectively). These mice were aged until they became moribund, and, as expected, SB transposition significantly accelerated tumour latency in mice of all genotypes (Figure 1a). The predominant tumour type of all genotypes was a widely disseminated CD3+ T-cell lymphoma (Figures 1b and c). A number of solid tumours, mainly undifferentiated sarcomas, were also observed, but only in Trp53−/− or Trp53−/+ mice (Figures 1b and c).

Genomic DNA from 36 Trp53−/−, 116 Trp53−/+ and 9 Trp53+/− SB-induced leukaemic/lymphomic tissues (typically spleen, thymus or lymph node) was extracted and subjected to a previously described linker-mediated PCR approach16 to amplify barcoded genomic fragments containing transposon-genome junction sequences. These products were then pooled and sequenced on the 454 platform, from which we generated 487,586 uniquely mapped sequence reads (approximately 3000 per tumour). After merging overlapping reads originating from the same sample and removing any on chromosome 1 (because SB
transposons frequently reintegrate into regions adjacent to the donor locus—a phenomenon known as ‘local hopping’\textsuperscript{16)}, we obtained 7538 (\textit{Trp53}\textsuperscript{+/-}), 21975 (\textit{Trp53}\textsuperscript{+/-}/\textit{C0}) and 1829 (\textit{Trp53}/\textit{C0}/\textit{C0}) unique, non-redundant insertion sites (for the respective tumour genotypes indicated in brackets). Using a previously described Gaussian Kernel Convolution statistical method for determining common insertion sites (\textit{CISs}),\textsuperscript{19,20} we identified 42, 63 and 9 \textit{CISs} in \textit{Trp53}\textsuperscript{+/-}, \textit{Trp53}\textsuperscript{+/-} and \textit{Trp53}\textsuperscript{-/-} tumours, respectively, (\textit{P} < 0.05 on a chromosome-adjusted scale; Figure 2 and Supplementary Table 1). Many of these genes have been previously implicated in the pathogenesis of T-cell lymphomagenesis/leukaemogenesis, including NOTCH1, \textit{PTEN} and \textit{IKZF1} (reviewed in Demarest \textit{et al.}\textsuperscript{21}). There were 12 \textit{CIS} genes in common between the \textit{Trp53}\textsuperscript{+/-} and \textit{Trp53}\textsuperscript{+/-}/\textit{C0} mice on a ‘jumping’ background according to the tumour type. Several mice had multiple tumour types. Numbers in brackets represent the percentage of mice developing a specific tumour type as a proportion of the genotype. (c) Representative photomicrographs of immunohistochemically-stained liver sections infiltrated by lymphomas of (v) B-cell origin (\textit{CD45R}\textsuperscript{+}) or (vi) T-cell origin (\textit{CD3}\textsuperscript{+}). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections that had undergone antigen retrieval (microwaving in high pH citrate buffer for 3 x 5 min) using rabbit anti-human polyvalent \textit{CD3} antibody (Dako, Ely, UK) and rat anti-mouse/human monoclonal B220/\textit{CD45R} antibody (BD Biosciences, Oxford, UK). The immunohistochemical signal was detected using a secondary biotinylated goat anti-rabbit or anti-rat antibody (Vector Laboratories, Burlingame, CA, USA), using the Vectorstain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. All sections shown are representative and images are at $\times$400 magnification.

![Figure 1](image-url)
(Mds1 and Evi1 complex locus), Myb, Notch1, Stat5b, Erg, Ikarf1, Raf1, Rasgrp1, Zmiz1, Pten, AB041803 and I12rb. Given that eight of these genes have also been identified as CISS in leukaemias/lymphomas from T2/Onc/Rosa26-SB11 mice on a wild-type background (Myb, Notch1, Erg, Ikarf1, Rasgrp1, Zmiz1, Pten and AB041803),27 they likely represent genes involved in lymphomagenesis/leukemogenesis in general, and do not contribute to promotion of tumourigenesis in the context of Trp53 heterozygosity.

