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Influence of Glutathione S-Transferase pi and p53 Expression on Tumor Frequency and Spectrum in Mice

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The role of glutathione S-transferase π (GSTπ) in tumor development has been previously suggested; however the exact function of this enzyme in carcinogenesis remains unclear. GSTπ has been identified as a modulator of cell signaling by interacting with and inhibiting c-Jun N-terminal kinase (JNK). This kinase has been in turn described as a regulator of p53 stability and transcriptional activity. To study the possible interaction between GSTπ and p53, we crossed GSTπ-deficient animals with $p53^{-/-}$ mice. Double knock out animals were viable but developed tumors within 6 months of age; the life span of these animals was however similar to that of $GST\pi^{+/-}/p53^{-/-}$ and $GST\pi^{+/+}/p53^{-/-}$. Mice heterozygous for p53 lived significantly longer than the $p53^{-/-}$ animals and developed tumors much later, and the expression of GSTπ did not significantly modify the life span of the animals. In contrast, in a wild-type p53 background, $GST\pi^{-/-}$ mice developed tumors with a significantly higher frequency than heterozygous and wild-type animals with a median tumor free life span 20 weeks shorter. In addition, in $p53^{+/+}$ background, one third of the $GST\pi^{-/-}$ animals developed lung adenomas, while less than 10% of $GST\pi^{+/-}$ and $GST\pi^{+/+}$ presented such tumors. GSTπ expression did not alter the expression of tumorigenesis markers such as COX-2 or ornithine decarboxylase in response to phorbol ester. Furthermore, GSTπ-deficient mouse embryo fibroblasts were more sensitive to H_2O_2-induced apoptosis. $P53^{-/-}$ cells, independent of GSTπ status, were more sensitive to UV and other DNA damaging agents than their wild-type counterparts. These results suggest that GSTπ may play a protective role in the development of spontaneous tumors.

Key words: glutathione S-transferase π; inhibitor; mitogen activated protein kinase; tumor development; p53

Glutathione S-transferases (GST) are ubiquitously expressed in plants and animals and have diverse roles in the conjugation of glutathione to electrophilic species. In mammals, 6 different isoforms α, μ, π, θ, ω and ζ have been identified.[1] GSTπ overexpression has been observed in many tumors as compared to the surrounding normal tissues and in various cancer cell lines resistant to anticancer agents.[2] However, the precise role that this enzyme plays in resistance to anticancer drugs remains ill-defined, particularly since GSTπ transfection does not always confer resistance to chemotherapeutic agents.[3] More recently, it has been shown that GSTπ acts as a regulator of mitogen-activated protein (MAP) kinases. GSTπ is an endogenous inhibitor of c-Jun NH_2-terminal kinase (JNK), mediated by interactions with the N-terminal region of the kinase.[4,5] Following oxidative stress, it has been hypothesized that GSTπ oligomerizes and disassociates from JNK, which then becomes phosphorylated.[4] GSTπ can also modulate the activation of p38 and extracellular-regulated kinase (ERK). For example, in NIH 3T3 cells, the forced expression of GSTπ inhibits JNK activity and activates ERK and p38 kinase.[6] In addition, in GSTπ deficient mouse embryo fibroblasts, basal activities of JNK and ERK are higher than in their wild-type counterparts.[4,7]

GSTπ expression has been shown to play a role in chemically induced tumorigenesis. GSTπ-deficient mice had a higher incidence of skin tumors following treatment by 7,12-dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (PMA) than their wild-type counterparts.[8] These observations could be correlated with the function of GSTπ in the metabolism of polycyclic aromatic hydrocarbons.[9] It is also well established that GSTπ is overexpressed with high frequency in a wide variety of tumors including breast, colon, oesophagus, kidney and lung, while its level in normal surrounding tissues is relatively low.[3] In contrast, GSTπ expression is lost in other cancer types including prostatic and hepatocellular carcinomas, a process associated with hypermethylation of the enzyme's promoter.[10] However, the involvement of GSTπ in the tumorigenesis process is still not well defined.

Over the past decades, the carcinogenesis process has been extensively studied. It has been shown that the lack of expression, or the mutation, of tumor suppressor genes such as p53 represent crucial steps in tumorigenesis. P53 is mutated in over 50% of human cancers.[11] In addition, p53-deficient mice develop tumors faster than heterozygote animals, which also have a higher tumor frequency than wild-type animals.[12] Presumably, the role played by p53 in tumor development is associated with its function as a sensor of DNA damage and its involvement in maintaining genome stability and integrity. Following treatment by DNA damaging agents, cells upregulate p53, mainly by increasing protein stability. As a transcription factor, this molecule can increase the expression of genes involved in cell cycle arrest, such as $p21^{WAF1}$, or in apoptotic pathways, including Bax and Fas.[13] Stability and consequently transcriptional activity of p53 are regulated by numerous proteins; among those, JNK plays a dual role on p53 cellular levels. In unstressed cells, JNK can interact with p53 and potentiate its proteasomal degradation. In contrast following stress, JNK can phosphorylate and stabilize p53 and then can potentiate its cellular function.[14,15]

Recent indications are that GSTπ has important cellular functions related to its ligand binding capacity to produce protein: protein interactions. In consequence, certain signaling pathways are regulated in a manner that influences cellular responses to chemical insults. We reasoned that the GSTπ phenotype may also alter the impact of tumor suppressor genes such as p53. As such, we initiated a series of crosses of deficient mouse strains to analyze the impact of null phenotypes on tumorigenesis and apoptosis in response to stress stimuli.

