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Applications of the human p53 knock-in (*Hupki*) mouse model for human carcinogen testing

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ABSTRACT **Tumor-driving mutations in the *TP53* gene occur frequently in human cancers. These inactivating mutations arise predominantly from a single-point mutation in the DNA-binding domain of this tumor suppressor gene (*i.e.*, exons 4–9). The *hu*man *p*53 *k*nock-*in* (*Hupki*) mouse model was constructed using gene-targeting technology to create a mouse strain that harbors human wild-type *TP53* DNA sequences in both copies of the mouse *TP53* gene. Replacement of exons 4–9 of the endogenous mouse *TP53* alleles in the *Hupki* mouse with the homologous normal human *TP53* gene sequences has offered a humanized replica of the *TP53* gene in a murine genetic environment. The *Hupki* mouse model system has proven to be an invaluable research tool for studying the underlying mechanisms of human *TP53* mutagenesis. The utility of the *Hupki* mouse model system for exploring carcinogen-induced *TP53* mutagenesis has been demonstrated in both *in vivo* animal experiments and *in vitro* cell culture experiments. Here, we highlight applications of the *Hupki* mouse model system for investigating mutagenesis induced by a variety of environmental carcinogens, including sunlight ultraviolet radiation, benzo[*a*]pyrene (a tobacco smoke-derived carcinogen), 3-nitrobenzanthrone (an urban air pollutant), aristolochic acid (a component of Chinese herbal medicine), and aflatoxin B1 (a food contaminant). We summarize the salient findings of the respective studies and discuss their relevance to human cancer etiology.—Besaratinia, A., Pfeifer, G. P. Applications of the human p53 knock-in (*Hupki*) mouse model for human carcinogen testing. *FASEB J*. 24, 2612–2619 (2010). www.fasebj.org**

Key Words: cancer · etiology · mutation · tumor suppressor gene

The pattern of somatic alterations in a human cancer genome is shaped by a number of factors, of which mutagen exposure holds great importance (1–3). Numerous mutagenic agents are present in the air we breathe, in the food we eat, and in the water we drink (4, 5). The complexity of human exposure to environmental mutagens can variably influence the compendium of somatic mutations occurring in human cancers (2, 3, 6, 7). For example, human nonmelanoma skin- and lung cancer genomes bear unique mutational signatures, which are attributable to exposure to sunlight ultraviolet (UV) radiation and tobacco smoke, respectively (8). Tumor genomes from nonmelanoma skin- and lung cancer patients, respectively, show a characteristic preponderance of C→T or CC→TT transitions at dipyrimidine sites (**Fig. 1**), and G→T transversions at methylated CpG dinucleotides (**Fig. 2**), which occur preferentially on the nontranscribed DNA strand (9–11).

The recent advent of next-generation sequencing platforms has massively reduced the cost and effort of cataloguing human cancer mutations (12, 13). Currently, high throughput next-generation sequencing projects are interrogating a variety of cancer genomes, including various types of human tumors and cancer cell lines (14–18). These projects are poised to identify unique somatic changes in the genome of cancer patients and/or cell lines with a history of exposure to specific mutagens. To address the issue of cancer etiology, however, the occurrence of these genetic mutations does need to be recapitulated experimentally. Demonstration of a link between exposure to certain mutagens and induction of specific mutations in genes that promote tumorigenesis when mutated should be done in validated experimental model systems and under well-defined and controlled exposure conditions (8). By helping draw causality inference, this approach can greatly improve our understanding of the etiology of human cancer.

