Table 1. Levels of DNA adducts in glandular stomachs of rats receiving sham or duodenal reflux surgery

Group	Weeks after surgery	Adduct level† (adducts/10^8 nucleotides)	
		O^6-CM-dG	3-ESA-dC
Sham	4	5.8 ± 2.3‡	3.1 ± 0.5
	8	5.9 ± 0.5	2.0 ± 1.0
Duodenogastric reflux	4	40.9 ± 9.4**	11.2 ± 1.0*
	8	56.3 ± 3.2**	8.9 ± 1.0**
Duodenogastric reflux + TPRO§	8	3.3 ± 3.4***	1.0 ± 0.3***

†Detection limit: 1 adduct/10^9 nucleotides.
‡Values are the mean ± SD of three analyses.
§TPRO treatment: 0.5%.
*$P < 0.05$, **$P < 0.01$: significantly different from the sham operation group.
***$P < 0.01$: significantly different from the duodenogastric reflux group.
TPRO, thioproline.

Moreover, TPRO inhibited the formation of O^6-CM-dG and 3-ESA-dC to a very large degree. The earlier demonstration that TPRO inhibited the development of gastric adenocarcinoma induced by gastroduodenal reflux in rats[17] is of obvious interest in this context.

It is likely that O^6-CM-dG and 3-ESA-dC are derived from nitrosation of glycocholic acid and taurocholic acid, respectively. Recently, there have been two articles describing O^6-CM-dG detection in human blood DNA and in exfoliated colonic cells by immunostaining systems, and it was also reported that red meat consumption enhances its formation.[29,30] Moreover, it was reported that O^6-CM-dG is in fact a potent mutagen producing G-A transitions and G-T transversions in adducted *p53* cDNA.[31] Thus, it is suggested that O^6-CM-dG could be implicated in cancer development via induction of mutations in the cancer related genes, including *p53*.

It has already been reported that both O^6-CM-dG and 3-ESA-dC are formed by reactions between *N*-nitroso bile acid conjugates (NO-GCA and NO-TCA) and DNA.[21,22] In the duodenogastric reflux model, all duodenal contents, including bile acid conjugates, flow back from the jejunum into the gastric corpus. In general, intragastric formation of nitrosamides could be mediated by acid-catalyzed reactions of amides with nitrite. Therefore, *N*-nitroso compounds, such as NO-GCA and NO-TCA, have been suggested to form in the glandular stomach of rats receiving reflux surgery.[5,6] In fact, it has been reported that taurocholic acid (TCA) is nitrosated in simulated gastric juice.[18] The pH value of gastric juice without reflux is about 2–3; however, under duodenogastric reflux conditions, it increases to 4–5.[11,32] It is also reported that *N*-nitrosation of taurocholic acid is catalyzed by increasing acidity and generally does not react at pH > 5.[18] Meanwhile, nitric oxide is suggested to be produced by activated macrophages in inflamed organs. Actually, on histological examination, gastritis, erosion, foveolar hyperplasia, and pyloric metaplasia were found in the mucosa adjacent to the anastomosis in our model at 4 or 8 weeks after surgery. Therefore, it is suggested that nitrosation of bile acid conjugates could be mediated by both acid catalysis and inflammatory responses in the stomach with duodenogastric reflux models.

As mentioned above, O^6-CM-dG and 3-ESA-dC could be detected in the glandular stomach of the sham operation animals; however, their levels were much lower than those of duodenogastric reflux animals. It has been reported that bile acids are detected in the gastric juice of not only reflux but also sham operation animals, with respective concentrations of 6.0 ± 5.5 mM/L and 0.4 ± 0.2 mM/L.[14] Moreover, Harrison *et al.* demonstrated that O^6-CM-dG is also formed from nitrosated glycine derivatives, including potassium diazoacetate and *N*-acetyl-*N'*-nitroso-*N'*-prolylglycine.[23,33] Similarly, we found that 3-ESA-dC is produced with a reaction mixture of nitrosated taurine and dC (own unpublished data). Thus, O^6-CM-dG and 3-ESA-dC might also be expected to be formed under sham operation conditions.

It is known that the incorporation of bile acids into the epithelial cells of the stomach is governed by the interaction between individual pKa values and the pH of the gastric juice.[11] The pKa values of glycine- and taurine-conjugated bile acids are around 4 and 2, respectively. As described above, the pH value of gastric juice without reflux is about 2–3; however, under duodenogastric reflux conditions, the pH value of gastric juice would be around 4–5. Since it has already been reported that glycocholic acid demonstrated more severe gastric epithelial cell damage than does taurocholic acid under neutral pH,[34] glycocholic acid might much more easily enter into epithelial cells of the stomach than taurocholic acid. In addition, *N*-nitrosation of the amido bonds of bile acid conjugates might not change the physical/chemical characteristics of simple bile acid conjugates, so both NO-GCA and NO-TCA could enter and form O^6-CM-dG and 3-ESA-dC.

In the light of the finding that TPRO inhibits the development of gastric and esophageal adenocarcinoma induced by gastroduodenal reflux in rats,[16,17] our data indicate that nitrosated bile acid conjugates could contribute to cancer development as endogenous mutagens/carcinogens. Since *N*-nitroso compounds, such as NO-GCA and NO-TCA, may be formed by nitrosation of glycocholic acid and taurocholic acid in the human body, they could play a crucial role in cancer development as endogenous mutagens/carcinogens. Further studies of the biological significance of both adducts are warranted.

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Mutagenicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6) and benzo[*a*]pyrene (BaP) in the gill and hepatopancreas of *rpsL* transgenic zebrafish

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ABSTRACT

We examined the *in vivo* mutagenicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6) and benzo[*a*]pyrene (BaP) by using transgenic (Tg) zebrafish carrying the mutational target gene *rpsL*. PBTA-6 is one of the PBTA-type compounds that were recently identified in highly mutagenic river water in Japan. BaP is a well-known contaminant that is frequently found in polluted water. Both compounds are potent mutagens, as determined by using the Ames test employing S9 mix and *Salmonella*. Adult *rpsL* Tg zebrafish were exposed to 0, 7, or 10 mg/L PBTA-6 or 0, 1.5, or 3 mg/L BaP for 96 h in a water bath and the mutations in their gills and hepatopancreata were measured 2–4 weeks later. At 3 weeks after exposure, 3 mg/L BaP significantly increased the *rpsL* mutant frequency (MF) in the gill and hepatopancreas by 5- and 2.3-fold, respectively, as compared to control fish. Sequence analysis showed that BaP mainly induced G:C to T:A and G:C to C:G transversions, which is consistent with the known mutagenic effects of BaP. In contrast, despite its extremely high mutagenic potency in *Salmonella* strains, PBTA-6 did not significantly increase the MF in the zebrafish gill or hepatopancreas. Although PBTA-6 is 300 times more mutagenic than BaP in the Ames test [T. Watanabe, H. Nukaya, Y. Terao, Y. Takahashi, A. Tada, T. Takamura, H. Sawanishi, T. Ohe, T. Hirayama, T. Sugimura, K. Wakabayashi, Synthesis of 2-phenylbenzotriazole-type mutagens, PBTA-5 and PBTA-6, and their detection in river water from Japan, Mutat. Res. 498 (2001) 107–115], calculation of the mutagenicity per mole of compound indicated that PBTA-6 was 33- and <3.7-fold less mutagenic in the zebrafish gill and hepatopancreas, respectively, than BaP.

1. Introduction

Phenylbenzotriazole (PBTA)-type compounds share the 2-[2-(acetylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole moiety and have been identified as major mutagens in the blue cotton/rayon-adsorbed substances of several rivers in Japan that run below textile-dyeing factories and sewage plants treating effluents from such factories [2]. These river samples have shown strong mutagenicity in the Ames test employing *Salmonella* and PBTA-type compounds have been found at times to account for 10–50% of the total mutagenicity [2]. Seven types of PBTA have been identified and are believed to form from corresponding dinitrophenylazo dyes after wastewater treatment. Since some of these contaminated rivers are used as sources of drinking water, it is important to determine whether PBTA-type compounds are genotoxic to human beings. However, while PBTA-type compounds have been shown to be highly mutagenic in the Ames test [2] and to induce abnormal nuclei in cultured hamster cell lines [3,4], their genotoxicity in higher organisms such as fish and mammals is poorly understood. Only one report, that of Masuda et al., showed that PBTA-6 injected intraperitoneally into goldfish was genotoxic, as determined by micronucleus and comet assays [5]. Since several lines of evidence suggest that the zebrafish is a useful model of carcinogenesis in humans as well as fish [6–8], and since fish

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are probably the species that are first affected by water-borne mutagens, we sought to determine the *in vivo* mutagenicity of PBTA-type mutagens by using a zebrafish system.