Material and methods

Chemicals and antibodies

Doxorubin, Melphalan, Cisplatin, Vinblastine, Paclitaxel, PMA and H_2O_2 were purchased from Sigma Chemical Co. (St. Louis, MO). TLK286 [(γ-glutamyl-α-amino-β-(2-ethyl-N,N,N,N-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl-(R)-(−)phenylglycine) and TLK199 (γ-glutamyl-*S*-(benzyl)cysteinyl-*R*-phenyl glycine diethyl ester] were provided by Telik (Palo Alto,

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CA). COX-2, ERK2, phospho-ERK and p38 antibodies were purchased from Santa Cruz (Santa Cruz, CA); Phopho-JNK1/2 and phospho-p38 were from Promega (Madison, WI); JNK1/2 antibody was from BD Biosciences (Bedford, MA) and GSTπ antibody was from MBL (Watertown, MA). Secondary antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Mouse strain

C57/BL6x123v GSTπ$^{-/-}$ mice, kindly supplied by Dr. C.J. Henderson (Cancer Research UK Molecular Pharmacology Unit, Dundee, UK),[8] were bred with C57/BL6x123v p53$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME). Offspring were genotyped and sacrificed when they became moribund or were presenting visible signs of advanced tumors. Necropsy was performed to assess the cause of sickness.

Mouse genotyping

Mouse genotype was determined by PCR using specific primers for genomic DNA or genomic inserts used for knocking out the genes. Primers used were 5′-GTGTTCCGGCTGTCAGCGCA-3′; 5′-AGCGTCTCACGACCTCCGTC-3′ and 5′-ACACACCTGTAGCTCCAGCAC-3′ for *p53*; 5′-GGACCTTCGCGGCAAATATG-3′ and 5′-CTGGTCACCCACGATGAAAG-3′ for GSTp1p2 and 5′-ACACATGTAAAGCATGTGCACCGAG-3′ and 5′-TTGGAATAAGGCCGGTGTGCGTTTG-3′ for the neocassette used to knock out *GSTp1* and *GSTp2* genes. PCR was performed on Genomic DNA extracted from mouse tail using DNeasy Tissue kit (Qiagen, Valencia CA) according to the manufacturer's instructions.

Mouse embryo fibroblasts

Wild-type, GSTπ$^{-/-}$, p53$^{-/-}$ and GSTπ$^{-/-}$/p53$^{-/-}$ timed pregnant mice were sacrificed, and the uterus was aseptically removed for dissection of the embryos. Embryos were harvested at 14 days. The head and liver were carefully removed and the remaining tissue was finely chopped and trypsinised. The cells were seeded in DMEM media containing 10% FBS, 4 mM Glutamine, 100 UI/ml Penicillin, 100 μg/ml Streptomycin and 1% non-essential amino-acids. Cultures were kept at 37°C for 48 to 72 hr. Primary MEF cell lines were then aliquoted and preserved in liquid nitrogen.

Cytotoxicity assay

Cells were plated at 2×10^3/ml in a 96-well plate and allowed to grow for 24 hr. Increasing concentrations of drugs were added to the cells and 72 hr later, cells were fixed by adding trichloroacetic acid (TCA) at a final concentration of 10% and incubated at 4°C for 1 hr. TCA was then washed with water and the plate was air-dried. Fixed cells were stained by adding 0.4% sulforhodamine B (SRB) in 1% acetic acid to each well and incubating at room temperature for 30 min. Excess SRB was washed out with 1% acetic acid and the plate air-dried; 10 mM of unbuffered Tris base was added to each well and the plate was read at 560 nm using a plate reader. Cell proliferation was determined as percentage of untreated control and IC_{50} for each drug and each cell line was determined.

Apoptosis assay

Percentages of apoptotic cells were determined by using the annexin V assay kit from Guava Biotechnologies, according to manufacturer's instructions. Briefly, cells were plated at 200×10^3/ml in a 6-well plate and allowed to grow for 24 hr. Cells were exposed to cytotoxic agents. At various time points, media and cells were collected by trypsinisation and centrifuged at 500*g* for 5 min. Pellets were resuspended in wash buffer and centrifuged again. Cells were resuspended in wash buffer and mixed with annexin V and 7-AAD reagents. Cells were incubated 20 min on ice in the dark and samples were then analyzed by using Guava personal flow cytometer. Percentages of annexin V positive cells were determined by using the software provided by Guava Biotechnologies.

