

ORIGINAL ARTICLE

Hipk2 cooperates with p53 to suppress γ -ray radiation-induced mouse thymic lymphomaJ-H Mao¹, D Wu², I-J Kim², HC Kang², G Wei^{3,4}, J Climent², A Kumar², FG Pelorosso², R DelRosario², EJ Huang³ and A Balmain²

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A genome-wide screen for genetic alterations in radiation-induced thymic lymphomas generated from $p53 +/−$ and $p53−/−$ mice showed frequent loss of heterozygosity (LOH) on chromosome 6. Fine mapping of these LOH regions revealed three non-overlapping regions, one of which was refined to a 0.2 Mb interval that contained only the gene encoding homeobox-interacting protein kinase 2 (*Hipk2*). More than 30% of radiation-induced tumors from both $p53 +/−$ and $p53−/−$ mice showed heterozygous loss of one *Hipk2* allele. Mice carrying a single inactive allele of *Hipk2* in the germline were susceptible to induction of tumors by γ -radiation, but most tumors retained and expressed the wild-type allele, suggesting that *Hipk2* is a haploinsufficient tumor suppressor gene for mouse lymphoma development. Heterozygous loss of both *Hipk2* and *p53* confers strong sensitization to radiation-induced lymphoma. We conclude that *Hipk2* is a haploinsufficient lymphoma suppressor gene.

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Introduction

The p53 tumor suppressor gene, one of most commonly mutated genes in a wide spectrum of human and animal tumors, controls cellular responses to DNA damage and forms a critical link to downstream effectors of growth arrest or cell death. We have previously described a genetic screen for modifiers of p53-dependent or -independent pathways leading to tumorigenesis in the mouse (Cai *et al.*, 2002; Mao *et al.*, 2004, 2007). This approach identified loci on chromosomes 3, 16 and 18, which undergo genetic alterations, specifically in tumors from mice carrying at least one functional p53 allele,

and are therefore likely to contain genes that mediate p53 signaling during tumor development. Additional loci on chromosomes 6, 12 and 19 showed genomic changes that were completely independent of the presence of p53. Detailed sublocalization of the commonest region of genomic alterations on chromosome 6 has enabled us to find a locus, which contains only one gene, the homeodomain interacting protein kinase 2 (*Hipk2*) gene.

Hipk2 is a recently identified serine/threonine nuclear kinase that is highly conserved in mammalian organisms. *Hipk2* acts as a multi-functional transcriptional regulator and corepressor that regulate cell growth, apoptosis, proliferation and development. HIPK2 has been found to be activated by a variety of DNA damaging agents, including ultraviolet light, ionizing radiation and genotoxic chemotherapeutic drugs (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002; Di Stefano *et al.*, 2004; Dauth *et al.*, 2007). In response to DNA damage, HIPK2 activates human p53 by promoting site-specific phosphorylation at Ser46 and subsequently activates apoptotic p53 target genes including PUMA, Noxa, DR5, PIG3, p53AIP1 and Bax (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002; Di Stefano *et al.*, 2004; Pistritto *et al.*, 2007). In addition, HIPK2 has also been shown to interact with and promote the degradation of CtBP in response to ultraviolet-induced DNA damage, and can induce p53 independent apoptosis (Zhang *et al.*, 2003). HIPK2 has also been shown to induce phosphorylation and degradation of c-Myb through the Wnt signaling pathway in the control of cell proliferation (Kanei-Ishii *et al.*, 2004) and to interact with several proteins containing the high mobility group I domain, a highly conserved domain in transcription factors of the lymphoid enhancer-binding factor 1/T cell factor (LEF1-TCF) family (Pierantoni *et al.*, 2001, 2007). Our previous study showed that germline deficiency of *Hipk2* increased susceptibility to two-stage chemically induced skin tumor development (Wei *et al.*, 2007), suggesting that *Hipk2* may act as a tumor suppressor gene. Herein, we demonstrate loss of heterozygosity (LOH) of *Hipk2* in radiation-induced tumors from both $p53 +/−$ and $p53−/−$ mice and show that *Hipk2* deficiency in mice promotes γ -radiation-induced tumor development.

