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Immunohistochemical Detection of the Placental Form of Glutathione S-Transferase in Dysplastic and Neoplastic Human Uterine Cervix Lesions[1]

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ABSTRACT

Expression of the human placental form of glutathione *S*-transferase (GST-π) in dysplasia (53 cases), carcinoma *in situ* (10 cases), and invasive carcinoma (46 cases) of human uterine cervix was investigated immunohistochemically with specific anti-GST-π rabbit antibody. While normal squamous epithelium was largely negative, the binding of antibody was appreciable in mild and moderate dysplasias, especially in the cytoplasm of cells demonstrating koilocytotic atypia. In severe dysplasia, the nuclei as well as the cytoplasm were strongly stained in all cell layers except for the superficial layer, and in carcinoma *in situ* both of them were also strongly stained in all cell layers. In invasive carcinoma, over 90% of cases exhibited strong cytoplasmic staining and in over 70% the nuclei were positive. GST activity towards 1-chloro-2,4-dinitrobenzene and GST-π protein content were significantly increased in all of 4 squamous cell carcinomas examined as compared to values for normal cervical epithelia. Two-dimensional gel electrophoresis followed by immunoblotting using the GST-π antibody demonstrated that, of many cytoplasmic proteins, only the GST-π subunit was specifically bound. These results indicate that GST-π is a potentially useful immunohistochemical marker for (pre)neoplasia of human uterine cervix. In addition, it was demonstrated that the cells in severe dysplasia, carcinoma *in situ*, and invasive carcinoma expressing GST-π were often characterized by staining with a monoclonal antibody to the v-H-*ras* gene product.

INTRODUCTION

GST-P[3] has been reported as one of the best markers for (pre)neoplastic cells in rat and hamster chemical hepato- and pancreatocarcinogenesis (1–5). GST-π, which is immunologically closely related to GST-P (2, 4, 6), has also been demonstrated to be useful for immunohistochemical detection of (pre)neoplastic lesions in the colon (7).

In the present study, the degree of expression and localization of GST-π in human cervical dysplastic and neoplastic tissues was investigated immunohistochemically using the polyclonal antibody raised against GST-π. Total GST activity toward 1-chloro-2,4-dinitrobenzene and GST-π protein content were also examined in cervical squamous cell carcinomas and compared with normal tissue values. In addition, binding of antibody to the v-H-*ras* gene product p21 was also investigated immunohistochemically to establish whether its expression is in any way related to increased GST-π.

MATERIALS AND METHODS

Antiserum. GST-π was purified from a human term placenta by chromatofocusing following *S*-hexylglutathione column chromatography and antiserum was raised as reported previously (6). The γ-globulin fractions, collected with 22–33% saturated ammonium sulfate solution, were used for the investigation. Antibody specificity was confirmed by the immunoblotting method following two-dimensional gel electrophoresis of crude extracts from tissues containing GST-π (see Fig. 6).

Immunohistochemical Staining Methods. The tissues examined were obtained at biopsy from 119 patients suspected of malignant lesions of the uterine cervix. The numbers of the specimens of dysplasia, carcinoma *in situ*, invasive carcinoma, and normal epithelium adhering to myoma specimen were 53, 10, 46, and 10, respectively. All of these samples were fixed in 90% ethanol. A further 5 carcinoma *in situ* specimens were fixed in 10% formalin:90% ethanol (1:1). Histological sections 6-μm thick were prepared in the usual manner, stained with hematoxylin and eosin, and examined by light microscopy. Serial sections from respective specimens containing lesions of interest were used for the immunohistochemical staining of GST-π according to the method of Hsu *et al.* (8). Biotin-bound anti-rabbit IgG goat immunoglobulin and avidin-biotin-peroxidase complex (Vectastain ABC Kit, PK 4001) were obtained from Vector Laboratories, Inc. (Burlingame, CA). Paraffin sections were passed through xylene and a series of graded alcohols (99% to 70%) and then treated sequentially with normal goat serum, the anti-GST-π rabbit antibody (1:3000), biotin-bound anti-rabbit IgG antibody (1:400), and avidin-biotin-peroxidase complex. The binding site of peroxidase was detected using diaminobenzidine as the substrate. Sections were then counterstained with hematoxylin. Negative control reactions were performed by substituting nonimmune serum for the GST-π antibody (γ-globulin fraction). For further purification of the GST-π antibody, the γ-globulin fraction was applied to a GST-π-bound Sepharose column (1.5 × 5 cm) and the antibody adsorbed to the column was eluted by 0.1 M acetic acid. This eluted fraction was used as the pure GST-π antibody preparation, and the breakthrough fraction was used as the control antibody preparation. γ-Globulin fraction antibodies to GST-α [GST-I in our previous paper (6)] and GST-μ prepared in rabbits (6) and a monoclonal antibody (rp-28) to v-H-*ras* gene product p21 (9) (generous gift of Dr. Kuzumaki, Hokkaido University) were also used for investigation of immunohistochemical binding.

