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Induction of aberrant crypt foci in DNA mismatch repair-deficient mice by the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP)

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Abstract

Disruption of the DNA mismatch repair (MMR) pathway results in elevated mutation rates, inappropriate survival of cells bearing DNA damage, and increased cancer risk. Relatively little is known about the impact of environmentally relevant carcinogens on cancer risk in individuals with MMR-deficiency. We evaluated the effect of MMR status (*Mlh1*^{+/+} versus *Mlh1*^{-/-}) on the carcinogenic potential of the cooked-meat mutagen, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) in mice. PhIP exposure did not obviously increase lymphoma or small intestinal tumorigenesis in either *Mlh1*-deficient or -proficient mice. In contrast, the frequency of aberrant crypt foci (ACF), a preneoplastic biomarker for colon tumorigenesis, was increased by PhIP, and the increase due to PhIP was significantly greater in *Mlh1*^{-/-} versus wild-type littermates. This apparent heightened susceptibility to induction of ACF parallels the previously reported hypermutability of *Mlh1*-deficient mice to PhIP and is consistent with the hypothesis that MMR-deficiency would increase the likelihood of PhIP-induced carcinogenic mutations. Further evaluation of the risk that consumption of heterocyclic amines may impart to MMR-deficient individuals therefore is warranted.

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1. Introduction

DNA mismatch repair (MMR) contributes to genomic stability through the correction of replication errors (thereby suppressing spontaneous mutation), and the activation of cell cycle arrest and apoptosis in

response to DNA damage (thereby preventing the survival of damaged cells and reducing induced mutation) [1,2]. In MMR, polymerase errors are identified by the heterodimeric MSH proteins (*MSH2* partnered with either *MSH6* or *MSH3*), and excision and resynthesis of the mistake-containing nascent strand is dependent on heterodimeric MLH proteins (*MLH1* partnered with either *PMS2* or *MLH3*). Heterozygous germline mutations in MMR genes (primarily *MSH2* and *MLH1*) underlie Lynch syndrome, an inherited predisposition to early onset

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colorectal and other internal cancers [3], whereas homozygous deficiency can result in severe hematological cancer [4,5]. Inactivation of MMR via epigenetic mechanisms (predominately hypermethylation of the *MLH1* promoter) is associated with up to 15% of sporadic colorectal, and other cancers [6]. Mice with homozygous mutations in MMR genes are cancer-prone [7,8], indicating the tumor suppressive functions of MMR proteins generally to be conserved in mammals. While the increased spontaneous mutation in MMR-deficient cells likely contributes significantly to increased cancer risk, the importance of the loss of MMR-dependent activation of cell cycle arrest and apoptosis in response to DNA damage is less understood.

Few studies have examined the impact of environmentally relevant carcinogens on cancer risk in individuals with MMR-deficiency. 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a mutagenic and carcinogenic heterocyclic amine (HCA) formed during the cooking of meat and fish [9,10]. Epidemiological studies suggest that colon cancer risk is increased for persons who consume high levels of HCAs [11,12], and PhIP has been classified by the National Toxicology Program as ‘reasonably anticipated to be a human carcinogen’ [13]. MMR may respond to PhIP-induced DNA damage, as cancer cell lines lacking MMR demonstrate reduced cytotoxicity, and increased frequency of induced mutation in response to PhIP [14–16]. Consistent with *in vitro* studies, we demonstrated that *Mlh1*-deficient mice were hypermutable by PhIP [17], exhibiting increased frequencies of induced-mutation (relative to repair-proficient littermates) in small intestinal and colon tissues. Analysis of mutational spectra revealed that G/C–T/A transversions, the ‘signature PhIP mutation’, were induced to similar levels regardless of *Mlh1* status. In contrast, *Mlh1*^{-/-} mice exhibited hypermutability to frameshifts, G/C–A/T transitions, and G/C–C/G transversions. Similar hypermutability was reported in *Msh2*-deficient mouse colon [18], indicating that MMR influences both the level and types of mutation induced by PhIP. MMR-deficiency therefore may increase the likelihood of PhIP-induced carcinogenic mutations, and exposure to PhIP may increase cancer risk for MMR-deficient individuals.