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Results

Sublocalization of LOH regions on chromosome 6

A substantial proportion of lymphomas induced in *p53*^{+/-} and *p53*^{-/-} mice by γ -radiation treatment show LOH on chromosome 6 (Mao *et al.*, 2004). Although most of the tumors showed large-scale losses involving the whole chromosome, several showed discrete patterns of loss that implicated at least three separate regions of deletion (Figure 1a). The smallest interval identified from our initial screen was a 0.2-Mb region between D6Mit91 and D6Mit222. This interval encodes only the *Hipk2* gene, a multi-functional transcriptional cofactor that regulates cell growth and proliferation. In a search for point mutations in the

Hipk2 coding region, the complete coding sequence was determined from 20 tumors from *p53*^{+/-} mice and a further 20 tumors from *p53*^{-/-} mice. No point mutations were found in any of the tumors. RNA was available from all lymphomas that showed LOH at the *Hipk2* locus, and in all cases, the remaining *Hipk2* allele was present (Figure 1b), indicating that no gene silencing had occurred. Further, we quantified the expression of *Hipk2* in radiation-induced thymic lymphoma by quantitative TaqMan analysis. Figure 1c shows that 50% of tumors with LOH (7/14) had clearly reduced *Hipk2* mRNA levels in comparison with normal thymus, or with tumors that retained both parental alleles (retention of heterozygosity) of *Hipk2*. We conclude that *Hipk2* is subject to genetic changes

