The associated contributions of p53 and the DNA mismatch repair protein Msh6 to spontaneous tumorigenesis

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DNA mismatch repair (MMR) is a highly conserved system that repairs DNA adducts acquired during replication, as well as some forms of exogenous/endogenous DNA damage. Additionally, MMR proteins bind to DNA adducted DNA and are not removed by MMR and influence damage-response mechanisms other than repair. Hereditary non-polyposis colorectal cancer, as well as mouse models for MMR deficiency, illustrate that MMR proteins are required for maintenance of genetic stability and tumor suppression. In both humans and mice, the phenotype associated with Msh6-associated tumorigenesis is distinct from that of Msh2. In this study, we hypothesized that Msh6+/−;p53+/− mice would display earlier tumor onset than their Msh6+/− or p53+/− counterparts, indicating that concomitant loss of these two tumor suppressors contributes to tumorigenesis via mechanisms that are only partially interrelated. We generated a Msh6+/−;p53+/− mouse model which succumbed to malignant disease at an accelerated rate and with a tumor spectrum distinct from both Msh6−/− and p53−/− models. Alteration of tumor phenotype in the Msh6+/−;p53+/− mice included a marked increase in microsatellite instability that was associated with loss of heterozygosity of the remaining p53 allele. Also, genetic instability was inversely correlated with survival. This manuscript marks the first in vivo investigation into the association between Msh6 and p53, and their combined role in the suppression of spontaneous tumorigenesis, cell survival and genomic stability. Our results support the hypothesis that p53 and Msh6 are functionally interrelated and that, with concomitant mutation, these tumor suppressors act together to accelerate tumorigenesis.

Introduction

The acquisition of genetic instability and the loss of growth suppression are defining characteristics of malignant cells. DNA mismatch repair (MMR) contributes to genetic stability by responding to post-replicative and meiotic DNA damage, as well as to endogenous and exogenous DNA damage (1). MMR proteins bind and process base mismatches, as well as insertion/deletion loops formed by polymerase stuttering through highly repetitive genomic DNA sequence (microsatellites). DNA lesions are recognized and bound by one of two MMR protein heterodimers; base mismatches are bound exclusively by MSH2/MSH6, large DNA loops are bound by MSH2/MSH3 and small DNA loops are bound by either heterodimer. Cells lacking MSH2 can form neither heterodimer and are completely deficient in MMR activity, whereas cells lacking MSH3 or MSH6 retain residual MMR activity provided by the remaining MMR heterodimer. Once bound, a second MMR protein heterodimer (e.g. MLH1/PMS2) is recruited, which in turn recruits other necessary proteins for strand repair (1). Left unrepaird, mismatches result in point mutations and insertion/deletion loops, resulting in expansion or contraction of microsatellites. Microsatellite instability (MSI) is a hallmark of MMR deficiency and can affect either coding or non-coding DNA. For example, several genes important to avverting tumorigenesis (e.g. TGFβR2, PTEN and APC) contain coding microsatellites that are disrupted in some cases of hereditary non-polyposis colorectal cancer (HNPPC) (2). Therefore, loss of MMR can initiate a mutator phenotype cascade, triggering loss of cellular control, accrual of additional mutations and eventual malignant transformation.

The importance of MMR function to the prevention of malignancy is illustrated by HNPPC, a cancer syndrome that predominantly affects the colon, endometrium, ovaries and stomach (OMIM #120435). Potential HNPPC patients can be identified using the Revised Amsterdam Criteria II (3): a family history of at least three cases of either colorectal cancer or other HNPPC-associated cancers (endometrial, small intestine, urinary tract, renal), spread over at least two generations, and at least one affected member diagnosed before the age of fifty. Typically, HNPPC patients inherit a heterozygous MMR gene mutation and are initially unaffected until the wild-type allele is mutated; 80% of carriers present with HNPPC by the age of seventy. The MMR genes MSH2 and MLH1 are most frequently mutated. In comparison, mutation of MSH6 results in an altered phenotype that has been dubbed ‘atypical’ HNPPC (4). Whereas patients with MSH2- or MLH1-associated HNPPC are characterized by an average age of onset of forty, a family history of colorectal carcinoma and tumors displaying readily detectable MSI, MSH6-associated HNPPC is characterized by a significantly later age of onset, a family history of endometrial carcinoma and tumors displaying low level or undetectable MSI (using the standard panel of five MSI markers) (4). Moreover, MSI observed for MSH6-associated tumors is located almost exclusively at mononucleotide repeats, as opposed to MSI observed for typical HNPPC that is located at both mononucleotides and dinucleotides (4). These phenotypic differences observed for MSH6-associated HNPPC result in many of these individuals falling outside of current diagnostic criteria and an underestimation of the impact of MSH6 mutation on human health (5–8). Therefore, investigations into the impact of Msh6 deficiency on the tumorigenesis of mammalian cells are warranted.

