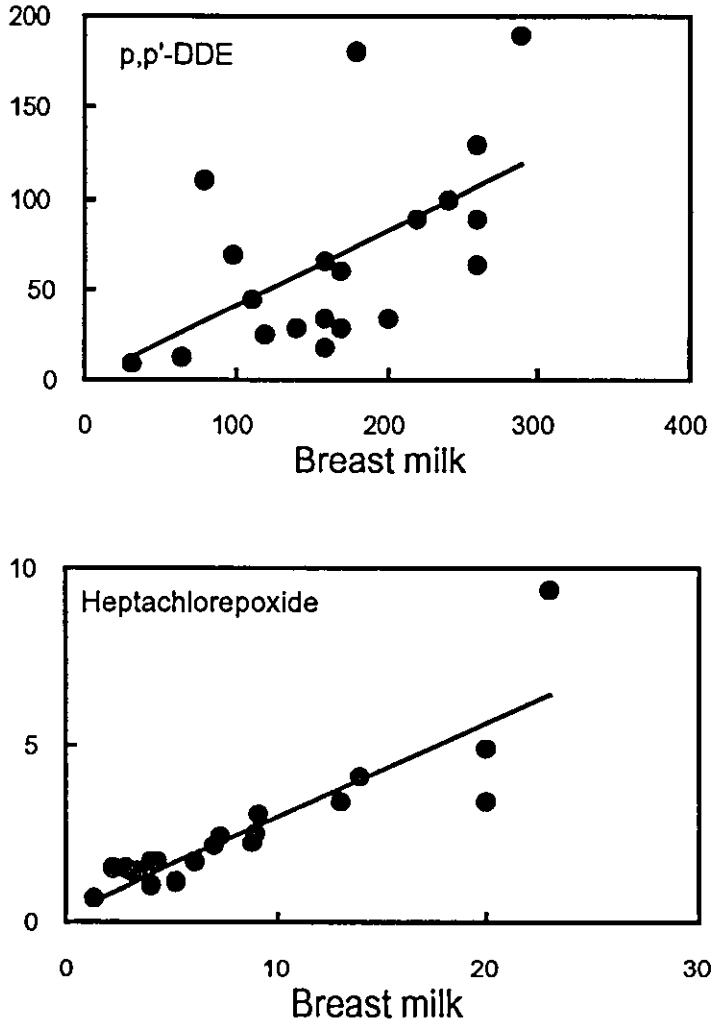

Fig. 1.: Relationship of p,p'-DDE (upper) and heptachlorepoxide (lower) between breast milk and placenta. ng/g-fat, N=20.

Acknowledgment

We thank all families who participated in this study. This research was funded by several grants from the Ministry of Health, Labor and Welfare (Risk Analysis Research on Food and Pharmaceuticals, H15-006), from the Ministry of Education, Culture, Sports, Science and Technology (B, #14370118), and from the Japan Public Health Association (Health Sciences Research Grant on Environmental Health), Japan.

References

1. Siddiqui M.K., Srivastava S., Srivastava S.P., Mehrotra P.K., Mathur N. and Tandon I. (2003) Int Arch Occupational Environ Health 76, 75-80.
2. Ribas-Fito N., Cardo E., Sala M., Eulalia de Muga M., Mazon C., Verdu A., Kogevinas M., Grimalt J.O. and Sunyer J. (2003) Pediatrics 111, e580-585.
3. Nagayama J., Okamura K., Iida T., Hirakawa H., Matsuda T., Tsuji H., Hasegawa M., Sato K., Yanagawa T., Igarashi H., Fukushige J. and Watabane T. (1998) Chemosphere 37, 1789-1793.
4. Dorner G. and Plagemann A. (2002) Neuroendocrinol Lett 23, 427-431.
5. Haradell L., Lindstrom G. and Van Bavel B. (2001) Environ Health Perspect 88, 141-144.
6. Nakai K., Suzuki K., Oka T., Murata K., Sakamoto M., Okamura K., Hosokawa T., Sakai T., Nakamura T., Saito Y., Kurokawa N., Kameo S. and Satoh H. (2004) Tohoku J Exp Med 202, 227-237.
7. Konishi Y., Kuwabara K. and Hori S. (2001) Arch Environ Contam Toxicol 40, 571-578.
8. Harris C.A., Woolridge M.W. and Hay A.W.M. (2001) Chemosphere 43, 243-256.

Tohoku J. Exp. Med., 2004, **204**, 221-228

Cervical Cytologic Examination During Physical Checkup of Pregnant Women: Cervical Cancer Screening in Women Under the Age of Thirty

YORIKO ABE, KIYOSHI ITO, CHIKAKO OKAMURA, HITOSHI NIIKURA, YUKIHIRO TERADA, TAKASHI MURAKAMI, TADAO TAKANO, NOBUYOSHI OZAWA,[1] HIROSHI NAGAI,[1] YUICHI WADA,[2] SHIGEKI UEHARA,[3] TAKANORI WATANABE,[4] SHINGO TANIGAWARA,[5] SHIGESATO WAGATSUMA,[6] YUJI YOSHIDA,[7] TSUKASA IGARASHI,[8] MASAKUNI SUZUKI,[9] KIMIO NAKAGAWA,[10] KUNIHIRO OKAMURA and NOBUO YAEGASHI

Department of Obstetrics and Gynecology, Tohoku University, Sendai, [1] Miyagi Prefecture Branch, Japanese Medical Association of Obstetrics and Gynecology, Sendai, [2] National Sendai Hospital, Sendai, [3] Tohoku Kousai Hospital, Sendai, [4] Sendai Municipal Hospital, Sendai, [5] Sendai Red Cross Hospital, Sendai, [6] Furukawa Municipal Hospital, Furukawa, [7] Sanuma Municipal General Hospital, Sanuma, [8] Miyako Prefectial Hospital, Miyako, [9] Suzuki Hospital, Iwadeyama, and [10] Nakagawa Obstetrics and Gynecology Hospital, Furukawa, Japan

ABE, Y., ITO, K., OKAMURA, C., NIIKURA, H., TERADA, Y., MURAKAMI, T., TAKANO, T., OZAWA, N., NAGAI, H., WADA, Y., UEHARA, S., WATANABE, T., TANIGAWARA, S., WAGATSUMA, S., YOSHIDA, Y., IGARASHI, T., SUZUKI, M., NAKAGAWA, K., OKAMURA, K. and YAEGASHI, N. *Cervical Cytologic Examination During Physical Checkup of Pregnant Women: Cervical Cancer Screening in Women Under the Age of Thirty.* Tohoku J. Exp. Med., 2004, **204** (3), 221-228 —— Recently, the Ministry of Health, Labor and Welfare proposed that cervical cancer screening should be conducted for women aged 20 to 29 years old in Japan. However, there are insufficient data available in Japan concerning the screening conducted for women under the age of 30. Therefore, we made a survey of the results of cervical cytologic examination for pregnant women. 28 616 pregnant women were examined as subjects of a study group. A group of 108 289 women, subjected to group screening for cervical cancer in Miyagi Prefecture, were studied as a control group. The rate of subjects who required close examination in the pregnant women's group was significantly higher than that in the mass screening group (1.12% vs. 0.84%). The rate of close examination was significantly higher in the women 19 years old or younger compared to those in the age group of 25 to 39 years old. The rate was also significantly higher in women aged 20 to 24 years old than those who are 25 to 34 years old. Of the 321

Received June 24, 2004; revision accepted for publication September 14, 2004.