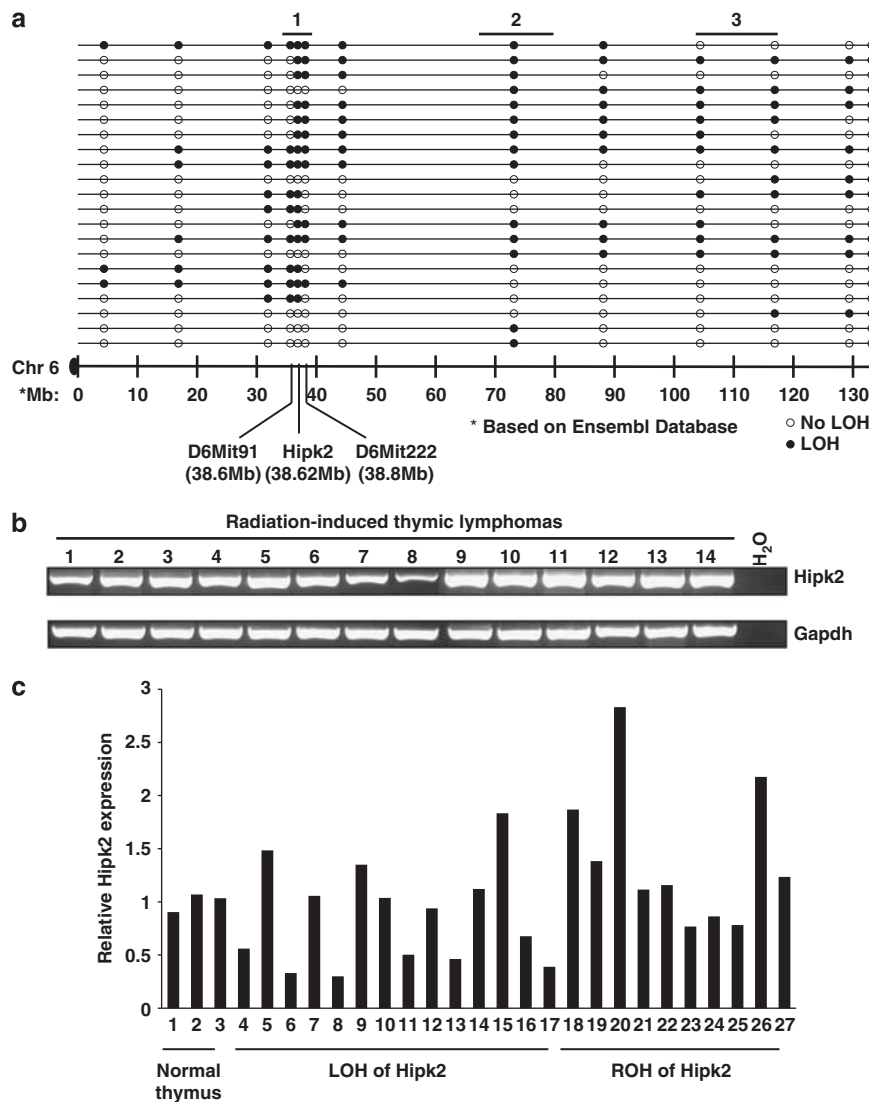


Figure 1 LOH of *Hipk2* in radiation-induced thymic lymphomas. (a) Detailed analysis of partial LOH patterns on chromosome 6 sublocalized three regions, denoted by the horizontal lines at the top of the figure. Physical mapping of markers and genes is based on the Ensembl database. Tumors showing either complete loss of chromosome 6 or no change are not shown in the Figure. LOH region 1 is centered on *Hipk2*. (b) mRNA expression of *Hipk2* in thymic lymphomas examined by RT-PCR, showing that all lymphomas examined retained expression of the remaining allele of *Hipk2* (c) Comparison of mRNA expression levels of *Hipk2* in tumors with LOH versus those with retention of heterozygosity using a quantitative TaqMan assay. The average expression level in tumors with LOH (lanes 4–17) is reduced, compared with those without LOH (lanes 18–27), and with normal thymus (lanes 1–3).

leading to reduced expression in a substantial proportion of lymphomas from p53 heterozygous mice.

Hipk2 cooperates with p53 in γ -radiation-induced tumorigenesis

To investigate whether loss of *Hipk2* accelerated γ -irradiation-induced tumorigenesis *in vivo*, 37 *Hipk2*^{+/-} and 31 wild-type mice were exposed to a single dose of 4-Gy γ -irradiation at 5 weeks of age. At the same time, 51 *Hipk2*^{+/-} and 34 wild-type mice were left untreated and observed concurrently to determine the spontaneous rate of tumor development. Within 75 weeks, only one unirradiated *Hipk2*^{+/-} mouse, none of the unirradiated wild-type mice and two irradiated wild-type mice developed tumors, in contrast to more than 40% of irradiated *Hipk2*^{+/-} mice (Figure 2a). We conclude that *Hipk2* is a functional tumor suppressor gene, loss of which increases susceptibility to tumors induced by radiation exposure. Importantly, loss of one allele of *Hipk2* also accelerated tumorigenesis in irradiated *p53*^{+/-} mice ($P=0.015$ for tumorigenesis in 29 *p53*^{+/-} mice versus 36

Hipk2^{+/-}*p53*^{+/-} mice; Figure 2b), in agreement with the observation of *p53*-independent deletions of this chromosomal region in mouse lymphomas (Mao *et al.*, 2004).

Hipk2 is a haploinsufficient tumor suppressor

Our observation of high frequency LOH, absence of point mutations and any evidence of silencing of the remaining allele suggested that *Hipk2* might function as a haploinsufficient tumor suppressor gene. In agreement with this interpretation, tumors from the *Hipk2*^{+/-}*p53*^{+/-} double heterozygous mice showed loss of the remaining *Hipk2* allele in only 1 of 20 tumors investigated, whereas loss of the remaining p53 allele was found in 18 of 20 tumors (Figure 3a). Expression of *Hipk2* was detected at the RNA level in tumors that had retained the wild-type allele, indicating that no gene silencing had taken place (Figure 3b). We conclude that *Hipk2* has many of the properties of a haploinsufficient tumor suppressor gene, in that loss of one copy confers predisposition without requiring complete inactivation of the remaining allele.

