Dietary Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine Induced
Prostate Carcinogenesis in CYP1A-humanized Mice

Guangxun Li1*, Hong Wang1*, Anna B. Liu1, Connie Cheung1, Kenneth R. Reuhl2, 
Maarten C. Bosland3 and Chung S. Yang1†

1Department of Chemical Biology and 2Department of Pharmacology & Toxicology; Center 
for Cancer Prevention Research, Ernest Mario School of Pharmacy, Rutgers, The State 
University of New Jersey, Piscataway, NJ 08854. 
3Department of Pathology, College of Medicine, University of Illinois at Chicago, Chicago, 
IL 60612.

*These two authors contributed equally.
Running Title: PhIP-induced prostate carcinogenesis in hCYP1A-mice
Key words: prostate cancer, PhIP, hCYP1A and mouse model

Abbreviation: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; HCA, heterocyclic 
amines; CYP, cytochrome P450; PIN, prostatic intraepithelial neoplasia; HgPIN, high-grade 
PIN; LgPIN, low-grade PIN IHC, immunohistochemistry; DNMT, DNA methyltransferase; 
GSTP1, glutathione S-transferase P1; 8-oxo-dG, 8-oxo-deoxyguanosine; DLP, dorso-lateral 
prostate; DLG, dorso-lateral glands

†To whom correspondence and requests for reprints should be addressed:

Dr. Chung S. Yang 
Department of Chemical Biology 
Ernest Mario School of Pharmacy 
Rutgers, The State University of New Jersey 
164 Frelinghuysen Road 
Piscataway, NJ 08854-8020 
Tel: (732) 445-5360; Fax: (732) 445-0687
Email: csyang@rci.rutgers.edu
Grant Support

This work was supported by grants from the U.S. National Institutes of Health (NIH) (RO1 CA120915, RO1 CA120915-S2, RO1 CA122474 and RO1 CA133021) and the John L. Colaizzi Chair endowment as well as the Shared facilities funded by National Cancer Institute Cancer Center Support Grant (CA72720) and National Institute of Environmental Health Center Grant (ES05022). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked as advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Conflict of Interest Statement: The authors have declared no conflict of interest.
Abstract

To develop a relevant mouse model for prostate cancer prevention research, we administered a dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), to CYP1A-humanized mice. In comparison to mouse Cyp1a2, human CYP1A2 preferentially activates PhIP to a proximate carcinogen. Following a single oral dose of PhIP (200 mg/kg body weight), we observed inflammation, atrophy of acini, low-grade prostatic intraepithelial neoplasia (PIN) (after 20 weeks) and high-grade PIN (HgPIN) (after 30 to 50 weeks) in dorso-lateral, ventral and coagulating anterior prostate glands of these mice. These lesions were androgen receptor positive and featured the loss of expression of the basal cell marker p63 and the tumor suppressor PTEN. Similar to human prostate carcinogenesis, glutathione-S-transferase P1 (GSTP1) expression was lost or partially lost in HgPIN. E-Cadherin expression was also lost in HgPIN. The expression of DNA methyltransferase 1 was elevated, possibly to enhance promoter hypermethylation for the silencing of GSTP1 and E-cadherin. Prostate carcinogenesis was promoted by a high-fat stress diet, resulting in HgPIN that developed earlier and in advanced lesions displayed features consistent with carcinoma in situ. This dietary carcinogen-induced prostate cancer model, recapitulating important features of early human prostate carcinogenesis, constitutes a new experimental system for prostate cancer research.
Introduction

Prostate cancer is the most frequently diagnosed malignancy and second leading cause of cancer-related death among men in the United States (1). Identifying the causes of this disease and developing strategies for its prevention are thus of great importance. Epidemiologic evidence links prostate cancer incidence and mortality with consumption of red meat and animal fat (2, 3), but the mechanism(s) by which a diet rich in red meat and fat leads to prostate cancer has not been fully established. One possible mechanism is related to the formation of heterocyclic amines (HCA) during the cooking of meat at high temperatures by grilling, broiling or frying. HCAs are well recognized as food-borne carcinogens of which 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant one (4-6). Epidemiological studies have associated PhIP exposure with increased risk for not only prostate cancer, but also colon and breast cancers (2, 7, 8). These cancers are common in Western countries and traditionally infrequent in Asian countries; however, the incidences of such cancers are increasing in Asia, possibly due to the westernization of the diet (9, 10).

PhIP has been shown to induce malignant lesions in rodents and primates (11-14). Exposure of laboratory rats to PhIP in the diet has been shown to induce several types of tumors, including carcinomas of the intestine, mammary gland, and prostate (15-18). For example, large oral doses of PhIP have been shown to cause epithelial cell damage, inflammation, prostatic intraepithelial neoplasia (PIN) and carcinomas in the rat prostate (11, 12, 16, 17). PhIP also induces lymphoid tissue tumors in mice (19), but its induction of prostate cancer has never been reported in mice.