Address for reprints: Kiyoshi Ito, Department of Obstetrics and Gynecology, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan.

e-mail: kito@mail.tains.tohoku.ac.jp

subjects who required close examination, 34 cases underwent treatment, and 17 cases were under age 30. Moreover, all three cases of microinvasive and/or invasive carcinoma were under the age of 30 years (23, 23, 27 years old, respectively). Our results suggest that screening for cervical cancer in pregnancy is a useful means to find cervical neoplasia in young women and is effective in reducing the cervical cancer morbidity rate. ——— cervical cancer; examination; pregnancy; young women; mass screening

In Japan, cervical cancer screening is conducted as a national project under the Health and Medical Service Law for the Aged, and early discovery has contributed to reduction of morbidity and mortality rates for cervical cancer. A study by the Ministry of Health, Labor and Welfare evaluating the effectiveness of cancer screening concluded that "there is sufficient evidence validating the effectiveness of cervical cancer screening by cytologic examination in women aged 30 or older" (Japan Public Health Association 2001).

On the other hand, the current system of cervical cancer screening has several problems. The first one is the low screening rate. For example, the rate of subjects undergoing cervical cancer screening in Miyagi Prefecture is the second highest in Japan (Health and Welfare Statistics Association 2000). Yet, this screening rate does not exceed 30% of the population (Sato et al. 1998; Miyagi Cancer Society 2003). In addition, the rate of subjects undergoing initial screening is only 10% of the total number of examination subjects (Sato et al. 1998; Miyagi Cancer Society 2003), although more than two thirds of cervical cancer cases are discovered in the initial screening. The second problem is the age of the subjects, i.e., current screening is limited to subjects aged 30 or older. In recent years, there has been an increase in incidence of cervical cancer among young women in Japan. While the overall morbidity rate of cervical cancer has been declining, the morbidity rate in the 20's and 30's age groups has been rising (Wright et al. 1994; Hamasaki and Yoshida 1998; Morimura et al. 1998; Sato et al. 1998; Ito et al. 2000; Miyagi Cancer Society 2003). For example, according to reports from the Miyagi Prefecture Cancer Registry, the age-adjusted morbidity rate for intraepithelial carcinoma in women under 30 has increased over the last 20 years from 0.4 to 3.3 per 100 000 women. Much research has identified that occurrence of cervical cancer is attributable to infection of the uterine cervix with human papillomavirus (HPV), and this infection route is known to be associated with sexual behavior (Yaegashi et al. 1989; Anttila et al. 1999; Yoshikawa et al. 1999; Woodman et al. 2001). It seems that recent changes in sexual behavior, such as decrease in age of first intercourse and an increase in the number of sexual partners, result in the spread of HPV infection among young women and lead to an increase in the cervical cancer morbidity rate among young people (Anttila et al. 1999). In the light of this, some countries have decided to begin cervical cancer screening at the age of 18, while others have decided to begin it at the age of first intercourse (Dickinson 2002; Disaia and Creasman 2002). Regardless of the fact that the same social background exists in Japan, cervical cancer screening is restricted to women aged 30 or older. It may be an important reason why cervical cancer morbidity rate has remained high among women under the age of 30 in Japan.

The question to be asked, therefore, is what extent would abnormal cases be discovered if cervical cancer screening were conducted in Japan on young women under the age of 30? Unfortunately, sufficient data is not available on this because the screening subjects in Japan had been 30 years old or older. In other words, the

current situation is that the facts concerning cervical cancer screening in women under the age of 30 in Japan are unknown.

On the other hand, a woman will most likely visit a gynecological care facility even under the age of 30 if there is possibility of pregnancy. It seems that such women often receive first medical consultation when they realize that they are pregnant and come to an obstetrics and gynecology department, and such an occasion is an opportunity for the care facility to conduct a cytologic examination in symptom-free women under the age of 30.

Here, in order to survey the results of cervical cancer screening for all women under the age of 30, we studied the results of cervical cytologic examination of pregnant women and the course of subjects with cytodiagnostic abnormalities.

Materials and Methods

Cervical cytologic examination was conducted, as screening for cervical cancer, in pregnant women who visited ten care facilities in Miyagi and Iwate prefectures (Tohoku University Hospital, Sendai National Hospital, Tohoku Kousai Hospital, Sendai Municipal Hospital, Sendai Red Cross Hospital, Furukawa Municipal Hospital, Sanuma Municipal General Hospital, Miyako Prefectural Hospital, Suzuki Hospital and Nakagawa Obstetrics and Gynecology Hospital) for pregnancy checkup over a ten-year period between 1993 and 2002. The cervical cytologic examination was conducted when the women underwent the initial pregnancy examination or during regular pregnancy checkups.

Subjects excluded from the study were women who were found to have cervical cytologic abnormalities before pregnancy and became pregnant during the follow-up period and women who were referred by other hospitals with cytologic abnormalities or pathological changes in the uterine cervix. The remaining 28 616 women (including 538 puerperant women) were employed as subjects to form the pregnant women's group in the study. The age distribution in the pregnant women's group was 560 subjects aged 19 or younger, 4159 subjects aged between 20 and 24, 11 034 subjects aged between 25 and 29, 9194 subjects aged 30 to 34, 3265 subjects aged 35 to 39 and 404 subjects aged 40 or older, and the mean age was 29.0 ± 4.88 years. The group included 15 753 subjects under the age of 30, which were 55.0% of the total. The cytologic evaluation was done based on the evaluation criteria of cervical cytologic examination of the Japanese Association of Obstetrics and Gynecology, and cases evaluated as Class IIIa, IIIb, IV or V were determined as cases requiring close examination (hereinafter, "required close examination"). The course of subjects who required close examination was followed up, and their histological diagnosis, method of treatment, and outcome were studied.

As control, 108 289 women who were subjected to cervical cancer mass screening (hereinafter, "mass screening") in Miyagi Prefecture in 2001 were used as a mass screening group. The mean age was 54.8 years old.

The χ^2 test was used to verify statistical difference. Because a relatively large number of subjects was studied, a significant difference was determined when the risk was $p < 0.01$.

Results

Rate of required close examination

Three hundred and twenty-one subjects out of 28 616 pregnant women group (1.12%) were found to belong to class III or higher in cytology (Table 1). In the mass screening group, 907 subjects out of 108 289 women (0.84%) were in class III or higher in cytology. The rate in the pregnant women's group was significantly higher than that in the mass screening group ($p < 0.001$).

Rate of required close examination by age stratification

The subjects were divided into age groups of five years, and the rate of required close examination was studied by age group (Table 2). In the pregnant women's group, the rates of required

TABLE 1. *Cytological results for the pregnant women's group and mass screening group*

	Pregnant women's group	Mass screeinig group
Cases (persons)	28 616	108 289
Close examination (persons)	321	907
Rate of close examination (%)	1.12	0.84
	$p < 0.001$	
Cytology		
IIIa	236	689
IIIb	79	190
IV	6	11
V	0	17

close examinations were 2.32% in age group of 19 or younger, 1.80% in age 20 to 24, 0.96% in age 25 to 29, 0.94% in age 30 to 34, 1.04% in age 35 to 39 and 1.73% in age 40 or older. The rate was significantly higher in age group of 19 or younger compared with the age group of 25 to 29, 30 to 34 and 35 to 39, respectively ($p < 0.01$). The rate was also significantly higher in the women aged 20 to 24 than the women in the 25 to 29 and 30 to 34 age groups ($p < 0.01$), though there was no significant difference compared with women 35 years old or older. When the pregnant women's group was divided into two groups, of women under the age of 30 and women aged 30 or older, and the two groups were compared, the rate was 1.23% (194 / 15 753) in the group of women under the age of 30 and 0.99% (127 / 12 863) in the group of women aged 30 or older. No statistically significant difference was found between them.

