Common tumour p53 mutations in immortalized cells from Hupki mice heterozygous at codon 72.

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Abstract
Codon 72 of human p53 gene is polymorphic, encoding arginine or proline. Here we report construction of a human p53 knock-in (Hupki) mouse encoding the codon 72 pro variant. The new strain was crossed with the original Hupki mice (codon 72 arg/arg) to obtain primary embryonic fibroblasts polymorphic at codon 72 or homozygous for codon 72 pro. The fibroblasts, cultured under standard conditions, immortalized within 12 weeks and acquired p53 mutations similarly to Hupki codon 72 arg/arg cells investigated previously. Sequencing of human p53 exons 4–9 in immortalized cultures revealed missense mutations found repeatedly in human tumours. In cell lines ensuing from benzo(a)pyrene-treated cultures the combined p53 mutation pattern from experiments with the 3 codon 72 genotypes showed a predominance of strand-biased G to T transversions (18 of 36 mutations), and mutations recurring at smokers’ lung tumour hotspot codons 157 and 273, supporting involvement of tobacco carcinogens in shaping the mutation signature in lung cancers of smokers. Mutations in cell lines from unexposed cultures did not cluster at these codons and G to T transversions were uncommon (2 of 52 mutations) [Fisher’s exact test P<0.0001]. Most mutations (13/16) in cell lines derived from cells polymorphic at codon 72 were found on the proline allele, with loss of the arginine allele.

Keywords
lung cancer; mutation hotspot; embryonic fibroblasts; senescence; polymorphism

We recently described a way to select and identify base changes induced by mutagens in human p53 sequences [Liu et al., 2004], based on the findings of several investigators that senescence bypass occurs readily in mouse cells, and requires only one crucial genetic step such as loss of p53 function [Harvey and Levine, 1991; Hahn and Weinberg, 2002]. The procedure we use scores mutations in immortalized cultures derived from mouse fibroblasts isolated from Hupki (human p53 knock-in) [Luo et al., 2001] mouse strain embryos (HUFs).
By repeatedly passaging a set of independent cultures, immortalized cell lines are readily established and can be directly sequenced for the presence of a p53 mutation. With this approach we showed that p53 mutant cell lines arising from cultures exposed to human carcinogens often harbour the type of base substitution predicted from the miscoding properties of the primary pre-mutagenic DNA adducts, and that mutations selected for in immortalization of Hupki cells in vitro correspond in large measure to major features of the human tumour p53 mutation spectrum [vomBrocke et al., 2006].

The human codon72 polymorphism

In human populations codon72 of the p53 gene is polymorphic, encoding either arginine or proline. Although the codon72*arg allele has been designated in the past as ‘wild-type’ and is the more prevalent allele in individuals of Northern European origin, codon72*pro is the more common allele in other ethnic groups [Pietsch et al., 2006]. The polymorphism at codon72 has received considerable attention because it affects certain critical biochemical properties of the p53 protein [Thomas et al., 1999; Dumont et al., 2003]. Numerous, often conflicting studies suggest an influence of the polymorphism not only on cancer susceptibility and response to therapy, but also on the frequency and biological impact of somatic p53 mutations [Pietsch et al., 2006; Storey et al., 1998; Marin et al., 2000; Bergamaschi et al., 2006].

Generation of a codon72 polymorphic variant of the Hupki mouse model.

The humanized p53 locus of the prototype Hupki strain we generated to obtain primary fibroblasts for mutagenesis studies encodes arginine at codon 72 [Luo et al., 2001; Jackson Laboratory designation for Hupki; Trp53*Tm1/holl, referred hereafter in this report as Hupki codon72*arg/arg]. Our first aim in the present study was to generate a second Hupki strain that encodes proline at codon 72 which could then be crossed with the previously constructed Hupki codon72*arg/arg strain. The two Hupki strains would allow preparation of HUFs for p53 mutagenesis experiments that present all human p53 codon 72 configurations in humans. Toward this end, a targeting vector and strategy were employed (Figure 1A) essentially as described previously to generate the prototype strain and germline p53 mutant derivative strains [Luo et al., 2001; Song et al., 2007]. The Hupki codon72*pro/pro strain we obtained (Figure 1B), which was viable and phenotypically normal, was then bred with the prototype Hupki*arg/arg strain, and F2+ heterozygote and homozygote 13.5 day embryos were harvested for preparation of fibroblasts for immortalization studies.

