Virchows Archiv (1995) 425:589–592

ORIGINAL ARTICLE

Kazuhiro Shimizu · Fumi Toriyama · Hikotaro Yoshida

The expression of placental-type glutathione S-tranferase (GST-π) in human cutaneous squamous cell carcinoma and normal human skin

Received: 20 May 1994 / Accepted: 26 September 1994

Abstract The expression of human placental-type glutathione S-transferase (GST-π) was investigated in human cutaneous squamous cell carcinoma (SCC) and normal skin using Northern blot and immunohistochemical analysis. In Northern blot examination, the expression of GST-π transcript was recognized in all instances, and SCC showed a significantly higher expression of GST-π than normal skin. In immunohistochemical examination, GST-π was stained well in the cytoplasm of all cells of the stratum granulosum, many cells of the stratum spinosum and a few cells of the stratum basale in normal skin. Some cells of the stratum spinosum and almost all cells of the stratum basale showed only a weakly positive or almost negative reaction for GST-π. No nuclear staining of GST-π was obvious in normal epidermal cells. In SCC, many cells showed strong positivity for GST-π in the cytoplasm, and some were obviously accompanied by nuclear staining of GST-π. These findings suggest that GST-π exists mainly in many cells in the upper layers of the normal epidermis and that GST-π is involved in the process of carcinogenesis.

Key words Placental-type glutathione S-transferase
Human skin · Squamous cell carcinoma
Northern blotting · Immunohistochemistry

Introduction

Numerous investigations concerning the function of glutathione S-transferase (GST) as a detoxifying enzyme have been conducted [3, 5, 10] since its discovery [2]. A variety of isozymes of GST in the rat have also been investigated. Sato et al. [24] detected placental form of GST (GST-P) in pre-neoplastic and neoplastic lesions in rat chemical hepatocarcinogenesis. Since then GST-P has been used as a (pre)neoplastic marker in rat hepatocarcinogenesis because it does not exit in normal hepatocytes. Human placental-type GST (GST-π), which corresponds to GST-P, was isolated from the human placenta [4] and the cloning of GST-π was performed [11]. A large number of reports have been published concerning the expression of GST-π in human malignant tumours [6, 7, 8, 9, 12, 15, 16, 17, 18, 19, 20, 26, 27]. The present study was conducted to compare the expression of GST-π in human cutaneous squamous cell carcinoma with that in normal skin using Northern blot and immunohistochemical analysis.

K. Shimizu (✉) · F. Toriyama
Division of Dermatology, Institute for Clinical Research,
Nagasaki Chuo National Hospital, Omura 856, Japan

H. Yoshida
Department of Dermatology,
Nagasaki University School of Medicine,
Nagasaki 852, Japan

Materials and methods

Anti-human GST-π antibody (rabbit immunoglobulin) was purchased from Bioprep Co. (#MED25PI, Dublin, Ireland). The antibody is polyclonal and cross-reacts with placental-type mouse and rat GST but not with α of μ-classes of enzyme (data from the manufacturer).

Human GST-π probe was purchased from Oncor (#1I005, Gaitherberg, Md.). Probe size is 725 bp cDNA fragment and the expected band in Northern analysis is at 0.7 kb (data from the company). For control hybridization on the amount of RNA, the complementary oligonucleotide probe for 28S ribosomal RNA was synthesized, and the sequence is as follows: 5′AA-CGATCAGAGTAGTGGTATTTCACC3′ [1].

We used ten squamous cell carcinomas and four normal skin samples obtained during surgical operations at Nagasaki Chuo National Hospital and Nagasaki University Hospital. The clinical data are summarized in Tables 1 and 2. Half of the resected tumours were fixed in 10% formalin and routinely embedded in paraffin. All tissues were examined histologically, and pathological diagnosis was performed. For Northern blot analysis, the rest of resected tumours were immediately immersed in a cold phosphate buffered saline (PBS) containing diethyl pyrocarbonate and trimmed. After trimming, these were snap-frozen in liquid nitrogen and stored at −70° C until RNA extraction. The four corners of normal skin grafts were used as controls. These were obtained from the thighs of patients with various skin conditions using an electric dermatome for the purpose of skin implantation. A portion of the control samples were fixed in 10% formalin and embedded in paraffin. The remainder were quickly treated for Northern blot analysis as described above.