Inactivating mutations in the *TP53* tumor suppressor gene are frequent events in human cancers (19–22), and *TP53* generally stands at the top of the list of the most frequently mutated genes even when all coding sequences of the human genome are analyzed (11). The vast majority of *TP53* mutations arise from a single point mutation in the segment encoding the DNA-binding domain of the TP53 protein (19, 20). These inactivating mutations render the mutant TP53 protein unable to carry out its normal functions, *i.e.*, transcriptional transactivation of downstream target genes that regulate the cell cycle and apoptosis (21, 22). Scrutiny of the available databases of *TP53* gene mutations in human cancers [*e.g.*, IRAC TP53 database (http://www-p53.iarc.fr/p53DataBase.htm), 27,132 entries; and UMD

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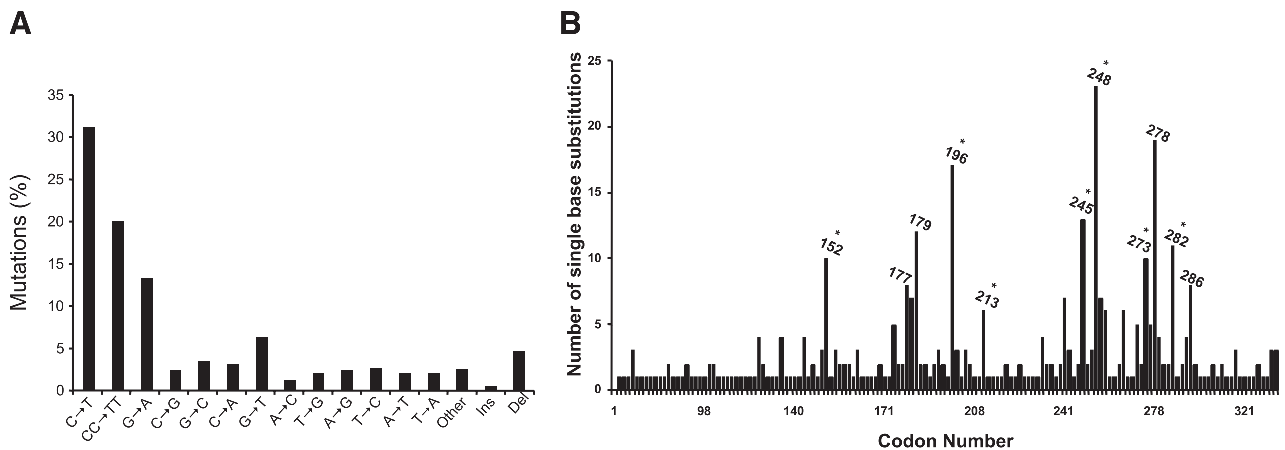

Figure 1. Mutation spectrum (*A*) and codon distribution (*B*) of the *TP53* tumor suppressor gene in human nonmelanoma skin tumors (basal cell and squamous cell carcinomas; n=541). Data were obtained from the *TP53* mutation database of the International Agency for Research on Cancer (http://www-p53.iarc.fr/p53DataBase.htm; R12 version) (79). Codons containing methylated CpG sequences are indicated by asterisks. Ins, insertions; Del, deletions.

TP53 mutation database (http://p53.free.fr/Database/p53_database.html), 28,513 entries] has revealed significant links between exposure to mutagenic compounds and *TP53* genetic alterations specific for certain types of human cancers (20, 23, 24). Attempts have been made to reproduce these putative links in experimental settings using a wide range of model systems, including bacterial Ames test (25, 26), functional analysis of separated alleles in yeast (FASAY; refs. 27, 28), reporter gene-based transgenic rodents, *e.g.*, BigBlue® system (29), or analysis of endogenous noncancer-related genes, *e.g.*, the house-keeping hypoxanthine phosphoribosyltransferase (*HPRT*) gene in mammalian or human tissues/cells (30). Although these model systems have all provided invaluable information on many aspects of mutagenesis-derived carcinogenesis, they all lack, in one way or another, crucially important factors that contribute to *TP53* mutations and human cancers (8). For instance, in these model systems, the known determinants of mutagenesis, *e.g.*, DNA-sequence contexts, DNA repair efficiency, and fidelity of replicative DNA polymerases, which are species/cell-type dependent, may not represent the respective parameters in the human *TP53* gene (8).