We developed a transgenic (Tg) zebrafish line carrying a shuttle vector plasmid (pML4) that could be used to detect *in vivo* mutagenicity [9]. The plasmid contains the *rpsL* gene [streptomycin (Sm)-sensitive gene] of *Escherichia coli* (*E. coli*), which serves as a mutational target gene, along with the kanamycin (Km)-resistant gene, which is used to recover the plasmid from the chromosomal DNA of the fish. These features allow the plasmid to be recovered from zebrafish tissues and placed in the appropriate host *E. coli* strain, which is then cultured with Sm and/or Km. Mutations induced in the *rpsL* gene result in the appearance of Sm-resistant colonies. The *rpsL* coding region is 375 bp long and easily sequenced. This Tg fish-based mutation assay involves exposing the Tg fish to compounds in a water bath and then, after waiting for the appropriate time for the mutations to develop, the gills and hepatopancreas are collected and the mutations in the *rpsL* gene in their chromosomal DNA are measured. This assay was used previously to show that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [10] and 6-chloro-9-[3-(2-chloroethylamino)-propylamino]-2-methoxyacridine (ICR-191) [11] increase the mutant frequency (MF) in the gill and hepatopancreas of *rpsL* Tg zebrafish. The mutations that were induced were characteristic of the mutations known to be generated by these compounds [10,11].

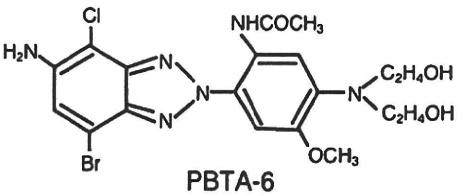
Of the various PBTA-type compounds that exist, PBTA-6 was examined in this study because this compound was detected in most contaminated river samples, sometimes at very high levels; the highest PBTA-6 level detected to date is 468 ng/g blue rayon, which accounts for 39% of the total mutagenicity of the sample as measured by the Ames test [2,12]. The *Salmonella*-based Ames test also revealed that PBTA-6 is 300-times more mutagenic than benzo[*a*]pyrene (BaP) toward *Salmonella* strain TA98 in the presence of an *in vitro* metabolic activation system derived from rat liver (S9 mix) [1]. BaP is a well-known positive control compound that requires S9 mix to exert its mutagenic activity in the Ames test. Since several reports have shown that zebrafish bear the appropriate enzymes or genes needed for metabolic activation [13–15], the *in vivo* zebrafish assay described above is expected to be useful for examining the mutagenicity of PBTA-6 as well as BaP. Furthermore, BaP is itself a wide-spread environmental contaminant that is also found in polluted water and is suspected to be a human carcinogen [16–18]. Analysis of BaP mutagenicity in the zebrafish assay is thus important in its own right.

Here, we first tested the ability of BaP to generate mutations in *rpsL* Tg fish to determine whether this assay can detect mutagens that require metabolic activation for their mutagenicity. While we have previously detected BaP mutagenicity by using *rpsL* Tg zebrafish embryos [9,19], we have not as yet conducted the assay using adult Tg fish. We show that BaP increased the MF and induced characteristic mutations in the gill and hepatopancreas of adult *rpsL* Tg zebrafish. In contrast, whereas PBTA-6 was shown previously to be highly mutagenic in *Salmonella*, we found that this compound is not mutagenic in zebrafish.

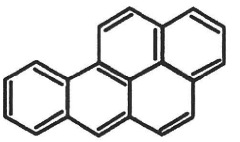
2. Materials and methods

2.1. Transgenic fish

Tg zebrafish carrying approximately 350 copies of the pML4 shuttle vector per haploid genome [9] were maintained as hemizygous fish over 10 generations in 0.1% Aqua Ocean salt at 24–28 °C with a 14-h light/10-h dark cycle. For the experiments, Tg fish were obtained by mating hemizygous Tg fish with non-Tg fish (AB line) and the Tg progeny were selected by dot blot hybridization. All experiments were performed according to the guidelines of the Animal Use and Care Committee of the National Institute for Environmental Studies.



PBTA-6



BaP

Fig. 1. Chemical structures of PBTA-6 and BaP.

2.2. Chemicals

PBTA-6 (CAS registry No. 392274-07-6), whose chemical structure is shown in Fig. 1, was synthesized as described previously by Watanabe et al. [1]. BaP (purity >98%, CAS No. 50-32-8) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan). PEG-40 hydrogenated Castor Oil (NIKKOL HCO-40 CAS No. 61788-85-0) was from Nikko Chemicals Co. Ltd. (Tokyo, Japan) while dimethyl sulfoxide (DMSO, CAS No. 67-68-5, purity >99.5%) was from Sigma–Aldrich Corporation (St. Louis, MO).

2.3. Exposure to BaP

While we previously detected BaP-induced mutations when we exposed *rpsL* Tg fish embryos to BaP solubilized with DMSO [9,19], it should be noted that we observed BaP precipitates in the solution used for exposure. These precipitates arose because BaP is poorly soluble in water. When we used acetone as a carrier, precipitates were also observed. Therefore, we here solubilized BaP by using both the detergent Castor Oil HCO-40 and DMSO. Thus, BaP was dissolved in DMSO containing 4 mg/L (w/v) HCO-40 (HCO/DMSO) in an ultrasonic bath to generate a 20 mg/L BaP stock solution. We performed pilot experiments to determine the maximum concentration of BaP that does not kill the fish, which was 3 mg/L. To generate the 1.5 and 3 mg/L BaP-containing solutions, 37.5 and 75 μL of the 20 mg/L stock BaP solution were added to 500 mL of rearing water with stirring, respectively. Precipitates were not observed in these solutions. The control fish were exposed to 500 mL of rearing water containing 75 μL of HCO/DMSO.

In the BaP-exposure experiment, ten 9–10-month-old *rpsL* Tg zebrafish were exposed for 96 h to 0, 1.5 or 3 mg/L BaP, respectively. Each of the three exposure groups contained equal numbers of males and females that were exposed separately in 500 mL of solution to prevent them from breeding during the exposure period. The fish were exposed at 21 °C without feeding in the dark (to avoid photolysis of BaP) with replacement of the entire exposure solution with a freshly prepared solution every 24 h. After exposure, the fish were rinsed well and maintained for 2 or 3 weeks at 21 °C in rearing water with feeding and a 14-h light/10-h dark cycle. After these mutation expression times, the gills and hepatopancreas of each fish were collected for the mutagenicity assay. Aliquots of the exposure solutions were sampled 0, 24, and 96 h after beginning exposure and the concentrations of BaP therein were measured by using a high-performance liquid chromatograph (L-7000, Hitachi, Tokyo, Japan). The actual concentrations of BaP in the 1.5 and 3 mg/L exposure solutions were 0.71–1.54 and 2.56–3.34 mg/L, respectively.

2.4. Exposure to PBTA-6

PBTA-6 was dissolved in DMSO to generate 7 and 10 mg/L solutions. The maximum concentration of PBTA-6 used for exposure was 10 mg/L as it is the highest concentration of PBTA-6 that can be dissolved well in water without the use of detergents. Three groups of eight or ten 9-month-old *rpsL* Tg zebrafish were exposed for 96 h to 0, 7, or 10 mg/L PBTA-6, respectively. One fish in the treated groups died in an accident that occurred when the exposure solution was being changed but all remaining fish survived during the exposure and expression periods. Each of the three exposure groups consisted of equal numbers of males and females that were exposed separately. The exposure solution volumes were 500 and 600 mL for groups of 4 and 5 fish, respectively. Exposure was performed in the dark at 21 °C without feeding. Each exposure solution was entirely replaced 48 h after commencing exposure with a newly prepared solution. After exposure, the fish were rinsed well and maintained for 3 and 4 weeks at 21 °C in rearing water as described above for the BaP-treated fish. In a pilot experiment where we performed the mutation assay after 2 weeks of expression time, we did not observe an obvious increase in MF.

Consequently, we performed the mutagenicity assay after 3 and 4 weeks. One or 2 fish of each group were fixed for morphological observation conducted in the other study.

2.5. Mutagenicity assay

After the 2- to 4-week expression times indicated above, the fish were anesthetized on ice and euthanased by bleeding out due to cutting of the dorsal aorta. The gills and hepatopancreata were then removed. Genomic DNA was extracted from these organs and the plasmid therein was rescued as described previously [9,10]. Briefly, genomic DNA was digested with *Ban*II, self-ligated with T4 DNA ligase, and introduced by electroporation using a MicroPulser (BioRad, California) into *E. coli RR1* (*rpsL20*), which is Km-sensitive and Sm-resistant. To increase the efficiency of transformation, we divided the mixture of ligated DNA and competent cells into 5–20 electroporation cuvettes (10–12 μL per electroporation cuvette, 0.1 cm (BioRad). After electroporation was performed, the transformed *E. coli* cells were combined and plated onto solid media containing Km (50 mg/L) or both Km and Sm (50 mg/L each). *E. coli* transformed with unaltered plasmids become Km-resistant and Sm-sensitive because the plasmid-borne Km marker and the wild-type *rpsL* gene are both dominant. In contrast, while *E. coli* cells transformed with plasmids bearing mutations in the *rpsL* gene remain Sm-resistant. Mutant frequency was calculated as the number of colonies resistant to both Km and Sm divided by the number of Km-resistant colonies. With some tissue samples, we failed to collect enough DNA or Km-resistant colonies for reliable MF data.