Immunoblot

Following treatments, cells were collected and washed twice in ice-cold PBS. The final pellets were resuspended in lysis buffer (20 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate and 1 mM Na_2VO_4) and incubated for 30 min on ice. Lysates were sonicated 3×10 sec and centrifuged for 30min at 10,000*g*. The resulting supernatants were considered as whole cell extracts. Protein concentrations were determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on SDS-PAGE gels and transferred overnight onto PVDF membranes (NEN, Boston, MA). Protein expression was determined by using specific primary and secondary antibodies. Briefly, after transfer, membranes were incubated in TBS-Tween containing 5% skim milk, washed with TBS-Tween and incubated with primary antibody in TBS-Tween containing 5% skim milk for 1 hr. Membranes were washed twice with TBS-Tween and incubated with secondary antibody in TBS-Tween containing 5% skim milk for 1 hr and finally washed 3 times with TBS-Tween. When phosphospecific antibodies were used, 5% skim milk was replaced by 10% IgG-free BSA (Jackson Immunoresearch Laboratories, West Grove, PA). Specific proteins were revealed by chemiluminescence using ECL or ECL+ Western blotting reagents from Amersham Pharmacia Biotech (Piscataway, NJ).

Results

Mouse genotyping

Mouse genotypes were determined by PCR. *P53* gene deletion was detected by using 3 primers; the first and second recognized

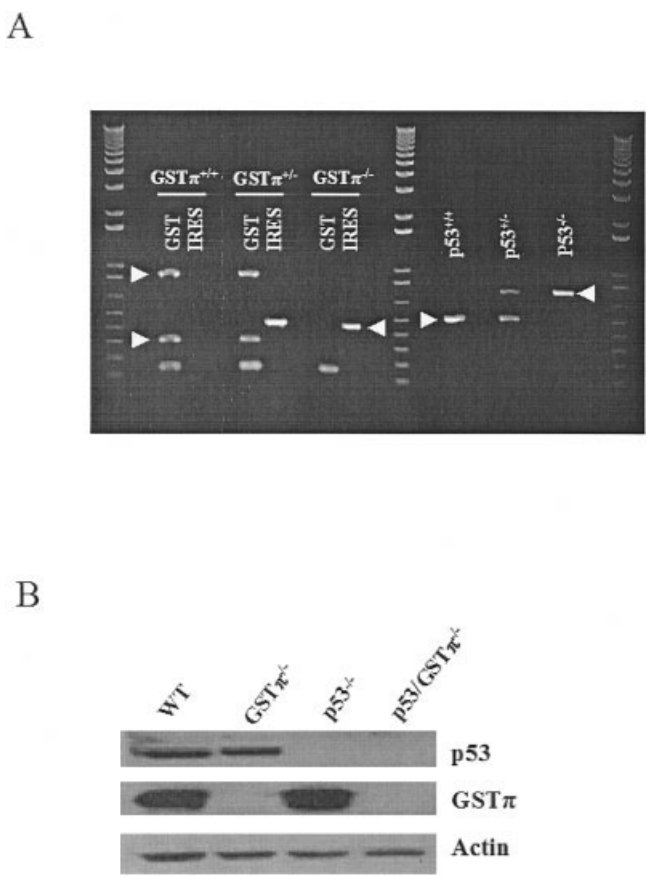

FIGURE 1 – (*a*) Agarose gel used for mouse genotyping by PCR. (*b*) Protein expression of p53 and GSTπ in wild-type, GSTπ$^{-/-}$, p53$^{-/-}$ and GSTπ$^{-/-}$/p53$^{-/-}$ mouse embryo fibroblasts.

the wild-type gene while the third detected specifically the insert used to delete the p53 genomic sequence. After PCR, a 500 bp band is detected if the animal is wild type for p53 and a 700 bp band is visualized if the animal is $p53^{-/-}$; both bands are detected for the heterozygote animal (Fig. 1*a*). As previously shown,[8] in mouse the *GSTπ* gene contains 2 isoforms, *GSTp1* and *GSTp2*, which are located at the same locus. In addition to some amino-acid changes, intron 5 is approximately 600 bp longer in the *GSTp1* than in *GSTp2* gene. We designed primers that recognized both *GSTp1* and *p2* on the flanking exons 4 and 5 to specifically amplify this genomic region. Furthermore we designed primers specific for the neocassette used to delete *GSTp1* and *p2*. For the wild-type animals, 2 bands at 920 and 320 bp are detected using GST-specific primers and no genomic fragment is amplified using oligonucleotides specific for the neocassette. In contrast, when the mouse is GSTπ-deficient, no band is detected with GSTπ specific primers, while a 450 bp band appears on the gel with primers specific for the neocassette. When the animal is heterozygote, bands are detected with both sets of primers (Fig. 1*a*). Expression of p53 and GSTπ in wild-type, $p53^{-/-}$, $GST\pi^{-/-}$ and p53/GSTπ-deficient MEFs was also determined by immunoblot. As shown in Figure 1*b*, p53 expression is only detected in WT and $GST\pi^{-/-}$ fibroblasts, while GSTπ is only expressed in WT and $p53^{-/-}$ cells.