More recently, a novel model system has been developed to investigate experimentally induced mutations in the human *TP53* gene, in its natural mammalian context. The human p53 knock-in (*Hupki*) mouse model has addressed the issue of DNA-sequence context by replacing exons 4–9 of the endogenous mouse *TP53* allele with the homologous normal human *TP53* gene sequence (31). In the following sections, we will discuss the *Hupki* mouse model system, which has the utility to detect both spontaneously arisen- and carcinogen-induced mutations in the human *TP53* gene *in vitro* (32–38) or *in vivo* (31, 39, 40).

Hupki MOUSE STRAIN

The *Hupki* mouse model system was constructed using gene-targeting technology to create a mouse strain that harbors human wild-type *TP53* DNA sequences from

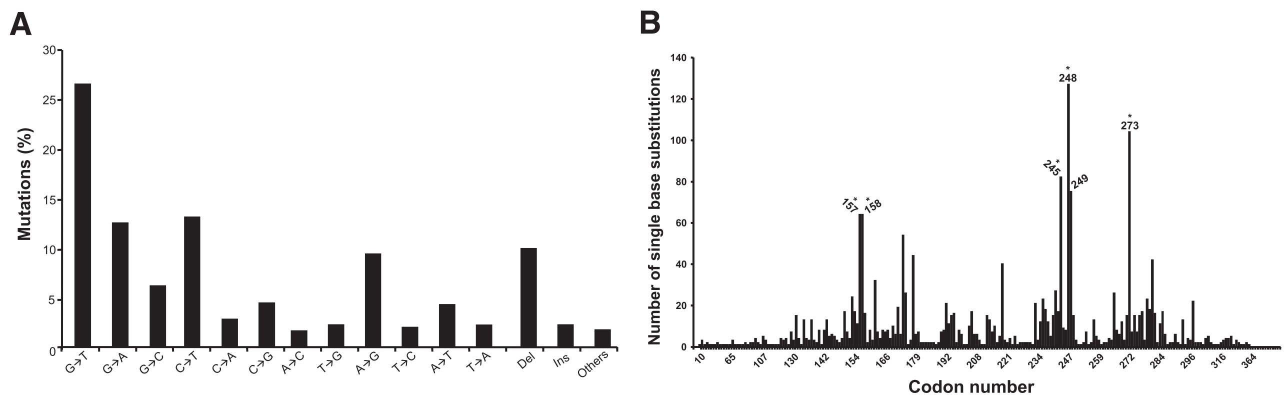

Figure 2. Mutation spectrum (*A*) and codon distribution (*B*) of the *TP53* tumor suppressor gene in tobacco smoke-associated lung cancer (n=2340). Data were obtained from the *TP53* mutation database of the International Agency for Research on Cancer (http://www-p53.iarc.fr/p53DataBase.htm; R12 version) (79). Entries with confounding exposure to asbestos, mustard gas, or radon were excluded. Codons containing methylated CpG sequences are indicated by asterisks. Ins, insertions; Del, deletions.

exons 4 to 9 in place of the homologous murine DNA sequences in both copies of the mouse *TP53* gene (31). The substituted segment encodes the polyproline domain and DNA-binding domain of wild-type human TP53, and the chimeric *TP53* gene remains under normal transcription regulation at the mouse locus. The *Hupki* mice develop normally, exhibit no apparent physiological defects, remain fertile, and show no susceptibility to spontaneous lymphomas, sarcomas, or other neoplasms, which are common in *TP53*-deficient or null mice (31). The *Hupki* mice retain a variety of normal *TP53* functions and characteristics, including nuclear accumulation of TP53 protein after exposure to DNA-damaging agents, transcriptional activation of known TP53 downstream targets, and induction of apoptosis in thymocytes after γ-irradiation, an outcome modulated by a functional *TP53* gene (31, 35, 41).