Although MMR and p53 are often found to have an inverse association in other forms of colorectal cancer (9–12), co-inactivation/mutation of MMR and p53 is rare in HNPPC. However, the use of MSI alone to select MMR-deficient samples probably has skewed such findings. Comparing samples with large MSI shifts to those with small MSI shifts (≥8 bp versus ≤6 bp), Oda et al. (13) found that only small MSI shifts were associated with p53 mutation (P = 0.006); both human HNPPC samples and MMR-deficient murine tumors are enriched by larger MSI shifts. Oda et al. (13) speculate that variable methodology and interpretation of MSI assays results in underestimation of the small MSI shifts in HNPPC. In addition, the association between MMR deficiency and p53 mutation appears to be tissue specific. MSI analyses of patient-matched glioblastomas and colorectal tumors from patients with the HNPPC variant Turcot syndrome revealed that only the glioblastomas demonstrated p53 gene mutation (14), thus demonstrating organ-specific tumorigenic pathways. In non-HNPPC-related colorectal cancer, the association of MMR/MSI status with prognostic indicators (such as p53 status) is dependent on the physical tumor location (12,15). For example, MSI is associated with tumor size in right-sided sporadic colon tumors, while MSI is associated with both p53 mutation and lymph node metastases in left-sided sporadic colon tumors (12). (HNPPC colon tumors are predominantly right-sided.) Therefore, the association between MMR

Abbreviations: CIN, chromosomal instability; LOH, loss of heterozygosity; MMR, DNA mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; PCR, polymerase chain reaction.

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deficiency and p53 mutation may be masked by tumor location and be underestimated due to sample selection criteria.

Investigations using colorectal and other tumor-derived cell lines have shown associations between MMR and p53. Specifically, data support roles for MMR both upstream and downstream of p53 in DNA damage-response pathways. For example, the absence of MSH6 (16–18) or MLH1 (16,19) is associated with diminished and delayed p53 activation in response to various forms of DNA damage. Also, using primary mouse embryonic fibroblasts, we demonstrated that Ultraviolet B-induced p53 activation was Msh2-dependent, but not Msh6-dependent (20,21). In contrast, p53 transactivates MSH2, supporting a role for MMR that is downstream of p53 (22–24). The observations that MMR- and p53-associated pathways are partially interrelated also is supported by in vivo experiments. Two independently generated mouse models have demonstrated synergism between the loss of Msh2 and the loss of p53 in the rate of tumorigenesis (25–27). The contributions of p53 and MMR to resisting malignant transformation and progression are far-reaching (summarized in ref. 28); the loss of either alters normal cell cycle control, apoptosis and DNA repair following cellular stress such as DNA damage, hypoxia or aberrant cellular proliferation. Therefore, loss of both MMR and p53 function may result in further propagation of cellular instability and malignant progression.

The generation of mouse models is an important tool for the study of potential tumor suppressors or genes identified as markers of a particular malignancy. In mouse models of MMR deficiency (reviewed in refs 29,30), mice haploinsufficient for a MMR mutation do not display a phenotype, and loss of the wild-type gene and subsequent tumorigenesis generally is not observed. In contrast, mice that are homozygous-null for a MMR gene display a reduced life span due to spontaneous tumorigenesis, usually lymphoma. Mice lacking Msh2 or Mlh1 primarily develop lymphomas, predominantly T-cell lymphomas, and in the absence of a thymus, evidence suggests that B-cell lymphomas predominate (31). Mice null for either Msh2 or Mlh1 exhibit the most severe phenotype with a median survival times of 6 months, while Msh6-null mice display a significantly later age of onset (11 months) (30). Mice lacking both Msh2 and Msh6 have a similar phenotype to the Msh2-null mice, consistent with established heterodimer biochemistry (30). Knockout and/or transgenic mice are frequently bred together to model compound losses and more closely approximate the accumulation of genetic mutations that precede malignant transformation. For example, Msh2 deficiency in mice carrying APC mutations results in a synergistic increase in the severity of gastrointestinal tumorigenesis over that observed for either of the single-gene models (30). Previously, we bred Msh2- and Msh6-null mice to athymic ‘nude’ mice to determine the diversification of the tumor spectrum in the absence of a thymus and found that hematological malignancies persisted, shifting from T-cell lymphoma to B-cell lymphoma (31).