2.6. DNA sequencing

To characterize the BaP-induced mutations in the gill, 105 and 75 Km- and Sm-resistant colonies derived from seven 3 mg/L BaP-treated (the mutation expression time was 3 weeks) fish and five control fish, respectively, were selected at random for sequence analysis. To assess BaP-induced mutagenesis in the hepatopancreas, 116 and 45 Km- and Sm-resistant colonies derived from eight 3 mg/L BaP-treated fish (the expression time was either 2 or 3 weeks) that showed higher MFs and five control fish, respectively, were selected at random.

To characterize the PBTA-6-induced mutations in the gill, 48, 80, and 48 Km- and Sm-resistant colonies derived from seven control fish, five 10 mg/L PBTA-6 treated fish (expression time, 3 weeks) and three 10 mg/L PBTA-6 treated fish (expression time, 4 weeks), respectively, were selected at random.

The mutant *rpsL* gene was sequenced as previously described [19].

2.7. Statistical analysis

Student's *t*-test was used to determine the significance of differences in MF between the control and each exposed group.

3. Results

3.1. BaP-mediated mutagenesis

To examine whether adult *rpsL* Tg fish can be used to detect the *in vivo* mutagenicity of compounds that require metabolic activation to exert their mutagenic activity, we first assessed the effect of exposing the fish to BaP.

In the gill, the MFs in fish that had been exposed to 1.5 and 3 mg/L BaP and provided with 3 weeks of expression time were 14.2×10^{-5} and 49.3×10^{-5}, respectively. These values are 1.4 and 5 times higher than the MFs of the control group and both differences were statistically significant (Table 1). Moreover, 3 mg/L BaP-exposed fish assessed after 3 weeks of expression time had a higher MF than when the expression time was only 2 weeks (49.3×10^{-5} *vs.* 14.8×10^{-5}, Table 1).

In the hepatopancreas, a significant increase in MF was observed after 2 weeks in the 3 mg/L-exposed fish (3-fold higher than in the control fish) and after 3 weeks in the 1.5 and 3 mg/L BaP-exposed fish (2.5- and 2.3-fold higher than in the control fish), respectively (Table 2).

3.2. PBTA-6-mediated mutagenesis

We then examined the MF in fish that had been exposed to 7 or 10 mg/L PBTA-6. In the gill, the MF in the fish exposed to 10 mg/L PBTA-6 was slightly higher at 3 weeks than that in the control gills, but this did not reach statistical significance (Table 3). When a longer expression time, namely, 4 weeks, was employed, the gill MF of the fish exposed to 10 mg/L PBTA-6 actually decreased (Table 3).

Table 1
The *rpsL* mutant frequency (MF) induced by BaP in the gill of transgenic (Tg) zebrafish

Treatment	Expression time	Fish no.	Sex	No. of Km-resistant colonies (A)	No. of Km- and Sm-resistant colonies (B)	MF ($\times 10^{-5}$)[a]	Mean S.D. ($\times 10^{-5}$)
Control	3 weeks	1	M	1,015,000	119	11.7	9.8 ± 3.1
		4	M	960,000	49	6.5	
		6	F	650,000	58	8.9	
		7	F	487,000	31	6.4	
		8	F	324,000	46	14.2	
		9	F	496,000	56	11.3	
1.5 mg/L	3 weeks	13	M	861,000	138	16.0	14.2 ± 3.7*
		15	F	810,000	80	9.9	
		16	F	321,000	43	13.4	
		17	F	437,000	85	19.5	
		18	F	542,500	66	12.2	
3.0 mg/L	3 weeks	21	M	245,000	293	119.6	49.3 ± 38.6*
		22	M	489,000	376	76.9	
		24	M	1,064,000	658	61.8	
		26	F	493,000	126	25.6	
		27	F	453,000	83	18.3	
		28	F	601,000	143	23.8	
		30	F	588,000	113	19.2	
3.0 mg/L	2 weeks	32	M	368,000	67	18.2	14.8 ± 2.1*
		33	M	365,000	58	15.9	
		34	F	268,000	33	12.3	
		35	F	256,000	33	12.9	
		36	F	946,000	141	14.9	
		37	F	773,000	113	14.6	
Mean no. of Km-resistant colonies				576,000			

* Indicates a significant difference between the control and exposed group ($P < 0.05$).

[a] Calculated as (B)/(A).

Table 2
The *rpsL* MF induced by BaP in the hepatopancreas of Tg zebrafish

Treatment	Expression time	Fish no.	Sex	No. of Km-resistant colonies (A)	No. of Km- and Sm-resistant colonies (B)	MF ($\times 10^{-5}$)[a]	Mean S.D. ($\times 10^{-5}$)
Control	3 weeks	3	M	281,000	9	3.2	3.4 ± 1.7
		5	M	396,000	4	1.0	
		6	F	677,000	35	5.2	
		7	F	323,000	11	3.4	
		8	F	525,000	11	2.1	
		9	F	262,000	14	5.3	
1.5 mg/L	3 weeks	11	M	107,800	9	8.3	8.6 ± 5.4*
		12	M	809,000	30	3.7	
		13	M	147,000	7	4.8	
		14	M	92,300	12	13.0	
		15	F	550,000	26	4.7	
		16	F	216,000	13	6.0	
		17	F	47,900	4	8.4	
		18	F	101,000	20	19.8	
3.0 mg/L	3 weeks	22	M	287,000	37	12.9	7.8 ± 4.7*
		23	M	419,000	23	5.5	
		24	M	353,000	51	14.4	
		25	M	538,000	61	11.3	
		26	F	337,000	14	4.2	
		27	F	237,000	11	4.6	
		28	F	160,000	2	1.3	
		30	F	656,000	53	8.1	
3.0 mg/L	2 weeks	32	M	807,000	94	11.6	10.1 ± 3.9**
		33	M	121,000	5	4.1	
		34	F	429,000	38	8.9	
		35	F	695,000	74	10.6	
		36	F	244,000	39	16.0	
		37	F	347,000	32	9.2	
Mean no. of Km-resistant colonies				363,000			

* Indicates a significant difference between the control and exposed group ($P < 0.05$).
** Indicates a significant difference between the control and exposed group ($P < 0.01$).
[a] Calculated as (B)/(A).

Table 3
The *rpsL* MF induced by PBTA in the gill of Tg zebrafish

Treatment	Expression time	Fish no.	Sex	No. of Km-resistant colonies (A)	No. of Km- and Sm-resistant colonies (B)	MF ($\times 10^{-5}$)[a]	Mean S.D. ($\times 10^{-5}$)
Control	3 weeks	1	M	228,000	31	13.6	8.4 ± 2.1
		2	M	516,000	60	11.6	
		3	M	718,000	73	10.2	
		6	F	310,000	27	8.7	
		7	F	200,000	6	3.0	
		8	F	147,000	5	3.4	
7 mg/L	3 weeks	11	M	83,900	12	14.3	8.3 ± 3.9
		12	M	156,000	12	7.7	
		13	M	81,300	9	11.1	
		15	F	272,000	10	3.7	
		16	F	337,000	18	5.3	
		17	F	482,000	36	7.5	
10 mg/L	3 weeks	31	M	354,000	46	13.0	10.4 ± 2.6
		32	M	241,000	26	10.8	
		33	M	293,000	38	13.0	
		36	F	532,000	66	12.4	
		38	M	317,000	21	6.6	
		37	F	303,000	23	7.6	
		39	F	83,900	8	9.5	
10 mg/L	4 weeks	41	M	552,000	30	5.4	4.7 ± 0.9
		42	M	985,000	34	3.5	
		43	M	1,045,000	43	4.1	
		47	F	393,000	20	5.1	
		48	F	1,155,000	65	5.6	
Mean no. of Km-resistant colonies				408,000			

[a] Calculated as (B)/(A).