A CIIS that was found in the Trp53+/− and Trp53−/− tumours, but not Trp53+/* tumours, was Rapgef6. The Rap1 guanine nucleotide exchange factor RAPGEF6 (also known as PDZGEF2) has a critical role in the maturation of adherens junctions.29 Although no immediate role for RAPGEF6 in tumourigenesis is evident, it has been shown to form protein complexes that result in the activation of Rap1A and control of cell adhesion/migration.24,25 Interestingly, apart from Rapgef6, the CIIS genes found in the Trp53−/− tumours were not found in tumours of the other genotypes. These included genes Usp42 (ubiquitin specific peptidase 42) and Wdr33 (WD repeat-containing protein 33). Although little is known about WDr33 gene, Usp42 gene has been recently identified as a fusion partner of RUNK1 in three cases of myeloid neoplasia, and the associated upregulated expression of USP42 suggests a role of this deubiquitinating enzyme in the pathogenesis of this leukaemia.26

There were also four CISS that were found to co-occur in tumours (Figure 2), specifically Notch1 and Pten, Notch1 and Ikarf1, Pten and Ikarf1 and Pten and Akt2 in Trp53+/* tumours. These genes have all been previously implicated in the pathogenesis of T-ALL.21,27 Our results are in keeping with the literature, as there is evidence for genetic co-operation of these genes in development of T-ALL. For example, loss of Ikarf1, a direct repressor of Notch target genes, and suppression of p53-mediated apoptosis are essential for development of T-ALL and Pten inactivation can compensate for some Notch-mediated processes in T-ALL.21 In addition, retroviral insertional mutagenesis recently identified Ikzf1, KrasG12D and Notch1 as a novel genetic pathway in T-lineage leukemogenesis.28

Quantitative PCR was performed on all tumours from Trp53+/* mice to identify those that had retained a wild-type copy of Trp53 and those that carried two copies of the targeted Trp53 allele (presumably having lost the wild-type allele by mitotic recombination; Figure 3a). From the 111 Trp53+/* tumours analysed, we identified 40 that had retained a wild-type Trp53 allele (defined as having a normalised wild-type allele content of >0.28 and a Trp53 allele content of <0.71) and 27 tumours that carried two targeted Trp53−/− alleles and no wild-type allele signal (defined as having a normalised wild-type allele content of <0.1 and a Trp53−/− allele content of >0.8). To determine if there were any somatic mutations in the intact wild-type copy of Trp53, genomic DNA from all 111 Trp53+/− tumours (as well as some tail samples to facilitate the identification of somatic mutations) underwent Trp53 sequencing on the Illumina platform (Illumina, San Diego, CA, USA) to scan for point mutations (using the primers shown in Supplementary Table 2). Paired-end sequencing of PCR amplified fragments was followed by base-calling with SAMTOOLS mpile-up,26 which identified three possible mutations, specifically MMU11:69400422 (T-C), MMU11:69403089 (G-A) and MMU11:69403110 (G-A) in single tumours. All other tumours appeared to have retained the wild-type Trp53 allele. A further two sequenced changes at MMU11:69401653 and MMU11:69401996 were discovered in 22 and 44 of the samples, respectively, and are therefore likely to be germline variants (as these mice were on a mixed C57BL/6J-129Sv background and the sequencing data was compared with the C57BL/6J reference genome). These data suggest point mutations of the wild-type Trp53 is infrequent in our model.

Taking the insertion sites found in tumours from Trp53+/* mice, we performed CIIS analysis in two ways. First, the tumours were divided into two groups: those that had either retained a wild-type copy of Trp53 or those that had lost the wild-type copy to identify the CIIS that were unique and common to each group (Figure 3b). We found a set of nine CIIS loci enriched in Trp53+/− mice that developed tumours despite retaining a wild-type copy of the gene, including AB041803, Akt2, Eras, Ikarf1, Jdp2, Myb, Rapgef6 and two intergenic regions. Second, we pooled the insertion sites from both groups together and then distinguished genotype-specific CIIS using a P-value generated by Fisher’s Exact test analysis.29 Using this more ‘stringent’ method of CIIS calling, we identified two CIIS that were ‘enriched’ in Trp53+/* tumours that had retained a wild-type copy of Trp53, specifically AB041803 and Jdp2. Little is known about AB041803 and as yet no role in tumourigenesis is evident. In addition, it was also found to be CIIS in leukaemia/lymphoma of wild-type mice (Supplementary Table 1).22 Thus we focused on Jdp2.