Next, we compared the pregnant women's group and the mass screening group. The rate of required close examination in the mass screening group was 1.78% for women aged 30 to 34, 1.12% for women aged 35 to 39 and 0.91% for women aged 40 to 44. When these rates were compared with the corresponding age groups in the pregnant women's group, the rate was significantly higher ($p < 0.001$) for the women aged 30 to 34 in the mass screening group. However, no statistically significant differences between the pregnant women's group and the mass screening group were found for the women aged 35 to 39 or for the women aged 40 or older. When groups were divided into two groups of women under the age of 30 and women aged 30 or older, the rate of required close examination in the pregnant women's group aged 30 or older had no significance compared with the corresponding age group in the mass screening group (0.99% vs. 0.84%). The rate of required close examination in the pregnant women's group of younger than 30 years old was significantly higher than that in the mass screening group ($p < 0.01$) (1.23% vs. 0.84%).

Follow-up of subjects requiring close examination

Followup was conducted on 321subjects who required close examination. Of these subjects, 60 could not be followed up because they changed hospitals, changed their address, and so on. Three cases of class IIIb and 57 cases of class IIIa were found. The remaining 261 were studied, and no abnormalities were found in close examination (spot histological examination) or repeated cytologic examination in 189 of these cases. Thirty-six cases in which atypical epitheliums were found are still under follow-up study. Two subjects were diagnosed in spot histological examination as having carcinoma in situ (CIS), but

TABLE 2. *The rate of close examination by age group*

Ages	Pregnant women's group			Mass screening group		
	Cases	Rate of close examination (%)		Cases	Rate of close examination (%)	
~19	560	13 (2.32)				
20~24	4,159	75 (1.80)	1.23			
25~29	11,034	106 (0.96)				
30~34	9,194	86 (0.94)		5,223	93 (1.78)	
35~39	3,265	34 (1.04)	0.99	8,605	96 (1.12)	0.84
40~44	404	7 (1.73)		11,477	105 (0.91)	

their whereabouts and the courses of their diseases are unknown. Thirty-four subjects are currently still under some kind of treatment, and the age, method of treatment and final histopathological diagnosis of these 34 cases are shown in Table 3. Seventeen of these subjects (50%) were under the age of 30. The method of treatment was conization in 25 cases, simple total hysterectomy in 7 cases, modified radical hysterectomy in 1 case, and radical hysterectomy in 1 case. The final histopathological diagnosis was moderate dysplasia in 2 cases, severe dysplasia in 10 cases, CIS in 19 cases, microinvasive carcinoma in 2 cases and invasive carcinoma in 1 case. The ages of all three cases of microinvasive and/or invasive carcinoma were 30 years old or younger (23, 23, 27 years old, respectively), and their cytologies were evaluated as class IIIb.

DISCUSSION

There has been an increase in the incidence of cervical cancer among young women in the developed countries (Wright et al. 1994; Hamasaki et al. 1998; Morimura et al. 1998; Anttila et al. 1999; Ito et al. 2000). Very recently, the Ministry of Health, Labor and Welfare proposed that cervical cancer screening should be conducted for women aged 20 to 29 years old even in Japan. However, there are insufficient data available in Japan to predict the results of screening possibly conducted on women under the age of 30. To the best of our knowledge, this is the first detailed report concerning about cervical cancer screening of symptom-free women under the age of 30.

Cervical cancer screening conducted on healthy pregnant women revealed that the rate of required close examination in the pregnant women's group was even higher than in the mass screening group, consisting of women aged 30 or older who undergo this screening in compliance with the current Health and Medical Service Law for the Aged. Our data in the pregnant women's group indicated that the rate of required close examination was significantly higher in the women aged 24 or younger than in older women. It is known that while the morbidity rate of cervical cancer has been declining every year, the morbidity rate of women under the age of 30 alone has been rising in Japan (Sato et al. 1998; Miyagi Cancer Society 2003). We believe that establishment of a screening system for women under the age of 30, who presently have the highest cervical cancer morbidity rate, will have the greatest impact on lowering the country's cervical cancer morbidity rate.

Previous reports concerning cervical cancer screening in pregnant women in Japan reported that the rate of required close examination was 1.2% (Abe et al. 2004), and the results of this study seem to confirm this rate. However, research samples in such reports were extremely small, numbering from 241 to 9051, and included cases of cytologic abnormalities identified before pregnancy. Our study was a joint research of sev-

TABLE 3. *The follow-up results in therapy cases*

Case	Age	Cytology	Histology	Treatment
1	23	IIIa	SD	Conization
2	27	IIIa → IV	CIS	Conization
3	27	IIIa → IV	CIS	Conization
4	28	IIIa → IIIb	CIS	Conization
5	30	IIIa → IIIb	SD	Conization
6	32	IIIa → IIIb	SD	Conization
7	23	IIIb	MIC	ATH
8	23	IIIb	MIC	ATH
9	27	IIIb	IC	RH+radiation
10	35	IIIb	CIS	Conization
11	36	IIIb	CIS	Conization
12	36	IIIb	CIS	Conization
13	34	IV	CIS	Conization
14	19	IV → IIIb	SD	Conization
15	26	IV	CIS	ATH
16	30	IV	CIS	VTH
17	34	IV	CIS	Conization
18	26	IIIb → IV	CIS	Conization
19	28	IIIb	SD	Conization
20	29	IIIb → IV	CIS	Conization
21	29	IIIb → IV	CIS	Conization
22	32	IIIb → IV	CIS	Conization
23	33	IIIb	SD	Conization
24	34	IIIb → IV	CIS	Conization
25	31	IV	CIS	Conization
26	36	IIIb	SD	ATH
28	25	IIIa	SD	Conization
27	33	IIIb	SD	Conization
29	31	IIIb	MD	Conization
30	29	IIIa → IIIb	MD	Conization
31	28	IV	CIS	Conization
32	29	IIIa → IIIb	CIS	ETH
33	39	IIIb	CIS	ATH
34	31	IIIa	SD	Conization

MD, moderate dysplasia; SD, severe dysplasia; CIS, carcinoma in situ; MIC, microinvasive carcinoma; IC, invasive carcinoma; ATH, abdominal total hysterectomy; VTH, vaginal total hysterectomy; ETH, extended total hysterectomy(modified radical hysterectomy); RH, radical hysterectomy.

eral facilities, ranging from general practitioner clinics to higher-order medical facilities, and was the largest-scale research with a subject population exceeding 28 000. The subjects in this research do not include all of the pregnant women who underwent cytologic examination but excluded patients for which cytologic abnormalities were identified before pregnancy. Therefore, this research reflects more accurately the results of cervical cancer mass screening of symptom-free subjects.