GST Activity Assay. The total GST activity in the cytoplasmic fraction (the supernatant obtained by centrifugation at 105,000 × *g* for 45 min) of 4 squamous cell carcinomas and 4 normal tissue specimens was determined using 1-chloro-2,4-dinitrobenzene as a substrate by the method of Habig et al. (10).

Determination of GST-π Protein Content. This was carried out by single radial immunodiffusion using the antibody to GST-π according to the method of Mancini *et al.* (11).

Two-Dimensional Gel Electrophoresis. This was carried out as described previously (6) and followed by immunoblotting with the anti-GST-π antibody for detection of GST-π as described previously (6).

RESULTS

Immunohistochemical Examination. Tissues in which over 50% of the component cells were stained immunohistochemically using the anti-GST-π antibody were evaluated as being positive for GST-π staining. Normal squamous epithelial cells of the human uterine cervix were almost negative for GST-π binding (Fig. 1), but occasionally the cytoplasm of the cells in the parabasal or intermediate layers showed a weak positive reaction. Mild and moderately dysplastic tissues demonstrated various intensities of GST-π staining. In some specimens, the cytoplasm of the cells with koilocytotic atypia (12) in the

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[3] The abbreviations used are: GST, glutathione *S*-transferase; GST-π, human placental form of GST; GST-P, rat placental form of GST (GST 7-7); HPV, human papillomavirus; p21, M_r 21,000 protein of v-H-*ras* gene product.

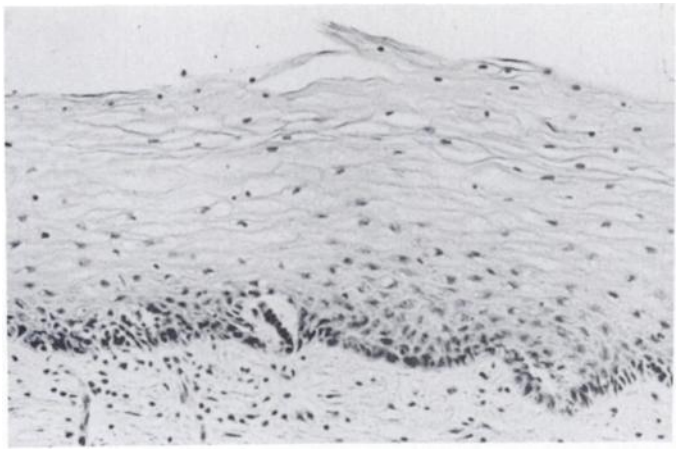

Fig. 1. Normal cervical squamous epithelium staining negative with the anti-GST-π antibody by avidin-biotin-peroxidase complex method. × 50.