Table 4
The *rpsL* MF induced by PBTA in the hepatopancreas of Tg zebrafish

Treatment	Expression time	Fish no.	Sex	No. of Km-resistant colonies (A)	No. of Km- and Sm-resistant colonies (B)	MF ($\times 10^{-5}$)[a]	Mean S.D. ($\times 10^{-5}$)
Control	3 weeks	1	M	226,000	3	1.3	4.4 ± 4.0
		3	M	190,000	4	2.1	
		6	F	159,000	4	2.5	
		7	F	116,000	13	11.2	
		8	F	99,000	5	5.1	
7 mg/L	3 weeks	11	M	227,000	7	3.1	3.9 ± 0.8
		12	M	249,000	9	3.6	
		13	M	200,000	8	4.0	
		16	F	157,000	8	5.1	
10 mg/L	3 weeks	32	M	136,000	5	3.7	4.0 ± 2.6
		33	M	243,000	19	7.8	
		37	F	168,000	7	4.2	
		38	M	244,000	1	0.4	
		39	F	360,000	14	3.9	
10 mg/L	4 weeks	43	M	1,204,000	37	3.1	1.7 ± 1.2
		46	F	139,000	2	1.4	
		47	F	391,000	8	2.0	
		48	F	348,000	0	0.1	
Mean no. of Km-resistant colonies				270,000			

[a] Calculated as (B)/(A).

In the hepatopancreas, no significant increase in MF was observed after either 3 or 4 weeks of expression time (Table 4). Thus, unlike BaP, PBTA-6 did not increase *rpsL* MF in either the zebrafish gill or hepatopancreas.

3.3. Characteristics of the mutations induced by BaP and PBTA-6

To characterize the BaP-induced mutations, we sequenced the mutated *rpsL* gene. In both the gill and hepatopancreas, BaP treatment increased the MF of G:C transversions (G:C to T:A and G:C to C:G) and G:C deletions (Tables 5 and 6). There was also an increase in the MF of 2 bp deletions in the gill (Table 5).

Although PBTA-6 exposure did not significantly increase the MF in the gill or hepatopancreas, we nevertheless observed a slightly increased gill MF in fish exposed to 10 mg/L and provided with 3 weeks of expression time. Consequently, we sequenced the mutated *rpsL* gene in the gill to determine whether peculiar types of mutations had been induced. Of all the mutations in the 48 and 80 samples from control fish and 10 mg/L PBTA-6-exposed fish with 3 weeks of expression time, respectively, fewer than 20% consisted of single base substitutions or deletions and over 45% consisted of deletions of 2 to over 100 base pairs. The individual fish differed greatly in terms of the spectrum of their mutations and a clear difference in spectrum between the control and treated group could not be detected (data was not shown).

3.4. Comparison of the mutagenicity of PBTA-6 and BaP in vivo (in Tg zebrafish) and in vitro (in Salmonella)

We compared the mutagenicity of PBTA-6 and BaP that was estimated here by using the *rpsL* Tg zebrafish assay to their published mutagenicity, which was estimated by Watanabe et al. [1] by using the Ames test. To do this, we converted the MF values arising from the *rpsL* Tg mutation assay to MF per 10 μM PBTA-6 or BaP. We also calculated the revertants per nmol in the Ames test. Comparison of these values revealed that while PBTA-6 was 300 times more mutagenic than BaP toward TA98 in the presence of S9 mix, it was 33 and at least 3.7 times less mutagenic than BaP in the *rpsL* Tg fish gill and hepatopancreas (given the MF detection limits), respectively (Table 7).

4. Discussion

4.1. Recovery of the shuttle vector and spontaneous mutant frequencies

The mean recoveries of the shuttle vector are presented in Tables 1–4 as numbers of mean Km-resistant colonies. In the BaP- and PBTA-6-exposure experiments, the recoveries were 576,000 and 408,000 in the gill and 363,000 and 270,000 in the hepatopancreas, respectively. These recoveries were much improved compared to those in our previous reports as they were approximately 2–6 times higher in each organ [10,11], by increasing the efficiency of transformation as described in Section 2.

The MFs of the control gills in the BaP and PBTA-6 exposure experiments were $9.8(\pm 3.1) \times 10^{-5}$ and $8.4(\pm 2.1) \times 10^{-5}$, respectively. The MFs of the control hepatopancreata in the BaP and PBTA-6 exposure experiments were $3.4(\pm 1.7) \times 10^{-5}$ and $4.4(\pm 4.0) \times 10^{-5}$, respectively. Thus, the spontaneous MFs measured in the current study were similar to those determined in our previous study, namely, $6.8(\pm 3.7) \times 10^{-5}$ in the gills and $3.0(\pm 3.0) \times 10^{-5}$ in the hepatopancreata [10]. Notably, as previously observed, the gill had an approximately two-times higher spontaneous MF than the hepatopancreas in both the BaP and PBTA-6 experiments. This may reflect a difference between these two organs in terms of their endogenous DNA lesion/repair-related metabolic activity.

4.2. BaP-induced mutagenesis

BaP is a known rodent carcinogen and probably also a human carcinogen. It has also been reported to induce hepatocellular carcinoma in fish exposed to it *via* diet, intraperitoneal injection, or water [20,21]. It is a promutagen that becomes mutagenic after metabolic activation by cytochrome P450 (CYP). In this study, we showed that exposure to water-borne BaP significantly increased the MF in the gill and hepatopancreas of zebrafish (Tables 1 and 2) and that the predominant types of base substitutions it induced were G:C to T:A and G:C to C:G transversions (Tables 5 and 6). These findings are consistent with those of Winn et al., who showed that BaP increased *cII* MF in the liver of lambda Tg

Table 5
Spectrum of *rpsL* gene mutations in the gill of Tg zebrafish exposed to BaP

Mutation type	Mutations						
	Control			Exposed			Difference in MF between the exposed and control groups[b] ($\times 10^{-5}$)
	No.[a]	%	MF ($\times 10^{-5}$)	No.	%	MF ($\times 10^{-5}$)	
Transition							
A:T→G:C	0	0	0.00	0	0	0.00	0.00
G:C→A:T	3	4.5	0.42	6	6.3	2.79	2.37
Transversion							
G:C→T:A	27	40.3	3.78	15	15.8	6.96	**3.18**
G:C→C:G	3	4.5	0.42	12	12.6	5.57	**5.15**
A:T→C:G	4	6.0	0.56	1	1.1	0.46	−0.10
A:T→T:A	3	4.5	0.42	1	1.1	0.46	0.04
2 bp	0	0	0.00	4	4.2	1.86	1.86
Frameshift							
A:T del	3	4.5	0.42	1	1.1	0.46	0.04
G:C del	0	0.0	0.00	9	9.5	4.18	**4.18**
2 bp del	4	6.0	0.56	17	17.9	7.89	**7.33**
4–19 bp del	15	22.4	2.10	11	11.6	5.11	3.01
~20 del	3	4.5	0.42	4	4.2	1.86	1.44
1 bp ins	0	0	0.00	5	5.3	2.32	2.32
Other ins	2	3.0	0.28	9	9.5	4.18	3.90
Total	67	100	9.38	95	100	44.1	34.7

[a] Number of colonies.
[b] Marked increases in MF are shown in boldface.

Table 6
Spectrum of *rpsL* gene mutations in the hepatopancreas of Tg zebrafish exposed to BaP

Mutation type	Mutations						
	Control			Exposed			Difference in MF between the exposed and control groups[b] ($\times 10^{-5}$)
	No.[a]	%	MF ($\times 10^{-5}$)	No.	%	MF ($\times 10^{-5}$)	
Transition							
A:T→G:C	1	2.4	0.07	0	0.0	0.00	−0.07
G:C→A:T	2	4.8	0.14	5	4.5	0.51	0.38
Transversion							
G:C→T:A	3	7.1	0.21	18	16.2	1.85	**1.64**
G:C→C:G	1	2.4	0.07	27	24.3	2.77	**2.70**
A:T→C:G	6	14.3	0.41	9	8.1	0.92	0.51
A:T→T:A	0	0.0	0.00	0	0.0	0.00	0.00
2 bp	0	0.0	0.00	8	7.2	0.82	0.82
Frameshift							
A:T del	1	2.4	0.07	0	0.0	0.00	−0.07
G:C del	3	7.0	0.20	16	14.4	1.64	**1.44**
2 bp del	1	2.3	0.07	9	8.1	0.92	0.86
4–19 bp del	16	37.2	1.07	5	4.5	0.51	−0.56
~20 del	6	14.0	0.40	3	2.7	0.31	−0.09
1 bp ins	0	0	0.00	8	7.2	0.82	0.82
Others	2	4.7	0.13	3	2.7	0.31	0.18
Total	42	100	2.9	111	100	11.4	8.5

[a] Number of colonies.
[b] Marked increases in MF are shown in boldface.