In the current study, Msh6-null mice were bred to p53-null mice in order to characterize the resultant effects on tumorigenesis and genetic instability. Although Msh2-null mice have previously been crossed with p53-null mice (25–27), it cannot be extrapolated that the crossing of Msh6-null and p53-null mice will result in the same phenotype. Msh6-null mice do have residual MMR activity and MSH6-associated HNPCC is significantly different than MSH2-associated HNPCC. We hypothesized that the Msh6-/-;p53-/- mice would display a more aggressive tumor etiology than their Msh6-, p53-/-, or p53-/- counterparts, thus indicating that concomitant loss of these two tumor suppressors contributes to tumorigenesis via mechanisms that are only partially interrelated. The observed shift in tumor spectrum away from thymic lymphoma (T cell), as developed in the Msh6-null mice, to the predominantly non-thymic early T cell lymphoma developed in the Msh6-/-;p53-/- mouse model indicates an underlying change in tumor progression towards a less differentiated cellular state. Moreover, we found the addition of p53 heterozygosity to the Msh6-null mouse model resulted in accelerated tumorigenesis that was related to increased genetic instability. These data support roles for MMR in the maintenance of genetic stability and in avoidance of tumorigenesis that are both p53 independent and p53 interrelated.

Materials and methods

Mice

The mouse experimental protocol was approved by the Health Science Animal Policy and Welfare Committee of the University of Alberta, and all animals cared for according to the guidelines of the Canadian Council on Animal Care. Mice on a C57Bl/6J background were a gift from Drs Edelmann and Kucherlapati (32), and p53 mice (33) were purchased from The Jackson Laboratory Bar Harbor, Maine (B6,129S-Tp53tm1RzJ). Msh6-/-;p53-/- mice, resulting from a Msh6-/- × p53-/- cross, were back-crossed to Msh6+/+ mice for six generations to obtain the Msh6-/-;p53+/+ mice used in this study. Mice were genotyped using ear notch biopsies according Edelmann et al. (32) and The Jackson Laboratory.

Tumor analysis

Mice displaying signs of poor health or a discernable tumor mass were euthanized by isoflurane overdose and subjected to necropsy. Samples of any observed tumor mass, as well as samples of thymus, kidney, liver and spleen were archived both in 10% buffered formalin for immunohistohistochemistry and at −80°C for DNA isolation. Brain and ear tissue also were archived at −80°C to provide a normal DNA sample and genotype confirmation. Mice that succumbed to poor health prior to euthanasia were inspected visually, but useful samples generally could not preserved; genotype was confirmed using ear tissue. Time of death and gross pathology were recorded for each mouse.

Histologic analyses were performed on 5 μm sections stained with haematoxylin and eosin using standard methods. Tumors were classified by the Pathology and Laboratory Medicine at the Cross Cancer Institute (Edmonton, Alberta, Canada) using standard diagnostic protocols and were interpreted by a hematopathologist (R.L.). Pax5 and CD3 were used to differentiate between B- and T-cell lymphomas, respectively.

Genetic instability assays

DNA was isolated from normal (brain or ear), tumor and organ samples using Qiagen DNA extraction columns (Qiagen, Valencia, CA); quantified; and diluted to 35 ng/μl.

Loss of heterozygosity (LOH) at the remaining p53 allele was determined using the p53 genotyping conditions outlined by The Jackson Laboratories. If the polymerase chain reaction (PCR) product demonstrated a maintenance of the wild-type allele, the DNA was subjected to small-pool PCR analysis. DNA was diluted and multiple reactions independently assembled (typically sets of eight, repeated at least once) and amplified using an increased number of cycles. DNA was deemed sufficiently diluted when ~50% of reactions failed to amplify (up to 1000-fold dilution). Using small-pool PCR. positivity for LOH was indicated by a loss of the wild-type band in a subset of the reactions, indicating the existence of a sub-population of cells with LOH at p53.

Instability at highly repetitive DNA sequence (MSI) was tested in the archived tumors and compared with matched normal DNA. Mononucleotide [J1043 (32), U12235 (31)] and dinucleotide [D1mit38, D7mit17 (34)] microsatellites were amplified using labeled and pigtailed primers. The four repeats were multiplexed using the QIAGEN Multiplex PCR Kit and analyzed on the ABI 3130x Genetic Analyser (Applied Biosystems Foster City, CA). Out of a set of 10 microsatellites representing mononucleotide to tetranucleotide repeats, we previously found these four microsatellites informative (MSI positive) for the analysis of MMR-deficient murine tumors (31).