In studying carcinogenesis and its prevention, animal models relevant to human cancers are essential. A dietary carcinogen-induced prostate cancer model in mice, however, is not available. A possible reason for the absence of PhIP-induced prostate cancer in the mouse is due to the lack of an efficient enzyme system to activate PhIP. Initial metabolic
activation of PhIP is carried out mainly by cytochrome P450 (CYP) enzymes, in particular \textit{CYP1A2} \cite{20}. Studies have shown that human \textit{CYP1A2} preferentially activates PhIP via N\textsuperscript{2}-hydroxylation \cite{20}, which leads to the eventual formation of an ultimate carcinogen (Fig. 1). The mouse \textit{Cyp1a2}, however mainly catalyzes the 4'-hydroxylation of PhIP, which is a detoxification pathways. Therefore, we used CYP1A-humanized (hCYP1A) mice, which express human \textit{CYP1A1} and \textit{CYP1A2} and are deficient in murine \textit{Cyp1a1} and \textit{Cyp1a2} \cite{21}, to develop mouse models that are relevant to human carcinogenesis. It is anticipated that the increased metabolic activation of PhIP in hCYP1A mice would result in a greater carcinogenic effect than in wild-type mice. We recently demonstrated the induction of colon cancer in hCYP1A mice at as early as 6 weeks after a single oral dose of PhIP (200mg/kg body weight) and treatment with dextran sulfate sodium (1.5% in drinking fluid) \cite{22}. The success in the development of this colon carcinogenesis model encouraged us to develop a PhIP-induced prostate carcinogenesis model.

There is strong evidence that prostatic inflammation and oxidative stress contribute to human prostatic carcinogenesis \cite{23-27}. Injury to the prostate epithelium elicits a stereotypical stress and regenerative response that can promote the development of cancer \cite{25, 28}. Molecular changes such as overexpression of cyclooxygenase-2 and \textit{p16}, and down-regulation of \textit{p27}, \textit{p63}, \textit{PTEN} and \textit{NKX3.1} have been observed in human prostate lesions \cite{24}. Glutathione S-transferase P1 (\textit{GSTP1}) is silenced through promoter methylation in subsets of cells \cite{29}. The continued proliferation of genetically unstable luminal cells and accumulation of genomic changes are thought to lead to progression towards invasive carcinoma \cite{24, 30}.

In this study, we demonstrated that administration of a single oral dose of PhIP to the hCYP1A mice induces prostate lesions and molecular alterations that resemble many features of human prostate carcinogenesis. In this report, we describe the development and
characterization of this prostate carcinogenesis model. This mouse model could be a very useful experimental system to study the dietary etiology and prevention of prostate cancer.
Materials and methods

Chemicals

PhIP was purchased from Toronto Research Chemicals (North York, Ontario, Canada) with a purity of over 99%. Before use, PhIP was dissolved in 20% (v/v) dimethyl sulfoxide (DMSO) in milliQ water (with HCl added to allow the PhIP to dissolve). Other chemicals were of the highest grade commercially available.

Animals and genotypes

Male and female Cyp1a2/Cyp1al<sup>tm2Dwn</sup> Tg (CYP1A1, CYP1A2)<sup>1Dwn/DwnJ</sup> and C57BL/6J mice (Cyp1a1, Cyp1a2) were purchased from Jackson Laboratories (Bar Harbor, ME) and used as founders to establish homozygous breeding colonies in our animal facility, as previously described (22). All mice were housed in our animal facility in accordance with protocol number 02-027 approved by the Rutgers University Institutional Animal Care and Use Committee. All mice were maintained under standard 12 hour light/12 hour dark cycles with water and diet provided <i>ad libitum</i> unless otherwise specified.

Animal treatments and sample collection

Twenty hCYP1A-mice and ten wild-type mice were treated with a single dose of PhIP (200 mg/kg body weight) by oral gavage at 6 weeks of age. Fifteen each of hCYP1A-mice and wild-type mice were included as vehicle (20% DMSO) controls. Mice were maintained on an AIN-93M diet without any further treatment. Body weight and food intake were measured weekly. To examine the progression of carcinogenesis, the PhIP-treated and control mice were euthanized by CO<sub>2</sub> asphyxiation at 20, 30, 40, or 50 weeks after PhIP treatment. Prostate and other organs were dissected, weighed and fixed in 10% buffered formalin. In a
second experiment, hCYP1A mice were treated with a single dose of PhIP and then divided into two groups: AIN 76A diet and the high-fat stress diet (Research Diets, New Brunswick, NJ). The high-fat stress diet was previously designed by Newmark et al. (31) based on the AIN76A diet, by increasing the fat (corn oil) content to 40% of the calories and decreasing the contents of calcium, vitamin D3 and phosphate to 0.5 mg/g, 0.11 IU/g, and 3.6 mg/g, respectively. The mice were sacrificed 24-38 weeks after PhIP treatment.