To determine whether deficiency for the *Mlh1* gene would affect the carcinogenic response to PhIP, we treated weanling *Mlh1*^{+/+} and *Mlh1*^{-/-} littermates with eight injections of 50 mg/kg PhIP or vehicle and subsequently monitored for the development of cancer. Although there was no obvious effect on hematological

cancer or small intestinal tumorigenesis, PhIP-exposure did induce ACF in both *Mlh1*-deficient and wild-type littermates. The induced frequency of ACF in *Mlh1*^{-/-} mice was fourfold greater than in *Mlh1*^{+/+} mice, similar to the increased frequency of induced mutation in *Mlh1*-deficient mouse colon reported previously [17], suggesting loss of MMR increases the likelihood PhIP-induced ACF. Although premature death due to lymphoma limited the analysis of colon tumors, a heightened susceptibility to induction of ACF in *Mlh1*^{-/-} mice is consistent with the hypothesis that MMR-deficiency increases risk of carcinogenesis in colon due to PhIP.

2. Methods

2.1. Animals and treatment

All procedures were approved by the Oregon State University IACUC. Heterozygous *Mlh1*-deficient C57Bl/6 mice were bred to obtain wild-type and *Mlh1*^{-/-} littermates [17], genotyped as described [19], and entered into the study as available over a period of approximately 5 months. Mice were housed in a positive-ventilation caging unit with automatic water (Thoren Caging Systems, Inc., Hazleton, PA), and fed a diet of laboratory chow (Picolab Rodent Diet 20, PMI Nutrition International, St Louis, MO). *Mlh1*^{+/+} and *Mlh1*^{-/-} littermates received eight intraperitoneal (i.p.) injections every other day of either 50 mg/kg PhIP (Toronto Research Chemicals, Ontario, Canada) or 10 ml/kg vehicle solution (0.9% sterile saline, pH 4.0) starting 2–3 days post-weaning as described [17]. Treatment groups were comprised of nearly equal numbers of male and female mice. PhIP-exposure by this protocol was not overtly toxic in either wild-type or *Mlh1*^{-/-} mice, as assessed by average weight gain in the cohort during the course of treatment (data not shown).

2.2. Tissue collection

Mice were monitored every 2–3 days and killed by carbon-dioxide inhalation followed by cervical dislocation when moribund (exhibiting signs of severe hunched posture, weakness and/or labored breathing), or at the end of the study following the death of the last *Mlh1*^{-/-} mouse. Upon necropsy, the intestinal tract was flushed with 4 °C sterile PBS and cut lengthwise. The colon was pinned flat onto Whatman 3 filter paper, and both colon and small intestine were fixed with 10% neutral buffered formalin. Mice found dead (three *Mlh1*^{+/+} and one *Mlh1*^{-/-} treated with PhIP, and one *Mlh1*^{+/+} treated with vehicle) were examined for lymphoma, but autolysis prohibited intestinal tumor analysis.

2.3. Pathogenic analysis

Adenomas identified in intestinal tissues using a Nikon SMZ-U Zoom 1:10 stereomicroscope were excised, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin. Adenomas were defined histologically as a focal area of disorganized epithelial tissue exhibiting increased mitotic activity and cellular atypia. An epithelial neoplasm was considered an adenocarcinoma only if it invaded the muscularis mucosae. Of 93 total adenomas identified by visual inspection, 65 were available for analysis, and 59 were confirmed histologically. To visualize ACF, formalin-fixed colons were stained with 0.2% methylene blue, and examined using the 6 \times and 10 \times objectives of a light microscope. ACF were identified by their thickened epithelia and enlarged luminal openings (two times larger than surrounding crypts), and by their elevation above adjacent normal crypts. The experimenter was blind to treatment for scoring intestinal adenomas and ACF.