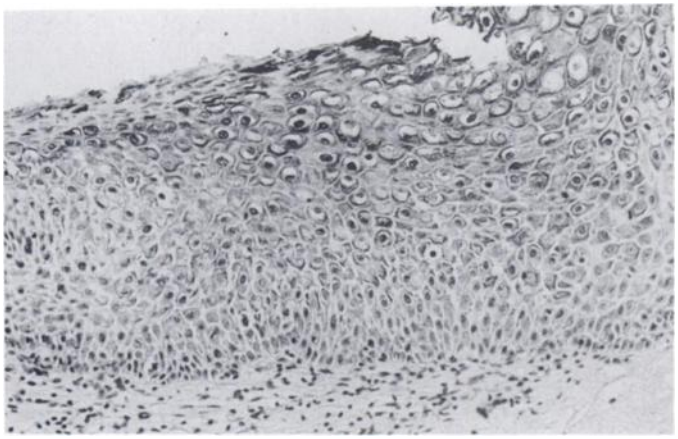

Fig. 2. Mild dysplasia stained with the anti-GST-π antibody. GST-π is weakly positive in the cytoplasm of epithelial cells with koilocytosis in the intermediate layer. × 50.

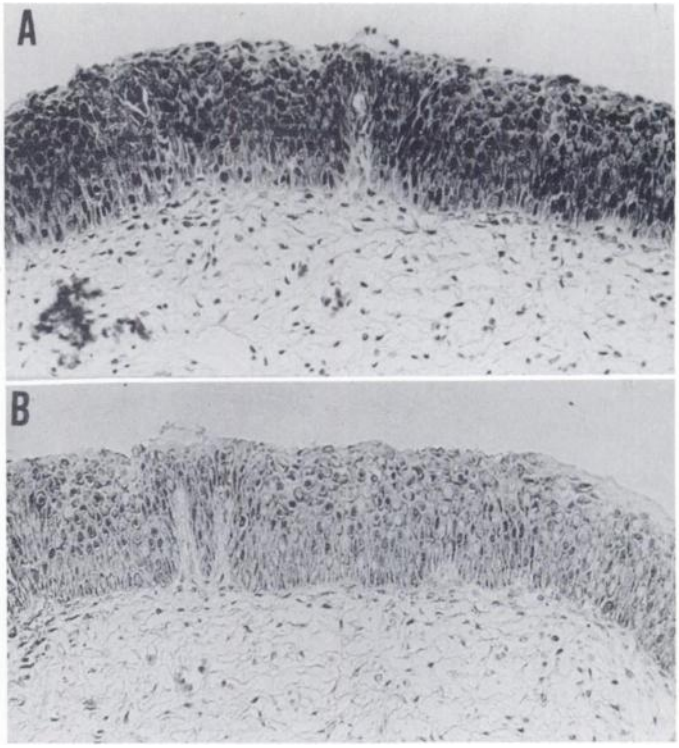

Fig. 3. *A*, severe dysplasia stained with the anti-GST-π antibody. GST-π is positive in both nuclei and cytoplasm of all cell layers except for the superficial layer. *B*, severe dysplasia stained with the antibody (rp-28) to v-H-*ras* gene product p21. The cytoplasm as well as plasma membranes is weakly positive. × 50.

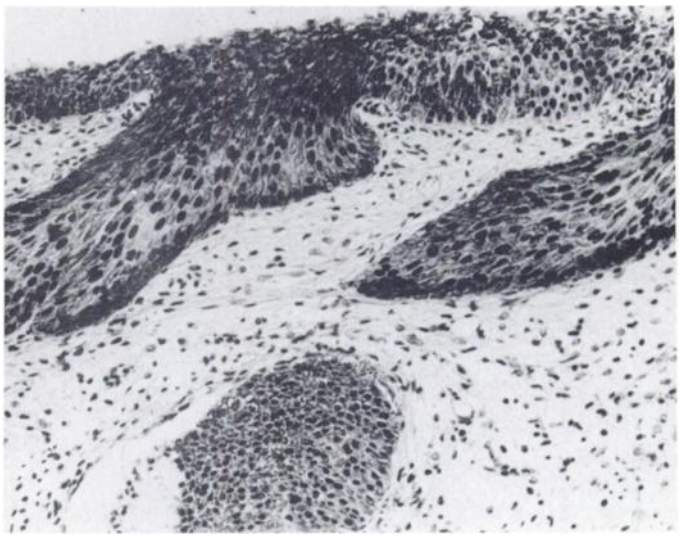

Fig. 4. Carcinoma *in situ* stained with the anti-GST-π antibody. Both cytoplasm and nuclei are strongly stained in all cell layers. × 50.