Table 7
Comparison of the mutagenicity of PBTA-6 and BaP that was determined by *in vitro* (*Salmonella*) and *in vivo* (zebrafish) assays

	Ames test[a] (revertants/nmol) *Salmonella typhimurium* strain		*rpsL* Tg fish mutation assay[b] [mutant frequency (10^{-5})/10 μM]	
	TA98	TA100	Gill	Hepatopancreas
PBTA-6	9170 (301)[c]	74.8 (0.58)	1.0 (0.03)	N.D.[d] (<0.27)[e]
BaP	30.5 (1)	128 (1)	33 (1)	3.7 (1)

[a] Calculated from the published data obtained by using the Ames test with S9 mix [1].
[b] The MFs in these columns were calculated from the MFs of the fish exposed to 10 mg/L (=19.5 μM) PBTA-6 or 3 mg/L (=11.9 μM) BaP at 3 weeks after exposure. The MFs of the corresponding control were subtracted from the MFs of the exposed fish and the increments of MFs were then converted to MFs per 10 μM. This conversion assumes that the increase in MF is linearly dependent on the concentration of the compound.
[c] Number of parenthesis indicates value relative to the value of BaP.
[d] Not detected.
[e] Given the detection limit, this value was estimated to be lower than 0.27.

medaka and that the increased types of mutations were G:C to T:A and G:C to C:G transversions [22]. Compared to the liver of lambda Tg medaka, *rpsL* Tg zebrafish seem to be less responsive to BaP, which might be explained by the different ways used to prepare the BaP solutions and/or the different activities of the enzymes participating in metabolic activation and/or the different degrees of BaP inactivation in the two fish species. Notably, we also showed by using *rpsL* Tg zebrafish embryos that BaP predominantly induces G:C to T:A and G:C to C:G transversions [19]. Thus, *rpsL* Tg zebrafish, both adult and embryos, can be used to detect mutagens that require metabolic activation. These BaP-induced mutations may represent the initial steps that eventually lead to fish cancers.

Significantly, studies with Tg mice have also shown that BaP usually generates G:C to T:A transversions or G:C to T:A and G:C to C:G transversions [23–27]. Single-base deletions of G:C base pairs were also induced by BaP treatment in both Tg fish (Tables 5 and 6) and Tg mice [25,27]. That fish develop similar BaP-induced mutations as mammals suggests that BaP may be activated and induces mutations in a similar manner in both fish and mammals. In line with this, it has been reported that the liver and gill of zebrafish show constitutive and inducible CYP protein or gene family expression [13–15].

The gill of the Tg zebrafish showed a much higher BaP-induced MF than the hepatopancreas (Tables 1 and 2). This probably reflects the greater exposure to BaP by the gill, which is the primary organ that takes up water-borne contaminants into the fish body. Furthermore, metabolic activation may be higher in the gill than in the hepatopancreas. It has also been shown that exposure to water-borne BaP induces CYP1A expression in the gill of several fish [28,29]. The study by Hawkins et al. [20] also showed that a medaka fish exposed to water-borne BaP developed a capillary hemangioma in its gill.

4.3. PBTA-6-induced mutagenesis

Since Tg zebrafish can be used to detect BaP mutagenicity, which requires metabolic activation, we had expected that PBTA-6, which also requires metabolic activation, is also potently mutagenic in the fish since the mutagenic potency of PBTA-6 in the Ames test was 300 times higher than that of BaP (Table 7). The result was quite different from what we had expected; we found that water-borne exposure of PBTA-6 did not significantly increase *rpsL* MF in the gill or hepatopancreas of zebrafish (Tables 3 and 4). This difference in mutagenicity between the zebrafish *in vivo* assay and the *Salmonella in vitro* assay may reflect differences in various steps that lead to mutagenesis, such as uptake, distribution, metabolic activation and inactivation of (pro)mutagenic compounds, and DNA repair mechanisms. This disparity between the *in vitro* and *in vivo* assay results highlights the importance of using an *in vivo* assay system to determine the genotoxic effects of compounds. This zebrafish *in vivo* assay also has the advantage over *in vitro* tests in that the zebrafish has recently been suggested to be a promising model for human carcinogenesis [6–8]. To further clarify the genotoxicity of PBTA-6, along with other PTBA-like compounds that are highly mutagenic *in vitro*, it will be necessary to conduct more extensive *rpsL* Tg fish studies that involve longer exposure times. This will provide a more reliable indicator of the genotoxicity and health risk presented by these compounds to aquatic species and mammals, including humans.

Conflict of interest

The authors declare that they have no conflict of interest.

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REVIEW

Genetic polymorphisms of human cytosol glutathione *S*-transferases and prostate cancer

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Glutathione S-transferases (GSTs) are involved in the metabolism of a wide range of carcinogenic chemicals. In humans, cytosol GSTs are divided into eight classes: α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), τ (GSTZ), σ (GSTS), ο (GSTO) and κ (GSTK). The allelic polymorphism of these enzymes is associated with variations in enzyme activity; hence, it may affect the concentration of activated carcinogenic chemicals in the body. In addition to the metabolism of chemical carcinogens, GSTs metabolize steroid hormones, compounds in the diet and other agents potentially involved in prostate carcinogenesis. Three genetic polymorphisms of GSTs, GSTM1*0 (null), GSTT1*0 (null) and GSTP1 A313G, have been well documented. No consistent associations between GSTM1, GSTT1 or GSTP1 genotypes and prostate cancer have been observed. Recent meta-analysis reports show that these polymorphisms of GSTM1, GSTT1 and GSTP1 are unlikely to be major determinants of susceptibility to prostate cancer.

Keywords: cancer, glutathione transferase, GST, methylation, polymorphism, prostate, SNP

It is generally accepted that cancer risk is determined by the interaction of environmental and genetic factors. Except for hereditary tumors, external carcinogenic exposure is involved in human tumorigenesis. Carcinogenic chemicals, however, undergo a complicated process of metabolism in the human body. Typically, these chemicals are activated by the so-called Phase I enzymes, resulting in the formation of electrophilic, reactive compounds [1]. The amount of active carcinogens is in good correlation with the risk of DNA damage and cancer formation. Detoxifying enzymes, Phase II enzymes, help in the removal of carcinogens from the body [2]. Most of these enzymes conjugate the carcinogenic chemical with a small molecule, making it less toxic and more water-soluble. Therefore, it seems logical to assume that the detoxifying capacity determines, to a certain extent, individual susceptibility to cancer. Among the Phase II enzymes, the glutathione *S*-transferase (GST) superfamily is suspected to have an influence on cancer susceptibility [3]. GSTs are likely to contribute to interindividual differences in responses to xenobiotics. This review describes the relationship between genetic polymorphisms of human cytosol GSTs and prostate cancer.

Prostate cancer

Carcinoma of the prostate is most common in Western countries and second only to lung cancer as a cause of death due to cancer [4]. As well as other malignancies, prostate carcinoma develops through the accumulation of somatic genetic and epigenetic alterations, resulting in the activation of tumor suppressor genes, caretaker genes and oncogenes. The overall lifetime probability of developing clinically evident prostate cancer by the age of 80 years is much lower than its prevalence at autopsy. The estimated lifetime risk for diagnosis of prostate cancer is 16%, and out of that 3–4% die due to this disease [5]. There is a large variation in prostate cancer incidence rates between racial/ethnic groups, being highest among Africans, intermediate among Caucasians and lowest among Asians [6]. This international variation suggests that both genetic and environmental factors play important roles in the etiology of prostate cancer.

Recently, a new hypothesis has been proposed, in which exposure to environmental factors, such as infectious agents and dietary carcinogens, and hormonal imbalances lead to injury of the prostate, resulting in carcinoma of the prostate [7,8]. Approximately 20% of all human malignancies in adults result from chronic inflammation. Although the cause of prostatic inflammation is unclear, there are various potential sources such as direct infection, urine reflux inducing chemical and physical trauma, dietary factors, estrogens and combinations of these. Epidemiological studies show significant associations between infection, inflammation and prostatic carcinoma, however, these are inconclusive. Many noninfectious mechanisms appear to lead to prostatic epithelial cell and stromal damage; for example, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) induces

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prostate inflammation and atrophy. These phenomena are thought to be related to cellular detoxification. Such environmental factors interact with a variable genetic predisposition related, in part, to differences in the metabolism of carcinogen xenobiotics. Genetic polymorphisms that affect the expression of genes regulating the activity of specific enzymes may modulate susceptibility to prostate cancers. Among the candidate genes to be studied in relation to these interindividual differences, cytosol GST may play a relevant role.

GST & genetic polymorphism

The glutathione transferases (EC 2.5.1.18) have historically also been called glutathione *S*-transferases, and it is this latter name that gave rise to the widely used abbreviation, GST. GSTs are prominent contributors to the cellular biotransformation of electrophilic compounds. They provide protection against the genotoxic and carcinogenic effects of numerous xenobiotics and compounds of endogenous origins. Originally, GSTs were found in the soluble cell fraction, and the enzymes occurring in the cytoplasm are usually referred to as cytosol GSTs.