Chk, pTEN, Bax, IgfII and Riz are important proteins for tumor suppression and growth regulation, and mutations within these genes are highly associated with HNPCC tumorigenesis (2). In addition to microsatellites in non-coding regions, six microsatellites from the coding regions of these five genes were analyzed for instability in tumors displaying other markers of instability. DNA was amplified (supplementary Table is available at Carcinogenesis Online) using standard conditions, purified (QIAquick Gel Extraction Kit, QIAGEN) and sequenced using the forward primer and the Big Dye Terminator cycle sequencing kit. Sequencing reactions were analyzed on 3130xl or 3100-Avant Applied Biosystems genetic analyzers and resulting sequence data were aligned with consensus sequence (GeneTool 2.0, BioTools) to identify contractions, expansions and point mutations.

Statistical analyses

Survival curves and statistical data analyses were performed using GraphPad Prism. Time-to-tumor (survival) data for the Msh6-/-, p53-/- and p53-/- mice were provided by Dr Edelmann (32) and Drs Olive and Jacks (35).

Results

Msh6-/-;p53+/+ mouse tumors

The Msh6-/- mouse model used in these experiments was originally described as developing gastrointestinal tumors (5 of 13 reported), as
| ID  | Age (months) | Gross pathology—visually affected organs | Histopathology | Diagnosis                  |
|-----|--------------|-----------------------------------------|----------------|---------------------------|
|     |              | Thymus | Liver | Kidney | Spleen | Adipose | Other | Thymus | Liver | Kidney | Spleen | Adipose | Other |
| 1   | 3.4          | X     | X     |        |        |         |       | +      | -     |        |        |         |       |       |
| 2   | 4.1          | X     |        |        |        |         |       | -      | +     | +      |        |         |       |       |
| 3   | 4.2          | X     |        |        |        |         |       | +      | -     | +      |        |         |       |       |
| 4   | 4.3          | X     | X     |        |        |         |       | -      | +     |        | -      |         |       |       |
| 5   | 4.9          | X     |        |        |        |         |       | -      | +     | +      |        |         |       |       |
| 6   | 4.9          | X     |        |        |        |         |       | -      | +     |        |        |         |       |       |
| 7   | 5.5          | X     | X     |        |        |         |       | +      | +     | +      |        |         |       |       |
| 8   | 5.7          |       | Paralysis |        |        |         |       | -      | -     | -      |        |         |       |       |
| 9   | 6.8          | X     | X     |        |        |         |       |        | +     | +      | +      |         |       |       |
| 10  | 6.9          | X     | X     |        |        |         |       | +      | +     |        |        |         |       |       |
| 11  | 7.0          | †     | X     | X     |        |         |       | †      | +     | -      | +      |         |       |       |
| 12  | 7.3          | X     |        |        |        |         |       |        | +     |        |        |         |       |       |
| 13  | 7.6          | X     | X     |        |        |         |       | -      | +     | -      |        |         |       |       |
| 14  | 8.0          | X     | X     |        |        |         |       | +      |        |        |        |         |       |       |
| 15  | 8.5          | X     | X     |        |        |         |       | +      | -     | -      | -      | +      |        |       |
| 16  | 8.7          | X     |        |        |        |         |       | -      | +     | +      |        |         |       |       |
| 17  | 8.9          | X     | X     |        |        |         |       | +      | +     |        |        |         |       |       |
| 18  | 9.5          | X     | X     |        |        |         |       |        | -     | +      |        |         |       |       |
| 19  | 9.9          | X     | X     |        |        |         |       | +      | +     | +      | +      |        |       |       |
| 20  | 10.6         | X     | X     |        |        |         |       | -      | +     | -      |        |         |       |       |
| 21  | 10.8         | X     |        |        |        |         |       | -      |        | +      | -      |        |       |       |
| 22  | 13.1         | Neck mass |        |        |        |         |       | -      | +     | -      | +      |         |       |       |
| 23  | 14.4         | X     | X     |        |        |         |       | -      | +     | -      |        |         |       |       |
| 24  | 1.2          | X     |        |        |        |         |       |        |        |        |        |         |       |       |
| 25  | 3.2          | X     |        |        |        |         |       |        |        |        |        |         |       |       |
| 26  | 3.5          | X     |        |        |        |         |       |        |        |        |        |         |       |       |
| 27  | 3.8          | X     |        |        |        |         |       |        |        |        |        |         |       |       |
| 28  | 3.8          | X     | X     |        |        |         |       |        |        |        |        |         |       |       |
| 29  | 4.2          | X     |        |        |        |         |       |        |        |        |        |         |       |       |
| 30  | 6.0          | X     | X     |        |        |         |       |        |        |        |        |         |       |       |
| 31  | 6.1          | X     | X     |        |        |         |       |        |        |        |        |         |       |       |
| 32  | 6.8          | X     |        |        |        |         |       |        |        |        |        |         |       |       |