2.4. Statistical analysis

Kaplan–Meier (non-parametric) survival curves were compared within genotypes with the log rank test, using Prism for Macintosh, version 4.0 (GraphPad Software, San Diego, CA). ACF incidences were compared with the Fisher's exact test using Prism. ACF counts were modeled without litter and gender as factors, after initial analysis within each treatment group revealed no evidence of those effects. ACF counts were compared between treatments and genotypes using a two-factor generalized linear model with identity link and with an over-dispersed Poisson distribution for the conditional response. The identity link was used to allow comparisons as differences on the original scale. For example, of primary interest is whether the effect of PhIP (as a difference on the original scale) differs between genotypes (which could be assessed as an interaction between treatment and genotype). The over-dispersed Poisson distribution captured the observed pattern of variation increasing with the mean, so that the model-based standard errors for each treatment reasonably matched the empirical standard errors from replicate animals. Analysis of ACF counts was done with SAS for Windows, version 9.1 (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Survival and hematopoietic tumorigenesis

Following exposure to PhIP, mice were monitored for signs of cancer over a period of 17 months. *Mlh1*^{-/-} mice were sacrificed when moribund, whereas wild-type mice generally were killed between the ages of 12 and 17 months, bracketing the age of the longest surviving

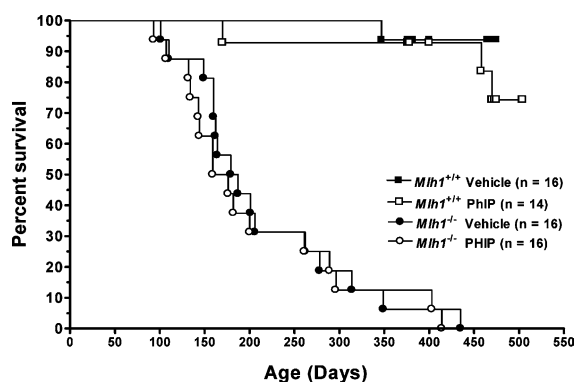


Fig. 1. Survival of *Mlh1*^{+/+} (squares) and *Mlh1*^{-/-} (circles) mice. Mice were treated with eight injections of 50 mg/kg PhIP (open symbols) or vehicle (filled symbols) and monitored for cancer development. *Mlh1*^{-/-} mice were euthanized when moribund, whereas wild-type mice generally were killed between the ages of 12 and 17 months, bracketing the age of the longest surviving *Mlh1*^{-/-} mouse. The median survival times for *Mlh1*^{-/-} mice treated with vehicle or PhIP were 183 days and 167 days, respectively. Number of mice included in the analysis is indicated parenthetically in the legend.

Mlh1^{-/-} mouse (Fig. 1). Survival, hematopoietic cancer development, and small and large intestinal tumorigenesis (see below) in vehicle-treated *Mlh1*^{-/-} mice generally were similar to previous reports of spontaneous tumorigenesis in *Mlh1*-deficient mice [20,21]. Morbidity in most *Mlh1*-deficient mice was associated with metastatic lymphoma (both T-cell and non-T-cell, data not shown) or leukemia affecting multiple organs (Table 1). There was, however, no significant difference in the median survival of *Mlh1*^{-/-} mice treated with PhIP (167 days) or vehicle (183 days) ($P=0.6$). Most wild-type mice in both treatment groups survived until sacrifice without obvious signs of disease (Fig. 1). There was no significant difference in survival (up to approximately 500 days, $P=0.3$), and the incidence of hematological cancer in PhIP- and vehicle-treated wild-type mice was similar (Table 1). Thus, PhIP exposure by the current protocol did not appear to increase risk of hematological cancer in wild-type or *Mlh1*-deficient mice.