The cytosol GST enzymes have a relatively wide range of substrates, for example, polycyclic aromatic hydrocarbon, monohalomethanes, heterocyclic amines, ethylene oxide and different pesticide solvents. In addition to the metabolism of chemical carcinogens, GST enzymes are involved in the intracellular transport of steroid hormones [9] and the isomerization of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione, the immediate precursor of testosterone [10].

In humans, cytosol GSTs are divided into eight classes: α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), τ (GSTZ), σ (GSTS), ο (GSTO) and κ (GSTK), each of which contains one or more of the homodimeric or heterodimeric isoforms [11,12]. There have been many reports of genetic polymorphisms for *GSTM1*, *GSTM3*, *GSTT1*, *GSTP1* and *GSTA1*, resulting in either decreased or altered enzyme activity. Owing to their role in metabolism, these polymorphisms may play an important role in prostate cancer susceptibility.

GSTM1

A total of five μ class genes (*M1–M5*) situated in tandem on chromosome 1p13.3 have been identified. *GSTM1* is expressed in human liver, stomach, brain and other tissues, while *GSTM2–M5* subunits have been detected in extrahepatic tissues. *GSTM1*, located on chromosome 1p13.3, detoxifies a number of electrophillic substances, including carcinogens such as polycyclic aromatic hydrocarbons, ethylene oxide, epoxides and styrene. *GSTM1* expression is hormonally controlled and induced by phenobarbital or by propylthiouracil [13]. Some lipid peroxidation products may be common substrates of both GSTM1 and GSTT1 [14].

Three polymorphisms of the *GSTM1* gene, namely *GSTM1*0*, *GSTM1*A* and *GSTM1*B*, have been identified (Table 1). *GSTM1*0* is a deleted allele, and the homozygous allele (*GSTM1*-null genotype) expresses no GSTM1 protein. Most studies of *GSTM1* polymorphism and cancer have compared the homozygous deletion genotype with the genotypes containing at least one functional allele. *GSTM1A* and *GSTM1B* differ by a C→G substitution at base position 534. This C→G substitution results in the substitution of Lys→Asn at amino acid 172 [35]. The Lys→Asn substitution results in no functional difference between the two alleles [36]. As a result, *GSTM1A* and *GSTM1B* are categorized together as non-null conjugator phenotypes.

Table 2 summarizes the frequency of homozygous deletion polymorphisms in *GSTM1*. The homozygous deletion genotype frequency in *GSTM1* ranges from 22.7 to 54.2% in Caucasians, from 27.1 to 46.7% in African–Americans and from 42.6 to 54.5% in Asians. We have to be cautious when interpreting the results since the frequencies of the polymorphisms were from controls of molecular epidemiological studies concerning prostate cancer. These prostate cancer studies used different study designs; hospital-based (for males with benign prostatic hyperplasia) or population-based.

GSTM3 is also located on chromosome 1p13.3. Alleles have been identified in *GSTM3*, with *GSTM3*A* and *GSTM3*B* differing in intron 6 by a three base pair (bp) deletion in *GSTM3*B*. Although the difference between *GSTM3*A* and *GSTM3*B* is intronic, its importance is suggested by the presence of a recognition motif for the versatile transcription factor YY1 in *GSTM3*B* but not in *GSTM3*A* [37]. Importantly, *GSTM3*B* and *GSTM3*A* are in linkage disequilibrium with *GSTM1*0* [10]. These results suggest that, in some cases, links between clinical phenotypes and *GSTM1* genotypes may reflect polymorphisms in *GSTM3* or, indeed, other μ class GST genes.

Table 1. Human soluble glutathione S-transferase polymorphisms relevant to prostate cancer.

Enzyme designation	Class	Gene	Chromosome location	Known alleles	Nucleotide change
GSTA1–1	α	GSTA1	6p12	GSTA1*A	T567, C69 and G52
				GSTA1*B	G567, T69 and A52
GSTM1–1	μ	GSTM1	1p13.3	GSTM1*0	Null
				GSTM1*A	C534
				GSTM1*B	G534
GSTM3–3	μ	GSTM3	1p13.3	GSTM3*A	Wild-type
				GSTM3*B	Three base pair deletion intron 6
GSTT1–1	θ	GSTT1	22q11.2	GSTT1*0	Null
				GSTT1*1	Present
GSTP1–1	π	GSTP1	11q13	GSTP1*A	A313 and C341
				GSTP1*B	G313 and C341
				GSTP1*C	G313 and T341

GSTT1

GSTT1 encoding for θ class is located on chromosome 22q11.2, and helps in catalyzing the conjugation of halomethanes in human erythrocytes [36]. Substrates of GSTT1 include industrial chemicals, such as methyl chloride, methyl bromide, dichloromethane, ethylene oxide and diepoxy butane, a reactive metabolite of 1,3-butadiene [39,40]. Two alleles have been identified at the *GSTT1* locus: one functional (*GSTT1*1*) and the other nonfunctional (*GSTT1*0*) [36]. Individuals with a homozygous deletion genotype are categorized as having the negative conjugator phenotype, while those who carry either one or both functional alleles are grouped as having the positive conjugator phenotype. The prevalence rates of the null genotype were 26.7 and 41.9–48.3% of the controls of African–American and Asian descent, respectively (Table 2). Among Caucasians, the frequency ranged from 13–58.7% (Table 2).

GSTP1

GSTP1, a member of the π gene family, is located at 11q13, expressed predominantly in the basal layer of the normal prostate epithelium [41]. GSTP1 metabolizes a variety of potential carcinogens, including cigarette smoke-derived chemicals such as benzo[a]pyrene diol epoxides and acrolein [10]. By contrast with most cancers, prostate carcinogenesis is associated with marked downregulation of *GSTP1*. The events leading up to the inactivation of GSTP1 during prostate carcinogenesis remain unclear. Several investigators have speculated that the early loss of GSTP1 function leads to increased vulnerability to oxidant and heterocyclic amine carcinogens, both implicated in prostate carcinogenesis [42,43]. Hence, heritable differences in GSTP1 function may also be associated with prostate cancer development. Two genetic polymorphisms, in exons 5 and 6, both resulting in an amino acid substitution, have been found. However, only the change in exon 5, that is the A→G polymorphism at nucleotide 313, results in an amino acid substitution (Ile105Val) in the substrate-binding site of GSTP1 [44]. The substitution was linked to a change in enzymatic activity [44]. The prevalence rates of the Val/Val homozygosity was 9–12.6% in Caucasian subjects, 13.3–19% in African–Americans and 0–5.4% in Asians, respectively (Table 2).

GSTA1

The α class GSTs, in other words GSTA1–5, are encoded by genes clustered within 6p12. Of all the GSTs, the α class isoenzymes are the most abundant GSTs found in the human liver, and are, notably, responsible for metabolizing the nitrogen mustard group of some anticancer drugs, and for binding endogenous compounds such as bilirubin in the liver [8]. Especially, *GSTA1*, *GSTA2* and *GSTA3* are widely expressed in human tissues, predominantly in the liver, while *GSTA4* is rarely expressed and *GSTA5* is normally undetectable [45,46]. Members of the α class possess high glutathione peroxidise activity, and play an important role in protecting cells against reactive oxygen species and the products of peroxidation. GSTA1 is also active towards several carcinogenic substrates, including polycyclic aromatic hydrocarbon epoxides [10], and the meat-derived carcinogen *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (*N*-acetoxy-PhIP) [47]. A polymorphism that

Table 2. Frequency of GSTM1, GSTT1, GSTP1 (Ile105Val) and GSTA2 genotypes in three major ethnic groups

First author	Country	Racial descent	GSTM1-null (%)	GSTT1-null (%)	GSTP1 (Ile/Val) (%)			GSTA1*B/*B			Ref.
					Ile/Ile *A/*A	Ile/Val *A/*B or *C	Val/Val *B or *C/*B or *C	*A/*A	*A/*B	*B/*B	
Autrup et al. (1999)	Denmark	Caucasian	53.5	15.3	42	58*					[15]
Rebbeck et al. (1999)	USA	Caucasian	47.6	31.2							[16]
Shepard et al. (2000)	USA	Caucasian			45.4	44.1	10.5				[17]
Steinhoff et al. (2000)	Germany	Caucasian	45	13	55	36	9				[18]
Gsur et al. (2001)	Austria	Caucasian	48.8	19.9	39.2	48.2	12.6				[19]
Kote-Jarai et al. (2001)	UK	Caucasian	50.0	23.7	51.2	38.5	10.3				[20]
Jeronimo et al. (2002)	Portugal	Caucasian			43.3	47.5	9.2				[21]
Kidd et al. (2003)	USA	Caucasian	53	15	57	43*					[22]
Acevedo et al. (2003)	Chile	Caucasian	22.7								[23]
Debes et al. (2004)	USA	Caucasian			38.2	49.0	12.9				[24]
Medeiros et al. (2004)	Portugal	Caucasian	54.2	58.7							[25]
Shankar et al. (2005)	India	Caucasian	35.4	20.1	57.6	38.9	3.5				[26]
Agalliu et al. (2006)	USA	Caucasian	47.5	16.9	43.2	45.7	11.1				[27]
Watson et al. (1998)	USA	Caucasian			42	51	7				[28]