Histopathology was not available for mice 24–32. These mice were diagnosed by gross pathology and in the context of those mice for which histological examination was possible (see text for explanation). Immunophenotyping data were not available for mouse 8. X, sample appeared abnormal visually; +, sample contained malignant cells; -, sample examined but did not contain malignant cells; blank, not notable visually or not investigated histologically. Samples listed under other were either visually abnormal or infiltrated with malignant cells. †, small thymic lymphoma upon visual inspection, not successfully embedded.

Mass of unknown origin filling chest cavity.

Tumor displayed both early T-cell and T-cell lymphoma.

Hard abdominal mass (2–3 cm) of unknown origin.
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well as B-cell lymphomas and T-cell lymphomas (five and three, respectively, of the eight tumors immunophenotyped) (32). We have housed this model in our animal facilities for 5 years and the Msh6 knockout mice have developed thymic lymphomas exclusively. The p53 model used in our studies was generated by Jacks et al. (33). The heterozygous mice (p53+/−) predominantly develop sarcomas and lymphomas (57 and 25%, respectively), while the homozygous-null mice (p53−/−) develop the same spectrum of tumors in reversed proportions (71% primarily T-cell thymic lymphomas and 25% sarcomas (33)).

During the collection of gross and histopathology for the Msh6−/−:p53−/− mice, two distinct phenotypes emerged (Table I). The mice belonging to the first phenotype comprised 25% of the sample population and shared the characteristics of rapid health decline (under 4.5 months), development of a visually apparent thymic lymphoma and a normal presentation of the liver and spleen (mice 1, 3, 24–29; Table I). For mice 1 and 3, the thymic lymphoma was confirmed as T-cell in origin. Many of these mice exhibited such rapid health decline that they were asymptotic prior to death, resulting in unusable samples for histopathology and DNA analysis (mice 24–32). On the basis of a visually apparent thymic lymphoma without involvement of either the spleen or liver, mouse 12 also belongs to this group, bringing the proportion of mice exhibiting this phenotype to 28%.

The remainder of the Msh6−/−:p53−/− mice did not display affected thymi, but did display enlarged spleens and/or enlarged livers; when available for histopathology, malignant infiltration was confirmed; of these mice, 53% developed lymphoma that was either B cell (mice 2, 16, 20) or T-cell (mice 4–7, 9–11, 13, 15, 17, 19, 21, 23) in origin and these mice comprised the second distinct phenotype. (The origin of lymphoma for mouse 8 was not determined.) The histology of this second group ranged from less aggressive, small lymphoid cells (low grade) to a single case of aggressive lymphoma. Six mice did not group with either of these phenotypes. Two mice (6%) developed sarcoma (mice 14, 18) and one mouse (3%) developed both lymphoma and sarcoma (mouse 22). Three mice (9%, mice 30–32) did not display a thymic lymphoma and histological diagnosis was not possible. However, these three mice did demonstrate early mortality and gross pathology consistent with malignancy.

Immunohistochemistry was performed with antibodies specific for Pax5 and CD3 to identify B- and T-cell lymphomas, respectively, and was not possible. However, these three mice did demonstrate early mortality and gross pathology consistent with malignancy.

Msh6−/−:p53−/− mice exhibit accelerated spontaneous tumorigenesis

We hypothesized that Msh6−/−:p53−/− mice would display spontaneous tumor onset earlier than their Msh6−/− or p53−/− counterparts, thus indicating that concomitant loss of these two tumor suppressors contributes to tumorigenesis via mechanisms that are only partially interrelated. Analysis of survival data revealed that the Msh6−/−:p53−/− mice succumbed to malignant disease at a significantly increased rate than the Msh6−/− mice or the p53−/− mice (P < 0.001), but not as rapidly as the p53−/− mice (P < 0.001; Figure 1a); median survival times for the Msh6−/−:p53−/−, Msh6−/−, p53−/− and p53−/− mice were 6.8, 22.9, 16.1 and 4.3 months, respectively. Comparing the survival times of the observed phenotypes (described in previous section of Results), it was confirmed that the Msh6−/−:p53−/− mice displaying only an affected thymus died significantly earlier than those mice with liver and/or spleen involvement and diagnoses of lymphoma (P < 0.0001) (Figure 1b).

