

Tumorigenic Effect of Nonfunctional p53 or p21 in Mice Mutant in the Werner Syndrome Helicase¹

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Abstract

Werner syndrome is an autosomal recessive disorder characterized by genomic instability and by the premature onset of a number of age-related diseases, including malignancy. To assess a potential collaboration between p21 or p53 cell cycle regulators and Wrn proteins, *Wrn* mutant mice were created and mated with *p21* or *p53* null mice to generate double mutants. The *p21* null/*Wrn* mutant mice did not show an acceleration of tumorigenesis during the first year of life, suggesting that the p53-dependent G₁-S cell cycle checkpoint (which operates via p21) is not involved in *Wrn*-abetted tumor suppression. In contrast, the *p53* null/*Wrn* mutant mice were particularly remarkable with respect to the rapidity with which they developed tumors. These mice were also distinguished by the variety of tumors they developed compared to those that developed in *p53* null mice. Such data suggest a genetic interaction between p53 and *Wrn* in which loss of *Wrn* provokes a more variable p53 response unrelated to its role in the G₁-S cell cycle checkpoint.

Introduction

Werner syndrome (WS)³ is a rare autosomal recessive disorder characterized by the premature onset of a number of processes associated with aging, including malignancy (1, 2). Lymphoid cells and cultured fibroblasts explanted from patients suffering from WS show several types of chromosomal rearrangements including deletions and variegated chromosomal translocations (3–6). These findings suggest that WS is a human genomic instability or mutator syndrome. The gene responsible for WS (*WRN*) was identified by positional cloning (7). It codes for a protein containing seven helicase consensus domains that are identical to the *Escherichia coli* RecQ gene (8) and to the Sgs1 yeast helicase (9, 10). The protein also contains a 3'-5' exonuclease activity in addition to its 3'-5' helicase activity (11–13). Recently, studies have demonstrated that the human WRN protein interacts with p53, which is a protein also important for genomic stability (14, 15).

The p53 protein is a transcription factor (16) whose level is increased in response to genotoxic stress such as DNA damage. This increase in p53 protein levels is thought to result in transcription of target genes that mediate many functions. Among its transcriptional targets is p21^{WAF1/CIP1} (17), an inhibitor of cyclin-dependent kinase complexes (18) and also an inhibitor of proliferating cell nuclear antigen (PCNA) activity as well as DNA synthesis (19, 20). p53 function is required for G₁ arrest (21) and it is believed that this arrest

allows cells time to repair DNA damage before being fixed as mutations. The p21 protein is also involved in this cell cycle arrest (22). It is induced by DNA damage and is found associated with inactive cyclin E/cyclin-dependent kinase 2 complexes which are essential for G₁-S phase transition (23).

In addition to cell cycle arrest, the ability of p53 to induce apoptosis is thought to be an important factor for its tumor suppressor function (24). Thus, cells lacking p53 function continue to proliferate, perpetuating potentially oncogenic mutations. Indeed, >70% of p53-deficient mice develop tumors, especially lymphomas and sarcomas, by 6 months of age (25, 26).

We recently created a deletion of part of the helicase domain of the murine homologue of the WS gene in embryonic stem cells to study tumor development in mice (27). These mutant mice were further crossed to *p53* null (25) or *p21* null mice (22) to assess genetically the effect of any G₁ cell cycle checkpoint control defect on tumorigenesis. In what follows, we show that *p53* null/*Wrn*^{Δhel} homozygous mutant mice show an acceleration of tumor formation as well as a change in the tumor spectrum compared to *p53* null. In contrast, the *p21* null/*Wrn*^{Δhel} homozygous mutant mice did not show an acceleration of tumorigenesis. These results suggest that alteration of a p53- and/or WRN-dependent pathway (possibly apoptotic) is more likely to be important in tumor progression than cell cycle checkpoint control in our mouse model.

Materials and Methods

Wrn mice lacking part of the helicase domain were created by a homologous recombination strategy into embryonic stem cells as described previously (27). Mice of all possible genotypes were generated by mating the chimeras with either Black Swiss or 129/SvEv mice and intercrossing the F₁ and F₂ generations. Animals were checked three times a week for any external mass, infection, bleeding, gasping, and overall decrease or change in activity or behavior. The date of such observation was recorded and animals were kept for an additional 2–4 weeks to assess any decline in the health status. If in each case the condition of the animal worsened, the mouse was sacrificed for histological examination of its organs as described previously (28).