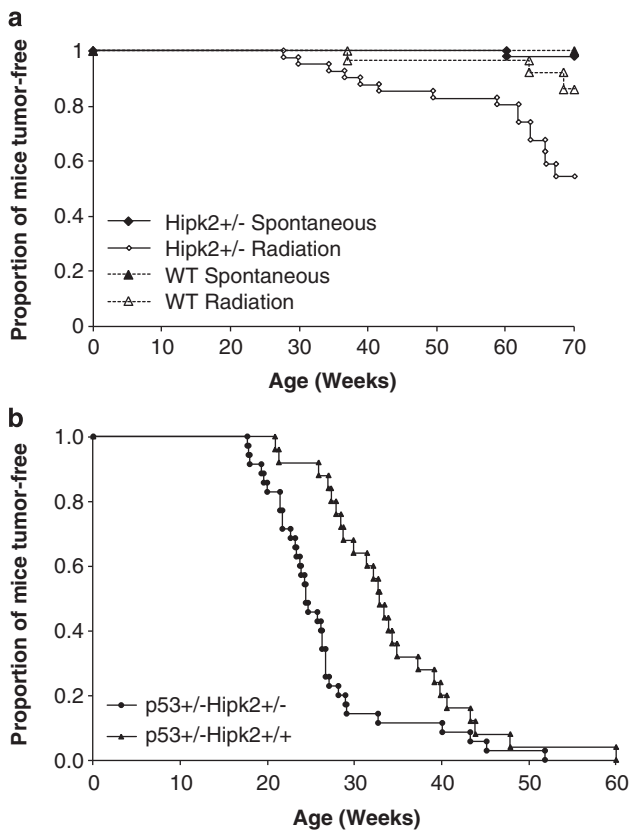


Figure 2 *Hipk2*-deficient mice are susceptible to radiation-induced tumor development. (a) Spontaneous and radiation-induced tumorigenesis in *Hipk2*^{+/-} and wild-type mice. 37 *Hipk2*^{+/-} and 31 wild-type mice were exposed to a single dose of a 4-Gy γ -irradiation at 5 weeks of age. At the same time, 51 *Hipk2*^{+/-} and 34 wild-type mice acted as unirradiated controls. (b) Radiation-induced tumorigenesis in double heterozygous *Hipk2*^{+/-}*p53*^{+/-} ($n=36$) and *p53*^{+/-}*Hipk2*^{+/+} ($n=29$) mice, showing the cooperative effects of partial deficiency in both genes.

Discussion

In this report, we have shown that LOH of the mouse *Hipk2* gene occurs frequently in radiation-induced thymic lymphomas. Most tumors retain and express one copy of the wild-type allele, demonstrating that the *Hipk2* gene has a haploinsufficient role in mouse lymphoma development. Deletion analysis of lymphoma DNA samples allowed us to localize the smallest common region of deletion to a 200-kb region containing only *Hipk2*, thus identifying this gene as the most viable candidate tumor suppressor.

A functional role for *Hipk2* in lymphomagenesis was demonstrated using mice carrying germline mutations in the *Hipk2* gene. *Hipk2* heterozygous mice are susceptible to radiation-induced tumorigenesis, but tumors retained and expressed the wild-type allele, in agreement with the hypothesis that loss of a single gene copy is sufficient to impact tumor susceptibility. Our observations of a haploinsufficient effect of *Hipk2* loss in lymphomagenesis suggests that chromosome changes resulting in copy number changes on human chromosome 7q34, where *HIPK2* is located, may have a role in development of human leukemias and solid tumors in which this chromosome undergoes somatic alterations (Mitelman *et al.*, 2010). Others have reported rare *HIPK2* mutations in AML (Li *et al.*, 2007), but there are also reports of amplification of *HIPK2* in some cancers (Deshmukh *et al.*, 2008), suggesting a complex role that is context dependent. This complexity is further underlined by apparently contradictory results on the effects of *Hipk2* on cell growth. Pierantoni *et al.* (2001) demonstrated that *Hipk2* can act as a suppressor of proliferation, but nevertheless cells from *Hipk2* knockout (KO) mice have variously been reported to exhibit decreased (Trapasso *et al.*, 2009) or increased (Wei *et al.*, 2007) cell growth