Frequency and spectrum of tumors in GSTπ and p53-deficient animals

We bred GSTπ-deficient animals with $p53^{-/-}$ mice and genotyped the offspring. We observed that pups essentially followed a Mendelian inheritance pattern (Table I). Double knock-out animals $GST\pi^{-/-}/p53^{-/-}$ were viable; however like $GST\pi^{+/-}/p53^{-/-}$ and $GST\pi^{+/+}/p53^{-/-}$ animals, mice developed tumors within 6 months of age. No significant difference in time-dependent tumor appearance was observed. Median survivals were 22.5, 19 and 22 weeks, respectively (Fig. 2, top panel). Mice heterozygous for p53 lived significantly longer than the $p53^{-/-}$ animals and developed tumors later; however the expression of GSTπ did not significantly modify the life span of the animals. The median survival was 61 weeks for $GST\pi^{-/-}$, 65 for $GST\pi^{+/-}$ and 66 for $GST\pi^{+/+}$ animals (Fig. 2, central panel). P53 wild-type mice showed signs of tumors significantly later than the p53-deficient animals. However, in this $p53^{+/+}$ background, $GST\pi^{-/-}$ mice developed cancers significantly sooner than the heterozygous or wild-type animals (Mantel-Cox test, $p<0.05$). The median survival was about 20 weeks shorter in $GST\pi^{-/-}$ mice than in heterozygous or wild-type animals (median survival: 95 *vs*. 116 and 115 weeks, respectively) (Fig. 2, bottom panel).

Figure 3 presents the spectrum of tumors developed by the mice. In a $p53^{-/-}$ background more than two thirds of the animals developed lymphomas independently of the expression of GSTπ. The other main type of cancer developed by these p53-deficient mice was soft tissue sarcoma. However, we also observed that a small percentage of $p53^{-/-}/GST\pi^{-/-}$ animals suffered from carcinomas and mesenteric tumors. A large percentage of mice heterozygous for p53 also developed lymphomas; however their percentage was lower than in p53-deficient mice (40 to 50% in $p53^{+/-}$ *vs*. 66 to 80% in $p53^{-/-}$). In the $p53^{+/-}$ animals, the second most common type of tumor was osteosarcomas in $GST\pi^{+/+}$ and $GST\pi^{+/-}$ mice and soft tissue sarcomas in $GST\pi^{-/-}$. Interestingly, in $GST\pi^{-/-}$ mice the percentage of soft tissue sarcomas was about twice that for the wild-type and heterozygous mice. In contrast, $GST\pi^{+/+}$ rodents presented a higher frequency of carcinomas than the GSTπ-deficient animals. Lymphomas also represented the main type of cancer in p53 wild-type animals. However, the percentage of lymphomas was higher in $GST\pi^{+/+}$ and $GST\pi^{+/-}$ mice than in $GST\pi^{-/-}$ animals (64 and 67 *vs*. 44% respectively). In addition, about one third of $GST\pi^{-/-}$ mice developed lung adenomas, while less than 10% of GSTπ wild-type or heterozygous animals suffered from such tumors.

TABLE I – EXPERIMENTAL AND THEORETICAL PERCENTAGES OF EACH GENOTYPE[1]

	$p53^{+/+}$	$p53^{+/-}$	$p53^{-/-}$
$GST\pi^{+/+}$	5.8 (6.25)	13.0 (12.5)	2.2 (6.25)
$GST\pi^{+/-}$	15.9 (12.5)	29.7 (25)	9.4 (12.5)
$GST\pi^{-/-}$	6.5 (6.25)	12.3 (12.5)	3.6 (6.25)

[1]Offspring from 3 independent matings made with $GST\pi^{+/-}/p53^{+/-}$ male and female mice were genotyped and the experimental percentages of each genotype were determined and compared with theoretical data (brackets) ($n = 138$).

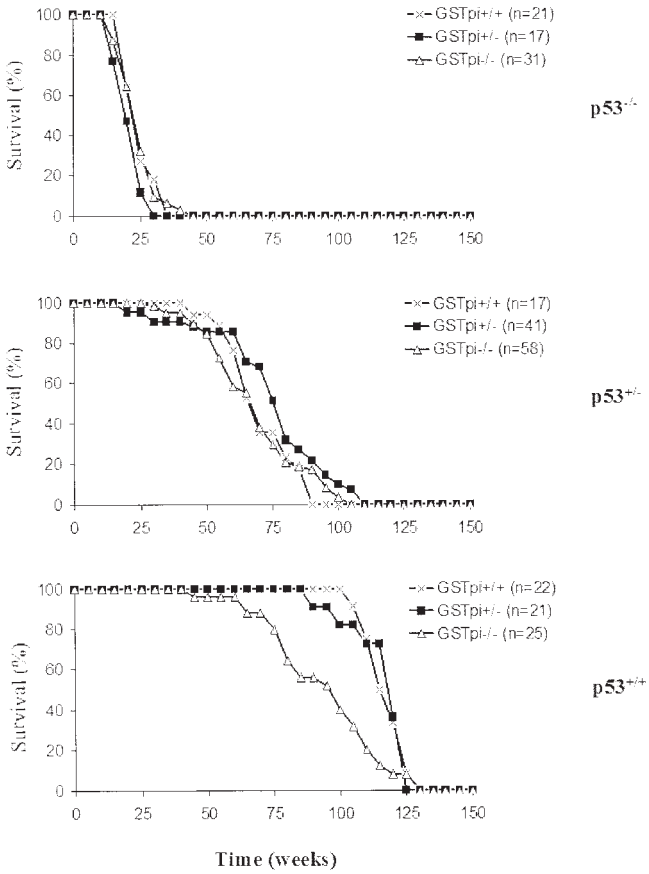

FIGURE 2 – Tumor-free survival of wild-type, heterozygous and $GST\pi^{-/-}$ mice with variable p53 background. Animal life span was assessed based upon time of sacrifice as a consequence of tumor burden.