In addition to its application for *in vivo* animal experiments (31, 39, 40), the *Hupki* model system is also amenable to *in vitro* cell culture experiments (32–38). Ideally, the origin of *TP53* mutations in human cancers should be determined using a mutagenesis assay in which suspected endogenous metabolites or exogenous carcinogens can target the DNA sequences of this tumor suppressor gene, thereby leading to mutant TP53 proteins that confer a selectable phenotype, preferably one that resembles the aberrant functions that are typical for human tumor *TP53* mutants. Theoretically, normal human cells would be the ideal cell type for such assay, were they not resistant to undergo immortalization or transformation *in vitro*. In practice, proliferative nonsenescent cultures are required for amplification and subsequently detection of the few phenotypically expressed *TP53* mutant cells, which are usually outnumbered by an overwhelming pool of wild-type nonmutant cells (even in cultures that have undergone numerous rounds of passaging) (8). Murine fibroblasts, in contrast to human cells, spontaneously undergo immortalization during *in vitro* culturing, and require only one key genetic defect, such as loss of *TP53* function, thus, allowing the selection of *TP53* mutant cells *in vitro* (42–44). Likewise, primary embryonic fibroblasts from the *Hupki* mice readily undergo immortalization during *in vitro* passaging, which allows for the selection of dysfunctional *TP53* point mutations that are characteristic of human tumors (32–38).

Given the importance of DNA sequence context in human *TP53* mutagenesis and carcinogenesis (8, 31), the murine *TP53* gene, however, cannot optimally represent its human counterpart gene due to the 15% discrepancy in base sequence in the DNA-binding domain and amino acid differences between these two species (35, 44, 45). This incomparability might explain, for example, the observation that whereas codon 248 (CGG) of the human *TP53* gene is the most prominent mutation hotspot in nonmelanoma skin cancers (Fig. 1*B*), the mouse equivalent codon (CGC) (45) hardly harbors any mutations in UVB-induced tumors (46). The *Hupki* model system has resolved the above issue of incomparability by genetically modifying the murine *TP53*, and creating a humanized replica of the *TP53* gene in the mouse genome (35). The system offers a promising venue for assaying spontaneous or experimentally induced human *TP53* gene mutations both *in vitro* (32–38) and *in vivo* (31, 39, 40). In the following paragraphs, we will highlight applications of the *Hupki* mouse model system for mutagenicity analysis of various physical or chemical carcinogens and discuss the advantages and disadvantages of this model system *in vitro* and *in vivo*.

Hupki MUTAGENESIS ASSAY *IN VITRO*

The *Hupki* mouse embryonic fibroblasts treated with benzo[*a*]pyrene (B[*a*]P), a prominent tobacco-derived carcinogen, harbored *TP53* mutations consisting of predominantly single base substitutions in the DNA-binding domain of this gene [29 of 36 (~81%) of all mutations] (32, 34, 36). G→T transversion mutations constituted half of all B[*a*]P-induced mutations, of which all but one (17 of 18) occurred at sites where the permutated guanines were positioned on the nontranscribed strand of the *TP53* gene. Distribution of the 29 B[*a*]P-induced mutations in the DNA-binding domain of the *TP53* gene revealed codons 157, 158, and 273 as the most frequently mutated sites. The overall pattern and distribution of B[*a*]P-induced mutations in the *Hupki* mouse model system (32, 34, 36) mirror the characteristic features of *TP53* mutations in lung tumors of smokers (Figs. 2*A* and **3*A***) (47–49).

The established cultures of *Hupki* mouse embryonic fibroblasts irradiated with UVC gave rise to point mutations in the DNA-binding domain of the *TP53* gene (33). Two of the seven induced mutations recovered from the UVC-irradiated cultures were at codons 248 and 273 (33), two major *TP53* mutational hotspots in human nonmelanoma skin cancers (Fig. 1*B*) (50). Of all UVC-induced mutations, three were C→T transitions on the nontranscribed strand of the *TP53* gene (33), a common feature of mutant *TP53* gene in sun-exposed human skin tumors (Fig. 1*A*) (10, 50).