Msh6−/−:p53−/− double knockout mice

Collection of tumorigenesis data from Msh6−/−:p53−/− mice was hampered on many fronts. First, the Msh6−/−:p53−/− females were poor mothers; problems with cannibalism and abandonment of the newborn pups was frequent, requiring the use of surrogate mothers. Also, the Msh6−/−:p53−/− pups were under-represented in successful litters with only three mice reaching the age of 6 weeks (two female, one male), the age at which litters were genotyped. However, in the course of generating mouse embryonic fibroblasts it was observed that Msh6−/−:p53−/− embryos were readily available at day 13 of gestation, suggesting that embryo failure occurs in the last half of gestation or that the pups were cannibalized at birth.

The tumor spectrum of the three Msh6−/−:p53−/− mice did not appear different from that of the Msh6−/−:p53−/− mice (Table II). Survival times and curves were not compared due to the small sample size.

Genetic instability assays

Forty-three malignant tissue samples from 17 Msh6−/−:p53−/− mice were analyzed for LOH at p53 and MSI at four microsatellites: JH103, U12235, D7mit17 and D7mit18. Out of an initial set of 10 microsatellites, including five mononucleotide repeats, we previously identified these four microsatellites as highly informative (MSI positive) for the analysis of MMR-deficient murine tumors (31). In all samples with detectable MSI (Table III), the mononucleotide repeat U12235 demonstrated instability, and in samples denoted as MSI (2) the second positive microsatellite was the dinucleotide repeat D7mit17. Mice that did not demonstrate MSI at these markers were categorized as.

![Fig. 1. Kaplan–Meier survival curve analysis. Top, The survival of the Msh6−/−:p53−/− mice (n = 32) was compared with historical p53−/− (n = 32), p53−/− (n = 41) and Msh6−/− (n = 32) mice (32,35) and found to be significantly different by log-rank test analysis (P < 0.0001). Bottom, The Msh6−/−:p53−/− mice are subdivided by disease phenotypes as described in the Results section.](https://academic.oup.com/carcin/article-abstract/28/10/2131/2476404)
Table II. Summation of visual and histological examination of Msh6−/−:p53+/− mice

| ID   | Age (months) | Gross pathology—visually affected organs | Histopathology—infiltrated organs | Diagnosis                      |
|------|--------------|------------------------------------------|----------------------------------|--------------------------------|
| 35   | 23           | X                                        | + + + -                          | 1. Sarcoma, poorly differentiated |
| 34   | 24.5         |ypical lymph node                         | + - - +                          | 2. T-cell lymphoma, early       |

X, sample appeared abnormal visually;+, sample contained malignant cells;−, sample examined but did not contain malignant cells; blank, not notable (visual) or not investigated (histology). Samples listed under other were either visually abnormal or infiltrated with malignant cells.

Table III. Genetic instability of Msh6−/−:p53+/− tumors

| ID   | Thymus | Liver | Kidney | Spleen | Other | Age (months) |
|------|--------|-------|--------|--------|-------|--------------|
| 1    | MSI (1) | LOH   | —      | —      | —     | 3.4          |
| 2    | MSI (2) | LOH   | MSS    | LOH    | —     | 4.2          |
| 3    | MSI (1) | LOH   | MSS    | LOH    | —     | 4.3          |
| 4    | MSI (2) | LOH   | MSS    | LOH    | —     | 4.9          |
| 5    | MSI (1) | LOH   | MSS    | LOH    | —     | 4.9          |
| 6    | MSI (2) | LOH   | MSS    | LOH    | —     | 5.5          |
| 7    | MSI (1) | LOH   | MSS    | LOH    | —     | 6.9          |
| 8    | MSI (1) | LOH   | MSS    | LOH    | —     | 7.0          |
| 9    | MSI (1) | LOH   | MSS    | LOH    | —     | 7.6          |
| 10   | MSI (1) | LOH   | MSS    | LOH    | —     | 8.0          |
| 11   | MSI (1) | LOH   | MSS    | LOH    | —     | 8.7          |
| 12   | MSI (1) | LOH   | MSS    | LOH    | —     | 8.9          |
| 13   | MSI (1) | LOH   | MSS    | LOH    | —     | 9.9          |
| 14   | MSI (1) | LOH   | MSS    | LOH    | —     | 10.6         |
| 15   | MSI (1) | LOH   | MSS    | LOH    | —     | 10.8         |
| 16   | MSI (1) | LOH   | MSS    | LOH    | —     | 13.1         |
| 17   | MSI (1) | LOH   | MSS    | LOH    | —     | 14.4         |

MSS denotes microsatellite stable at all four markers; MSI denotes microsatellite unstable at the indicated number of markers; LOH denotes loss of heterozygosity at the p53 locus; HET denotes loss of the wildtype p53 allele.