The rate of Japanese women who experience childbirth even once in their lives, as calculated from the rate of first childbirth for each age, is 64% (Kaneda 2003). If cervical cancer screening were conducted whenever a woman came to a clinic or hospital for a physical checkup in pregnancy, about two thirds of the total population of women in Japan would undergo cervical cancer screening. In addition, it seems that many women under the age of 30 get their first chance to be checked in an obstetrics and gynecology department when they become pregnant. It therefore appears that pregnancy would be the best opportunity to introduce women for the first time to cervical cancer screening. In other words, cervical cancer screening of the pregnant women is a useful means to find cervical carcinoma in young women. We expect this research to change the age range of subjects for cervical cancer screening.

Acknowledgments

This study was intended and subsidized by the Miyagi chapter of Japan Obstetrician and Gynecologist Society, and was researched by Tohoku University's Department of Obstetrics and Gynecology, School of Medicine. We express our appreciation of the opportunity granted to us for this study.

We wish to thank Miyagi Prefecture Cancer Society, National Sendai Hospital, Tohoku Kousai Hospital, Sendai Municipal Hospital, Sendai Red Cross Hospital, Suzuki Hospital, Sanuma Hospital, and Nakagawa Obstetrics and Gynecology Hospital for their cooperation in this study.

References

Abe, Y., Okamura, C., Niikura, H., Terada, Y., Murakami, T., Ozawa, N., Nagai, H., Wada, Y., Uehara, S., Watanabe, T., Tanigawara, S., Wagatsuma, S., Yoshida, Y., Igarashi, T., Suzuki, M., Nakagawa, K., Ito, K., Okamura, K. & Yaegashi, N. (2004) Study of cervical cytologic examination during physical checkup of pregnant women: Cervical cancer screening in women under the age of thirty. *Tohoku Journal of Obstetrics and Gynecology,* **51**, 24-29. (in Japanese)

Anttila, A., Pukkala, E., Soderman, B., Kallio, M., Nieminen, P., Hakama, M. (1999) Effect of organised screening on cervical cancer incidence and mortality in Finland, 1963-1995: recent increase in cervical cancer incidence. *Int. J. Cancer,* **83**, 59-65.

Dickinson, J.A. (2002) Cervical screening: time to change the policy. *Med. J. Australia,* **176**, 547-550.

Disaia, P.J. & Creasman, W.T. (2002) Preinvasive disease of the cervix. In: *Clinical Gynecologic Oncology,* P.J. Disaia & W.T. Creasman, 6th ed, St. Louis, London, Philadelphia, Sydney, Toronto, Mosby, Inc., 1-2.

Hamasaki, C. & Yoshida, K. (1998) Epidemiology and Prevention of Uterine Cancer. *Obstet. Gynecol.,* **65**, 1686-1690. (in Japanese)

Health and Welfare Statistics Association (2000) Report of senile health and welfare project by the division of statistics and information secretary of health and walfare. 328-333. (in Japanese)

Ito, T., Ishizuka, T., Suzuki, K., Ikoma, Y., Saito, J., Onuma, M., Miwa, T., Hashiba, Y., Kuno, N., Horibe, N., Mizuno, K., Ishikawa, K. & Kazeto, S. (2000) Cervical cancer in young Japanese women. *Arch. Gynecol. Obstet.,* **264**, 68-70.

Japan Public Health Association (2001) A study by the Ministry of Health, Labour and Welfare. The efficacy evaluation of cancer screening, 7-9. (in Japanese)

Kaneda, I. (2003) Maternal and child health statistics of Japan. *Mother's and Children's Health and Welfare Association,* 48-50. (in Japanese)

Miyagi Cancer Society: Annual Report, 2002 (2003) Sendai, Japan, Miyagi Cancer Society.

Morimura, Y., Hoshi, K., Tomita, T., Hanyu, T., Sekimoto, S., Sato, A. (1998) A twenty-year period in mass screening for uterine cervical cancer in Fukushima Prefecture. *Fukushima J.*

Med. Sci., **44**, 59-64.

Sato, S., Matsunaga, G. & Yajima, A. Mass screening for cancer of the uterine cervix in Miyagi Prefecture, Japan: Effects and problems. (1998) *Acta Cytol.*, **42**, 299-304.

Woodman, C.B., Collins, S., Winter, H., Bailey, A., Ellis, J., Prior, P., Yates, M., Rollason, T.P. & Young, L.S. (2001) Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet*, **357**, 1831-1836.

Wright, T.C., Kurman, R.J. & Ferenczy, A.F. (1994) Precancerous lesion of the cervix. In: *Blaustein's Pathology of the Female Genital Tract.* 4th ed., edited by R.D. Kurman, NewYork, Berlin, Heidelberg, London, Paris, Tokyo, HongKong, Barcelona, Budapest, Springer-Verlag, pp. 229-277.

Yaegashi, N., Yajima, H., Shikano, K., Sato, S. & Yajima, A. (1989) Detection of human papillomavirus type 52b in cervical dysplastic tissue. *Tohoku J. Exp. Med.*, **159**, 215-219.

Yoshikawa, H., Nagata, C., Noda, K., Nozawa, S., Yajima, A., Sekiya, S., Sugimori H., Hirai, Y., Kanazawa, K., Sugase, M., Shimizu, H. & Kawana, T. (1999) Human papillomavirus infection and other risk factors for cervical intraepithelial neoplasia in Japan. *Br. J. Cancer*, **80**, 621-624.

Steroid Sulfatase and Estrogen Sulfotransferase in Human Endometrial Carcinoma

Hiroki Utsunomiya,[1] Kiyoshi Ito,[1] Takashi Suzuki,[2] Takako Kitamura,[1] Chika Kaneko,[2] Taisuke Nakata,[3] Hitoshi Niikura,[1] Kunihiro Okamura,[1] Nobuo Yaegashi,[1] and Hironobu Sasano[2]

[1]Departments of Obstetrics and Gynecology and [2]Pathology, Tohoku University School of Medicine, Sendai; and [3]Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

ABSTRACT

Purpose: **Intratumoral metabolism and synthesis of estrogens are considered to play important roles in the pathogenesis and/or development of human endometrial carcinoma. Steroid sulfatase hydrolyzes biologically inactive estrogen sulfates to active estrogens, whereas estrogen sulfotransferase sulfonates estrogens to estrogen sulfates. However, the status of steroid sulfatase and/or estrogen sulfotransferase in human endometrial carcinoma has not been examined.**

Experimental Design: **We first examined the expression of steroid sulfatase and estrogen sulfotransferase in 6 normal endometrium and 76 endometrial carcinoma using immunohistochemistry to elucidate the possible involvement of steroid sulfatase and estrogen sulfotransferase. We then evaluated the enzymatic activity and the semiquantitative analysis of mRNA using reverse transcription-PCR in 21 endometrial carcinomas. We correlated these findings with various clinicopathological parameters including the expression of aromatase, 17β-hydroxysteroid dehydrogenase type 1 and type 2.**

Results: **Steroid sulfatase and estrogen sulfotransferase immunoreactivity was detected in 65 of 76 (86%) and 22 of 76 (29%) cases, respectively. Results of immunoreactivity for steroid sulfatase and estrogen sulfotransferase were significantly correlated with those of enzymatic activity and semiquantitative analysis of mRNA. No significant correlations were detected among the expression of the enzymes involved in intratumoral estrogen metabolism. There was a significant correlation between steroid sulfatase/estrogen sulfotransferase ratio and clinical outcomes of the patients. However, there were no significant differences between steroid sulfatase or estrogen sulfotransferase and estrogen receptor, progesterone receptor, Ki67, histologic grade, or clinical outcomes of the patients.**