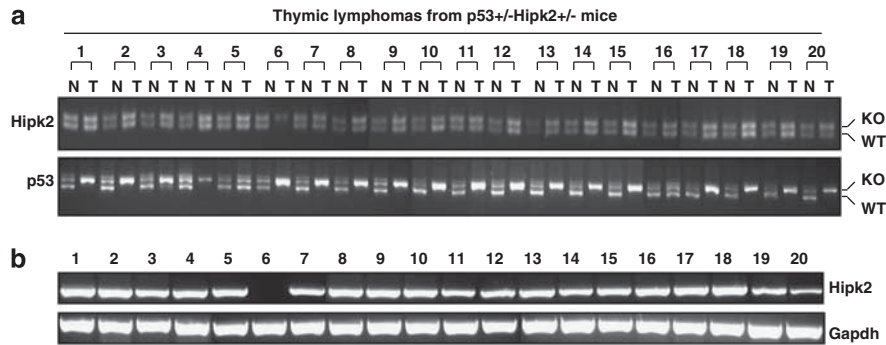


Figure 3 *Hipk2* is a haploinsufficient tumor suppressor gene. **(a)** LOH analysis of thymic lymphomas from *Hipk2*+/*p53*+/*p53*-/*Hipk2*- mice on chromosome 6 and 11. PCR analysis was carried out on 20 matched normal—tumor DNA samples to investigate loss of the wild-type alleles of *Hipk2* (top panel) and *p53* (bottom panel). Only one tumor (number 6) showed loss of the wild-type *Hipk2* allele, but 18/20 showed loss of the wild-type *p53* allele. **(b)** Expression analysis of *Hipk2* in thymic lymphomas from *Hipk2*+/*p53*+/*p53*-/*Hipk2*- mice by RT-PCR analysis. Only tumor number 6 that had lost the wild-type *Hipk2* allele showed complete loss of *Hipk2* expression.

in vitro. The reasons or these discrepancies are unclear, and may be related to the specific nature of the KO alleles or the genetic background from which the cells were derived. Our data demonstrate conclusively that *Hipk2* is a tumor suppressor gene *in vivo*; however, the fact that tumors retain and express one wild-type allele suggests that complete *Hipk2* loss may in some way be detrimental to tumor growth. The mechanistic basis for the positive or negative effects of *Hipk2* on cell growth and tumor development remains to be established.

Although HIPK2 has been implicated in regulating p53-dependent cell growth and apoptosis (D’Orazi *et al.*, 2002; Hofmann *et al.*, 2002), our studies on radiation-induced tumor development in the *Hipk2/p53* double heterozygous mice show that the activities of these two genes in lymphomagenesis are independent, as germline deletion of one copy of both genes shows additive effects on lymphoma susceptibility.

Independent effects of loss of *p53* and of *Hipk2* were also evident during the somatic events involved in tumor progression, as loss of one *Hipk2* allele was also found in tumors from *p53*-/*p53*- mice. Documented p53-independent functions of HIPK2 include induction of apoptosis upon DNA damage, through proteasomal degradation of CtBP (Zhang *et al.*, 2003), but whether this mechanism is linked to the tumor suppressor role of *Hipk2* in lymphoma susceptibility and progression remains to be determined.

Materials and methods

Mice and tumor induction

p53 and *Hipk2* single or double heterozygous KO mice were generated by crossing *Hipk2*+/*p53*- heterozygous KO (Wei *et al.* 2007) with *p53*+/*p53*- heterozygous KO mice (C57BL/6J), purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice at 5 weeks of age of both sexes were exposed to a single dose of 4-Gy whole body γ -irradiation and monitored daily until moribund, then killed and autopsied. Mice were bred and treated under University of California at San Francisco (UCSF) Laboratory Animal Resource Center (LARC) regulations.

Preparation of DNA

Thymic lymphomas and normal tissues were minced and placed in a microfuge tube containing 0.5 ml lysis buffer (100 mM Tris-HCl, pH 8.5; 5 mM ethylene diamine tetraacetic acid, 0.2% sodium dodecyl sulfate, 200 mM NaCl). Proteinase K was added at a final concentration of 100 μ g/ml and incubated at 55 °C overnight. The lysate was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (GibcoBRL, Carlsbad, CA, USA). DNA in the lysate was precipitated by addition of an equal volume of isopropanol. The DNA precipitates were solubilized in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM ethylene diamine tetraacetic acid).