Table 1 Relevant clinical information on the patients with squamous cell carcinoma (*M*, male; *F*, female; *S.C.C.*, squamous cell carcinoma; *Mod.*, moderately; *diff*, differentiated)

Patient	Age	Sex	Site	Size	Histology	Remarks
1	62	M	Nose	26×18×10 mm	Pseudoglandular S.C.C.	von Recklinghausen
2	78	F	Thigh	18×13×5 mm	Mod. diff. S.C.C.	
3	82	M	Ear	14×12×4 mm	Mod. diff. S.C.C.	
4	92	F	Chest	45×30×10 mm	Well diff. S.C.C.	
5	90	M	Cheek	24×20×3 mm	Well diff. S.C.C.	with Actinic keratosis
6	92	F	Cheek	40×40×25 mm	Mod. diff. S.C.C.	with Actinic keratosis
7	71	M	Ear	30×27×4 mm	Mod. diff. S.C.C.	
8	69	M	Cheek	23×13~10×5 mm	Pseudoglandular S.C.C.	recurrent
9	74	F	Hand	17×15×5 mm	Well diff. S.C.C.	recurrent
10	78	M	Heel	28×25×3 mm	Mod. diff. S.C.C.	

Table 2 The sites of skin grafts taken as normal skin samples from patients with various skin diseases and the relevant clinical information (*M*, male; *F*, female; S.C.C., squamous cell carcinoma)

Patient	Age	Sex	Site	Background
11	78	M	Thigh	S.C.C.
12	69	M	Thigh	S.C.C.
13	61	F	Thigh	Necrotizing Fasciitis
14	74	F	Thigh	S.C.C.

Total RNA was extracted by the standard guanidium isothiocyanate/caesium chloride method [14], and the samples were stored in ethanol at −70° C until use. Fifty micrograms of the total RNA of each sample were electrophoresed on 1.0% agarose gel containing 8% formaldehyde. The quality of loaded RNA was confirmed by the visualization of agarose gel stained with ethidium bromide (EB). The samples were then transferred onto a GeneScreen Plus nylon filter membrane (du Pont, Boston, Mass., USA).

The filter was baked for 2 h at 80° C in a vacuum. After prehybridization, hybridization was performed at 60° C overnight using a human GST-π probe labelled with the random primers DNA labelling system (BRL, Gaitherberg, Md., USA) and [α-^{32}P] dCTP. The hybridization solution contained 1% sodium dodecyl sulphate (SDS), 1 M sodiumchloride, 10% dextran sulphate and 100 μg/ml denatured salmon sperm DNA. The filter was washed several times with 0.1×SSC–0.1% SDS (1×SSC consists of 0.15 M sodium chloride and 15 mM sodium citrate) at 65° C. The filter was exposed to a Fujix imaging plate for one day at room temperature and analysed with a BAS 2000 bioimaging analyser (Fujix, Tokyo, Japan).

After that, the filter was stripped and rehybridized at 42° C with complementary oligoprobe for 28 S ribosomal RNA labelled with T4 polynucleotide kinase and [γ-^{32}P] ATP. The filter was washed several times with 0.1×SSC–0.1% SDS at 42° C and also exposed to an imaging plate for three days at room temperature and analysed with a BAS 2000.

For immunohistochemical analysis , the alkaline phosphatase method was adopted. Deparaffinized tissue sections (5 μm thick) were immersed in 0.05 M TRIS-buffered saline (TBS) containing 1% bovine serum albumin for 15 min at room temperature to diminish the non-specific binding of the secondary antibody. After washing in 0.05 M TBS for 5 min, each section was treated overnight with anti-GST-π antibody (1:500) at 4° C. After washing, the sections were treated with anti-rabbit IgG labelled with alkaline phosphatase (1:500) at room temperature for 1 h purchased from Organon Teknika Corp. (Durhman, N.C., USA). After washing, they were immersed in a prewarmed alkaline substrate buffer (ASB) containing 1 mM levamisol for 5 min to bring about conversion into substrate buffer and to block internal phosphatase activity, and subsequently they were treated at 30° C with ASB (ASB consists of 0.1 M TRIS-HCl (pH 9.5), 0.1 M sodium chloride and 50 mM magnesium chloride) containing 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride purchased from Gibco BRL (Gaitherberg, M.D., USA). Human full-term placenta was used as a positive control, while negative control staining was obtained by replacement of the primary antibody with nonimmunized rabbit serum.