Conclusions: **Results of our study demonstrated that increased steroid sulfatase and decreased estrogen sulfotransferase expression in human endometrial carcinomas may result in increased availability of biologically active estrogens and may be related to estrogen-dependent biological features of carcinoma.**

INTRODUCTION

Endometrial carcinoma is one of the most common malignancies in developed countries, and its incidence has increased recently (1). *In situ* estrogen metabolism, including its synthesis and degradation, has been considered to play a very important role in the development and/or progression of various human estrogen-dependent neoplasms including endometrial carcinoma (2). In endometrial carcinoma, *in situ* 17β-estradiol availability has been demonstrated to be closely related to the pathogenesis and development of endometrial proliferative disorders including endometrial hyperplasia and carcinoma, especially of the endometrioid type (3). Aromatase catalyzes circulating androgens, which are androstenedione and testosterone, into estrone (E1) and 17β-estradiol, respectively (4, 5). The enzyme 17β-hydroxysteroid dehydrogenase catalyzes the reversible interconversion of E1 and 17β-estradiol. 17β-Hydroxysteroid dehydrogenase type 1 catalyzes the 17β-reduction of biologically weak E1 to strong 17β-estradiol (6–8), whereas 17β-hydroxysteroid dehydrogenase type 2 preferentially catalyzes the oxidation of 17β-estradiol to E1 (9).

A major circulating form of plasma estrogen is estrogen sulfate (E1S and E2S), a biologically inactive form of estrogen. E1S and E2S have a relatively long half-life in the peripheral blood (10), where serum levels of E1S and E2S are known to be 10-fold higher than those of unconjugated E1 or 17β-estradiol (11). It was reported recently that *in situ* estrogen activity in breast cancer, which is estrogen dependent as well as endometrial cancer, may be mainly regulated by the status of intratumoral steroid sulfatase (12, 13). Steroid sulfatase hydrolyzes biologically inactive estrogen sulfates to active estrogens. Estrogen sulfotransferase (*SULT 1E1* or *STE* gene) is a member of the superfamily of steroid sulfotransferases and sulfonates estrogens to estrogen sulfates (14–16). Therefore, it is suggested that estrogen sulfotransferase, especially the balance between the levels of intratumoral steroid sulfatase and estrogen sulfo-

Received 1/7/04; revised 4/1/04; accepted 4/8/04.
Grant support: Grant-in-aid for Cancer Research 7–1 from The Ministry of Health and Welfare, Japan, a grant-in-aid for scientific research area on priority area (A-11137301) from The Ministry of Education, Science and Culture, Japan, a grant-in-aid for Scientific Research (B-11470047) from Japan Society for the Promotion of Science, and a grant from The Naitou Foundation and Suzukenn Memorial Foundation.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Hiroki Utsunomiya, Northwestern University, Reproductive Biology Research, Department of Obstetrics and Gynecology, 303 East Chicago Avenue, Tarry Building, Suite #13–756, Chicago, IL 60611. Phone: 312-503-5399; Fax: 312-503-5394; E-mail: u-kichi@pg8.so-net.ne.jp.

transferase, may also play an important role in the regulation of *in situ* estrogen levels in human endometrial carcinoma. Steroid sulfatase and estrogen sulfotransferase activities have been examined in estrogen-dependent neoplasms such as endometrial and breast cancers (17). However, to date, steroid sulfatase and estrogen sulfotransferase mRNA and protein expressions have not been examined in human endometrial carcinoma. In addition, the comparison among the enzymes involved in intratumoral estrogen production and metabolism has not been reported in human endometrial carcinoma. Therefore, in this study we first examined the expression of steroid sulfatase and estrogen sulfotransferase in 6 normal cycling endometrium and 76 endometrial carcinomas using immunohistochemistry to elucidate the possible involvement of steroid sulfatase and estrogen sulfotransferase. We then studied the enzymatic activity and semiquantitative analysis of mRNA by reverse transcription-PCR (RT-PCR) for steroid sulfatase and estrogen sulfotransferase in endometrial carcinomas. We subsequently correlated these findings with the results of Aromatase, 17β-hydroxysteroid dehydrogenase type 1, type 2, and clinicopathological parameters to study the biological and/or clinical significance of steroid sulfatase and estrogen sulfotransferase.

MATERIALS AND METHODS

Tissue Preparation. Six normal cycling human endometria (3 proliferative phase and 3 secretory phase) and 76 endometrial endometrioid adenocarcinomas (33 well differentiated, 26 moderately differentiated, and 17 poorly differentiated; 44 stage I, 14 stage II, 17 stage III, and 1 stage IV) were obtained from surgical pathology files of Tohoku University Hospital. This study was approved by the Ethical Committee of Tohoku University School of Medicine. We obtained nonpathological endometria from hysterectomy specimens performed due to carcinoma *in situ* of the uterine cervix. Endometrial carcinoma specimens were obtained from hysterectomy. All of the patients examined had not received irradiation or chemotherapy before surgery. The histopathological classification in each specimen was evaluated according to Fédération Internationale des Gynaecologistes et Obstetristes histologic grading system for endometrial carcinoma in 1988 (18).

The specimens were all routinely processed (i.e.,10% formalin fixed for 24 to 48 h), paraffin embedded, and thin sectioned (3 μm).

Antibodies. Rabbit polyclonal antibody for estrogen sulfotransferase (PV-P2237) was purchased from Medical Biological Laboratory (Nagoya, Japan). This antibody was raised against the synthetic NH_2-terminal peptide of human estrogen sulfotransferase corresponding to amino acids 1–13. The affinity-purified monoclonal antibody for steroid sulfatase (KM1049) was raised against the enzyme purified from human placenta, which recognized the steroid sulfatase peptide corresponding to amino acids 420–428. 17β-Hydroxysteroid dehydrogenase type 1 antibody (polyclonal) and 17β-hydroxysteroid dehydrogenase type 2 antibody (monoclonal) were kindly provided by Dr. Matti Poutanen at the University of Oulu (Oulu, Finland) and Dr. Stefan Andersson at the University of Texas Southwestern Medical Center (Dallas, TX), respectively. Aromatase antibody (polyclonal) was provided by Dr. Nobuhiro Harada at the Fujita Health University School of Medicine (Aichi, Japan). Monoclonal antibodies for estrogen receptor (ER1D5), progesterone receptor (MAB429), and Ki67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA), and DAKO (Carpinteria, CA), respectively. Utilization of these antibodies for immunohistochemistry has been reported previously (19).

Immunohistochemistry. Immunohistochemical analyses were performed using the streptavidin-biotin amplification method using EnVision (DAKO Co. Ltd., Carpinteria, CA) for steroid sulfatase and using a Histofine kit (Nichirei, Tokyo, Japan) for estrogen sulfotransferase, Aromatase, estrogen receptor, progesterone receptor, Ki67, 17β-hydroxysteroid dehydrogenase type 1, and type 2. The dilutions of the primary antibodies used in this study were as follows: 1:1,500 estrogen sulfotransferase, 1:9,000 steroid sulfatase, 1:800 17β-hydroxysteroid dehydrogenase type 1, 1:5 17β-hydroxysteroid dehydrogenase type 2, 1:750 Aromatase, 1:2 estrogen receptor, 1:30 progesterone receptor, and 1:50 Ki67.