3.2. Colonic tumorigenesis and ACF development

Several *Mlh1*^{-/-} mice older than 250 days developed colon adenomas (Table 2). Of five *Mlh1*^{-/-} mice in each treatment group older than 250 days at time of sacrifice, one tumor was found in a single mouse treated with vehicle, whereas three tumors were found in two mice exposed to PhIP (Table 2). Although the small number of surviving *Mlh1*^{-/-} mice of this age precluded a definitive analysis, these findings suggested

Table 1
Incidence of hematopoietic cancers in PhIP-treated *Mlh1*^{-/-} and *Mlh1*^{+/+} mice

Genotype	Treatment	Median survival (months)	Lymphoma ^a (%)	Leukemia ^b (%)	Total hematopoietic cancers (%)
<i>Mlh1</i> ^{-/-}	Vehicle	6.1	14/16 (88)	2/16 (13)	16/16 (100)
	PhIP	5.6	14/15 (93)	0/15 (0)	14/15 (93) ^{c,d}
<i>Mlh1</i> ^{+/+}	Vehicle	14.5	4/15 (27)	1/15 (7)	5/15 (33) ^d
	PhIP	14.6	4/14 (29)	1/14 (7)	5/14 (36) ^d

^a Lymphomas were identified in thoracic masses, in liver, spleen, and kidney in *Mlh1*^{-/-} mice, and in liver, spleen, and kidney in *Mlh1*^{+/+} mice, and included both T-cell and non-T cell types, as assessed by immunohistochemical staining for the cell surface marker CD3 (data not shown).

^b Leukemia types were myelogenous or mast cell, and were identified in liver and/or spleen.

^c One mouse free of hematopoietic cancer became moribund at 13.5 months due to histiocytic sarcoma present in liver, spleen, kidney, and uterus.

^d One additional mouse not available for histology.

a possible increase in colon tumor risk due to PhIP and supported the need to evaluate other potential markers of colonic tumorigenesis.

We therefore determined the effect of *Mlh1*-deficiency on PhIP-induction of aberrant crypt foci (ACF), a monoclonal, preneoplastic lesion thought to represent an early step in colorectal carcinogenesis [22,23]. In *Mlh1*^{-/-} mice, PhIP exposure increased the incidence of ACF from 31% (5/16) to 87% (13/15) ($P=0.003$) and the average frequency of ACF from 0.4 ± 0.2 to 2.7 ± 0.6 ($P < 0.0001$), indicating a PhIP-induced frequency of approximately 2.3 ACF per mouse (PhIP-treated frequency minus vehicle-treated frequency) (Fig. 2). In contrast, the PhIP-induced frequency of ACF in wild-type littermates was 0.6 per mouse ($0.7 \pm 0.3 - 0.1 \pm 0.07$; $P=0.04$), associated with a corresponding increase in incidence from 7% (1/15) to 45% (5/11) ($P=0.05$). The approximately fourfold greater PhIP-induced frequency of ACF in *Mlh1*^{-/-} mice versus *Mlh1*^{+/+} mice was significant (2.3 versus 0.6; $P < 0.01$), suggesting that MMR suppresses the induction of ACF by PhIP. Although not quite significant statistically, vehicle-treated *Mlh1*^{-/-} mice also had an observed higher incidence (31 versus 7%; $P=0.17$) and average frequency of ACF (0.4 ± 0.2 versus 0.1 ± 0.07 ; $P < 0.08$) than vehicle-treated

wild-type mice, suggesting a trend towards higher spontaneous risk of ACF similar to mice lacking *Msh2* [24].

3.3. Small intestinal tumorigenesis

Adenomas or adenocarcinomas of the small intestine were detected in 75% (12/16) of vehicle-treated *Mlh1*^{-/-} mice with an average of 2.7 ± 0.7 tumors per mouse (Table 2). PhIP-exposure did not increase the incidence (47%; 7/15) or average number of tumors (2.8 ± 1.4 per mouse). There also was no apparent difference in the incidence or number of carcinomas in PhIP- versus vehicle-treated *Mlh1*^{-/-} mice, suggesting that PhIP exposure did not increase the likelihood of progression of adenomas to carcinomas. Wild-type mice were free of small intestinal tumors except for two PhIP-treated wild-type mice that each developed a single adenoma.