**Histopathological analysis**

After fixation in formalin, the entire prostate lobes (anterior, dorso-lateral complex, and ventral) were embedded in paraffin wax and serially sectioned at 4-µm. Histopathological analyses of the entire prostate lobes were performed on multiple sections per block.

Lesions were identified as inflammation, atrophy, PIN and carcinoma according to the criteria commonly used to score prostate lesions in different transgenic mouse models (32, 33). The areas with inflammation were expressed as percentages of the total luminal area. PIN lesions were classified as either low-grade PIN (LgPIN) or high-grade PIN (HgPIN) based on the consensus criteria described by Shappel et al. (33). LgPIN was characterized by glands lined by 1-3 layers of epithelial cells displaying minimal pleomorphism or hyperchromasia, slight nuclear enlargement with little atypia, infrequent mitosis and essentially normal glandular profiles with only occasional hints of papillary epithelial proliferation. HgPIN was characterized by extensive intra-glandular epithelial proliferation, forming papillary or cribriform structures consisting of epithelial cells displaying significant nuclear atypia and hyperchromasia, cellular pleomorphism, and increased frequency of mitoses. Carcinoma *in situ* was characterized by extensive epithelial proliferation with marked nuclear atypia, increased nuclear/cytoplasmic ratio, loss of epithelial polarity with
extensive ‘piling up’ of cells, and an increased frequency of mitoses with papillary and cribriform growth patterns as well as gland-within-gland structures, but no evidence of invasive growth.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on 4-μm thick paraffin-embedded sections after antigen retrieval. The antibodies used were anti-p63 (mouse monoclonal, Abcam, Cambridge, MA), anti-PTEN (rabbit monoclonal, Cell Signaling, Danvers, MA), anti-GSTP1, anti-androgen receptor (AR) (rabbit monoclonal, Epitomics, Burlingame, CA), anti-E-cadherin (rabbit monoclonal, Cell Signaling, Danvers, MA), anti-DNA (cytosine-5)-methyltransferase 1 (DNMT1) (mouse monoclonal, Abcam, Cambridge, MA), anti-8-8-oxo-deoxyguanosine (8-oxo-dG) (mouse monoclonal, JalCA, Japan), and anti-CD45 (mouse monoclonal, eBioscience, San Diego, CA). The number of positively stained cells (or the immunostaining intensity) and the total number of cells were determined by using an Image-Pro Plus Image Processing System (Version 5.0) (Media Cybernetics, Bethesda, MD) or Aperio ScanScopeR GL system (Aperio, Vista, CA).

**Statistical analysis**

One-way analysis of variance (ANOVA) followed by Dunnett’s test was used for comparison of differences between treatment groups and the control group. Student’s *t*-test was used to determine the difference between two groups. For comparisons of lesions incidence, Fisher’s exact test was used. Differences were considered statistically significant when *p* < 0.05 in two-tailed comparisons.
Results

Administration of PhIP leads to prostate carcinogenesis in hCYP1A mice.

To assess the carcinogenic action of PhIP, hCYP1A mice and wild-type mice were given a single dose of PhIP (200mg/kg body weight) by oral gavage. After the treatment with PhIP, there was an initial slight drop in body weight of the hCYP1A and wild-type mice, but after a week the mice resumed normal growth.

The histopathological lesions of the dorso-lateral, ventral and anterior prostate glands were analyzed. Special attention was paid to the dorso-lateral prostate (DLP), which is considered to correspond to the human peripheral prostate zone, the most common site of human prostate cancer (30, 34). Representative examples are shown in Fig. 2. Infrequent minimal inflammation, but no proliferative lesions were found in control hCYP1A mice that did not receive PhIP treatment at week 30 (Fig. 2A), and weeks 40 and 50 (not shown). At week 20, more frequent and extensive inflammation was found in the dorso-lateral glands (DLG) of PhIP-treated hCYP1A mice (Fig. 2B). The inflammation was often associated with atrophic glands in LgPIN at week 30 (Fig. 2C). LgPIN lesions were observed displaying large papillary foci with layers of atypical epithelial cells at week 30 (Fig. 2D) and papillary extension into the glandular lumen of atypical epithelial cells with nuclear hyperchromasia at week 40 (Fig. 2E). Some PIN lesions appeared to show an epithelial nest extending beyond the basal lamina. Mononuclear (CD45+) leukocytes were often found infiltrating into the area surrounding the HgPIN (Supplemental Fig. 1A and Fig. 2D), indicating the occurrence of inflammation. CD8+ T-cells were also observed outside the DLG (supplemental Fig. 1B).