intermediate layers was only weakly positive (Fig. 2), while in others the atypical nuclei, together with the cytoplasm in parabasal and intermediate layers, were also stained. In severe dysplasia, the nuclei and cytoplasm of all but superficial layer cells were strongly positive for GST-π staining (Fig. 3*A*). In the carcinoma *in situ* specimens fixed in ethanol, both the nuclei and cytoplasm demonstrated strong antibody binding in all cell layers (Fig. 4). In formalin-ethanol-fixed specimens, however, while cytoplasmic staining was strong, that of the nuclei was much weaker. In invasive carcinoma, the cytoplasm (over 90% of cases) and the nuclei (over 70% of cases) both exhibited strong staining (Fig. 5). There was no difference in GST-π expression with regard to histological differentiation of the squamous cell carcinomas examined. The degree of expression of GST-π in various categories of dysplastic and neoplastic tissues is summarized in Tables 1 and 2. With progression from mild to moderate dysplasia, the positive evaluation of GST-π in nuclei increased first in parabasal and then in intermediate layers. Furthermore, in carcinoma *in situ* with very high levels of nuclear staining even the superficial layers became positive. Invasive carcinoma, in contrast, demonstrated a higher percentage of cases with cytoplasmic than nuclear binding. The control staining using nonimmune serum and the breakthrough antibody fraction proved negative, and the immunohistochemical findings obtained with the purified GST-π antibody preparation were the same as those obtained with the crude GST antibody (γ-globulin fraction) (data not shown). GST-α and GST-μ were stained in the cytoplasm in all of the specimens with no significant differences evident between normal, dysplastic, and neoplastic tissues (data not shown). The nuclei were not stained with these other antibody preparations.

GST Activity and GST-π Protein Content. The total GST activity and GST-π content were significantly increased in all of 4 squamous cell carcinomas as compared with values for 4 normal cervical epithelial tissue specimens (Table 3).

Two-Dimensional Gel Electrophoresis and Immunoblotting. Only the subunit of GST-π protein was stained of the large number of proteins in the cytoplasmic fraction from invasive

Table 1 *Expression of glutathione S-transferase-π form in dysplasia and neoplasia of human uterine cervix*

Tissues in which over 50% cells were stained immunohistochemically using the anti-GST-π antibody were evaluated as positive for GST-π. N and C indicate the nucleus and cytoplasm, respectively. Values indicate the number of specimens positive for GST-π.

	No. of specimens examined	Parabasal layer		Intermediate layer		Superficial layer	
		N	C	N	C	N	C
Normal squamous epithelium	10	0 (0)[a]	1 (10)	0 (0)	6 (60)	0 (0)	1 (10)
Mild to moderate dysplasia	41	21 (51)	39 (95)	11 (27)	39 (95)	1 (2)	13 (31)
Severe dysplasia	12	12 (100)	10 (83)	9 (75)	9 (75)	3 (25)	4 (33)
Carcinoma *in situ*	10	10 (100)	8 (80)	10 (100)	7 (70)	7 (70)	6 (60)
Invasive carcinoma	46	34 (73)	44 (96)	35 (76)	45 (98)	34 (74)	44 (96)

[a] Numbers in parentheses, percentage.

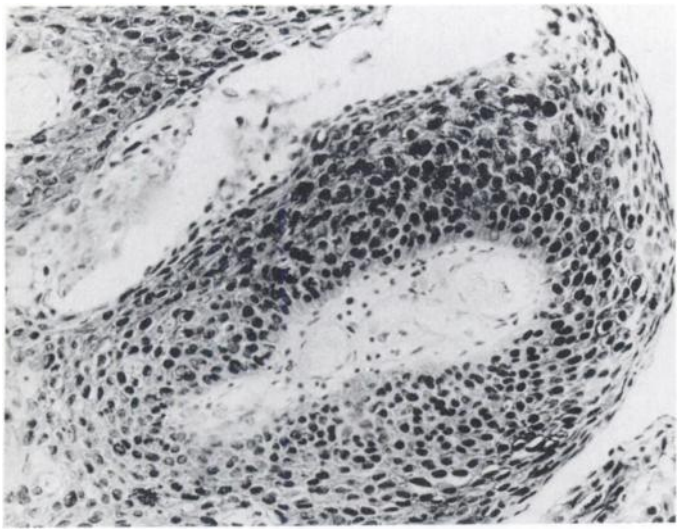

Fig. 5. Invasive carcinoma stained with anti-GST-π antibody. Both nuclei and cytoplasm are strongly stained. × 50.