4. Discussion

We report the first comparison of the carcinogenic response of MMR-deficient and -proficient mice to PhIP. Although there was no obvious effect on survival (generally reflective of the development of lethal lymphoma) or on small intestinal tumorigenesis, the

Table 2
Intestinal tumors in PhIP-treated *Mlh1*^{-/-} and *Mlh1*^{+/+} mice

Genotype	Treatment	Small intestinal adenoma incidence (%)	Small intestinal carcinoma incidence (%)	Number of tumors (adenomas plus carcinomas) per mouse (mean \pm SEM)	Colon adenoma incidence (%)
<i>Mlh1</i> ^{-/-}	Vehicle	12/16 (75)	4/16 (25) ^a	2.7 ± 0.7	1/16 (6) ^b
	PhIP	7/15 (47)	4/15 (27)	2.8 ± 1.4	2/15 (13) ^c
<i>Mlh1</i> ^{+/+}	Vehicle	0/15 (0)	0/15 (0)	None detected	0/15 (0)
	PhIP	2/11 (18)	0/11 (0)	0.1 ± 0.3	0/11 (0)

^a A single tumor from this group was adenoma mixed with carcinoma and is designated as 'carcinoma'.

^b This mouse was 8 months old when moribund and had one colon tumor.

^c These mice were both 15 months old when moribund; one mouse had one tumor, the other had two tumors.

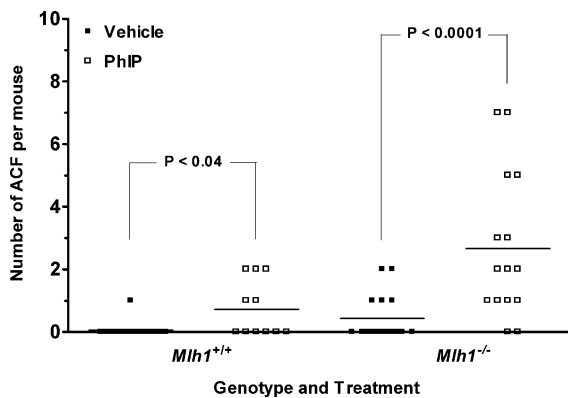


Fig. 2. Induction of ACF by PhIP in *Mlh1*^{-/-} and *Mlh1*^{+/+} mice. Number of ACF per mouse in vehicle (filled symbols) and PhIP-exposed mice is plotted. The bars indicate the mean. Standard error of the means (SEM) are reported in the text. Vehicle-treated *Mlh1*^{+/+} mice, *n*=15, PhIP-treated *Mlh1*^{+/+} mice, *n*=11, vehicle-treated *Mlh1*^{-/-} mice, *n*=16, PhIP-treated *Mlh1*^{-/-} mice, *n*=15.

induced frequency of ACF was significantly greater in *Mlh1*^{-/-} versus *Mlh1*^{+/+} mice, suggesting that loss of MMR increases the likelihood of PhIP-induced ACF. The increased induced frequency of ACF in *Mlh1*^{-/-} mice parallels the previously reported three-fold greater induction of mutation by PhIP in the colon of MMR-deficient (both *Mlh1*^{-/-} [17] and *Msh2*^{-/-} [18]) versus -proficient mice, suggesting that MMR-dependent suppression of PhIP-induced mutation helps prevent the development of ACF. One potential confounding factor is that wild-type mice were significantly older at the time of analysis than the *Mlh1*-deficient mice (Fig. 1). As carcinogen-induced ACF may regress after cessation of exposure [23], additional ACF induced by PhIP-treatment might have been missed. However, the incidence and frequency of ACF detected in the PhIP-exposed wild-type mice are similar to or greater than those previously reported in normal mice after 4 weeks of dietary exposure, or 10 weeks after i.p. exposure of PhIP [25,26], suggesting that earlier assessment would not have altered the current conclusions. Effects on PhIP-induced colon tumorigenesis in *Mlh1*^{-/-} mice could not be assessed directly due to a limited number of colon tumors detected in the animals surviving past 8 months. Analysis of colon tumors will require additional studies with larger groups of animals. However, the induction of ACF in *Mlh1*^{-/-} mice is consistent with the hypothesis that PhIP-exposure would increase risk of colon carcinogenesis in individuals with MMR-deficiency.