The epithelial lesions in the DLP of the PhIP-treated hCYP1A mice are summarized in Table 1. Inflammation was first identified at week 20 (data not shown) and became extensive frequent by weeks 30, 40 or 50 in PhIP-treated hCYP1A mice (Table 1). The differences in the extent of inflammation between hCYP1A and wild-type mice at week 30 or
40 were highly significant (Table 1). We found HgPIN in 60% of mice at week 30 and in 100% of mice at weeks 40 and 50, at which time approximately 38% of the glands contained HgPIN. The differences in HgPIN incidence and extent between hCYP1A and wild-type mice at Week 40 were highly significant (Table 1). In wild-type mice only LgPIN were observed 30 or 40 weeks after PhIP-treatment (data not shown). The results are consistent with the time-dependent development of LgPIN and progression to HgPIN in PhIP-treated hCYP1A mice.

In the ventral prostate glands, the PIN lesions were less severe than those in DLG and some were associated with inflammation (Supplemental Fig. 2). In the anterior prostate glands, HgPIN lesions were infrequent. No abnormalities were observed in the pelvic lymph nodes. Tumor development was not observed in other organs: the small intestine, colon, lung, liver, kidney, lymph nodes, or spleen in PhIP-treated hCYP1A-mice.

**High-fat diet accelerates PhIP-induced prostate carcinogenesis.**

The mice on the high-fat stress diet had a 26% higher body weight than those on the AIN76A diet (32.9g vs. 26.2g on average) when sacrificed on Week 27. The high-fat stress diet appeared to accelerate lesion development (Table 1). HgPIN lesions were observed in mice on the high-fat stress diet as early as week 24, with papillary proliferations filling about 75% of the glandular lumen (Fig. 2H), and at week 31, showing papillary epithelial cell growth filling almost 90% of the glandular lumen (Fig. 2I). The HgPIN lesions at week 38 showed cribriform and tufting epithelial growth patterns and marked cellular atypia with features consistent with carcinoma in situ (Fig. 2J-L). In contrast, in 6 PhIP-treated hCYP1A mice fed the standard (low-fat) AIN76A diet, most lesions were LgPIN (Fig. 2G) and only a few HgPIN lesions were observed at week 24-27, consistent with the result found in the PhIP-treated hCYP1A mice on the standard (low-fat) AIN93M diet at week 30-40. The
differences in HgPIN incidence and extent between hig-fat stress diet-fed mice and mice on control diet at Week 24-27 were highly significant (Table 1). This result suggests that the high-fat stress diet promotes prostate carcinogenesis in PhIP-treated hCYP1A mice.

**Key molecular changes in human prostate cancer are found in PhIP-induced lesions in hCYP1A mice.**

Androgen receptor (AR) is expressed in epithelial cells of human normal prostate, PIN, and carcinomas (30). Positive nuclear staining was found in the luminal prostate epithelial cells in untreated mice (Fig. 3A) and PhIP-induced LgPIN (Fig. 3B) and HgPIN (Fig. 3C). Compared to the level in normal epithelial cells, AR protein immunostaining intensities were progressively higher at weeks 30 and 40 (Fig. 3D). This result is consistent with the upregulation of AR commonly found in human prostate cancer (35).

p63, a homolog of the p53 tumor suppressor, is a marker for prostate basal cells (36). Basal cells with p63 positive staining were seen along the basement membrane surrounding DLG epithelium of untreated mice (Fig. 3E). p63 positive basal cells in the DLG were reduced in number and distributed aberrantly in the PhIP-induced LgPIN at week 30 (Fig. 3F) and week 40 (Fig. 3G). Quantitative analysis showed that the number of p63 positive cells decreased with time (Fig. 3H). Absence of basal cells is a hallmark of most epithelia malignancies and it is a major diagnostic criterion for human prostate cancer (36); similarly, we observed loss of p63-expressing basal cells in the PhIP-induced prostate lesions in our mouse model.

PTEN is a tumor suppressor that is frequently lost at advanced stages of many human malignancies, including prostate cancer (30). We found uniform PTEN expression in the normal prostate glands (Fig. 3I), partial loss of PTEN expression in the epithelial cells in LgPIN lesions at week 30 (Fig. 3J), and markedly reduced PTEN expression in HgPIN at
week 40 (Fig. 3K). Quantitative analysis showed that the PTEN-staining intensity was significantly lower at week 30 and appeared even lower at week 40 (Fig. 3L).