Results

Ethidium bromide staining showed that the quality of loaded RNA was good. A 0.7 kb transcript band corresponding to GST-π mRNA was confirmed in 14 instances, that is, 10 SCCs and four controls. A 5.0 kb transcript band for 28 S ribosomal RNA was also confirmed in all 14 instances (Fig. 1).

The transcript levels were quantitated by BAS 2000. The normalized intensity of GST-π transcript band was calculated by comparison with that of the respective 28 S r-RNA band (GST-π expression/28 S r-RNA). The ratio of each normalized value of GST-π to the control mean values was also calculated. The distribution of each ratio and the mean values of SCCs and controls are shown in Fig. 2.

The SCCs showed a mean value of 3.45 with a range between 1.56 and 7.08, while the controls showed a mean value of 1.00 with a range between 0.77 and 1.36. Mann-Whitney's U test revealed a statistically significant difference between SCCs and controls ($P<0.01$).

The cytoplasm of all cells in the stratum granulosum and many cells in the stratum spinosum of normal skin responded well to GST-π staining, but some cells in the latter stained poorly. The cytoplasm of almost all cells in the stratum basale were weakly positive or almost nega-

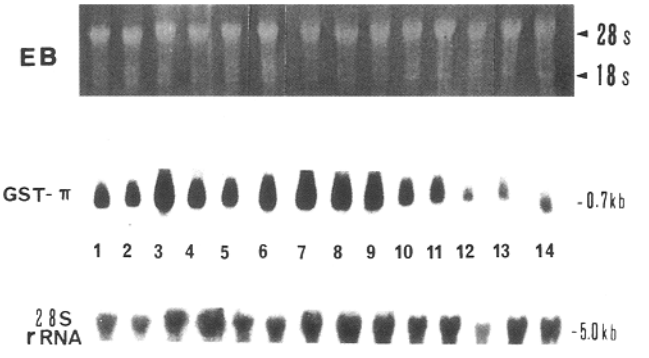

Fig. 1 Northern blot examination of total RNA (50 μg in each well) for analysis of GST-π transcripts at 0.7 kb. The numbers correspond to patient numbers. The upper panel represents transferred blots after visualization of the agarose gel with ethidium bromide (*EB*). The lower panel represents 28S ribosomal RNA bands at 5.0 kb

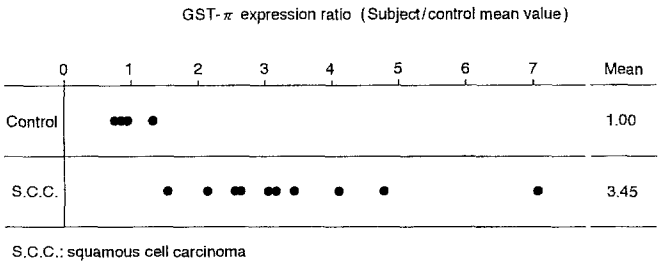

Fig. 2 The ratio of each GST-π transcript level normalized by the respective 28S r-RNA band to control mean value along with the mean values of squamous cell carcinomas and controls. A statistically significant difference ($P<0.01$) was confirmed by Mann-Whitney's U test

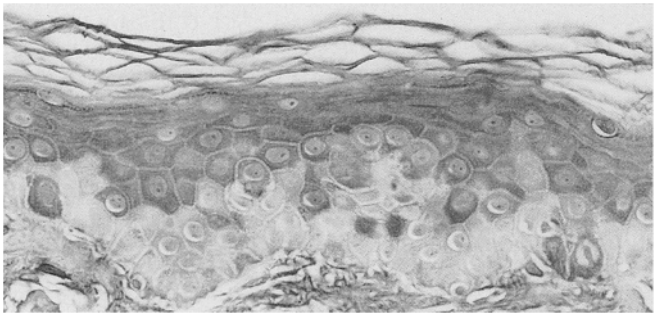

Fig. 3 Immunohistochemical staining of GST-π in normal skin. GST-π is expressed diffusely in the cytoplasm of all cells in the stratum granulosum, many cells in the stratum spinosum and a few cells in the stratum basale. No nuclear staining of GST-π is evident. (×350)

tive, but the cytoplasm of a few cells stained well. No cells with nuclear staining of GST-π were apparent in the epidermis (Fig. 3).