In our previous work with the prototype Hupki codon72*arg/arg mice, we observed that in vitro senescence, immortalization after prolonged culturing, and selection of dysfunctional p53 mutations occurred in a manner comparable to that described previously for wild-type murine embryonic fibroblasts (MEFs) with a normal, unmodified murine p53 locus [Liu et al., 2007]. Our next aim in the present study was to determine whether HUF codon72*arg/pro and HUF codon72*pro/pro primary cultures also displayed this wild-type behaviour in immortalization studies. As anticipated, both codon72*arg/pro and codon72*pro/pro HUF primary cultures showed typical features of senescence by passage 6 (after several weeks in culture, under standard cell culture conditions) and readily underwent immortalization, often
within 12 weeks (Figure 1c). When seeded (p.1) at $2 \times 10^4$ cells/cm$^2$, grown under standard conditions (5% CO$_2$ in normal DMEM medium supplemented with 10% FCS), and serially passaged when confluent at 1:2 to 1:4 dilution, most cultures (>95%) immortalized spontaneously. Established cell lines from 7 independent experiments, each consisting of 24 primary cultures (with some attrition due to contamination during the 3–4 months in culture) were screened for the presence of a p53 mutation. Approximately one in every 5 cell lines harboured a single mutation, typically a missense base substitution in the DNA binding domain corresponding to a mutation also found in multiple human tumours, (Table 1A). As seen in HUF codon72$^{arg/arg}$ control experiments, G:C to C:G substitutions were the most common sequence change in the cell lines from untreated cultures. We are currently investigating whether these ‘spontaneous’ mutations are induced by specific reactive oxygen species components arising from the unphysiologically high oxygen levels of standard culture conditions, recently shown to be responsible for triggering senescence in primary fibroblast cultures [Parrinello et al., 2003].

B(a)P mutagenesis in the codon72 polymorphic Hupki model

Human tumour p53 mutation patterns vary by cancer type and patient exposure group, explained in part by the fact that different risk factors elicit distinct mutation spectra [Olivier et al., 2002; Hainaut and Hollstein 2000]. The p53 mutation profile of smokers is unequivocally different from the pattern in tumours of non-smokers [Hainaut and Pfeifer, 2001]. The tobacco smoke mutation signature in lung cancers is characterized by a high prevalence of G to T base substitutions on the non-transcribed strand, clustered at specific locations in the p53 coding sequence, notably at codons 157 and 273. A landmark DNA adduct mapping study showed a correlation between preferred sites of adducted bases in the human p53 gene sequence of human bronchial epithelial cells exposed to benzo(a)pyrene (BaP) and hotspots of mutation in lung tumours of smokers, supporting the notion that exposures to BaP and other polycyclic aromatic hydrocarbons in tobacco smoke may indeed be directly responsible for inducing characteristic mutation signatures found in smokers’ lung cells [Denissenko et al., 1996]. The next key test of this hypothesis is to perform mutation mapping in the human p53 gene of cells exposed experimentally to tobacco carcinogens. It is important to use the human sequence as mutagen target because base sequence context is a fundamental determinant of mutation spectra. This requirement was met in our preliminary mutagenesis experiments with codon72$^{arg/arg}$ HUFs, and showed that G to T p53 mutations were prevalent in immortalized cell lines from the BaP-exposed HUFs [Liu et al., 2005].

In the present study, our final objective was to generate a composite BaP mutation spectrum in the Hupki model representing all human codon72 configurations. To this end, we exposed independent sets of HUF$^{arg/pro}$ and HUF$^{pro/pro}$ cultures to 1uM BaP for 6–9 days, and then passaged the cultures successively to obtain immortalized cell lines to be sequenced for the presence of a p53 mutation. Fifteen cell lines harboured p53 point mutations, 3 of which had 2 mutations each (Table 1B). Half the mutations (9/18) from BaP-treated HUF$^{arg/pro}$ or HUF$^{pro/pro}$ were G to T transversions (Table 1B), most of which (8 of 9) were oriented with the pre-mutated guanine on the non-transcribed strand, consistent with results in cell lines
from BaP-treated HUF$_{\text{arg/arg}}$ cultures reported previously which also showed predominance of G to T substitutions [Liu et al., 2005].