Discussion

Recent studies have demonstrated that MSH6-associated tumors often fall outside of current diagnostic criteria and, consequently, their prevalence may be greatly underestimated; 60–100% of MSH6-associated HNPCC cases are excluded based solely on Amsterdam criteria II (5–8). Compounding factors include an average age of onset for MSH6 mutation carriers that is 10 years later than for typical HNPCC patients (8,36), reduced gene penetrance (5,8,36,37) and a greater prevalence of non-HNPCC tumors (7). Moreover, MSH6 has only a 15% sensitivity for the detection of MSH6 mutation carriers (7,8); MSH6-associated HNPCC tumors typically display a lower level of MSI that is predominantly detected in mononucleotide repeats.

As outlined in the Introduction, recent data infer that both the contribution of MSH6 mutation and the association between MMR deficiency and p53 mutation are underestimated in human tumorigenesis. Experiments using tumor-derived cell lines and mouse models have shown associations between MMR and p53, and place MMR both upstream and downstream of p53 in tumor-suppressing pathways (16–27,38–40). Heterozygosity for p53 has been shown to cooperate with loss of Msh2, resulting in earlier onset of tumorigenesis.
altered MSI profiles in mouse models (25–27). However, these Msh2-null mice have complete MMR inactivation. We questioned the consequences of p53 heterozygosity in the context of residual MMR activity present in the Msh6-null mice. This manuscript marks the first in vivo investigation into the association between Msh6 and p53 and their combined roles in the suppression of spontaneous tumorigenesis, survival and genomic stability. We hypothesized that Msh6-/-;p53/-/- mice would display earlier tumor onset and altered tumor spectrum compared with their Msh6-/- or p53-/-/- counterparts, thus indicating that concomitant loss of these two tumor suppressors contributes to tumorigenesis via mechanisms that are only partially interrelated.

An expanded understanding of the key roles Msh6 plays in protecting against tumorigenesis, and the ability of Msh6 to compensate for Msh2 mutation, can be gained from the comparison of the altered phenotype of the Msh6-/-;p53-/-/- mice described here, to the phenotype of previously published Msh2-/-;p53-/-/- mouse models. Cranston et al. (26,27) and Toft et al. (25) independently generated Msh2/p53 mouse models; however, these studies were not focused on detailed tumor spectrum and only the latter included Msh2-/-;p53-/-/- mice. Toft et al. (25) observed comparable tumor spectrums for the Msh2-/-;p53-/-/- and the Msh2-/-/- mice, with 95% of the mice developing lymphoma (immunophenotyping not reported). In contrast to our studies with Msh6, they concluded that heterozygosity for p53 did not alter the Msh2-null tumor spectrum. The immunophenotyping and genetic instability profiling described in this manuscript both confirms and extends the state of understanding of the roles of p53 and MMR in spontaneous tumorigenesis.

Unlike their Msh6-/- progenitors, the Msh6-/-;p53-/-/- mice predominantly developed early T-cell lymphomas that did not involve the thymus; extensive back-crossing ensures that the altered phenotype of the Msh6-/-;p53-/-/- mouse is attributable to modulation of the p53 status. We have maintained the Msh6 mouse model in our animal facilities for over 5 years, and the Msh6-/-/- mice develop thymic lymphomas exclusively. The p53 model used in our studies developed both sarcomas and lymphomas; p53-/-/- mice predominantly develop sarcomas and lymphomas (57% and 25%, respectively), while p53-/-/- mice develop the same spectrum of tumors in reversed proportions (71% primarily T-cell thymic lymphomas and 25% sarcomas (33)). Therefore, the predominant tumor spectrum of the Msh6-/-;p53-/-/- mice described here is different from those of the Msh6-/-/-;p53-/-/- and p53-/-/- mouse models. The observation that the tumor spectrum of the Msh6-/-;p53-/-/- mouse model is different from that of the p53-/-/- model is particularly notable given the high levels of LOH at p53 observed in the Msh6-/-;p53-/-/- derived tumors (Table III, discussed below).

In addition to an altered tumor spectrum, we found that the rate of tumorigenesis in the Msh6-/-;p53-/-/- mice was increased significantly over that of either the Msh6-/-/- or p53-/-/- mice (median age of onsets of 6.4, 9.9 and 16.1 months, respectively), but did not reach that observed in the p53-/-/- mice (4.3 months; ). The pattern of our results is different than those observed for similar experiments using Msh2-/-/- mice; Toft et al. (25) found that the rate of tumorigenesis for the Msh2-/-/-;p53-/-/- mice was more rapid than those of either the Msh2-/-/- or p53-/-/- mice. This early onset of tumorigenesis is consistent with current consensus that loss of Msh2 is more critical in the development of malignancy than loss of Msh6. The Msh2-/-/-;p53-/-/- mice are deficient in both the Msh2/Msh6 and Msh2/Msh3 heterodimers, whereas the Msh6-/-;-p53-/-/- mice retain the Msh2/Msh3 heterodimer and residual MMR activity, probably accounting for differing survival times.