In SCC, the GST-π was not expressed clearly in the cytoplasm of all cells. But although the cytoplasm of some cells was only weakly positive or almost negative, that of the majority of cells was strongly positive. Some cells clearly showed nuclear staining of GST-π (Fig. 4).

Discussion

Riou et al. [22] reported, on the basis of Northern blot analysis, that although GST-π is expressed in normal epithelium its expression is enhanced in dysplasia and in carcinoma of the uterine cervix. Also using Northern blot analysis, Konohana et al. [13] stated that GST-π was expressed in the human skin but that no quantitative difference was recognized in the expression of GST-π between cultured human keratinocytes and the A431 SCC cell line. No other reports has been published concerning GST-π expression in human cutaneous SCC. In the present study, a high expression of GST-π was confirmed in human cutaneous SCC using RNA extracted from human specimens obtained rapidly during operations. Therefore, the disparity between the results of Konohana et al. [13] and our results is considered to be the difference between in vitro and in vivo experiments. It is a fact that GST-π is expressed in normal human skin and that some samples show a value of GST-π close to that of

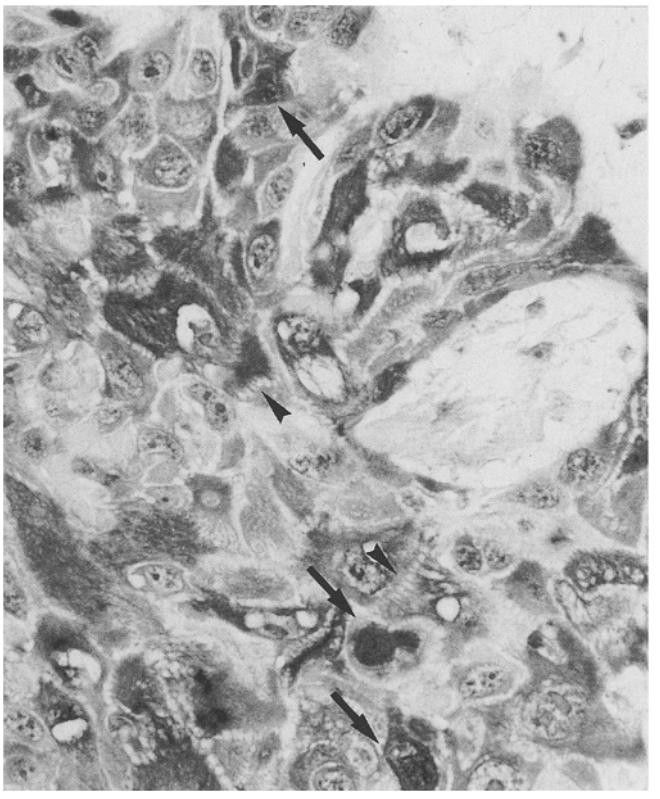

Fig. 4 Immunohistochemical staining of GST-π in squamous cell carcinoma. Many cells show strongly positive staining of GST-π in the cytoplasm. Some cells show nuclear staining of GST-π (*arrows*). Note the intercellular bridges between tumour cells (*arrow heads*). (×500)

SCC but we have confirmed that the expression is much higher in human cutaneous SCC than in normal human skin, using Northern blot analysis. Like SCC originating from other organs therefore, SCC from the human skin is considered to show a high expression of GST-π transcript.