carcinoma by immunoblotting with the anti-GST-π antibody following two-dimensional gel electrophoresis (Fig. 6), indicating that the antibody is monospecific for GST-π and that GST-π is expressed in invasive carcinoma at a level detectable by Coomassie brilliant blue staining.

Expression of v-H-*ras* Product. In severe dysplasia (Fig. 3*B*), carcinoma *in situ* and invasive carcinoma, v-H-*ras* gene product p21 stained positively in the cytoplasm as well as in plasma membranes with the monoclonal antibody rp-28, and the portions of tissue positive for this antigen were almost the same as those stained with the anti-GST-π antibody.

DISCUSSION

Thus far, a number of markers such as tissue polypeptide antigen (13) and TA-4 (14, 15) have been reported for human uterine cervical dysplasia and squamous cell carcinoma. However, the functions and significance of these proteins in the (pre)neoplastic lesions have not been clarified. An acidic GST form, which is the dominant GST form in human term placenta, has been purified in our laboratory from the placenta and demonstrated on the basis of several properties (6) to be identical to the GST-π reported by Guthenberg and Mannervik (16). Immunohistochemically, early placental cytotrophoblasts were strongly stained using the anti-GST-π antibody, while in term placenta mainly syncytiotrophoblasts were positive.[4] GST-π has been shown to be immunologically related to GST-P, which has proved to be one of the best markers for early detection of preneoplastic and neoplastic cells in rat chemical hepatocarcinogenesis (1–3). It was further demonstrated by us that GST-π is potentially useful for immunohistochemical detection of human (pre)neoplastic tissues in the colon (7). The results of the present study clearly indicate that GST-π may similarly be of assistance in immunohistochemical diagnosis of preneoplastic lesions in human uterine cervix.

Since Zur Hausen *et al.* (17, 18) detected HPV types 16 and 18 in human uterine cervical carcinomas by DNA hybridization, a relationship between HPV and uterine cervical carcinoma has been suggested. Moreover, in cells with cytological koilocytosis or koilocytotic atypia reported by Koss *et al.* (12), HPV particles were detected immunohistochemically (19, 20); therefore, koilocytosis has been considered to indicate HPV infection. In this study, it was evident that GST-π is expressed mainly in the cytoplasm of mildly dysplastic cells but also becomes elevated in the nuclei with irregular shapes, *i.e.*, with koilocytotic atypia. In severe dysplasia, both nuclei and cytoplasm were strongly stained, and in carcinoma *in situ* and squamous cell carcinoma the percentage of GST-π positive cases approached 100. Intranuclear binding of GST-π, like that demonstrated earlier for rat GST-P (3, 4), is noteworthy. Since the nuclei were not stained by the control reaction using nonimmune serum or the breakthrough antibody preparation from the crude anti-GST-π antibody preparation, and similar immunohistochemical findings were obtained using either pure GST-π antibody prepared by GST-π bound affinity column chromatography or the crude GST-π antibody preparation (γ-globulin fraction), it is unlikely that nuclear binding is artifactual. Positive staining with crude antibody preparations to GST-α and GST-μ was limited to the cytoplasm in all samples examined. The results therefore indicate that GST-π or the antigen(s) related to GST-π are indeed expressed in the nuclei.

It has been suggested that GST-P is related to the drug resistant properties of preneoplastic foci (21) during chemical carcinogenesis, and a similar mechanism has been proposed for multidrug resistant breast cancer cells (22). However, the actual role and functions of GST-π in (pre)neoplastic cells remain to be clarified.