Results

Like other members of the RecQ helicase superfamily, the mouse WS protein contains seven conserved helicase motifs and motifs III and IV are encoded by two separate exons as in the human gene homologue (29). To assess *in vivo* the importance of the helicase domain, we designed a homologous recombination construct to replace a 4.1-kb genomic fragment containing these exons with a *neo* cassette in embryonic stem cells (27). These embryonic stem cells were used to generate mutant mice. As described previously, a stable mutant protein is synthesized from the homozygous mutant mice, presumably, with a nonfunctional helicase domain. Therefore, these homozygous mice are referred as *Wrn*^{Δhel/Δhel} mice. Fifty-four *Wrn*^{Δhel/Δhel} mice were observed for a period of 1 year as described in "Materials and Methods." More than 90% of live-born *Wrn*^{Δhel/Δhel}

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³ The abbreviations used are: WS, Werner syndrome; PCNA, proliferating cell nuclear antigen.

mice appeared normal during the first year of life. Ten percent of the mice died of an unknown cause or showed severe cardiac fibrosis upon dissection (data not shown). All 16 wild-type animals observed during the same period of time were healthy (data not shown). Several different tumors and other pathological findings were observed in homozygous mutant mice older than 15 months of age (Table 1). Approximately 62% of the mice had developed some type of hyperplasias or tumors in one of their organs by 24 months of age (Table 1 and data not shown). This is without considering the other symptoms that were or were not associated with cancer such as infections, inflammations, prolapses, fibrosis, and so forth (see Table 1). The examination of more wild-type mice of similar age are required to determine whether there is a significant difference in aging or tumorigenesis between both cohorts.

To determine the effect of the *p21* defect on tumorigenesis, *Wrn* mice were crossed to *p21* null mice to generate *p21* null/*Wrn*^{Δhel/Δhel} mice. After 1 year of observation, only 10% of these mice (3 of 23 animals) looked ill. Upon dissection of these sick animals, severe cardiac fibrosis similar to that of the *Wrn*^{Δhel/Δhel} mice was detected. Only, one *p21* null/*Wrn*^{Δhel/Δhel} mouse developed an hemangiosarcoma before the age of 12 months. Fifty-six percent of the *p21* null/*Wrn*^{Δhel/Δhel} mice older than 15 months of age had developed hyperplasias or tumors similar to those detected in *Wrn*^{Δhel/Δhel} mice with the same latency (data not shown). Thus, there was no acceleration of tumorigenesis on this *p21* null/*Wrn*^{Δhel/Δhel} cohort compared to the *Wrn*^{Δhel/Δhel} cohort.

To assess the joint role of *p53* and *Wrn* in tumor progression, *p53* null and *Wrn*^{Δhel/Δhel} mice were crossed to generate *p53* null/*Wrn*^{Δhel/Δhel} mice. These were carefully followed up for over 6 months and scored for the occurrence of tumors. Fig. 1 shows that *p53* null/*Wrn*^{Δhel/Δhel} mice developed tumors more rapidly than *p53* null mice. Half of the *p53* null/*Wrn*^{Δhel/Δhel} mutants had developed some type of illness or tumor by 3 months of age. Approximately 50% of the *p53* null mice had developed a tumor by the age of 4–5 months. Histological analysis of the sick mice revealed that *p53* null/*Wrn*^{Δhel/Δhel} mutants developed a variety of unusual tumors compared to the *p53* null or *Wrn*^{Δhel/Δhel} mice in our colony (Table 2). Several sarcomas of