Analysis of human HNPCC samples has revealed that MSI in MSH6-deficient tumors is restricted predominantly to mononucleotide repeats and is often not detectable using standard panels of MSI loci. The Msh6-/-/- mice used to generate the Msh6-/-;-p53-/-/- mouse model develop thymic lymphomas that are MSS (M.R.Campbell and S.E.Andrew, unpublished data; (32)]. However, we found that in a context of p53 heterozygosity, 70% of the Msh6-/-;p53-/-/- mice developed at least one tumor with demonstrable MSI. This is in concordance with Toft et al. (25) which reported a similar MSI amplification in Msh2-/-;p53-/-/- derived tumors. It should be noted that there is no evidence for p53 deficiency promoting MSI in a MMR proficient context. Taken together, these data suggest an interrelation between p53 heterozygosity, increased MSI and a shift in the spontaneous tumor spectrum.

We analyzed the p53 locus by PCR to determine if loss of the wild-type copy of p53 was increased in a MMR-deficient context. We found that 82% of the Msh6-/-;p53-/-/- mice contained at least one tumor that exhibited LOH at this locus (Table III), proportions similar to the 75% LOH exhibited by the p53-/-/- mice (33). In comparison, Toft et al. (25) did not detect altered p53 status or function in a significant proportion of Msh2-/-;p53-/-/- derived tumors. It is unclear why LOH at p53 was not observed in the Msh2-/-;p53-/-/- mouse model. These data suggest that addition of p53 heterozygosity to an MMR-null background is sufficient to increase the propensity for MSI and that, in this genetically unstable context, LOH of the wild-type p53 can be expected.
MSI and CIN are products of genetic instability that historically have been considered mutually exclusive in colorectal cancer; dogma has been that sporadic colorectal cancers are MSS/CIN+, while MMR-associated colorectal cancers are MSI/CIN−. However, overlap between MSI and CIN recently has been demonstrated in sporadic colorectal cancer (41). Additionally, our laboratory has demonstrated a role for Msh2 in the maintenance of CIN; Msh2−/− mouse embryonic fibroblasts display increased chromosome aneuploidy, centromere amplification and defects in telomere capping (42). We found a strong, significant, inverse correlation between survival time and genetic instability (MSI and LOH combined, Figure 2C). Moreover, we found a significant association (P = 0.0051, Figure 2B) between the acquisition of MSI and LOH at the p53 loci. These findings underline the impact of concomitant MMR and p53 dysfunction to the tumorigenic pathway. This association is supported by the observation that defects in MMR and p53 are both early events in uterine carcinosarcoma (43) and, together, are sufficient to promote spontaneous transformation of murine epithelial cells (44). Moreover, association of MMR with p53, both upstream and downstream, is supported by several studies. For example, (i) transcription of MSH2 has a p53-dependent component (22–24); (ii) activation of p53 is MMR-dependent following some forms of DNA damage (16–27,38–40); (iii) MMR deficiency may promote p53 mutation (38–40) and (iv) decreased levels of wild-type p53 amplify MSI [25 and this study].

Finally, in this study, there are several instances of multiple tumors occurring. Current data suggest that both the contribution of Msh6 and p53 in tumorigenesis is underesti-
mated following some forms of DNA damage (16–27,38–40); (iii) MMR deficiency may promote p53 mutation (38–40) and (iv) decreased levels of wild-type p53 amplify MSI [25 and this study].

In summary, we report the first in vivo analysis of a Msh6−/−; p53−/− mouse model and show that tumorigenesis was advanced and tumor spectrum altered by the addition of heterozygosity at p53 to a Msh6-null background. The compounding effects of these genetic losses resulted in amplification of MSI and, in this genetically unstable context, LOH of the wild-type p53 occurred. Current data suggest that both the contribution of MSH6 mutations to HNPC and the association between MMR deficiency and p53 mutation is underestimated (see Introduction). Our results support the hypothesis that p53 and Msh6 are functionally interrelated and that, with concomitant mutation, these tumor suppressors act together to accelerate tumorigenesis.

Supplementary material
Supplementary Table can be found at http://carcin.oxfordjournals.org/

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