The *Hupki* mouse embryonic fibroblasts were treated with aristolochic acid (AA) (32, 33, 38), a plant extract potentially involved in Chinese herbs nephropathy and possibly leading to urothelial cancer development (51). Twenty of the 37 AA-induced *TP53* mutations (54%) were A→T transversion mutations (52) (Fig. 3*B*), reflecting the hallmark mutation detected in the urothelial tumor cells of patients with documented AA exposure (53, 54). The induced A→T transversion mutations were presumably due to the permutated adenines located almost exclusively on the nontranscribed strand of the *TP53* gene (32, 33, 38). This finding is consistent with the preferential formation of AA-adenine adducts found in the DNA of AA-treated *Hupki* cells and nephropathy patients (51, 54–56), as well as in the DNA from target organs of AA-exposed rats (57, 58). Recently, Grollman *et al.* (53) have also identified AA-adenine and -guanine adducts in the renal cortex of nephropathy patients and in the transitional cell tumors of patients with upper urinary tract malignancies who were residents of the endemic (Balkan) villages in which chronic dietary exposure to AA is prevalent. Mutation analysis of the *TP53* gene in this population showed a

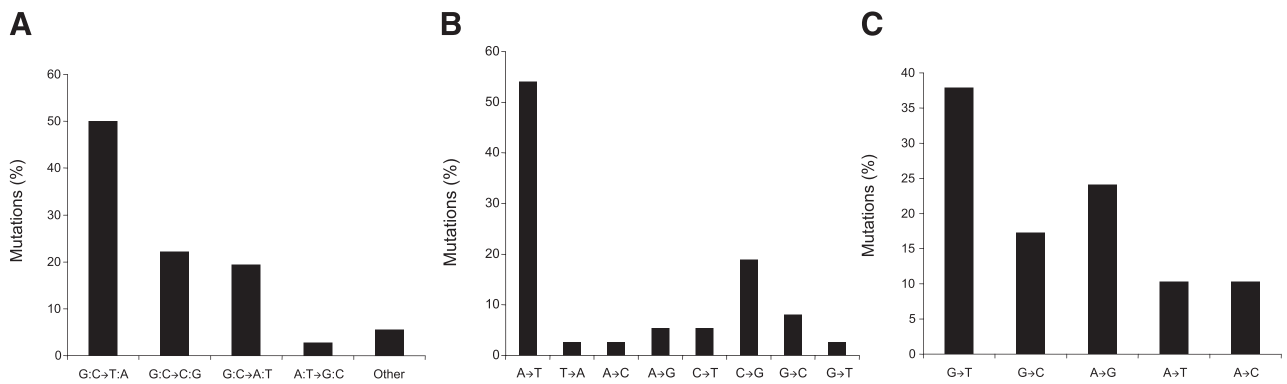

Figure 3. Induced mutation spectra of the *TP53* tumor suppressor gene in the *Hupki* mouse model system. *A*) B[*a*]P-treated *Hupki* mouse embryonic fibroblasts (*n*=36) (data compiled from refs. 32, 34, 36). *B*) AA-treated *Hupki* mouse embryonic fibroblasts (*n*=37) (data compiled from refs. 32, 33, 38, 52). *C*) 3-NBA-treated *Hupki* mouse embryonic fibroblasts (*n*=29) (data obtained from ref. 37).

predominance of mutations at A:T base pairs accounting for 89% of all detected mutations, with the vast majority (15 of 19) being A:T→T:A transversions (53).

The *Hupki* mouse embryonic fibroblasts were treated with 3-nitrobenzanthrone (3-NBA) (37), a member of the class of nitropolycyclic aromatic hydrocarbons, present in particulate fraction of diesel exhaust (59), and a ubiquitous urban air pollutant (60, 61). The established cultures of 3-NBA-treated cells harbored *TP53* mutations in the DNA-binding domain of this gene, which consisted mainly of base substitutions (22 of 29, ~76%) (37). Of these, G:C→T:A transversions were the major type of mutations (10 of 22, ~46%) followed by A:T→T:A transversions (3 of 22, ~14%) (Fig. 3*C*). This ratio of G:C→T:A to A:T→T:A transversions (3:1) perfectly mirrored the ratio of dG/dA adduct formation (75:25%) determined in similarly treated cells with 3-NBA or its reactive metabolite, *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) (37). A similar correlation in ratios of 3-NBA-derived purine adducts to transversion mutations was previously found in liver tissues of the MutaMouse, where the proportion of induced dG to dA adducts was 6 to 1 and that of corresponding G:C→T:A and A:T→T:A mutations was 5 to 1 (61). G:C→T:A transversions were also the predominant type of mutations found in *N*-OH-3-ABA-treated shuttle vector plasmids propagated in human cells (62), as well as in livers of 3-NBA-treated MutaMice (61) and in the lungs of gpt-Δ transgenic mice following inhalation of diesel exhaust (63).