The antigen-antibody complex was visualized with 3.3′-diaminobenzidine solution [1 mmol/L 3.3′-diaminobenzidine, 50 mmol/L Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2], and counterstained with hematoxylin. Tissue sections of full-term placenta were used as positive control for steroid sulfatase, and normal liver was also used as positive control for estrogen sulfotransferase. As for negative controls, normal rabbit or mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of Immunoreactivity. For evaluation of steroid sulfatase and estrogen sulfotransferase immunoreactivity, we determined the labeling index (LI: *i.e.*, the percentage of positive cells) according to the report by Sasano *et al.* (20). As in previous studies, two of the authors (H. U. and T. S.) independently divided the cases into the following two groups: +, > 5% positive cells and −: <5% positive-cell immunoreactivity (20, 21). Scoring of 17β-hydroxysteroid dehydrogenase type 1, type 2, estrogen receptor, progesterone receptor, and Ki67 in gland or carcinoma cells was reported previously (21).

Enzyme Assay. Twenty one carcinoma cases of fresh-frozen tissues (*i.e.*, the cases immediately frozen in liquid nitrogen and stored at −80°C) were available for examination of enzymatic assay. Estrogen sulfotransferase was assayed as described previously (15). The samples were homogenized at 4°C in phosphate buffer [100 mmol/L KCl, 10 mmol/L KH_2PO_4, 10 mmol/L Na_2HPO_4, and 1 mmol/L EDTA (pH 7.5)], and centrifuged for 15 minutes at 1000 × *g*. The upper layer was used as the enzyme source. Approximately 0.2 mg of protein were added in each assay, and the reaction contained 50 mmol/L Tris-HCl (pH 7.4) and 7 mmol/L $MgCl_2$, and E1 contained [^{3}H]E1 at 20 nM. Reactions were started with the addition of 3′-phosphoadenosine 5′-phosphosulfate to final concentration of 20 μmol/L in a final volume of 0.125 ml. The reaction mixtures were incubated at 37°C for 30 minutes, and the reactions were terminated with the addition of 4.0 mL of chloroform followed by the addition of 0.375 mL 0.25 mol/L Tris-HCl (pH 8.7) to alkalinize the solution. The reaction mixtures were centrifuged at 600 × *g* for 5 minutes to separate E1S (aqueous phase) from E1 (organic phase). Synthesis of the tritiated E1S

was determined with a liquid scintillation counter (Beckman, LC-6500). The steroid sulfatase activity was assayed according to Utaaker and Stoa (22) with slight modifications.

Briefly, enzyme solution (~0.2 mg protein) was mixed with E1S containing [6,7-^{3}H]E1S (1.6×10^5 dpm, 0.5 pmol/L) at 20 μmol/L and added to a reaction volume up to 15 mL with PBS (-) containing 25 mmol/L sucrose and 4 mmol/L Nicotinamide. The reaction mixture was incubated at 37°C for 60 minutes in a shaking water bath. The enzyme reaction was terminated with the addition of toluene and mixed by vortex mixer for 1 minute. The reaction mixtures were centrifuged at $600 \times g$ for 5 minutes to separate E1S (aqueous phase) from E1 (organic phase). The toluene layer was collected, and [^{3}H] radioactivity was measured by liquid scintillation counter (LC-6500, Beckman), which is equivalent to E1 formed. Incubation condition of these assays was designed so that the formation of product was linear.

Semiquantitative Analysis of mRNA. Twenty one specimen of fresh-frozen tissues (*i.e.*, the cases immediately frozen in liquid nitrogen and stored at −80°C) were available for examination. Total RNA was extracted by homogenizing frozen tissue samples in 1 mL of TRIzol reagent (Life Technologies, Inc., Grand Island, NY) followed by a phenol-chloroform phase extraction and isopropanol precipitation. All of the RNA samples were quantified by spectrophotometry and stored at −80°C until they were processed for reverse transcription. The SUPERSCRIPT Preamplification system reverse transcription kit (Life Technologies, Inc.) was used in the synthesis and amplification of cDNA. cDNA was synthesized from total RNA (2 μg) using 25 ng/μL oligo(dT)$_{12-18}$ primer (Life Technologies, Inc., Gaithersburg, MD) on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Inc., Watertown, MA). To test for the presence of genomic DNA contamination, we performed the reverse transcription step in the absence of SUPERSCRIPT II RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc.,) followed by PCR. RT-PCR products lacking reverse transcriptase in the initial reverse transcription step were run on an ethidium bromide-stained 2% agarose gel. No band was observed in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) using the DNA binding dye SYBER Green I (Roche Diagnostics GmbH) for the detection of PCR products. The primer sequences used in this study are as follows: estrogen sulfotransferase [NM005420; FWD 5′-AGAGGAGCTTGTGGACAGGA-3′ and REV 5′-GGCGACAATTTCTGGTTCAT-3′] (14), steroid sulfatase [M16505; FWD 5′-AGGGTCTGGGTGTGTCTGTC-3′ and REV 5′-ACTGCAACGCCTACTTAAATG-3′] (23), and glyceraldehyde-3-phosphate dehydrogenase [M33197; FWD 5′-TGAACGGGAAGCTCACTGG-3′ and REV 5′-TCCACCACCCTGTTGCTGTA-3′] (24). PCR was set up using 2 mmol/L $MgCl_2$, 10 pmol/L of each primer, and 2.5 units of TaqDNA polymerase (Life Technologies, Inc.). An initial denaturing step of 95°C for 1 minute was followed by 40 cycles, respectively, of 95°C for 0 seconds; 15 seconds annealing at 60°C (steroid sulfatase and glyceraldehyde-3-phosphate dehydrogenase) and 58°C (estrogen sulfotransferase); and extension for 15 seconds at 72°C. The fluorescence intensity of the double strand-specific SYBER Green I, which reflects the amount of formed specific PCR products, was read by the LightCycler at 85°C after the end of each extension step. After PCR, these products were resolved on a 2% agarose ethidium bromide gel. Images were captured with Polaroid film under UV transillumination. In initial experiments, PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 310 Genetic Analyzer, Perkin-Elmer Corp., PE Applied Biosystems, Foster City, CA) to verify amplification of the correct sequences. As a positive control, frozen tissues of placenta were used for steroid sulfatase (25), and liver (HuH7 human hepatocellular carcinoma cells) was used for estrogen sulfotransferase (16). Negative control experiments lacked cDNA substrate to check for the presence of exogenous contamination DNA. No amplified products were observed under these conditions. The mRNA levels of steroid sulfatase and estrogen sulfotransferase in each case are summarized as a ratio of glyceraldehyde-3-phosphate dehydrogenase and evaluated as a ratio (%) compared with that of each positive control. Although conventional quantitative PCR requires the utilization of a purified plasma cDNA in the construction of a standard curve, we found that we were able to semiquantify our PCR products with the Light Cycler using purified cDNA of known concentrations. Other studies to date have used a similar protocol to semiquantify PCR products with the LightCycler (26, 27).