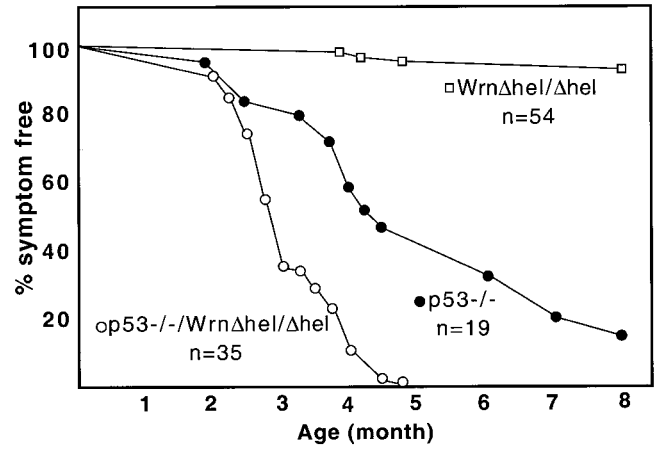


Fig. 1. Percentage of symptom-free animals from *Wrn*^{Δhel/Δhel}, *p53* null, and *p53* null/*Wrn*^{Δhel/Δhel} mutant mice over a period of 8 months. Animals were checked three times a week for any external mass, infection, bleeding, gasping, and overall decrease or change in activity or behavior. The dates of such observations were recorded at the first appearance of a symptom in each mouse. Mice were kept for an additional 2 weeks to assess any decline in health status and for pathological examination. The number of animal (*n*) in this survey are indicated for each genotype.

Table 2 Incidence of each phenotype in *p53* null and *p53* null/*Wrn*^{Δhel/Δhel} mutant mice

Symptoms	% <i>p53</i> ^{-/-} (n = 19) ^a	% <i>p53</i> ^{-/-} / <i>Wrn</i> ^{Δhel/Δhel} (n = 35) ^a
Hind limb paralysis	10	3
Malignant lymphoma ^b	10	31
Thymoma ^b	25	26
Myeloid hyperplasia ^b	5	9
Myeloid leukemia ^b	20	3
Hemangiosarcoma	10	17
Hemangiosarcoma (several foci)	5	34
Hemangiosarcoma of heart	— ^c	3
Rectal adenoma	—	17
Cortical hyperplasia thymus	—	34
Sarcoma (jaw)	—	3
Sarcoma of salivary gland	—	3
Spindle cell sarcoma (jaw)	—	3
Pericytoma (jaw)	—	6
Chondrosarcoma of mouth	—	3
Ovarian teratoma	—	3
Myocarditis	—	3
Hydronephrosis/pyelonephritis	—	3
Unknown cause of death	15	3
Animals with multiple types of tumors	—	34

^a Number of mice analyzed.
^b Symptoms common to *p53*^{-/-} null, *p53*^{+/-}/*Wrn*^{Δhel/Δhel}, and *p53*^{-/-}/*Wrn*^{Δhel/Δhel} mutant mice.
^c —, none detected.

Table 1 Phenotype of *Wrn*^{Δhel/Δhel} mutant mice

Symptoms	% <i>Wrn</i> ^{Δhel/Δhel} (n = 54) ^a
Myocarditis/aortitis	4
Perivascular lymphoid infiltrate	6
Myocardial fibrosis	59
Harderian hyperplasia or displasia	4
Myeloid hyperplasia ^b	9
Myeloid leukemia ^b	6
Lymphoma ^b	9
Thymoma ^b	6
Granulocytic sarcoma	2
Hepatoblastoma	2
Hepatoma	9
Granuloma in pancreas	2
Bronchial adenoma	2
Lung adenocarcinoma	2
Mammary keratoacanthomas	2
Mammary carcinoma	2
Prolapse or obstruction bladder	6
Gastric or intestinal polyps	2
Inflammation of lung, gut, or bladder	17
Inflammation of uterus	9
Polycystic endometrium	4
Hyperplasia prostatic apparatus	2
Granulosa cell tumor ovary	2
Cystadenoma ovary	2
Unknown cause of death	20
Animals with multiple types of tumors	17

^a Number of mice analyzed.
^b Symptoms common to *p53*^{-/-} null, *p53*^{+/-}/*Wrn*^{Δhel/Δhel}, and *p53*^{-/-}/*Wrn*^{Δhel/Δhel} mutant mice.

the mouth, including the salivary glands, were noticed in the *p53* null/*Wrn*^{Δhel/Δhel} mice. Such tumors included rare pericytoma, spindle cell sarcoma, and chondrosarcoma. The *p53* null mice mainly developed myeloid leukemias, hemangiosarcomas, thymomas, or malignant lymphomas (Table 2) (25). Several *Wrn*^{Δhel/Δhel} mice also developed leukemias, thymomas, or lymphomas but none of them developed hemangiosarcomas (Table 1). These results indicate that deletion of the helicase domain of the *Wrn* protein affects tumor spectrum.