Sublocalizing LOH on chromosome 6 by PCR of microsatellite markers

LOH was determined by PCR of microsatellite markers, with normal and tumor DNA from the same mice. Map position was based on the Ensembl (http://uswest.ensembl.org/Mus_musculus). The primers for microsatellite marker within the *Hipk2* gene are: F: AGCTTAGATGGTCTGTGGAA; R: GCAGGTCTCTACTAGCATGG.

PCR primer pairs for markers were purchased from Qiagen Operon (www.operon.com). PCR reactions were set up in a total volume of 20 μ l, containing 2 μ l of 10 \times PCR buffer (Bioline, Tauton, MA, USA), 1.6 μ l of 2.5 mM dNTPs (Pharmacia Biosystem Ltd, Piscataway, NJ, USA), 1 μ l (6.6 μ M) of each primer, 0.6 μ l of 50 mM MgCl₂ (Bioline), 11.7 μ l dH₂O, 0.1 μ l Taq polymerase (Bioline) and 2 μ l (40 ng/ μ l) tumor or normal tissue DNA. Amplifications were initially denatured at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C or 52 °C for 30 s and 72 °C for 30 s, and then a final incubation at 72 °C for 5 min. The PCR products were then mixed with loading buffer and electrophoresed in 4% (3% NuSieve/1% agarose) agarose gel with 0.5 mg/ml ethidium-bromide, photographed and saved in image file for analysis of density of bands.

Mutational analysis of the *Hipk2* coding region

Total RNA was extracted from tumor tissue samples with the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription PCR reactions were performed using Thermoscript RT-PCR system (GibcoBRL) according to the manufacturer’s instructions. Mutation analysis of the *Hipk2* gene was performed by PCR amplification of cDNA from thymic lymphomas by using the following primers:

F1: TCAAGTGCCTTCTGTAGTGTGAA; R1: CATAAA GGTTCCTGCTCCAACATC; F2: GAGTGCTTCCAGCAC AAGAAC; R2: TAACAGGTCAATGAACTCCCG; F3: GG ACAAAGACAAGTGGTTTTTCAA; R3: TGGTAGTTT AGTATGGAGACTTCGG; F4: CCAATCCCGAAGTCTC CATA; R4: TGGTTGTTGCCTCATCACAT; F5: GCATG CAGCTGTGATTCT; R5: GTGTCCAAGGTGTTGGT GC; F6: CAAGCGTGTCAAGGAGAACA; R6: AGCTCCC TGAAGAGTGTCCA; F7: CAGTCACCATTTCGCTCTC CT; R7: GGTCTCTTCTCCGCTCTCAA.

PCR products were purified by gel electrophoresis and sequenced by Quintarabio (www.quintarabio.com).

Detection of Hipk2 mRNA expression using Taqman assay

Total RNAs from all tumors and three normal thymuses were used to assess *Hipk2* mRNA expression by TaqMan Assays-on-Demand (Mm00439329_m1), purchased from Applied Biosystems (Carlsbad, CA, USA). *Hipk2* expression was measured using the ABI PRISM 7700 sequence detection system (Applied Biosystems). PCR reactions were performed in triplicate for each sample and experiments were repeated a minimum of three times. Ct values were normalized against β -actin RNA ($\Delta\text{Ct} = \text{Ct of Hipk2} - \text{Ct of } \beta\text{-actin}$). The relative *Hipk2* expression was calculated by $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct} = (\Delta\text{Ct of sample}) - (\text{average } \Delta\text{Ct of three normal thymuses})$.

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Statistical analysis

The Mann–Whitney *U*-test was used to compare the expression levels of *Hipk2* between tumors with LOH and retention of heterozygosity. The Kaplan–Meier method was used to compare the survival time post-radiation between different groups of genotypes of mice using the SPSS statistical package (SPSS, Armonk, NY, USA).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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