Transcription factor JDP2 (also known as JUNDM2) is an AP-1 repressor protein30 that has a paradoxical role in tumour formation. Overexpression of Jdp2 has been shown to potentiate hepatocellular carcinoma in mice32 and retroviral insertions predicted to activate the gene have been reported in mouse lymphoma models.33,34 In contrast, downregulation of JDP2 has been associated with a poor prognosis in pancreatic cancer.35 Loss of Jdp2 has also been associated with resistance to replicative senescence.36,37 and Jdp2 expression has been shown to suppress cell-cycle progression by downregulation of cyclin-A2.38 However, hypomethylation of the Jdp2 promoter or upregulation of Jdp2 expression in common myeloid progenitors and in granulocyte-macrophage progenitors has led to suggestions that it functions as a regulator of myelopoiesis.39 Here, we find that transposon insertions in the Jdp2 promoter occur exclusively in tumours from Trp53+/* mice that retain a wild-type allele of Trp53. These
insertions clustered in the promoter of Jdp2 (Figure 3c) and were mostly orientated so that the transposon was inserted in the same transcriptional orientation as the gene, suggesting that these insertions were functioning to drive overexpression (with a single insertion orientated on the reverse strand relative to the gene, which may represent an enhancer insertion\textsuperscript{40}). RT-PCR on RNA from these tumours showed splicing of the T2Onc transposon splice donor site directly onto Jdp2 exons 2 and/or 3 (Supplementary Figure 1). Indeed insertions in this exact location have been shown to activate Jdp2 expression\textsuperscript{34}, and consistent with this, qPCR on RNA from tumours containing insertions in Jdp2 showed a trend towards having higher expression levels of Jdp2 and lower expression levels of Trp53, relative to Trp53\textsuperscript{+/+} tumours with no insertions in Jdp2 (randomly selected from mice on this study that had not lost the Trp53 allele by mitotic recombination; Figure 3d).