**Composite spectrum:**

The new data on BaP-exposed and control codon72$_{\text{arg/pro}}$ and codon72$_{\text{pro/pro}}$ HUFs were combined with previous data on codon72$_{\text{arg/arg}}$ HUFs to produce the composite profiles shown in Figure 2. In 5 experiments with BaP-treated HUFs isolated from 5 different embryos representing all codon72 genotypes, a total of 18 G to T transversions were found among the 36 p53 mutations, 17 of which were oriented with the pre-mutated guanine on the non-transcribed strand. Among the 52 mutations in cell lines from control (untreated) cultures, only 2 were G to T transversions (G to T mutations, BaP vs. untreated: Fisher’s exact test: p <0.0001), and they were found in codon 176 and codon 275, not at lung cancer mutation hotspots (see Table 2). Frequent strand-biased G to T transversion is a distinguishing feature of lung tumour p53 mutations in smokers, particularly when occurring at hotspot codons 157, 158, 273 [Pfeifer and Hainaut, 2003], and DNA repair has been shown to play an important role in this [Denissenko et al., 1998]. In cell lines from BaP-treated HUFs, codons 157 and 273 are also recurrent sites of G to T mutations in independent experiments (codon 157: 5 occurrences, 3 are G to T; codon 273: 5 occurrences, 2 are G to T), whereas no such mutations were found in control lines. The concordant BaP-associated p53 hotspots at codons 157 and 273 in the Hupki model and smokers’ lung tumours are notable because separate experiments and embryos with different codon72 genotypes contributed to the final tally, thus they cannot be ‘jackpot’ mutations. Given that the diol epoxide of BaP preferentially binds at codons with a methylated cytosine [Denissenko et al., 1997; Chen et al., 1998], differences in methylation status among cell types may shift mutation frequencies at specific CpG mutation sites, depending on the cell system under study, possibly accounting for the lack of G to T mutations at the codon 248 hotspot in the Hupki cell lines.

It has been proposed that cancer type-specific hotspot p53 mutations observed in human tumours are due to tissue-specific impact of the respective mutant protein on neoplastic development, enhancing its selection in a particular tissue/cell type. The recapitulation of codon 157 as BaP hotspot in HUFs suggests a simpler scenario in this case, whereby base sequence context and preferential binding of an electrophile to a specific base conspire to produce an exposure-associated mutation hotspot. It will be interesting to learn whether acrolein, a direct-acting tobacco mutagen, recently shown to bind to guanines at lung cancer p53 gene hotspots, also induces codon 157 mutations in the HUF assay [Feng et al., 2006].

Enhanced risk of cancer due to p53 codon72 genotype, and preferential mutation or LOH in individuals polymorphic at codon72 have been investigated repeatedly in studies with clinical samples; however, a consistent picture has yet to emerge. In two of 3 large non-small cell lung cancer cohorts, a higher mutation frequency on codon72 proline alleles was reported [Hu et al., 2005; Szymanowska et al., 2006; Nelson et al., 2005]. In the present experimental study, 13 of 16 cell lines from HUF codon72$_{\text{arg/pro}}$ heterozygous cultures with p53 mutation and LOH harboured the mutation on the proline allele with loss of the arginine allele (Table 1B and data not shown), but the trend was not statistically significant.
Regardless of p53 codon72 status, there is general agreement among studies of various cancer types that in cancers with mutant p53, the non-mutated allele is often deleted in tumours, indicating selective pressure to escape residual p53 function. The precise mutation, in particular the degree of dominant negative activity, may be an important factor influencing loss of the unaffected allele [Dearth et al., 2007]. Exposure to cigarette smoke increases LOH at tumour suppressor loci, and may provide an additional, independent contribution to deletion events [Zienolddiny et al., 2001]. Further mutagenesis studies using the codon72<sup>arg/pro</sup> HUFs may help to clarify the factors involved in preferential LOH of either allele. In addition, the HUF cell line panel representing human p53 DNA-binding domain mutations in <i>cis</i> with codon72<sup>arg</sup> or codon72<sup>pro</sup> provides a tool to explore the impact of the polymorphism on mutant p53 behaviour.