GSTP1 is a detoxifying enzyme, and the changes of its expression have been studied extensively in human prostate carcinomas (37). GSTP1 expression was elevated in epithelial cells in atrophic DLG of PhIP-treated hCYP1A mice at week 20 as compared to the control group (Fig. 4B). However, loss of GSTP1 expression was observed in LgPIN and HgPIN at week 30 (Fig. 4C). In HgPIN at week 40, extensive or complete loss of GSTP1 expression was observed (Fig. 4D). The percentage of GSTP1-positive cells was decreased significantly in HgPIN (Fig. 4E). In anterior prostate HgPIN, extensive loss of GSTP1 expression was also observed at week 50 (data not shown).

E-Cadherin is an epithelial cell adhesion molecule involved in the maintenance of the normal tissue architecture and its dysregulation is strongly associated with human prostate cancer progression (38, 39). In untreated mice, a high level of E-cadherin was found uniformly on plasma membrane of luminal epithelial cells (Fig. 5A). In PhIP-treated hCYP1A mice, partial loss of E-cadherin membrane expression occurred in LgPIN lesions at week 30 (Fig. 5B). E-Cadherin membrane expression was further reduced in HgPIN epithelial cells, with heterogeneous expression occurring at week 40 (Fig. 5C). Quantification of E-cadherin membrane-staining intensity in DLG showed that E-cadherin expression was reduced on week 30 and appeared to be further reduced at week 40 (Fig. 5D).

To assess whether the loss of expression of E-cadherin and GSTP1 was associated with hypermethylation of the CpG islands in the promoter regions of these genes, we used methylation-specific qPCR to quantitate CpG island methylation status using DNA samples extracted from unstained tissue slides of prostate tissue with HgPIN or normal tissue. CpG island methylation in Cdh1 and Gstp1/2 genes was higher in tissues containing HgPIN than in normal prostate tissues, but only the increase of Cdh1 was statistically significant.
(Supplemental Fig. 3). The variation in Gstp1/2 CpG island methylation observed is probably due to the heterogeneous distribution of GSTP1-negative and -positive cells because we were not able to selectively dissect the area containing cells of interest. These preliminary data suggest that the promoter hypermethylation is involved in the loss of expression of E-cadherin and GSTP1, and the conclusion needs to be confirmed in future studies by bisulfate genomic sequencing.

The expression of DNMT1 is upregulated in PhIP-induced prostate lesions.

The loss of GSTP1 expression has been attributed to the silencing of the Gstp1 gene by hypermethylation of the CpG islands located in Gstp1/2 gene, and such hypermethylation has been observed frequently during the development of carcinoma (29, 40-44). Therefore, we examined the expression of DNMT1. DNMT1 was expressed at low levels in a small number of prostate epithelial cells in untreated mice (Fig. 5E). The number of cells stained-positive for nuclear DNMT1 staining in PhIP-treated hCYP1A mice increased in LgPIN at week 30 and HgPIN lesions at week 40 (Figs. 5F & G). Quantitative analysis indicated that the percentage of DNMT1-positive cells was significantly higher at weeks 30 and 40 (Fig. 5H). Taken together, the increase of DNMT1 and the time-dependent loss of E-cadherin and GSTP1 expression suggest that gene silencing by promoter hypermethylation is involved in the PhIP-induced prostate carcinogenesis in hCYP1A mice.

Oxidative stress is increased in the PhIP-induced prostate lesions.

To examine the possible involvement of oxidative stress in prostate carcinogenesis, we used a DNA oxidative product, 8-oxo-dG, as a marker. Low levels of basal 8-oxo-dG nuclear staining was observed in DLG epithelial cells of untreated mice (Fig. 5I). Strong 8-oxo-dG nuclear positive staining, however, was found in atypical prostate epithelial cells at
weeks 30 and 40 (Fig. 5J & 5K). The percentage of 8-oxo-dG-positive cells was significantly increased at week 30 and perhaps further increased at week 40 (Fig. 5L). This result suggests the involvement of oxidative stress in prostate carcinogenesis in the PhIP-treated hCYP1A mouse.
Discussion

The present study demonstrates the induction of prostate lesions in hCYP1A mice by a dietary carcinogen, PhIP. The histopathogenesis of LgPIN and HgPIN in the DLP, the loss of expression p63 and PTEN, as well as the silencing of Gstp1 and Cdh1 in this model closely resemble features of human prostate carcinogenesis. The promotion of prostate carcinogenesis by the high-fat stress diet provides biological plausibility for the involvement of dietary fat in human prostate cancer. To our knowledge, this is the first report on the development of proliferative prostate lesions, which closely mimics human prostate cancer in histopathogenesis and molecular changes, in a mouse model induced by a suspected human dietary carcinogen.