In this paper, the immunohistochemical staining of GST-π was shown in normal skin, but as far as we know there has been no other report concerning the immunohistochemical analysis of GST-π in normal human skin. Raza et al. [21] conducted the immunohistochemical analysis of GST-π using Sencar mouse skin. They mentioned that GST-π is located in the sebaceous glands and outer root sheath of the hair follicles but did not refer to the staining pattern in the epidermis. Konohana et al. [13] stated in their report that anti-GST-π did not produce a linear basement immunofluorescence pattern of bullous pemphigoid (data not shown) and they clarified that GST-π mRNA was expressed throughout the epidermis and epidermal appendages as well as the dermis using the in situ hybridization method. The present staining pattern is thought to be consistent with the latter author's morphological data. A few cells of the stratum basale, many cells of the stratum spinosum and all cells of the stratum granulosum show a positive reaction for GST-π in the cytoplasm. This staining pattern may lead to the speculation that GST-π is naturally distributed in many cells in the upper layers of the skin to prevent toxic inju-

ries, because the skin is the organ which borders on the atmosphere and GST-π is originally a detoxifying enzyme.

In an immunohistochemical analysis of the uterine cervix, which also possesses a squamous epithelium, Shiratori et al. [25] mentioned that the normal squamous epithelial cells showed a weakly positive GST-π staining in the cytoplasm in the intermediate layer but seldom showed a positive reaction in the parabasal or superficial layers. They also noticed the intra-nuclear binding of GST-π, which was absent in the normal epithelium in over 70% of cases of invasive carcinoma. There are many similarities between the study described above and our own with regard to the staining pattern of GST-π in the normal epithelium, and the few differences such as the staining pattern in the superficial layer may be attributable to tissue specificity. We also noticed nuclear staining of GST-π in SCC, which is not an obvious finding in normal skin. It is considered that the GST-π or GST-π related antigen mentioned by Shiratori et al. [25] is expressed in the nuclei. As Sato [23] speculated, it is possible that GST-π in the nuclei either is involved in the process of carcinogenesis, for example as a carrier protein or acts to detoxify genotoxic substances from the cytosol because it is originally a detoxifying enzyme.

In conclusion, the immunohistochemical staining pattern of GST-π was demonstrated in normal human skin and the nuclear binding of GST-π was clearly detected in cutaneous SCC. The expression of GST-π transcript was significantly higher in cutaneous SCC than in normal human skin.

Acknowledgements We are grateful to Dr. M. Kishikawa (Pathology, Nagasaki University School of Medicine) for critical comments, and Dr. Y. Urata (Biochemistry, Nagasaki University School of Medicine) for technical advice.