Whibley *et al.* (64) have recently shown that primary murine embryonic fibroblasts from wild-type and *Hupki* mice alike undergo *in vitro* spontaneous immoratalization consequent to successive passaging in culture. The authors demonstrated that basic features of oxidative-stress-induced senescence and subsequent immortalization of wild-type mouse embryonic fibroblasts are preserved in the counterpart cells from the *Hupki* mice. While wild-type mouse embryonic fibroblasts entered and exited senescence slightly later than their counterpart *Hupki* cells, reactive oxygen species (ROS) levels and the extent of DNA damage, determined by staining with a ROS-labile dye and the alkaline comet assay, respectively, were similar in the two cell lines at corresponding passages. *TP53* mutations and *p19* deletions occurred in a significant proportion of spontaneously immortalized cells in both wild-type and *Hupki* embryonic fibroblasts, and the frequency of these events did not differ significantly between these two genotypes (64). Notably, *TP53* mutations arising during spontaneous immortalization of the *Hupki* fibroblast cultures correspond to human cancer *TP53* mutations (64) that are known to be deficient in transcriptional activity (65). Although a limited number of *TP53* mutation data was available from the immortalized wild-type mouse embryonic fibroblasts as compared to that from the counterpart *Hupki* cells (12 *vs.* 64 entries, respectively), the spectra of *TP53* mutations found in the respective genotypes were similar in that G:C→C:G transversions constituted the most common type of base substitutions (**Fig. 4**) (64). This type of base alteration occurs at high frequency (up to ~30%) in human breast cancer (1, 17, 66), although the driving force behind this mutagenic event is currently unknown (11). The prevalence of G:C→C:G transversions in the spectra of *TP53* mutations in both wild-type and *Hupki* immortalized mouse embryonic fibroblasts remains a matter of further investigation (M. Hollstein, University of Leeds, Leeds, UK; personal communication, February 2, 2010).

Hupki MUTAGENESIS ASSAY *IN VIVO*

Luo *et al.* (35) have demonstrated that UVB-irradiated *Hupki* mice exhibit characteristic molecular pathology features of sunlight-associated human skin cancers (67, 68), including development of clones of epidermal cell patches with *TP53*-immunoreactive nuclei; formation of UV-induced cyclobutane pyrimidine dimers at skin cancer mutational hotspots in the *TP53* gene, which colocalize with the respective lesions induced in UVB-exposed human keratinocytes; and induction of signature C→T transition mutations in the respective *TP53* mutational hotspots (35). Screening of the UVB-irradiated *Hupki* mouse epidermal cells for mutations at codons 247–248 and 278–279, two major skin cancer