An important feature of this model is its close resemblance to human prostate cancer in terms of epithelial cell origin and the major site of neoplasia, the DLP, which correspond to the human peripheral prostate zone, in which over 70% prostate cancers arise (30, 34). The observed prostate inflammation, atrophy, and LgPIN and HgPIN lesions are also similar to those lesions observed in men (7, 25, 37, 41). Our results are consistent with the sequence of events in the development of inflammatory proliferative epithelial lesions, LgPIN, and HgPIN that have been observed in men. Although frankly invasive carcinoma was not observed, some of the epithelial lesions had features suggestive of carcinoma in situ. With time and with the promotion by a high-fat diet, it is conceivable that invasive carcinoma will develop.

Similar to human prostate cancer, in this mouse model positive staining of AR was observed in prostate lesions, as well as several key molecular changes that occur in human prostate cancer. The loss of p63-expressing basal cells in PIN lesions in this model is similar to what is found in human cancer (36). Moreover, the loss of PTEN is another key feature of this model that resembles human prostate cancer. Whether this loss of expression is due to a
PTEN gene mutation or to epigenetic mechanisms remains to be investigated. The presently observed initial overexpression and subsequent loss of expression of GSTP1, closely resemble the alterations in expression of this important enzyme in human prostatic cancer development (40). Similarly, the expression of E-cadherin was down-regulated during the PhIP-induced carcinogenesis process, as seen in human prostate cancer (38, 39). The silencing of GSTP1 and E-cadherin is likely to be caused by DNA hypermethylation (Supplemental Fig. 2), a notion also supported by the observed induction of DNMT1 expression. In preliminary studies, we found elevated levels of DNMT1 as early as on 1 and 3 days after PhIP administration (data not shown). It is not known whether the elevated level of DNMT1 continues to weeks 30 and 40 or not. If not, DNMT1 could be induced at the later stage, possibly by inflammation and resultant oxidative stress.

Our finding that PhIP induces prostate carcinogenesis in hCYP1A mice, but not in the wild-type mice, illustrates the role of the human CYP1A2 in the activation of PhIP in vivo and further strengthens the concept that PhIP is a human prostate carcinogen. It has been established that PhIP is activated by CYP1A2 and N-acetyl transferase (or sulfotransferase) to an ultimate carcinogen, which reacts with DNA to form DNA-adducts (45, 46). The DNA-adducts conceivably lead to oncogenic mutations that may be the driving force of carcinogenesis (46). Based on our results, we propose that epigenetic changes involving DNMT1 induction and gene silencing by DNA hypermethylation are also key events in the PhIP-induced prostate carcinogenesis.

In the present study, prostate carcinogenesis is induced in hCYP1A mice by a single oral dose of PhIP at 200 mg/kg (i.e., about 3 mg per mouse). This dose is much lower than the dietary doses used previously in a rat model of HgPIN and carcinomas (12, 16), in which 100 and 400 ppm of PhIP in the diet was fed to rats for a period of 12 months (12, 16). Since the human CYP1A2 is more effective than the corresponding rodent gene in activating PhIP,
it is not surprising that the lower dose of PhIP was effective to induce prostate carcinogenesis in hCYP1A mice. In this study, we observed the early stages of prostate cancer development, which are not as advanced as some of the more aggressive, well-established, genetically-engineered mouse models for prostate cancer (30, 47). These models help to demonstrate the roles of designated oncogenes or tumor suppressor genes in prostate carcinogenesis, but the molecular events which trigger these genetic changes are not known. Our present model offers the opportunity to discover early molecular events that lead to these known oncogenic changes.

The roles of high-fat diet and obesity in contributing to prostate cancer are complicated and have remained inconclusive (48), but a recent meta-analysis has showed obesity is a risk factor of prostate cancer-specific mortality and its recurrence following initial treatment (49). Our present study suggests that a high-fat stress diet promotes the development of prostate cancer. This result is consistent with the epidemiological observation that a Western-style diet is associated with risk of prostate cancer (3, 48, 50). We used the high-fat stress diet, which also contained lower levels of calcium and vitamin D than the AIN76A diet (31). The high-fat stress diet was designed by Newmark et al. (31) to mimic the human Western-style diet. It is unclear whether the cancer promoting effects are caused by the high-fat content or by the low levels of calcium and vitamin D, or a combination of these dietary factors. Nevertheless, our results indicate that PhIP-induced prostate carcinogenesis in mice can be promoted by dietary factors and that the present model is an excellent experimental system to investigate the roles of fat, calcium, vitamin D, and conceivably other dietary factors in prostate carcinogenesis.