Sensitivity of mouse embryo fibroblasts to anticancer drugs

P53-deficient MEFs were more sensitive than wild type to the tubulin stabilizing agent paclitaxel (~3-fold) and to DNA damaging agents doxorubicin (~15-fold), cisplatin (~5-fold), melphalan (~16-fold) and the prodrug TLK286 (~3-fold) (Table II). In contrast, no differences in the cytotoxicity of H_2O_2, vinblastine and the GSTπ inhibitor TLK199 were observed in $p53^{+/+}$ and $p53^{-/-}$ cells. Wild-type and GSTπ-deficient MEFs were equally sensitive to all the chemotherapeutic agents tested. In addition, in a p53-deficient background, the expression of GSTπ did not modulate cell sensitivity to the various agents tested.

H_2O_2-induced apoptosis and activation of MAP kinases

Wild-type and GSTπ-deficient fibroblasts were exposed to cytotoxic concentrations of H_2O_2 (100 μM) and percentages of

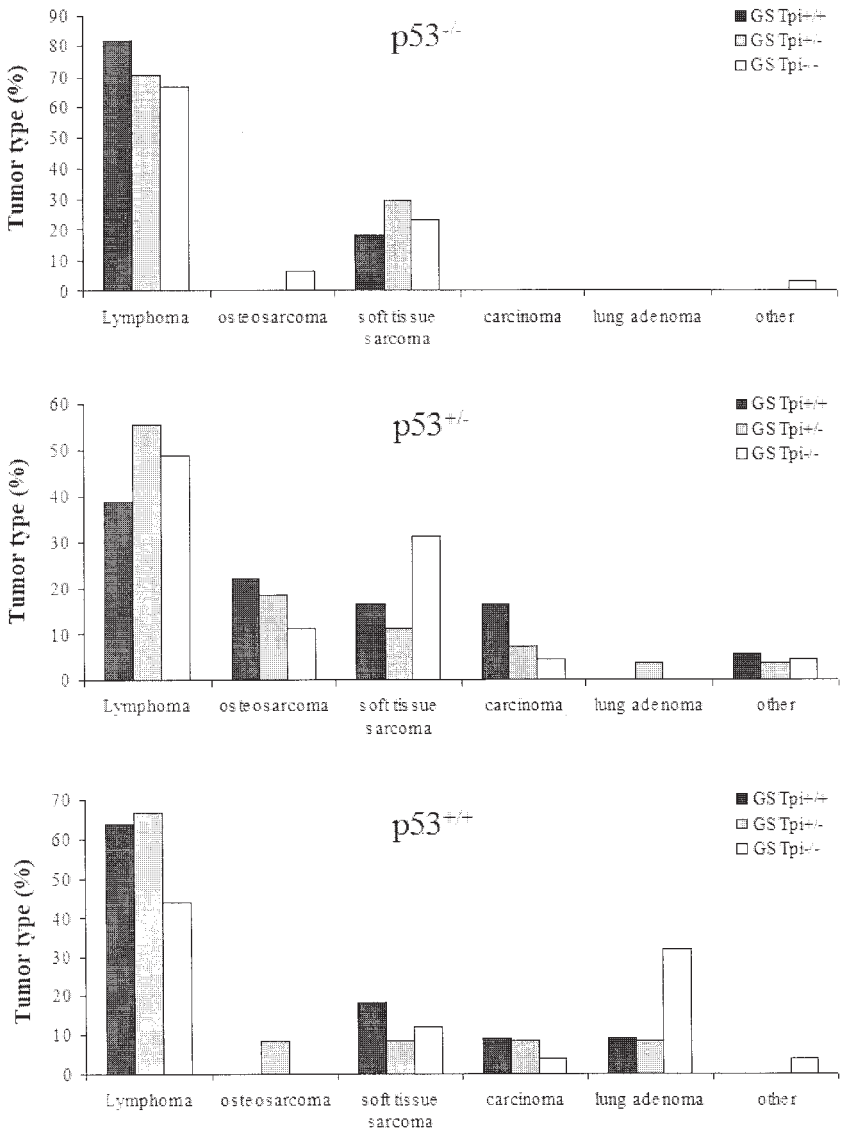

FIGURE 3 – Tumor spectra in wild-type, heterozygous and GST$\pi^{-/-}$ mice with variable p53 background.

TABLE II – SENSITIVITY OF WILD-TYPE, GST$\pi^{-/-}$, p53$^{-/-}$ AND GST$\pi^{-/-}$/p53$^{-/-}$ MOUSE EMBRYO FIBROBLASTS TO ANTICANCER DRUGS[1]