*GSTP1 Val allele (Ile/Val and Val/Val)

Table 2. Frequency of GSTM1, GSTT1, GSTP1 (Ile105Val) and GSTA2 genotypes in three major ethnic groups

First author	Country	Racial descent	GSTM1- null (%)	GSTT1-null (%)	GSTP1 (Ile/Val) (%)			GSTA1*B/*B			Ref.
					Ile/Ile *A/*A	Ile/Val *A/*B or *C	Val/Val *B or *C/*B or *C	*A/*A	*A/*B	*B/*B	
Sweeney et al. (2003)	USA	Caucasian						35.8	47.5	16.7	[29]
Ning et al. (2004)	USA	Caucasian						29.6	54.3	16.1	[30]
London et al. (1995)	USA	African–American	27.1								[31]
Watson et al. (1998)	USA	African–American			35	46	19				[28]
Agalliu et al. (2006)	USA	African–American	46.7	26.7	6.7	80.0	13.3				[27]
Sweeney et al. (2003)	USA	African–American						29.8	57.4	12.8	[29]
Ning et al. (2004)	USA	African–American						39.7	49.2	11.1	[30]
Murata et al. (1998)	Japan	Asian	42.6								[32]
Watson et al. (1998)	USA	Asian			67	30	3				[28]
Nakazato et al. (2003)	Japan	Asian	50.5	41.9	72.4	27.6	0				[33]
Komiya et al. (2005)	Japan	Asian	54.5	48.3	72.9	23.7	5.4	81.0	17.0	2.0	[34]

*GSTP1 Val allele (Ile/Val and Val/Val)

influences the hepatic expression of *GSTA1* has recently been described [45,48]. Liver cytosols from individuals who carried the variant *GSTA1*B* allele, which consists of several linked SNPs in the proximal promoter region of *GSTA1*, had reduced levels of GSTA1 enzyme [49]. The gene has a polymorphic SP1-binding site that results in fourfold higher mean hepatic expression of *GSTA1*A* compared with *GSTA1*B* [45,48].

There are a few reports regarding GST α expression in normal and malignant prostate tissues, demonstrating low levels of GST α expression in high-grade prostatic intraepithelial neoplasia and prostate cancers. Increased expression of *GSTA1* was also detected in proliferative inflammatory atrophy (PIA), suggesting the involvement of localized increases in oxidative stress. Recently, using antibodies for Ya, Yc and Yk-type subunits, an immunohistochemical study was conducted in normal and malignant prostate tissues, showing low levels of GST α expression in prostate cancers and heterogeneous staining patterns. These suggest that a lack of detoxification activity plays an important role in carcinogenesis of the prostate.

Association of GST polymorphism with prostate cancer susceptibility

The genetic polymorphism of GSTs has been investigated in many molecular epidemiological studies as a susceptibility factor for prostate cancer development. For each study, we abstracted the publication date, country where the study was conducted, control source and the numbers of cases and controls (Table 3). Healthy subjects recruited from hospitals as controls were categorized as 'hospital-healthy'.

The 13 available case-control studies investigated the association between *GSTM1* polymorphism and prostate cancer (Table 3). Three case-control studies reported associations between the *GSTM1*-null genotype and prostate cancer risks [23,26,27]. In a large, case-control study (559 cases, 523 controls), Agalliu *et al.* demonstrated that the *GSTM1*-null genotype was associated with an increased risk of prostate cancer (OR: 1.54; 95% CI: 1.19–2.01) [27]. Among *GSTM1*-null individuals, the relative risk increased linearly with increasing pack-years of smoking (p-value for trend = 0.007), with the highest OR observed for heavy smokers (>30 pack-years). Two other studies in Chile [23] and India [26], which are consistent with Agalliu's data, showed a statistically significant OR of 1.94 and 2.24 for prostate cancer among *GSTM1*-null subjects, respectively. However, ten other studies did not recognize an increased risk associated with a lack of *GSTM1* activity. In addition, Kelada *et al.* observed no interaction between smoking and the *GSTM1* genotype [50]. Moreover, the *GSTM1*-null genotype did not have a familial prostate cancer risk [33].

To our knowledge, there are 11 reports investigating the associations between *GSTT1* polymorphism and prostate cancer (Table 3). Eight case-control studies reported no associations between the *GSTT1*-null genotype and prostate cancer risk. However, two studies reported significant associations between the *GSTT1*-null genotype and prostate cancer risk [18,26]. Intriguingly, in one of those reporting American patients with carcinoma of the prostate, a significantly increased frequency of *GSTT1* non-null genotype was found [50]. Furthermore, a Japanese study yielded similar results of a significantly higher frequency of the *GSTT1* non-null genotype among smokers [32].

There are 12 case-control studies that investigated the association between *GSTP1* polymorphisms and prostate cancer. The results of these studies are inconsistent. Three of them reported that *GSTP1* Ile/Val or Val/Val genotypes might be associated with prostate cancer risk [19,20,26]; the Ile/Val or Val/Val genotypes have been associated with a significant increase in the risk of prostate cancer in North Indian populations [26]. Kote-Jarai *et al.* found that patients in the UK with the *GSTP1* Val/Val genotype were at a higher risk of early-onset prostate cancer [20]. On the contrary, Gsur *et al.* reported that there was a significant decrease in the Val allele (Ile/Val and Val/Val) among cases compared with controls [19]. This result suggests that the Val allele is associated with a decreased risk of prostate cancer in Austrians. However, in nine other studies the *GSTP1* Ile105Val polymorphism was not associated with a statistically significant increase in the risk of prostate cancer.

Thus, it is evident that the association of *GSTM1*, *GSTT1* and *GSTP1* polymorphisms with prostate cancer risk differs widely among different populations, suggesting the significance of ethnic differences and environmental factors on prostate cancer susceptibility.

Recently, Ntais *et al.* reported a meta-analysis that included data from 11 studies with *GSTM1* genotyping (2063 prostate cancer cases and 2625 controls), ten studies with *GSTT1* genotyping (1965 cases and 2554 controls) and

Table 3. Summary of case–control studies on GSTM1, GSTT1, GSTP1 (Ile105Val) and GSTA1 genotypes and prostate cancer incidence

Study	Country	Control source	Racial descent	Cases (n)	Controls (n)	GSTM1-null, OR (95% CI)	GSTT1-null, OR (95% CI)	GSTP1 (any Val), OR (95% CI)	GSTA1*B/*B, OR (95% CI)	Ref.
Murata et al. (1998)	Japan	Hospital	Asian	115	204	1.3 (0.82–2.04)				[32]
Autrup et al. (1999)	Denmark	Healthy	Caucasian	153	288	1.27 (0.85–1.90)	1.31 (0.77–2.19)	0.80 (0.54–1.19)		[15]
Kelada et al. (2000)	USA	Hospital	Caucasian	276	499	1.00 (0.69–1.29)	1.61 (1.14–2.28)			[50]
Shepard et al. (2000)	USA	Hospital	Caucasian	590	803			0.81 (0.63–1.06)		[17]
Steinhoff et al. (2000)	Germany	Hospital	Caucasian	91	127	1.20 (0.71–2.05)	2.31 (1.17–4.59)	1.09 (0.66–1.77)		[18]
Gsur et al. (2001)	Austria	Hospital (BPH)	Caucasian	166	166	0.86 (0.55–1.36)	0.78 (0.43–1.42)	0.24 (0.09–0.61)		[19]
Kote-Jarai et al. (2001)	UK	Healthy	Caucasian	275	280	1.29 (0.92–1.81)	1.05 (0.71–1.55)	Ile/Val 1.30 (0.99–1.69) Val/Val 1.80 (1.11–2.91)		[20]
Jeronimo et al. (2002)	Portugal	Healthy + BPH	Caucasian	105	141			1.02 (0.59–1.75)		[21]
Kidd et al. (2003)	USA	Participants of the ATBC cancer prevention study	Caucasian	206	194	0.64 (0.43–0.95)	0.74 (0.42–1.33)	1.10 (0.72–1.69)		[22]
Acevedo et al. (2003)	Chile	BPH	Caucasian	102	128	1.94 (1.04–3.63)				[23]
Nakazato et al. (2003)	Japan	Hospital	Asian	81 (familial)	105	0.98 (0.58–1.66)	0.87 (0.49–1.55)	Ile/Val 0.97 (0.50–1.36) Val/Val 9.31 (0.47–184)		[33]

*GSTT1 non-null, OR (95% CI).