In summary, we have established and characterized a novel mouse model for prostate carcinogenesis using a dietary carcinogen, PhIP. This mouse model recapitulates many of the features in the histological and molecular changes commonly found in human prostate
cancer. Further studies with this model have the potential to lead to discovery of novel mechanisms of prostate carcinogenesis. It would also serve as a relevant model for studying the effects of dietary factors in the causation and prevention of prostate cancer.
Acknowledgments

We thank Ms. Yu-Hai Sun for her technical assistance in the preparation of histological slides.
References


Legends

Fig. 1. Differential metabolic pathways of PhIP by CYP1A2 and Cyp1a2.

Fig. 2. PhIP-induced prostate inflammation and PIN in hCYP1A mice. (A): Normal dorso-lateral glands (DLG) of an untreated mouse at week 30. (B): DLG with focal stromal inflammation (star) at week 20. Images C - L were from DLG of PhIP-treated mice. (C): Epithelial atrophy (arrows) with focal chronic stromal inflammation (star) at week 30. (D): LgPIN at week 30 showing papillary proliferation and atypical epithelial cells displaying enlarged nuclei and prominent nucleoli (arrow head). (E): HgPIN at week 40 showing papillary extension into glandular lumen, atypical epithelial cells with nuclear hyperchromasia. (F): LgPIN at week 40 showing an epithelial nest extending beyond the basal lamina (arrow). (G): LgPIN at week 24 showing a papillary proliferation of LgPIN into the glandular lumen. Images H to L were from high-fat fed groups of PhIP-treated mice. (H): HgPIN at week 24 showing papillary proliferation filling about 75% of the glandular lumen. (I): HgPIN at week 31 showing tufting patterns and papillary epithelial cell growth that involves almost 90% of the glandular lumen. (J): HgPIN at week 38 showing cribriform and tufting epithelial growth patterns, and marked atypia with features consistent with carcinoma in situ. (K) and (L): High-power view image of (J) illustrating the histological and cytological details. Scale bars, 50 µm.

Fig. 3. AR, p63 and PTEN expression in DLG in hCYP1A mice. (A): Normal nuclear expression of AR in an untreated mouse at week 30. (B): Increased AR expression in the epithelial cells in PhIP-induced LgPIN at week 30. (C): Highly increased AR expression in the epithelial cells in HgPIN at week 40. (D): Quantification of AR expression, with intensity shown as mean ± SD; n=5 for the untreated (U), week 30 (W30) or week 40 (W40) group.
(E): Basal cell expression of p63 (brown color) along the basement membrane in DLG of an untreated mouse. (F & G): p63 stained basal cells in PhIP-induced LgPIN at week 30 and HgPIN at week 40, respectively, showing that the p63 expression was reduced and frequently disorganized. (H): Quantification of p63 positive cells, with positive cells per glands shown as mean ± SD (n=5 per group). (I): Uniform PTEN nuclear expression in the normal prostate epithelium of an untreated mouse at week 30. (J): Partial loss of PTEN expression in the epithelial cells in PhIP-induced LgPIN at week 30. (K): Loss of PTEN expression in HgPIN at week 40. (L): Quantification of PTEN expression, with staining intensity shown as mean ± SD (n=5 per group). For this and other figures, different superscripts (a, b, and c) indicate statistically significant differences between group (one-way ANOVA; p < 0.05). Scale bars, 50 μm.

Fig. 4. Reduced GSTP1 expression in PhIP-induced DLG lesions in hCYP1A-mice. (A): Nuclear and cytosolic basal expression of GSTP1 (brown color) in normal DLG of an untreated hCYP1A mouse. (B): Elevated nuclear and cytosolic GSTP1 expression in the epithelial cells in DLG at week 20. (C): Loss of nuclear GSTP1 expression in epithelial cells in LgPIN and HgPIN at week 30. (D): Extensive loss of GSTP1 expression in hgPIN, whereas most of normal luminal epithelial cells showed positive staining at week 40. (E): Quantification of GSTP1 expression normal and DLG with LgPIN and HgPIN shown as mean ± SD (n=10 per group).

Fig. 5. E-Cadherin and DNMT1 expression and oxidative stress in DLG in hCYP1A-mice. (A): E-cadherin membrane expression in luminal epithelial cells in an untreated (U) mouse. (B): Partial loss of E-cadherin plasma membrane expression in PhIP-induced LgPIN at week 30 (W30). (C): Lower and heterogeneous E-cadherin plasma membrane expression in luminal
epithelial cells in HgPIN at week 40 (W40). (D): Quantification of E-cadherin membrane expression, with staining intensity shown as mean ± SD (n=8 per group). (E): DNMT1 expression in luminal epithelial cells in an untreated mouse. (F): Increased DNMT1-positive stained cells in luminal epithelial cells in PhIP-induced LgPIN at week 30. (G): Highly increased DNMT1-positive stained cells in HgPIN at week 40. (H): Quantification of DNMT1 nuclear expression, with percentage of positive stained cells shown as mean ± SD (n=7 per group). (I): Basal 8-oxo-dG nuclear staining in prostate epithelial cells of an untreated mouse. (J&K): Elevated 8-oxo-dG nuclear staining in atypical epithelial cells of PhIP-induced HgPIN at week 30 and 40, respectively. (L): Quantification of 8-oxo-dG nuclear staining, with percentage of positive stained cells shown as mean ± SD (n=6 per group).
Fig. 1.