Statistical Analysis. Values for patient age and LIs of estrogen receptor, progesterone receptor, and Ki67 were presented as mean ±95% confidence interval. Association between steroid sulfatase/estrogen sulfotransferase and these parameters was evaluated using Welch's t test. We also studied the statistical differences between patients for steroid sulfatase/estrogen sulfotransferase and histologic grade in a cross-table using the χ^2 test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method. P values < 0.05 were considered as significant.

RESULTS

Normal Cycling Endometrium. Estrogen sulfotransferase immunoreactive protein was detected only in the cytoplasm of glandular cells in the secretory phase (Fig. 1*A*). Estrogen sulfotransferase immunoreactivity was not detected in either epithelium or stromal cells of proliferative phase endometrium (Fig. 1*B*). Steroid sulfatase immunoreactivity was not detected in any of the cases examined.

Endometrial Carcinoma. Steroid sulfatase and estrogen sulfotransferase immunoreactivity was detected in the cytoplasm of carcinoma cells (Fig. 2, *A* and *B*) but not in stromal cells. Steroid sulfatase and estrogen sulfotransferase immunoreactivity was detected in 65 of 76 (86%) and 22 of 76 (29%) cases, respectively. There were no significant correlations between steroid sulfatase or estrogen sulfotransferase and estrogen receptor LI, progesterone receptor LI, Ki67 LI, histologic grade, or clinical outcomes of the patients (Tables 1 and 2). However, there was a significant positive correlation between steroid sulfatase/estrogen sulfotransferase ratio and clinical outcomes of the patients [$P < 0.05$; alive (3.93 ± 2.95) *versus* dead (8.35 ± 4.53)]. There were

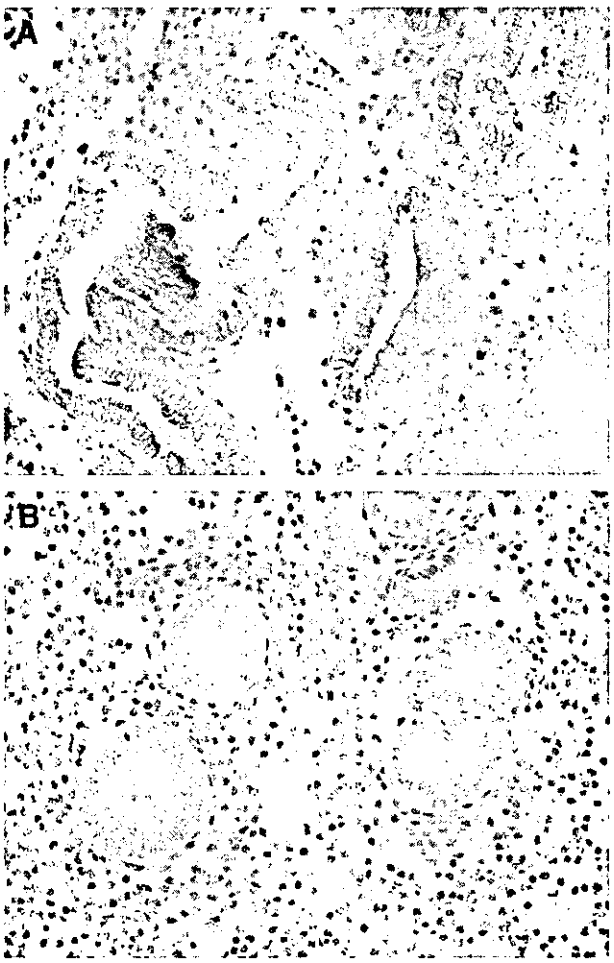

Fig. 1 Immunohistochemistry for estrogen sulfotransferase in normal endometrium obtained during secretory phase (*A*) and proliferative phase (*B*). Original magnification, ×200.

no associations among the expression of steroid sulfatase, estrogen sulfotransferase, Aromatase, 17β-hydroxysteroid dehydrogenase type 1, and type 2 (Table 3).

For enzyme activity, the results of all of the cases are summarized in Table 4. There was a statistically significant positive correlation between immunoreactivity and enzyme activity for steroid sulfatase [$P < 0.01$; + (0.48 ± 0.22 nmol/mg/hour) *versus* − (0.18 ± 0.10 nmol/mg/hour)] and estrogen sulfotransferase [$P < 0.05$; + (0.41 ± 0.46 nmol/mg/hour) *versus* − (0.09 ± 0.03 nmol/mg/hour)].

For RT-PCR, the results of all cases are summarized in Table 4. There was a statistically significant positive correlation between steroid sulfatase immunoreactivity and results of steroid sulfatase semiquantitative analysis of mRNA (RT-PCR) [$P < 0.05$; + (42.2 ± 22.3) *versus* − (20.2 ± 9.3)]. There was also significant positive correlation between estrogen sulfotransferase immunoreactivity and results of estrogen sulfotransferase RT-PCR analysis [$P < 0.05$; + (35.0 ± 28.1) *versus* − (15.9 ± 7.4)].

DISCUSSION

Estrogens, especially 17β-estradiol, have been demonstrated to contribute greatly to the development and progression of the great majority of endometrial carcinoma (28, 29). In addition, *in situ* estrogen metabolism, including its synthesis and degradation, has been considered recently to play a very important role in the development and progression of various human estrogen-dependent neoplasms including endometrial carcinoma especially in the postmenopausal subjects (2).

Two principal pathways are implicated in the last steps of 17β-estradiol formation in humans. One is through the aromatization of androstenedione or testosterone to E1 or 17β-estradiol, which was considered a major pathway for peripheral estrogen production (4, 5). However, conversion of E1S and E2S into E1 and 17β-estradiol has been also postulated as a source of peripheral estrogen production (30, 31). For instance, the concentrations of E1S and E2S in breast cancer tissues have been reported to be significantly higher than the circulating plasma levels (32). In addition, quantitative determinations of estrogens in breast cancer tissues demonstrated that the "estrogen sulfatase pathway" is 40–500 times higher than that of the "aromatase pathway" (32, 33). Therefore, peripheral steroid sulfatase/estrogen sulfotransferase is considered to play very

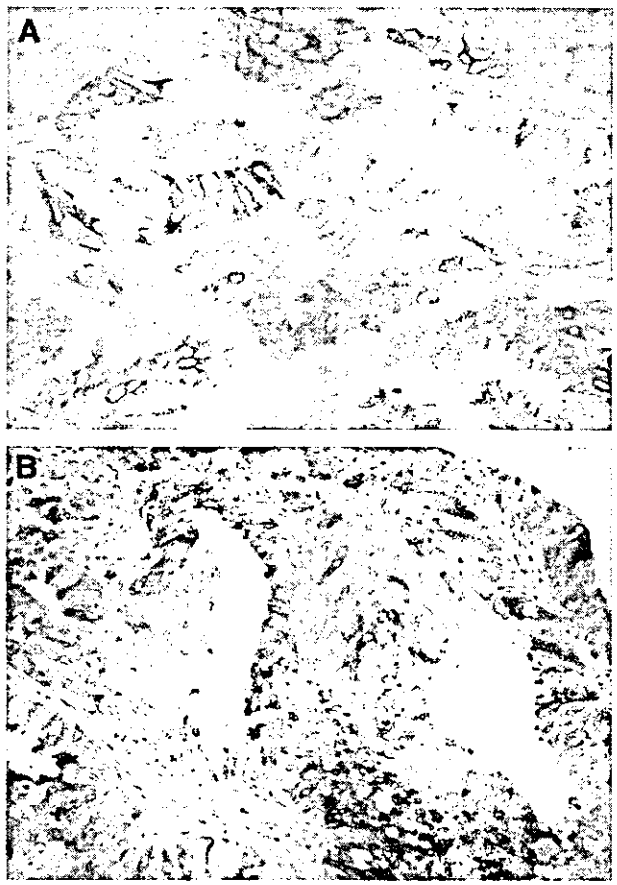

Fig. 2 Immunohistochemistry for steroid sulfatase (*A*) and estrogen sulfotransferase (*B*) in endometrial endometrioid adenocarcinoma. Original magnification, ×200.