Using monoclonal antibody to a v-H-*ras* oncogene product (p21), cytotrophoblasts in early placenta (data not shown), dysplastic cells in the cervix epithelium, and malignant cells in carcinoma *in situ* and invasive carcinoma were stained, although the binding reaction was observed mainly in the cytoplasm and plasma membranes. Carcinoma *in situ* was most strongly stained. The present findings suggest that the elevation of GST-π in human cervical carcinogenic process may be related, on one hand, to the infection with some types of HPV and, on the other, to the expression of H-*ras* oncogene. Further investigations are indicated.

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[4] Unpublished data.

Table 2 *Intensity of GST-π expression in dysplasia and neoplasia of human uterine cervix*

	Parabasal layer	Intermediate layer	Superficial layer
Normal epithelium	−[a] (−)[b]	± (−)	− (−)
Mild to moderate dysplasia	± (±)	+ (±)	− (−)
Severe dysplasia	+ (+)	++ (++)	± (±)
Carcinoma *in situ*	++ (++)	++ (++)	+ (+)
Invasive carcinoma	++ (++)	++ (++)	++ (++)

[a] The intensity of the staining was classified as follows: −, negative; ±, weak positive; +, positive; ++, strongly positive.
[b] In parentheses, intensity of GST-π staining of nuclei.

Table 3 *GST-π activity and content in human placenta and squamous cell carcinoma of human uterine cervix*

	GST activity[a] (units/g)	GST-π content[b] (μg/g)
Early placenta (4)[c]	5.8 ± 2.0[d]	41.4 ± 31.5
Term placenta (5)	14.8 ± 3.4	106.3 ± 29.3
Squamous cell carcinoma		
No. 1	17.2	173.8
No. 2	68.8	348.8
No. 3	43.8	360.6
No. 4	14.2	190.0
(4)	36.0 ± 25.6[e]	268.3 ± 100.1[e]
Normal (4)	5.0 ± 1.0	39.3 ± 6.3

[a] GST activity towards 1-chloro-2,4-dinitrobenzene in 105,000 × *g* supernatant.
[b] By single radial immunodiffusion.
[c] Numbers in parentheses, number examined.
[d] Mean ± SD.
[e] Mean ± SD of squamous cell carcinomas.

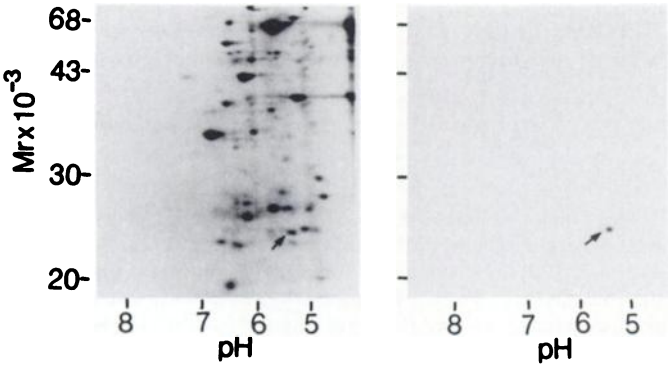

Fig. 6. Immunoblotting following two-dimensional gel electrophoresis of cytosolic proteins in invasive carcinoma. *Left*, protein staining with Coomassie Brilliant Blue R-250. *Left ordinate*, molecular weights of the marker proteins, as described previously (6). *Right*, specific immunoblotting reaction (*arrow*) with the anti-GST-π antibody.

28) to v-H-*ras* gene product (p21) and Dr. Malcolm A. Moore for assistance in preparing this manuscript.