Co-transfection of JDP2 cDNA in an overexpression vector with a mouse Trp53 proximal promoter construct in murine NIH3T3 (Figure 3e) and human HEK293T cells (data not shown) resulted in significant repression of Trp53 promoter activity, confirming that overexpression of JDP2 functions directly on the Trp53 promoter to repress Trp53 expression. The ability of JDP2 to repress transcription of the p53 promoter is reported to occur via its binding to an atypical AP-1 site, termed the PF-1 site\textsuperscript{41}. When we mutated (deleted) this binding site in the proximal Trp53 promoter, this completely abrogated the suppressive effects of JDP2 (Figure 3e), confirming that overexpression of JDP2 mediates repression of Trp53 through the PF-1 site in the proximal promoter. Furthermore, overexpression of JDP2 in HEK293T cells was shown to repress endogenous TP53 expression (Figure 3f).
Figure 3. Identification of driver mutations associated with loss of Trp53 by mitotic recombination or with the retention of a wild-type copy of Trp53. (a) SYBR Green quantitative real-time PCR (ABI, Carlsbad, CA, USA) was performed on tumour genomic DNA to quantify the relative proportions of Trp53 wild type and Trp53-/- alleles in genomic DNA extracted from the leukaemias/lymphomas and data were normalised to the single-copy genes β-Actin and Gapdh (primers are detailed in Supplementary Table 3). Red triangles represent tumours from Trp53 mice, blue squares represent tumours from Trp53+/+ mice, and black circles represent tumours from Trp53+/− mice. Of the tumours from Trp53+/− mice, open squares are those that have retained a wild-type copy of Trp53, closed dark blue squares are those that have lost the wild-type copy of Trp53, and closed light blue squares are those with a mixture of Trp53+/− and Trp53−/− cells and were thus excluded from further analyses. (b) Common insertion sites (CISs) were identified in tumours from Trp53−/− mice that had retained a wild-type copy of Trp53 (dotted blue circle) and those that had lost the wild-type copy (solid blue circle) as described previously.21,22 CISs were called using a genome wide cut-off of P < 0.05. Asterisk indicates the CIS was also found in the other genotype/circle, but below the P < 0.05 cut-off. Double asterisk indicates the CISs were in intergenic regions that is, not located within ± 150K base pairs of a gene and were given the label 'CIS' followed by the chromosome and the peak circling of the Gaussian kernel; there were two regions for ‘tumours retaining a wild-type copy of Trp53’: CIS:737371163_15k and CIS:75854217_15k, and one for ‘tumours without a wild-type copy of Trp53’: CIS:737326623_15k. (c) Location and orientation of the transposon insertions (blue triangles) associated with the Jdp2 CIS (the exons of Jdp2 are represented as boxes). One tumour was found to harbour multiple independent transposon insertion events (indicated with dotted lines). (d) Quantitative PCR (qPCR) was performed on five tumours containing insertions in Jdp2 and nine randomly selected Trp53−/− T-cell tumours (without insertions in Jdp2). RNA was extracted using the RNeasy Minikit (Qiagen), DNAse-treated (Turbo DNase, Ambion, Warrington, UK) and reverse transcribed (RNA to cDNA EcoDry Random Hexamers, Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Quantitative PCR was performed in triplicate using SYBR Green PCR MasterMix (Applied Biosystems, Carlsbad, CA, USA) and the CT for Trp53 and Jdp2 were normalized to the 'control' (average of five housekeeping genes: Gapdh, β-Actin, Hprt1, Ripl32 and Rpl13a) using the 2−ΔΔCT method.20 Primers used for qPCR are given in Supplementary Table 4. (e) Transient overexpression of JDP2 in NIH3T3 cells resulted in a significant repression of Trp53 proximal promoter activity. The 375 bp mouse Trp53 proximal promoter (Trp53-Luc) was PCR amplified from tail genomic DNA (using primers: F: 5′-AAAAAAAAAGGTACCGGTCCACTTACGATAAAAAC-3′ and R: 5′-AAAAAAAAAGGTACCGGTCCACTTACGATAAAAAC-3′) and cloned into the pGL3-BASIC vector (Promega, Southhampton, UK). The mutated mouse Trp53 proximal promoter sequence in which the 7 bp F1 site (5′-TGACTCT-3′) was removed (mutTrp53-Luc) was synthesized (GeneArt-Invitrogen, Paisley, UK) and cloned into the pGL3-BASIC vector. A full-length human Jdp2 cDNA was obtained from Origene (Rockville, MD, USA). NIH3T3 cells grown in 96-well plates were transfected with (i) either 100 ng Trp53-Luc (black lines) or mutTrp53-Luc (grey lines), (ii) 20 ng pRL-SV40 (an internal control reporter; Promega) and (iii) either 50 ng Jdp2 cDNA or empty vector according to the manufacturer's instructions (Lipofectamine 2000; Invitrogen). Firefly and Renilla luciferase were measured 50 h later using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). The firefly light units were normalised to the Renilla light units. All data were normalised to the average value of the 'control' transfection (Luc vector plus empty vector) and were presented as fold-change relative to the control. Experiments were performed in triplicate on at least three independent occasions and the data analysed by two-tailed Student's t-test. (f) Transient overexpression of JDP2 in HEK293T cells represses Trp53 expression. HEK293T cells (Grrophon Eco, Allele Biotechnology, San Diego, CA, USA) were seeded in 12-well plates and transfected with 2 μg Muc-DDK-tagged ORF clone of JDP2 (pCMV6Entry; Origene) or empty vector, according to the manufacturer's instructions (Lipofectamine 2000, Invitrogen). Experiments were performed in triplicate. RNA was extracted 48 h post-transfection and reverse transcribed as described above. Quantitative PCR was performed in triplicate using SYBR Green PCR MasterMix (Applied Biosystems) and the CT for TP53 and JDP2 were normalized as described above. Primers used for qPCR are given in Supplementary Table 4.
In conclusion, we show that overexpression of Jdp2 in tumours that are heterozygous for Trp53 precludes the need for loss of the wild-type allele of Trp53 during the process of leukaemogenesis. Jdp2 overexpression is the first genetic mechanism that has been identified as being responsible for tumour formation in the context of Trp53 heterozygosity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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