Drug	Wild type	GST$\pi^{-/-}$	p53$^{-/-}$	GST$\pi^{-/-}$/p53$^{-/-}$
Doxorubicin (nM)	42.3 ± 0.3	29.7 ± 0.8 (0.7)	2.4 ± 0.5 (0.06)	2.8 ± 0.1 (0.07)
Melphalan (μM)	70.4 ± 16.6	60.5 ± 5.4 (0.9)	4.3 ± 2.2 (0.06)	7.3 ± 3.5 (0.1)
Cisplatin (μM)	3.8 ± 0.2	5.0 ± 0.6 (1.3)	0.9 ± 0.1 (0.2)	0.9 ± 0.1 (0.2)
TLK286 (μM)	27.5 ± 8.1	30.9 ± 2.8 (1.1)	9.1 ± 1.1 (0.3)	9.6 ± 1.1 (0.4)
H_2O_2 (μM)	25.9 ± 2.7	23.3 ± 1.9 (0.9)	20.5 ± 0.2 (0.9)	23.8 ± 5.5 (0.9)
Vinblastine (nM)	2.9 ± 0.4	6.4 ± 0.8 (2.2)	3.4 ± 0.5 (1.2)	3.8 ± 0.9 (1.3)
Paclitaxel (nM)	1.0 ± 0.2	1.2 ± 0.3 (1.3)	0.3 ± 0.1 (0.3)	0.3 ± 0.1 (0.3)
TLK199 (μM)	36.8 ± 4.4	49.5 ± 0.3 (1.4)	45.6 ± 7.2 (1.2)	48.4 ± 9.9 (1.3)

[1]Results are expressed as concentration of drug inducing 50% of growth inhibition. Resistance levels as compared to wild type are shown in parentheses.

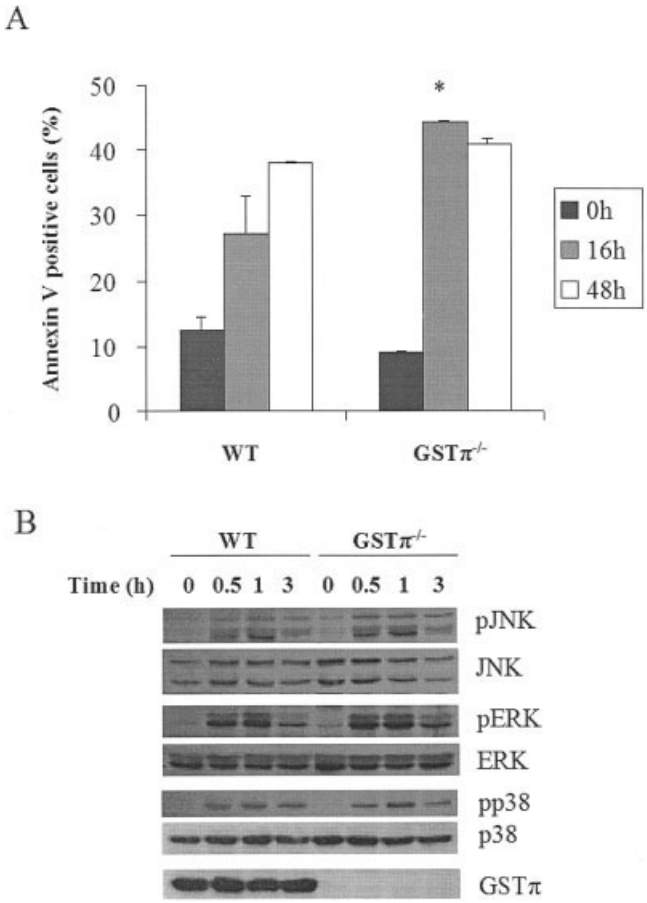

FIGURE 4 – (*a*) Effect of GSTπ expression on H_2O_2-induced apoptosis. Cells were treated with 100 μM hydrogen peroxide for 16 and 48 hr; apoptotic cell percentages were determined by annexin V assay as described in Material and Methods. *Significantly different from wild-type 16 hr (Student's *t*-test, $p<0.05$). (*b*) MAP kinase activation in response to H_2O_2. Cells were exposed to 100 μM hydrogen peroxide and at various time points, cells were collected and MAP kinase expression and phosphorylation were determined by immunoblot.

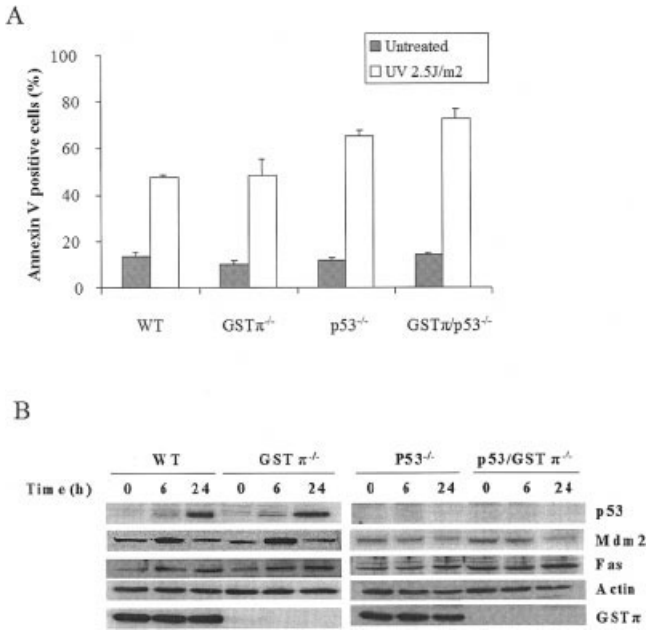

FIGURE 5 – (*a*) Effect of GSTπ and p53 expression on UV-induced apoptosis. Cells were treated with UV (2.5 J/m^2) for 12 hr, apoptotic cell percentages were determined by annexin V assay as described in Material and Methods. (*b*) P53 and p53-inducible gene expression in response to UV. Cells were exposed to UV and at various time points, cells were collected and expression of specific proteins was determined by immunoblot.