§GSTA1*A/*B or *B/*B, OR: 1.49, 95% CI: 0.96-2.32.

ATBC: Finnish male paticpants of the Alpha Tocophenol Beta Carotene (ATBC) cancer prevention study; BPH: Benign prostatic hypertrophy.

Table 3. Summary of case–control studies on GSTM1, GSTT1, GSTP1 (Ile105Val) and GSTA1 genotypes and prostate cancer incidence

Study	Country	Control source	Racial descent	Cases (n)	Controls (n)	GSTM1-null, OR (95% CI)	GSTT1-null, OR (95% CI)	GSTP1 (any Val), OR (95% CI)	GSTA1*B/*B, OR (95% CI)	Ref.
Debes et al. (2004)	USA	Healthy	Caucasian	438 (familial) 499 (sporadic)	510			1.00 (0.74–1.37) 0.84 (0.65–1.09)		[24]
Medeiros (2004)	Portugal	Healthy	Caucasian	150	185	1.20 (0.75–1.90)	0.87 (0.51–1.51)			[25]
Ning et al. 2004	USA	Healthy	Caucasian	254	81				0.80 (0.36–1.78)	[30]
			African–American	93	63			0.81 (0.25–2.61)		
Shankar et al. (2005)	India	No information	Caucasian	127	144	2.24 (1.37–3.65)	1.89 (1.09–3.28)	2.48 (1.51–4.08)		[26]
Komiya et al. (2005)	Japan	Healthy	Asian	190	294	0.76 (0.52–1.12)	1.39 (0.95–2.03)*	0.87 (0.57–1.35)	1.33 (0.39–4.51)§	[34]
Agalliu et al. (2006)	USA	Healthy	Caucasian	559	523	1.54 (1.19–2.01)	1.04 (0.73–1.47)	Ile/Val 0.95 (0.72–1.25), Val/Val 1.10 (0.71–1.69)		[27]

*GSTT1 non-null, OR (95% CI).

§GSTA1*A/*B or *B/*B, OR: 1.49, 95% CI: 0.96-2.32.

ATBC: Finnish male paticpants of the Alpha Tocophenol Beta Carotene (ATBC) cancer prevention study; BPH: Benign prostatic hypertrophy.

12 studies with *GSTP1* genotyping (2528 cases and 3076 controls) [51]. The random effects OR was 1.08 (95% CI: 0.95–1.25) for the *GSTM1*-null genotype versus the *GSTM1*-nondeleted genotype, and 0.90 (95% CI: 0.73–1.12) for the *GSTT1*-null genotype versus the *GSTT1*-nondeleted genotype. There was no evidence that the *GSTP1* Val allele modified the risk of prostate cancer. The summary of OR was 1.05 (95% CI: 0.90–1.21). No association was observed in subjects of Caucasian descent (OR = 1.02), and there was only one study subject of Asian descent (OR = 1.25).

There have been only two case-control studies of *GSTA1*. Ning *et al.* reported that the OR associated with risk of prostate cancer according to *GSTA1* genotype was not significant within race and after adjustment for age [30]. Komiya *et al.* stated that the frequency of *GSTA1*A/*B* or **B/*B* genotype individuals among cases increased to 26.3% compared with the control groups (19.0%) [34]; however, this difference did not reach statistical significance (OR 1.49; 95% CI: 0.96–2.32) after adjustment for age and smoking status. The apparent lack of interaction between the *GSTA1* genotype and prostate cancer risk suggests a low impact of hepatic GST expression on the risk of the disease. For example, this may be due to the importance of 2-amino-1-methyl-6-phenylimidazo [4,5] PhIP activation locally in the target tissue, activation of *N*-hydroxy-PhIP by paths other than O-acetylation [52], and the low importance of PhIP in the etiology of prostate cancer.

Agalliu *et al.* found evidence suggesting that carriers of the *GSTM3*B/*B* genotype have a fivefold increased risk of onset of prostate cancer (OR: 5.5; 95% CI: 1.2–25.8) [27]. However, their report is only one study that is relevant to prostate cancer, and no other reports regarding the *GSTM3* genotype and prostate cancer have been found.

Although some substrates are metabolized by specific GST isoenzymes [10], they have overlapping substrate specificities; therefore, a combination of unfavorable genotypes could theoretically confer a high risk. In a few studies, an increased risk was observed when a combination of the variant GST genotypes was present [20,26,34]. Kote-Jarai *et al.* [20] and Shanker *et al.* [26] demonstrated that the combination of GST genotypes might define a specific high-risk genotype (*GSTM1*, *GSTT1*-null and *GSTP1* Val allele) in the UK and India. On the other hand, Komiya *et al.* observed that the OR of carrying the combined genotyping of *GSTA1*A/*B* or **B/*B* and *GSTT1* nondeletion was 2.08 (95% CI: 1.14–3.80), with the combined genotyping of *GSTA1*A/*A* and *GSTT1*-null as a reference in Japanese [34].

Future perspective

A number of independent studies have demonstrated the importance of polymorphisms in xenobiotic metabolism as risk factors in the development of cancer associated with chemical exposure. According to these results, many case-control studies have been undertaken regarding *GST* genes and the risk of prostate cancer. However, the evidence from recent studies has not been consistent. The reasons for this disparity in risk are mostly unclear; however, we can speculate on three possibilities.

The first possible reason for these discrepancies, the biochemical evidence for the putative relationship of GST polymorphisms with prostate cancer, is equivocal. It is unlikely that specific environmental carcinogens whose effect might also be modifiable by GST genotype have a high attributable risk for prostate cancer. The second reason is that, in addition to the metabolism of chemical carcinogens, GSTs are involved in the intracellular transport and the synthesis of steroid hormones [12], and compounds found in the diet are potentially involved in prostate carcinogenesis. Population differences in androgen levels and dietary factors have been implicated as a possible explanation. The third reason for these discrepancies may be insufficient study power or bad study design, or a bias against publishing the absence of correlations. Bartsch *et al.* provided state-of-the-art reviews of the application of biomarkers and the design and analysis of molecular epidemiological studies [53]. The prerequisites for proper study design and conduct include:

- A clear definition of representative study populations and controls
- A sample size adequate to provide enough statistical power
- Proper documentation (or measurement) of exposure
- Avoidance of confounding data because of the use of mixed ethnicity study subjects
- Study of only the gene polymorphisms that have been demonstrated to lead to altered phenotypic expression

The rigor and size of study designs will need to increase, as multiple comparisons and the power issue dictate. In parallel with these studies, a clearer understanding of the genetic bias of the polymorphisms has emerged, together with more accurate and less invasive methods for the screening of populations.

Expert opinion

Three genetic polymorphisms of GSTs, that is *GSTM1*0* (null), *GSTT1*0* (null) and *GSTP1 A313G*, have been well documented. However, associations between other polymorphisms of GSTs, that is to say *GSTA*, *GSTZ*, *GSTS*, *GSTO* and *GSTK*, and prostate cancer are not well known. It will be interesting to know whether polymorphisms in these genes influence susceptibility to prostate cancer.

Of particular interest are studies of gene–environment interactions and gene–gene–environment interactions. To date, molecular epidemiology studies of prostate cancer have rarely looked at a variety of potential gene–environment interactions or explored associations and interactions with more than one genetic polymorphism. Future studies that examine the association among several genetic polymorphisms should take into account risk factors for prostate cancer, such as diet and other environmental exposures, as well as possible biological pathways. In addition, some studies had limited power to detect more associations between *GST* polymorphisms and prostate cancer risk; further well-designed and large-scale studies are needed to reveal these associations.

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Executive summary

Prostate cancer

- Carcinoma of the prostate is most common in Western countries, and second only to lung cancer as a cause of death due to cancer.

Glutathione S-transferases families

- In humans, cytosol glutathione S-transferases (GSTs) are divided into eight classes: α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), τ (GSTZ), σ (GSTS), ο (GSTO) and κ (GSTK).

Glutathione S-transferase substrates

- GSTs metabolize numerous harmful chemicals produced endogenously and in the environment.

Genetic polymorphisms of glutathione S-transferase

- Cytosol GSTs display polymorphisms in humans, which is likely to contribute to interindividual differences in responses to xenobiotics.

Association of glutathione S-transferase polymorphisms with prostate cancer susceptibility

- Three genetic polymorphisms of GST: GSTM1*0 (null), GSTT1*0 (null) and GSTP1 A313G, have been well documented.
- No consistent associations between GSTM1, GSTT1 or GSTP1 genotypes and prostate cancer have been observed.

Conclusions

- Further well-designed, large-scale studies are required to detect the precise associations between GST polymorphisms and prostate cancer risk.
- Research is needed on the associations between the polymorphisms of GSTA, GSTZ, GSTS, GSTO and GSTK and prostate cancer.

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