ACF exhibiting microsatellite instability have been identified in human patients with Lynch syndrome, suggesting that ACF may be a premalignant lesion of

MMR-deficient cancer [27]. However, the significance of ACF as biomarker for PhIP-induced cancer in mice has been questioned [25] as the mouse colon is not a strong target tissue for PhIP carcinogenesis [25]. In rats, a subset of PhIP-induced ACF do progress to adenoma [23]; these ACF appear dysplastic histologically, and are associated with cellular accumulation of β -catenin and specific mutations activating β -catenin (*Ctnnb1*) or inactivating the tumor suppressor *adenomatous polyposis coli* (*Apc*) [28,29]. The types of mutations and the genes targeted in PhIP-induced ACF in mice are likely to be similar to those in rats, as β -catenin and *Apc* mutant mice show increased spontaneous and PhIP-induced ACF [25,30,31]. The rapid induction of adenocarcinoma in mice following a single exposure of PhIP (200 mg/kg) and post-treatment with dextran sodium sulfate [32] indicates that PhIP-initiated colonic tumors can develop in mice given an appropriate tumor-promoting environment. The robust induction of ACF in *Mlh1*^{-/-} mice, possibly in combination with appropriate tumor promoters, represents a potentially useful model system for identification of the molecular events associated with the development and progression of PhIP-induced ACF and colon cancers.

The increase in PhIP-induced ACF in *Mlh1*^{-/-} mice was not matched by a corresponding increase in small intestinal tumors. This lack of significant increase in small intestinal tumorigenesis was not predicted by the similar induction of mutation by PhIP in both small intestine and colon in *Mlh1*^{-/-} mice [17]. However, the small intestine is a minor target of PhIP-induced tumorigenesis, for example, requiring long-term exposure in nucleotide-excision repair (NER)-deficient mice or long-term exposure in combination with a high-fat diet in repair-proficient mice [36,37]. In addition, most small intestinal tumors in mice are initiated very early in life [38], and significant induction of such tumors by PhIP in genetically susceptible *Apc* mutant mice requires neonatal exposure [39]. Similarly, induction of small intestinal tumorigenesis in *Mlh1*-deficient mice might require long-term, or neonatal exposure.

An increased risk of carcinogenesis due to PhIP also was not apparent in the lymph system (lymphoma), in contrast to the significant sensitivity of MMR-deficient mice to lymphoma induced by alkylating agents [33–35]. However, PhIP-exposure by the current protocol also did not significantly induce mutation in the thymus of *Mlh1*-deficient (or wild-type) mice [17], and lymphoma in wild-type or in NER-deficient mice apparently requires long-term exposure [36,37]. Thus,

PhIP is a weak lymphomagen even in the absence of important DNA repair pathways.

We have demonstrated an induction of ACF by PhIP in *Mlh1*^{-/-} mice that correlates with a previously described hypermutability in *Mlh1*-deficient mouse colon. These findings are consistent with the hypothesis that loss of MMR increases the likelihood of PhIP-induced carcinogenic mutations. As such, exposure to PhIP may be a significant concern for individuals at risk for MMR-deficiency. Such risk is not limited to those with an inherited deficiency, as MMR activity can be down-regulated by cellular environments such as chronic oxidative stress [40] or hypoxia [41]. Reduced exposure to PhIP is a modifiable lifestyle factor that may reduce intestinal carcinogenesis, particularly in susceptible populations. Additional studies of the risk that may be imparted to MMR-deficient individuals when they consume heterocyclic amines are warranted.

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