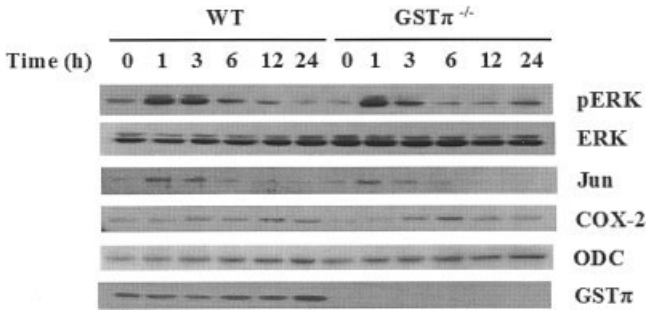

FIGURE 6 – Effect of GSTπ expression on PMA-induced carcinogenesis markers. Cells were exposed to 100 ng/ml PMA. At various time points, cells were collected and protein expression and phosphorylation were determined by immunoblot.

apoptotic cells determined using the annexin V assay. Basal levels of apoptosis were similar in untreated, wild-type and GSTπ$^{-/-}$ cells (about 10%). Despite their equal sensitivity to H_2O_2 as determined by SRB assay, after 16 hr of exposure to hydrogen peroxide, higher percentages of apoptotic cells were observed in GSTπ-deficient cells than in their wild-type counterparts (44.4 ± 1.2 *vs*. 27.1 ± 6.2, respectively). However, after 48 hr, levels of apoptosis, while greater than at 16 hr, were similar in both wild-type and GSTπ-deficient cells (Fig. 4*a*). H_2O_2 (100 μM) stimulated the phosphorylation of ERK, JNK and p38 kinase after 30 min and activation lasted at least up to 3 hr. The GSTπ phenotype did not alter the time course or the intensity of kinase activation (Fig. 4*b*).

UV irradiation-induced apoptosis and p53 activation

Wild-type and deficient MEFs were exposed to 2.5 J of UV for 12 hr and percentages of apoptotic cells determined. P53-deficient cells were more susceptible to apoptosis than wild type, independent of GSTπ expression (Fig. 5*a*), confirming the key role of p53 in protection against DNA damaging agents. UV stimulation of p53 expression was similar in wild-type and GSTπ$^{-/-}$ cells, while no p53 expression was observed in p53$^{-/-}$ cells (Fig. 5*b*). The expression of p53 inducible genes mdm2 and Fas was also determined after UV treatment. Following irradiation, their levels were increased in both WT and GSTπ-deficient MEFs. In contrast, following similar treatments, the expression of mdm2 and Fas was not modified in p53-deficient cells confirming that their expression was under the control of p53.

PMA-induced expression of Jun, COX-2 and ODC

To study the possible function of GSTπ in modifying p53 impact on tumor formation, the induction of genes associated with cellular transformation such as c-Jun, COX-2 and ornithine decarboxylase (ODC) was studied in response to PMA. Cells were serum-starved for 24 hr and then exposed to PMA (100 ng/ml) (Fig. 6). At various time-points, cells were collected, activation of ERK and expression of c-Jun, ODC and COX-2 was determined by immunoblots. In both wild-type and GSTπ-deficient cells, PMA stimulated ERK phosphorylation within 1 hr and lasted up to 3 hr. The basal expression of this kinase did not change over the course of the experiment. In response to PMA, expression of c-Jun, ODC and COX-2 increased. While the increased expression of

c-Jun was transient (up to 3 hr), the upregulation of ODC and COX-2 was more sustained and lasted up to 24 hr. However, the pattern of activation of those proteins was similar in both wild-type and GST$\pi^{-/-}$ cells.

Discussion

GSTπ expression has been implicated in chemically induced tumorigenesis, where it has been shown that GSTπ-deficient mice were more sensitive to skin papillomas induced by DMBA and PMA.[8] Since GSTπ can detoxify metabolites of polycyclic aromatic hydrocarbons [9], it was suggested that these results were the consequence of the catalytic activity of GSTπ. However, oxidative stress can also play an important role in tumor development in response to carcinogens.[16,17] Since GSTπ can protect cells against oxidative stress induced by hydrogen peroxide, possibly by regulating MAP kinase pathways,[6] the absence of the protective role of this enzyme could also explain the elevated frequency of chemically induced tumors in GST$\pi^{-/-}$ animals. Increased sensitivity to oxidative stress has also been observed in GSTπ-deficient mouse embryonic fibroblasts. Despite their apparent similar sensitivity to hydrogen peroxide, GST$\pi^{-/-}$ cells undergo apoptosis faster than the wild type. This could be explained by the fact that GSTπ is an endogenous inhibitor of JNK through ligand binding with its c-terminus. The GSTπ null phenotype leads to an upregulation of JNK activity in GST$\pi^{-/-}$ mice and MEFs as compared to wild type.[4,18] Since this kinase plays an important role in hydrogen peroxide-induced apoptosis,[19] increased JNK activity in GSTπ-deficient cells could cause the faster induction of apoptosis.