A number of *p53* null/*Wrn*^{Δhel/Δhel} mice (34%) developed several foci of hemangiosarcomas in different organs. In contrast, <11% of the *p53* null mice had more than one focus of hemangiosarcoma. Moreover, 34% of the *p53* null/*Wrn*^{Δhel/Δhel} mutants simultaneously developed multiple types of tumors. Only 17% of *Wrn*^{Δhel/Δhel} mice and none of the *p53* null mice under survey developed multiple tumors (Tables 1 and 2). These observations indicate that *p53* null/*Wrn*^{Δhel/Δhel} mice rapidly develop aggressive tumors as well as un-

usual types of tumors compared to *p53* null or *Wrn*^{Δhel/Δhel} mice in our mixed genetic background (Black Swiss × 129/SvEv).

Discussion

The WS gene product contains both an exonuclease and a helicase domain. To assess the importance of the helicase domain, two exons coding for part of this domain were deleted by homologous recombination in mouse embryonic stem cells, leaving all other exons intact (27). Further analysis with an antibody against the mouse *Wrn* protein indicated that a stable mutant protein was being synthesized in mutant mice. Although the smaller mutant contains an intact nuclear localization signal, it does not copurify with a protein complex containing PCNA and topoisomerase I (30). This WRN/PCNA/topoisomerase I complex might be part of the bigger multiprotein DNA replication complex. Indeed, it has recently been shown that the human WRN protein interacts functionally with the DNA polymerase δ in yeast (31).

The possibility that the *Wrn*^{Δhel} mutant protein has a dominant-negative effect cannot be ruled out at this point. However, it is interesting to note that heterozygous mutant embryonic stem cells are more sensitive to topoisomerase inhibitors than wild-type cells, but less sensitive than homozygous cells (27). This intermediate sensitivity of heterozygous cells to a specific DNA-damaging agent parallels the findings that human heterozygous *WRN* mutant cells have an intermediate phenotype compared to homozygous and wild-type individuals in terms of sensitivity to a specific DNA-damaging agent and genomic instability (32, 33). It is, thus, possible that a deletion of part of the helicase domain might alter the three-dimensional structure of the helicase and its potential association with other cellular proteins that are part of a complex (30). Our results suggest that the *Wrn*^{Δhel} mutant protein has no obvious dominant-negative activity. However, the *Wrn* protein may have other, as yet unknown, functions of which our mutation might affect only a subset.

The *Wrn* mutant mice with a *p53*-deficient background were remarkable with respect to the rapidity with which they developed tumors. Moreover, the *p53* null/*Wrn*^{Δhel/Δhel} mice were further remarkable by virtue of the variety of tumors to which they give rise as compared to *p53* null or *Wrn*^{Δhel/Δhel} mice. Leukemias, thymomas, and lymphomas were the only tumor types common to all three cohorts of mice. In addition, several *p53* null/*Wrn*^{Δhel/Δhel} mice rapidly and simultaneously developed multiple tumor types (Table 1), indicating a synergistic effect of the *Wrn* and *p53* mutations in accord with previous findings with a *Wrn* null mutation (34). These data also indicate that a simple deletion of the helicase domain of the *Wrn* protein is sufficient to induce accelerated tumorigenesis on a *p53* null background.

The *p53*-*Wrn* genetic interaction might be accounted for by suggesting that the *p53* defect allows cells to escape apoptosis which would be the normal consequence of DNA damage. Lesions in the DNA may cause the DNA replication fork to stall or to create breaks in the DNA. The *Wrn* protein, like RecQ in *E. coli*, may be required for ensuring that structural abnormalities arising during recombinational repair at the replication fork are corrected at a high level of fidelity (35). Deletion of the helicase domain in the *Wrn* protein would increase genomic instability in already unstable *p53* null-proliferating or precancerous cells in certain tissues. This increase in genomic instability would give rise to additional mutations accelerating tumor progression and aggressive behavior on a *Wrn*^{Δhel/Δhel} mutant background. Interestingly, it has been shown recently that human WS cells have an attenuation of the *p53*-dependent apoptotic pathway (14). A similar behavior in mice would certainly promote tumorigenesis.

In summary, *p53* protein is an important component of cell cycle checkpoints. At least a portion of this *p53*-dependent cell cycle control is manifest via *p21* induction which is, in turn, responsible for the G₁-S cell cycle checkpoint. The fact that *p21* null/*Wrn*^{Δhel/Δhel} mice do not undergo accelerated tumorigenesis suggests that the G₁-S checkpoint is not a focus of *p53*-dependent tumor suppression. More likely, the key suppressor pathway in our mouse model is via the induction of apoptosis which is also dependent on *p53*.

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