CYP1A-humanized mouse (Human CYP1A2) → Human CYP1A2 → N²-Hydroxy-PhIP → PhIP-DNA Adducts → Mutation → Carcinogenesis

PhIP → Mouse Cyp1a2 → 4′-Hydroxy-PhIP → Detoxification
Fig. 4.
Fig. 5.

[Image: Diagram showing immunohistochemical staining of E-cadherin, DNMT1, and 8-oxo-dG in different conditions.]

- **E-cadherin**
  - Panels A, B, C: Staining intensity%.
  - Panels D: Graph showing staining intensity with conditions U, W30, W40.

- **DNMT1**
  - Panels E, F, G: % Positive cells.
  - Panels H: Graph showing % Positive cells with conditions U, W30, W40.

- **8-oxo-dG**
  - Panels I, J, K: % Positive cells.
  - Panels L: Graph showing % Positive cells with conditions U, W30, W40.

Note: The diagrams are labeled with lowercase letters (a, b, bc) indicating statistical significance between groups.
Table 1. PhIP-induced prostate inflammation and PIN in the DLG of hCYP1A mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Termination (Week)</th>
<th>Inflammation (%) a</th>
<th>HgPIN incidence (%) b</th>
<th>% glands with HgPIN c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n=5)</td>
<td>AIN93M</td>
<td>30</td>
<td>4.5 ± 0.87</td>
<td>0/5 (0)</td>
<td>0</td>
</tr>
<tr>
<td>hCYP1A (n=5)</td>
<td>AIN93M</td>
<td>30</td>
<td>13.6 ± 2.15 d</td>
<td>3/5 (60) d</td>
<td>8.4 ± 3.8 d</td>
</tr>
<tr>
<td>Wild-type (n=5)</td>
<td>AIN93M</td>
<td>40</td>
<td>4.8 ± 0.93</td>
<td>0/5 (0)</td>
<td>0</td>
</tr>
<tr>
<td>hCYP1A (n=5)</td>
<td>AIN93M</td>
<td>40</td>
<td>15.5 ± 2.96 d</td>
<td>5/5 (100) d</td>
<td>37.6 ± 3.7 d</td>
</tr>
<tr>
<td>hCYP1A (n=3)</td>
<td>AIN93M</td>
<td>50</td>
<td>12.3 ± 1.73</td>
<td>3/3 (100)</td>
<td>38.7 ± 7.3</td>
</tr>
<tr>
<td>hCYP1A (n=6)</td>
<td>AIN76A</td>
<td>24-27</td>
<td>nd</td>
<td>2/6 (33)</td>
<td>5.8 ± 3.7</td>
</tr>
<tr>
<td>hCYP1A (n=7)</td>
<td>High-fat</td>
<td>24-27</td>
<td>nd</td>
<td>7/7 (100) e</td>
<td>42.7 ± 3.9 e</td>
</tr>
<tr>
<td>hCYP1A (n=4)</td>
<td>High-fat</td>
<td>38</td>
<td>nd</td>
<td>4/4 (100) e</td>
<td>59.5 ± 11.2 e</td>
</tr>
</tbody>
</table>

All the mice were administered one dose of PhIP (200 mg/kg, i.g.). n, number of mice in the group. nd, not determined.

a Percent of area involved (mean ± SE). Student’s t-test was used to analyze the difference between the treated group and the corresponding wild-type mice.

b Percent of mice with HgPIN. Fisher’s exact test was used to analyze the difference between the treated group with the corresponding control group.

c Percent of glands with HgPIN (mean ± SE). Student’s t-test was used to analyze the difference between the treated group and the corresponding control group.

d Significantly different from the corresponding control group with the wild-type mice (p<0.01).

e Significantly different from the corresponding control group with AIN76A-fed mice at 24-27 weeks (p<0.01).
Dietary Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine Induced Prostate Carcinogenesis in CYP1A-humanized Mice

Guangxun Li, Hong Wang, Ba Liu, et al.

Cancer Prev Res  Published OnlineFirst May 11, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-12-0023

Supplementary Material  Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2012/05/11/1940-6207.CAPR-12-0023.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.