Since p53 plays a key role in genome stability and tumor development,[20,21] we decided to study the possible effect of GSTπ expression on tumor development in p53-deficient mice. The phenotype of p53-deficient mice has been well documented.[12] For example, the majority of animals of the C57/BL6x123v mouse strain develop lymphomas independently of the p53 status, although no obvious explanation for this observation is apparent. P53 knockout animals develop tumors (mainly lymphomas) within 6 months of birth, while heterozygous animals show signs of cancer before 1.5 years of age. Expression of GSTπ in p53-deficient animals did not significantly modify the life span and the tumor spectrum in these animals. These results suggest that the influence of p53 on tumor development may mask the possible role of GSTπ in this process. In addition, while p53$^{-/-}$ mice developed mainly lymphomas and soft tissue sarcomas, the tumor spectrum in heterozygous and wild-type animals was more diverse. These results may imply that some tumor types such as osteosarcomas, carcinomas or lung adenomas may take longer to develop and consequently cannot be detected in animals with such a short life span as p53$^{-/-}$ mice. In p53$^{+/-}$ mice, a larger percentage of soft tissue sarcomas was observed in GST$\pi^{-/-}$; such a result may suggest involvement of GSTπ in the development of such tumors. However, the fact that this occurs only in p53$^{+/-}$ animals would argue against a generality to this conclusion. Furthermore, we observed that in a p53 wild-type background, about one third of the GST$\pi^{-/-}$ mice developed lung adenomas, while much lower percentages of wild-type or heterozygous animals showed evidence of such cancer. These observations infer a role for GSTπ in the protection against lung tumor development. These results are consistent with epidemiological studies showing that individuals deficient in GSTπ expression or expressing a catalytically less active form of GSTπ are more susceptible to lung cancer.[1,22,23] Since the lung is the first organ exposed to aerosol carcinogens and polycyclic aromatic hydrocarbons (PAH) are detoxified by GSTπ,[9] it is plausible that the absence of this enzyme could lead to a decrease in carcinogen detoxification and consequently an increase in tumor development. However, ambient PAH exposure should be low in the animal facility environment and could not totally explain the increased frequency of lung adenomas in GSTπ-deficient animals. In addition, GST$\pi^{-/-}$ animals developed tumors faster than with 1 or 2 alleles of the gene, suggesting that there may be some quantitative relationship between GSTπ levels and the transformation process in lung. Those results suggest a protective role of GSTπ during carcinogenesis.

The known involvement of p53 in DNA repair[20,21] is consistent with the higher sensitivity of p53$^{-/-}$ mouse embryo fibroblasts (independent of GSTπ status) to DNA damaging agent such as doxorubicin, melphalan, cisplatin, TLK286 or UV irradiation. Presumably in the absence of p53, the capacity to repair DNA would be compromised and cell exposure to chemotherapeutic agents would result in accumulation of DNA damage leading to cell death. In contrast, discrete accumulation of DNA alterations associated with mild environmental stress would not significantly impact the viability of p53$^{-/-}$ cells and would lead to accumulation of DNA mutations, which could lead to tumor formation.

In unstressed cells, JNK can interact with p53 and potentiate its degradation by the proteasome; in contrast, in response to stress activation, JNK can phosphorylate p53 at threonine 81 and increase its stability.[14,15] Because of the inhibitory role of GSTπ on JNK function, it might be possible that optimal tumor suppression properties of p53 are only observed in the presence of GSTπ. In GSTπ-deficient animals, p53 functions might be altered and this could, at least in part, be responsible for the increased tumor frequency in these animals. However, the shorter life-span of GSTπ-deficient animals might also be independent of p53 expression. Indeed, despite the demonstrated role of p53 mutations in the etiology of many cancers, its involvement is generally in the latter stages of tumor development.[16] Mutations in various genes including Ras family members are believed to be among the earliest steps in transformation. Since GSTπ plays a role in cellular response against reactive oxygen species involved in DNA base alteration, the absence of this enzyme may lead to an increased oxidative stress which could contribute to mutations and tumor formation.

In addition, JNK has been implicated in the control of cell proliferation and transformation. JNK expression is required for cell transformation induced by various oncogenes including Ras[24] and Bcr-Abl.[25] JNK-deficient MEFs have reduced proliferation potential,[26] and JNK activity is required for c-kit-mediated proliferation in mast cells.[27] Furthermore, it was shown that JNK2-deficient mice were more resistant to chemically induced skin carcinogenesis.[28] These results are consistent with our previous work showing that GSTπ null MEFs, which exhibited a higher JNK activity, proliferated faster than their wild-type counterparts.[7] These observations suggest that increased proliferation, probably associated here with the proliferative and oncogenic properties of JNK, may alter the cellular capabilities to repair DNA damage and in consequence favor DNA mutation, a key step in the carcinogenic process.

Because of the role of GSTπ in the regulation of MAP kinase pathways, we studied the influence of its expression on PMA-induced expression of COX-2 and ODC, 2 markers of carcinogenesis.[29–31] Our data indicate that GSTπ status does not impact directly on the activation of ERK or the expression of COX-2 and ODC following PMA treatment, suggesting that these pathways are not directly involved in the spontaneous occurrence of lung tumors in these animals.

In conclusion, our results suggest that GSTπ expression may influence tumor development in response to specific stimuli that are associated with spontaneous tumor incidence.

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