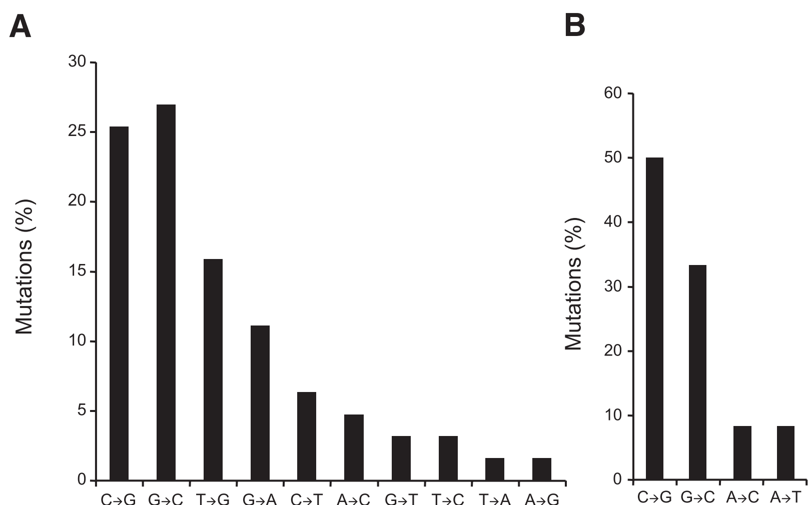

Figure 4. Spontaneous mutation spectra of the *TP53* tumor suppressor gene in immortalized *Hupki* (*A*) and wild-type (*B*) mouse embryonic fibroblasts. Total numbers of *TP53* mutations in wild-type and *Hupki* immortalized cells were 12 and 64, respectively. Data were obtained from ref. 64.

TP53 mutational hotspots (Fig. 1*B*), showed that 58 of the 59 mutant clones (98%) harbored a transition mutation, including 9 (15%) with tandem CC→TT mutations (35). All of the UVB-induced mutations in codons 278–279 and ~28% of mutations in codons 247–248 occurred at sites where the premutated pyrimidine dinucleotides were on the nontranscribed strand of the *TP53* gene (**Fig. 5**) (35). The overall pattern of *TP53* mutations found in UVB-irradiated *Hupki* mice (35) corresponds to the unique features of *TP53* mutations in sunlight-associated human skin tumors (69–71).

Tong *et al.* (40) have used the *Hupki* mice to investigate the effect of local DNA sequence on *TP53* codon 249 mutation, a prevalent occurrence in human hepatocellular carcinoma associated with synergistic exposure to aflatoxin B1 (AFB_1) and hepatitis B virus (HBV) infection (72). A single intraperitoneal injection of AFB_1 to the *Hupki* mice and counterpart wild-type animals showed that the mice expressing the humanized *TP53* were more prone to hepatocellular carcinoma development and death, compared to mice expressing the murine *TP53*, without acquiring any mutations in the *TP53* gene (40). These findings support the notion that the specificity of *TP53* codon 249 mutation in human hepatocellular carcinoma is not solely dependent on DNA sequence context of this gene, and other determining factors, *e.g.*, concomitant HBV infection, may synergistically be involved in this process (73). Currently, work is underway to address this question in a more relevant model system, *i.e.*, the Hupki × Chisari's HBV cross-strain mice (M. Hollstein, personal communication, November 18, 2008).

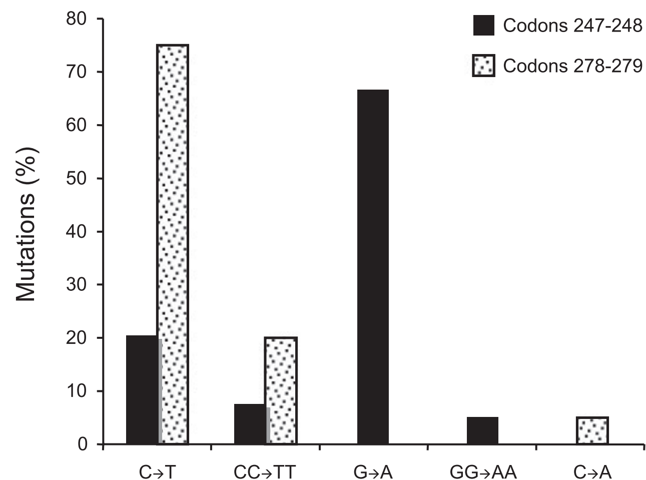

Figure 5. Mutation spectrum of the *TP53* tumor suppressor gene in UVB-irradiated *Hupki* mice. Restriction enzyme digestion-coupled to PCR amplification of codons 247–248 and codons 278–279 followed by DNA sequencing were performed to establish the spectrum of mutations in the respective codons, two major *TP53* mutational hotspots in human nonmelanoma skin cancers (see Fig. 1*B*) (50). Total numbers of mutant clones at codons 247–248 and 278–279 were 39 and 20, respectively.