References

1. Barbu V, Dautry F (1989) Northern blot normalization with a 28 S rRNA oligonucleotide probe. Nucleic Acids Res 17:7115
2. Booth J, Boyland E, Sims P (1961) An enzyme from rat liver catalysing conjugation with glutathione. Biochem J 79:516–524
3. Chasseaud LF (1979) The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. Adv Cancer Res 29:175–274
4. Guthenberg C, Mannervik B (1981) Glutathione-transferase(transferase π) from human placenta is identical or closely related to glutathione S-transferase(transferase ρ) from erythrocytes. Biochim Biophys Acta 661:255–260
5. Hara A, Yamada H, Sakai N, Hirayama H, Tanaka T, Mori H (1990) Immunohistochemical expression of glutathione S-transferase placental type (GST-π), a detoxifying enzyme, in normal arachnoid villi and meningiomas. Virchows Arch 417:493–496
6. Harrison DJ, Kharbanda R, Bishop D, McLelland LI, Hayes JD (1989) Glutathione S-transferase isoenzymes in human renal carcinoma demonstrated by immunohistochemistry. Carcinogenesis 10:1257–1260
7. Hirata S, Odajima T, Kohama G, Ishigaki S, Niitsu Y (1992) Significance of glutathione S-transferase-π as a tumor marker in patients with oral cancer. Cancer 70:2381–2387
8. Holmes J, Wareing C, Jacobs A, Hayes JD, Padua RA, Wolf CR (1990) Glutathione-s-transferase pi expression in leukaemia: a comparative analysis with mdr-1 data. Br J Cancer 62:209–221
9. Howie AF, Forrester LM, Glancey MJ, Schlager JJ, Powis G, Beckett GJ, Hayes JD, Wolf CR (1990) Glutathione S-transferase and glutathione peroxidase expression in normal and tumour human tissues. Carcinogenesis 11:451–458
10. Jakoby WB (1978) The glutatione S-transferases: a group of multifunctional detoxification proteins. Adv Enzym 46:383–414
11. Kano T, Sakai M, Muramatsu M (1987) Structure and expression of a human class π glutathione S-transferase messenger RNA. Cancer Res 47:5626–5630
12. Klone A, Weidner U, Hußnatter R, Harris J, Meyer D, Peter S, Ketterer B, Sies H (1990) Decreased expression of the glutathione S-transferases alpha and pi genes in human renal cell carcinoma. Carcinogenesis 11:2179–2183
13. Konohana A, Konohana I, Schroeder WT, O'Brien WR, Amag M, Greer J, Shimizu N, Gammon WR, Siciliano MJ, Duvic M (1990) Placental glutathione-S-transferase-pi mRNA is abundantly expressed in human skin. J Invest Dermatol 95:119–126
14. Maniatis T, Fritsch EF, Sambrook J (1984) Molecular cloning: a laboratory manual, 8th edn. Cold Spring Harbor Laboratory, New York, pp 187–210
15. Mannervik B, Castro VM, Danielson UH, Tahir MK, Hansson J, Ringborg U (1987) Expression of class Pi glutathione transferase in human malignant melanoma cells. Carcinogenesis 8:1929–1932
16. Moorghen M, Cairns J, Forrester LM, Hayes JD, Hall A, Cattan AR, Wolf CR, Harris AL (1991) Enhanced expression of glutathione S-transferases in colorectal carcinoma compared to non-neoplastic mucosa. Carcinogenesis 12:13–17
17. Murray GI, Paterson PJ, Weaver RJ, Ewen SWB, Melvin WT, Burke MD (1993) The expression of cytochrome P-450, epoxide hydrolase, and glutathione S-transferase in hepatocellular carcinoma. Cancer 71:36–43
18. Peters WHM, Wormskamp NGM, Thies E (1990) Expression of glutathione S-transferases in normal gastric mucosa and in gastric tumors. Carcinogenesis 11:1593–1596
19. Randall BJ, Angus B, Akiba R, Hall A, Cattan AR, Proctor SJ, Jones RA, Horne CHW (1990) Glutathione S-transferase (placental) as a marker of transformation in the human cervix uteri: an immunohistochemcial study. Br J Cancer 62:614–618
20. Ranganathan S, Tew KD (1991) Immunohistochemical localization of glutathione S-transferase α, μ and π in normal tissue and carcinomas from human colon. Carcinogenesis 12:2383–2387
21. Raza H, Awasthi YC, Zaim MT, Eckert RL, Mukhtar H (1991) Glutathione S-transferases in human rodent skin: multiple forms and species-specific expression. J Invest Dermatol 96:463–467
22. Riou G, Barrois M, Zhou D (1991) Expression of anionic glutatione S transferases (GST-π) gene in carcinomas of the uterine cervix and in normal cervices. Br J Cancer 63:191–194
23. Sato K (1988) Glutathione S-transferases and hepatocarcinogenesis. Jpn J Cancer Res (Gann) 79:556-572
24. Sato K, Kitahara A, Sato K, Ishikawa T, Tatematsu M, Ito N (1984) The placental form of glutathione S-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. Gann 75:199–202
25. Shiratori Y, Soma Y, Maruyama H, Sato S, Takano A, Sato K (1987) Immunohistochemical detection of the placental form of glutathione S-transferase in dysplastic and neoplastic human uterine cervix lesions. Cancer Res 47:6806–6809
26. Tidefelt U, Rosenborg AE, Paul C, Hao XY, Mannervik B, Eriksson LC (1992) Expression of glutathione transferase π as a predictor for treatment results at different stages of acute non lymphoblastic leukemia. Cancer Res 52:3281–3285
27. Tsutsumi Mugisaki T, Makino T, Miyagi N, Nakatani K, Shiratori T, Takahashi S, Konishi Y (1987) Oncofetal expression of glutathione S-transferase placental form in human stomach carcinomas. Jpn J Cancer Res (Gann) 78:631–633