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**Abbreviations:**

- **Hupki** human p53 knock-in
- **BaP** benzo(a)pyrene
- **HUF** Hupki embryonic fibroblasts

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Figure 1.
Generation of new Hupki strain encoding proline at codon 72.
A. Targeting strategy. The CGC to CCC base substitution at codon 72 of the human p53 genomic DNA fragment (exons 4–9) of the Hupki targeting construct [Luo et al., 2001] was introduced by site-directed mutagenesis. Homologous recombinants from electroporated ES 14.1 cells were injected into C57Bl/6 blastocysts, producing chimeric offspring that were bred to generate progeny transmitting the Hupki codon72^pro variant in Exon 4 (asterisk) through the germline. Mice from these colonies were bred with the Hupki codon72^arg/arg strain to generate Hupki codon72^arg/pro heterozygote mice. B. Southern blot analysis of genomic DNA from tails of Hupki codon72^pro/pro and Hupki codon72^arg/pro mice and sequencing of exon 4 confirmed the genotypes. Lane a: WT strain 129, with endogenous
murine p53 (mp53\textsuperscript{WT}). Lane b, Hupki codon72\textsuperscript{arg/arg} (prototype strain; Luo et al., 2001). Lane c, Hupki codon72\textsuperscript{arg/pro} heterozygote. Lane d, Hupki codon72\textsuperscript{pro/mp53\textsuperscript{WT}} heterozygote. Lane e, Hupki codon72\textsuperscript{pro/pro}. Fragment I: Hupki allele; Fragment II: mp53\textsuperscript{WT} allele; [Fragment III: mouse pseudogene]. DNA was digested with Bgl II and BamHI, and probed with sequences corresponding to murine p53 exon 11. Electropherograms showing the p53 codon72 site in genomic DNA from Hupki\textsuperscript{arg/arg}, pro\textsuperscript{pro}, and arg\textsuperscript{pro} mouse tails are shown on the right, panels 1–3. C. Phase contrast photomicrographs of primary (p.1), senescent (p.7) and immortalized (p.17) HUF codon72\textsuperscript{pro/pro} cultures. (40x) (Panels 1–3).
Figure 2. P53 Mutation patterns in immortalized cell lines from untreated and from benzo(a)pyrene-treated HUF cultures (all codon72 genotypes combined).
A. Type of base substitutions in human p53 coding sequences in immortalized cell lines from untreated HUF cultures (clear bars) and B(a)P treated (filled bars) cultures, all codon72 genotypes [Data are combined from this report (HUFarg/pro and HUFpro/pro Table 1), and from published data on HUFarg/arg cells [Liu et al., 2004, 2005 and Feldmeyer et al, 2006]. G to T mutations (*) are far more prevalent in p53 mutant cell lines from B(a)P treated cultures than in cell lines from untreated cultures, (2/52 vs. 18/36; Fisher's exact test, p<0.0001) and reveal a strong strand bias: 17/18 G to T mutations occurred at sites where the pre-mutated guanine was positioned on the non-transcribed strand.
B. Distribution of the 29 base substitutions along the p53 coding sequence in cell lines derived from B(a)P-treated HUF
cultures (all HUF genotypes combined). (Note: Of 36 p53 sequence changes in cell lines from B(a)P treated cultures, 29 were single base substitutions in codons shown here. Additional single base substitutions were at splice sites (not depicted).
Table 1.

P53 mutations in cell lines from codon72\textsuperscript{arg/pro} and codon72\textsuperscript{pro/pro} HUFs.

| Location of mutation | No. of mutations | No. of missense mutations\textsuperscript{1} | No. of missense mutations found in \geq 5 human tumours | Frequently mutated codons |
|----------------------|------------------|----------------------------------------|--------------------------------------------------|-----------------------------|
| Exon 4               | 1                | 1                                      | 1                                                | 176 (3x)                     |
| Exon 5               | 10               | 10                                     | 9                                                | 245 (6x)                     |
| Exon 6               | 2                | 2                                      | 2                                                |                             |
| Exon 7               | 10               | 9                                      | 9                                                | 281 (4x)                     |
| Exon 8–9             | 6                | 6                                      | 6                                                |                             |
| Splice (intron)      | 2                | 28                                     | 27                                               |                             |
| TOTAL                | 31               | 28                                     | 27                                               |                             |

\textsuperscript{1}G:C to C:G mutations were the most common (58%) followed by G:C to A:T transitions (16%). Two G:C to T:A transversions (6%) were found.