ADVANTAGES AND DISADVANTAGES OF THE Hupki MOUSE MODEL SYSTEM

The *Hupki* mouse embryonic fibroblasts have proven to be an invaluable *in vitro* model system for mutagenicity testing of various physical or chemical carcinogens (32–38). The system has appeared metabolically competent by converting all the tested chemicals to their reactive metabolites (32–38), and expressing a number of phase I and II metabolic enzymes, including Cyp 1b1, Cyp 1a2, microsomal epoxide hydrolase, NAD(P)H:quinone oxidoreductase, and nitroreductase (33). However, the *Hupki* system has yet to resolve its inherent challenges, including time- and labor-intensiveness, two common drawbacks of the currently available *in vitro* mutagenesis assays (8). The latter hinders generation of a sizable mutation database. Another limitation of the *Hupki* model system, both *in vitro* and *in vivo*, is the intrinsic possibility that the *Hupki* TP53 protein may perform suboptimally in the genetic environment of the murine cells, either due to the absence of other human-specific proteins that interact with TP53, or because of blocking of its function by mouse-specific proteins. Also, uncertainties remain about the ability of the *Hupki* TP53 protein to function exactly as expected in a genetically engineered environment. For exam-

ple, species-specific differences in phosphorylation sites within the substituted polyproline domain of the *Hupki* model system may lead to a differential post-translational regulation of TP53 function in this model system (39, 74, 75). Moreover, the ability of the *Hupki* TP53 protein to participate in processes of DNA repair and recombination (76, 77) remains a matter of uncertainty. Despite the overall conservation in evolution of DNA repair mechanisms, differences exist between humans and mice, such as the efficiency of the global genomic repair subpathway of nucleotide excision repair (78). Such discrepancies may prove problematic because promutagenic lesions in the *Hupki TP53* gene are subject to the murine DNA repair machinery (33). Altogether, although the *Hupki TP53* model system has recapitulated many aspects of *TP53* mutagenesis and human cancer (31–40), future studies will determine the extent to which this model system can precisely delineate human *TP53* mutagenesis and carcinogenesis.

CONCLUSIONS

Inactivating mutations in the *TP53* tumor suppressor gene occur frequently in a variety of human cancers (19–22). The pattern (spectrum) and frequency distribution (hotspots) of mutations in the *TP53* gene are tumor-specific and reflective of past mutagen exposure (11). Thus, investigating human *TP53* mutagenesis in relation to exposure to mutagenic agents can provide information on the underlying etiology of human cancers. The *Hupki* mouse model system was constructed using gene-targeting technology to create a humanized replica of the *TP53* gene in mouse, thus allowing for experimental recapitulation of human *TP53* mutagenesis (31). The utility of the *Hupki* mouse model system for studying the etiologic involvement of suspect environmental mutagens in human *TP53* mutagenesis has been demonstrated in a number of *in vivo* animal experiments (35, 40) and/or *in vitro* cell culture experiments (32–34, 36–38, 52). For the most part, these investigations have reproduced the respective human *TP53* mutagenesis data obtained from populations with documented exposure to mutagens of interest. A recent study has also shown the validity of the *Hupki* mouse model system for portraying upstream events leading to, and downstream events caused by, human *TP53* mutagenesis (64). Further studies of such design must determine the accuracy of the *Hupki* mouse model system for representing the events preceding, and those following, human *TP53* mutagenesis *in vivo*. Another important area of research, which awaits further exploration, is establishing the status of CpG methylation in the substituted segment of the *Hupki* mouse genome. The methylated CpGs (mCpGs) in this segment in the human genome constitute the single most significant mutational target in the *TP53* gene (11). The importance of mCpGs in human *TP53* mutagenesis is borne out by the observation that *TP53* mutational hotspots in certain types of human cancers localize almost exclusively to mCpG-containing codons (*e.g.*, the majority of both lung and colon cancer mutational hotspots have mCpGs in their sequence contexts, or nonmelanoma skin cancer mutational hotspots cluster at pyrimidine-mCpG sequence contexts) (11). FJ

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