REFERENCES

1. Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M., and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. Gann, *75:* 199–202, 1984.
2. Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I., and Sato, K. Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. Proc. Natl. Acad. Sci. USA, *82:* 3964–3968, 1985.
3. Tatematsu, M., Mera, Y., Ito, N., Satoh, K., and Sato, K. Relative merits of immunohistochemical demonstrations of placental, A, B and C forms of glutathione *S*-transferase and histochemical demonstration of γ-glutamyl transferase as markers of altered foci during liver carcinogenesis in rats. Carcinogenesis (Lond.), *6:* 1621–1626, 1985.
4. Moore, M. A., Satoh, K., Kitahara, A., Sato, K., and Ito, N. A protein cross-reacting immunohistochemically with rat glutathione *S*-transferase placental form as a marker for preneoplasia in Syrian hamster pancreatic and hepatocarcinogenesis. Jpn. J. Cancer Res., *76:* 1–4, 1985.
5. Obara, T., Makino, T., Ura, H., Yokose, Y., Kinugasa, T., Moore, M. A., Sato, K., and Konishi, Y. Comparative histochemical investigation of the glutathione *S*-transferase placental form and γ-glutamyltranspeptidase during *N*-nitrosobis(2-hydroxypropyl)amine-induced pancreatic carcinogenesis in hamsters. Carcinogenesis (Lond.), *7:* 801–805, 1986.
6. Soma, Y., Satoh, K., and Sato, K. Purification and subunit-structural and immunohistochemical characterization of five glutathione *S*-transferases in human liver, and the acidic form as a hepatic tumor marker. Biochim. Biophys. Acta, *869:* 247–258, 1986.
7. Kodate, C., Fukushi, A., Narita, T., Kudo, H., Soma, Y., and Sato, K. Human placental form of glutathione *S*-transferase (GST-π) as a new immunohistochemical marker for human colonic carcinoma. Jpn. J. Cancer Res., *77:* 226–229, 1986.
8. Hsu, S. M., Raine, L., and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J. Histochem. Cytochem., *29:* 577–580, 1981.
9. Kuzumaki, N., Oda, A., Yamagiwa, S., Taniguchi, N., Kobayashi, H., and Oikawa, T. Establishment of four mouse hybridoma cell lines producing monoclonal antibodies reactive with *ras* oncogene product p21. J. Natl. Cancer Inst., *77:* 1273–1279, 1986.
10. Habig, W. H., Pabst, M. J., and Jakoby, W. B. Glutathione *S*-transferases. J. Biol. Chem., *249:* 7130–7139, 1974.
11. Mancini, G., Carbonara, A. O., and Hermans, J. F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, *2:* 235–254, 1956.
12. Koss, L. G., and Durfee, G. R. Unusual patterns of squamous epithelium of the uterine cervix: cytologic and pathologic study of koilocytotic atypia. Ann. NY Acad. Sci., *63:* 1245–1261, 1956.
13. Einerth, Y. Cryosurgical treatment of dysplasia and carcinoma *in situ* of the cervix uteri. Acta Obstet. Gynecol. Scand., *57:* 361–365, 1978.
14. Kato, H., Morioka, H., Tsutsui, H., Aramaki, S., and Torigoe, T. Value of tumor-antigen (TA-4) of squamous cell carcinoma in predicting the extent of cervical cancer. Cancer (Phila.), *50:* 1294–1296, 1982.
15. Kato, H., Morioka, H., Aramaki, S., Tamai, K., and Torigoe, T. Prognostic significance of the tumor antigen TA-4 in squamous cell carcinoma of the uterine cervix. Am. J. Obstet. Gynecol., *145:* 350–354, 1983.
16. Guthenberg, C., and Mannervik, B. Glutathione *S*-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, 1981.
17. Durst, M., Gissmann, L., Ikenberg, H., and Zur Hausen, H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA, *80:* 3812–3815, 1983.
18. Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W., and Zur Hausen, H. A new type of papilloma-virus DNA, its presence of genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J., *3:* 1151–1157, 1984.
19. Kurman, R. J., Shah, K. H., Lancaster, W. D., and Jenson, A. B. Immunoperoxidase localization of papillomavirus antigens in cervical dysplasia and vulvar condylomas. Am. J. Obstet. Gynecol., *140:* 931–935, 1981.
20. Ferenczy, A., Braun, L., and Shah, K. V. Human papilloma-virus (HPV) in condylomatous lesions of cervix. Am. J. Surg. Pathol., *5:* 661–670, 1981.
21. Farber, E. Cellular biochemistry of the stepwise development of cancer with chemicals. G. H. A. Clowes Memorial Lecture. Cancer Res., *44:* 5463–5474, 1984.
22. Cowan, K. H., Batist, G., Tulpule, A., Sinha, B. K., and Myers, C. E. Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. Proc. Natl. Acad. Sci. USA, *83:* 9328–9332, 1986.