B. P53 Mutations in cell lines established from HUF codon72\textsuperscript{arg/pro} and codon72\textsuperscript{pro/pro} cultures exposed to benzo(a)pyrene.

| Unique cell line ID | Mutated codon | WT codon sequence | Mutated codon sequence | Base change | Amino acid change | Primary culture codon72 genotype | Cell line mutant codon72 genotype |
|---------------------|---------------|-------------------|------------------------|-------------|------------------|----------------------------------|----------------------------------|
| L84                 | 36            | CCG               | xCG                   | del C       | P/-              | R/P                             | (het)*                            |
| L82                 | 119           | GCC               | GCC                   | C → G       | A/G              | R/P                             | (het)*                            |
| L83                 | 135           | TGC               | TGG                   | C → G       | C/W              | R/P                             | (het)                            |
| L75                 | 141           | TGC               | TGG                   | C → G       | C/W              | P/P                             | P                                |
| L87                 | 144           | CAG               | CAT                   | G → T       | Q/H              | R/P                             | (het)                            |
| L76                 | 153           | CCC               | CT-                   | C → T, delC | P/-              | R/P                             | (het)*                            |
| L79                 | 157           | GTC               | TTC                   | G → T       | V/F              | P/P                             | P                                |
| L82                 | 157           | GTC               | TTC                   | G → T       | V/F              | R/P                             | (het)*                            |
| L85                 | 170           | ACG               | ACx                   | del G       | T/-              | R/P                             | (het)                            |
| L76                 | 216           | GTG               | TTG                   | G → T       | V/L              | P/P                             | P                                |
| L74                 | 248           | CGG               | CCG                   | G → C       | R/P              | P/P                             | P                                |
| L86                 | 273           | CGT               | GGT                   | C → G       | R/G              | R/P                             | P                                |
| L81                 | 273           | CGT               | TGT                   | C → T       | R/C              | R/P                             | P                                |
| L78                 | 273           | CGT               | CTT                   | G → T       | R/L              | P/P                             | P                                |
| L84                 | 273           | CGT               | CTT                   | G → T       | R/L              | R/P                             | (het)*                            |
| L77                 | 281           | GAC               | GAA                   | C → A       | D/E              | P/P                             | P                                |
| L77                 | 282           | CGG               | CTG                   | G → T       | R/L              | P/P                             | P                                |
| L88                 | intron 4      | ag                | at                    | G → T       | splice           | R/P                             | P                                |

Abbreviations: ‘n’ in the final column indicates the cell line harbours more than one p53 mutation; het, heterozygous mutation.
Table 2.
Prevalence of G to T mutations at selected codons of p53 in human lung, brain and colon tumours

A. Cancer type | No. of mutations in IARC Database | G to T mutations at codon 157 | G to T mutations at codon 273
---|---|---|---
lung | 2544 | 53 (2.08%) | 52 (2.04 %)
brain | 1522 | 7 (0.46%)* | 4 (0.26 %)*
colon | 974 | 5 (0.51%)** | 3 (0.31 %)*

*Fisher’s exact test p<0.0001 ** p<0.0005

B. Cancer type | No. of mutations in IARC Database | G to T mutations at codon 176 | G to T mutations at codon 275
---|---|---|---
lung | 2544 | 12 (0.47 %) | 6 (0.23 %)
brain | 1522 | 6 (0.39 %) ns | 8 (0.52 %) ns
colon | 974 | 4 (0.41 %) ns | 1 (0.10 %) ns

ns, not significant

The prevalence of G to T transversions at the hotspot codons 157 and 273 in human lung tumours harbouring a p53 mutation is significantly higher than the prevalence of these mutations in brain or colon tumours (A). For comparison, note that at codons 176 and 275, which are not lung tumour hotspots, the G to T prevalence is not exceptional in lung tumours (B).