Table 1 Correlation between STS immunoreactivity and other parameters in human endometrial carcinoma

	STS expression		
	+ (n = 65)	− (n = 11)	P
ER LI (%)	50.7 ± 13.7	52.1 ± 9.8	N.S.
PR LI (%)	41.9 ± 9.8	37.4 ± 13.9	N.S.
Ki67 LI (%)	31.0 ± 16.8	24.0 ± 12.8	N.S.
Age (years)	48.4 ± 7.6	44.1 ± 8.1	N.S.
Grade 1	32	3	
Grade 2	20	4	
Grade 3	13	4	N.S.
Overall survival (5 years)	87.5%	87.5%	N.S.
Disease-free survival (5 years)	80.0%	80.0%	N.S.

NOTE. All data except for grade and survival are presented as mean ± 95% confidence interval.

Abbreviations: STS, steroid sulfatase; N.S., not significant; PR, progesterone receptor; ER, estrogen receptor.

Table 2 Correlation between EST immunoreactivity and other parameters in human endometrial carcinoma

	EST expression		
	+ (n = 22)	− (n = 54)	P
ER LI (%)	48.0 ± 12.2	54.6 ± 15.8	N.S.
PR LI (%)	44.2 ± 12.0	36.2 ± 9.1	N.S.
Ki67 LI (%)	27.6 ± 12.9	30.3 ± 11.6	N.S.
Age (years)	45.3 ± 8.4	46.8 ± 7.2	N.S.
Grade 1	10	23	
Grade 2	6	20	
Grade 3	6	11	N.S.
Overall survival (5 years)	91.2%	85.3%	N.S.
Disease-free survival (5 years)	83.3%	79.4%	N.S.

NOTE. All data except for grade and survival are presented as mean ± 95% confidence interval.

Abbreviations: EST, estrogen sulfotransferase; N.S., not significant; ER, estrogen receptor; PR, progesterone receptor.

important roles in estrogen metabolism and actions in human breast cancer.

Steroid sulfatase is known as a single enzyme that hydrolyzes several sulfated steroids, including not only E1S and E2S but also dehydroepiandrosterone sulfate and cholesterol sulfate (34, 35). Recent studies demonstrated that the bioactive androgen 5α-dihydrotestosterone is locally produced from dehydroepiandrosterone (36, 37) in human breast cancer tissues. A great majority of estrogen receptor-positive breast carcinomas are also positive for androgen receptor (38). *In vitro* studies also demonstrated that 5α-dihydrotestosterone decreases estrogen-induced cell proliferation in human breast cancer cells (39). Therefore, steroid sulfatase may possibly be involved in the local production of both androgens and estrogens in endometrial carcinoma as well as breast carcinomas. Additional investigations are required to clarify the biological significance of these interesting findings.

Estrogen sulfotransferase expression has been reported to be induced by progesterone in the uterus (40–42). In addition, estrogen sulfotransferase activities were reported to be highest during the secretory phase of the menstrual cycle in the porcine uterus, which suggests a possible correlation between uterine estrogen sulfotransferase activity and plasma progesterone levels (43). However, we reported previously that 17β-hydroxysteroid dehydrogenase type 2 was also present in the endometrial glandular epithelium of secretory phase, but 17β-hydroxysteroid dehydrogenase type 1 was not detected in any of the phases (21). Estrogen sulfotransferase in endometrial carcinoma was markedly diminished compared with normal endometrium of secretory phase, which is in the same manner as pattern of 17β-hydroxysteroid dehydrogenase type 2 expression

Table 3 Correlation between immunoreactivity of the enzymes

	STS LI	EST LI	17βHSD Type2 LI	STS LI
STS				
Positive cases		22.6 ± 38.4	6.8 ± 4.1	7.9 ± 4.3
Negative cases		11.5 ± 11.7	11.1 ± 13.1	8.6 ± 3.9
		N.S.	N.S.	N.S.
EST				
Positive cases	36.2 ± 16.6		12.0 ± 7.74	7.3 ± 9.1
Negative cases	45.1 ± 20.9		8.2 ± 9.63	8.1 ± 4.0
	N.S.		N.S.	N.S.
17βHSD Type2				
Positive cases	50.2 ± 26.2	13.5 ± 12.0		9.1 ± 5.0
Negative cases	35.7 ± 21.6	21.1 ± 35.4		7.3 ± 2.2
	N.S.	N.S.		N.S.
Arom.				
Positive cases	44.5 ± 30.0	14.6 ± 9.4	9.0 ± 4.2	
Negative cases	38.5 ± 19.3	24.6 ± 37.0	9.5 ± 7.8	
	N.S.	N.S.	N.S.	

NOTE. All data are presented as mean ± 95% confidence interval.

Abbreviations: STS, steroid sulfatase; EST, estrogen sulfotransferase; N.S., not significant; Arom., aromatase; 17βHSD, 17β-hydroxysteroid dehydrogenase.

Table 4 Twenty one cases for enzyme assay and RT-PCR

	Enzyme Assay (nmol/mg protein/hr)		mRNA level (%)		Immunoreactivity	
Case	STS	EST	STS	EST	STS	EST
1	0.84	0.11	58.3	28.4	+	−
2	0.29	0.05	11.4	6.3	+	−
3	0.57	0.07	25.0	12.9	+	−
4	0.39	0.12	43.3	24.0	+	−
5	0.16	0.21	27.7	33.3	−	+
6	0.10	1.31	11.3	89.5	−	+
7	0.10	0.08	13.5	11.1	+	−
8	0.30	0.10	7.7	13.1	−	−
9	0.75	0.06	81.1	21.6	+	−
10	0.60	0.10	44.0	18.8	+	+
11	0.43	0.07	16.7	9.9	+	−
12	0.38	0.12	40.5	3.8	+	−
13	0.29	0.06	22.3	16.4	−	−
14	0.14	0.13	31.7	9.7	−	+
15	0.39	0.12	52.8	25.0	+	−
16	0.17	0.07	47.1	13.7	+	−
17	0.69	0.40	30.6	32.2	+	+
18	0.63	0.06	87.9	11.1	+	−
19	0.08	0.11	19.1	15.9	−	−
20	0.29	0.29	34.0	26.4	+	+
21	0.66	0.07	50.4	25.0	+	−

Abbreviations: EST, estrogen sulfotransferase; STS, steroid sulfatase.

in human endometrium (21). These results suggest that the expression of estrogen sulfotransferase and 17β-hydroxysteroid dehydrogenase type 2, both of which decrease *in situ* estrogen activity, may be regulated by progesterone.

It was reported that steroid sulfatase activities in endometrial cancer tissues were significantly higher than those in normal endometrial tissues (17). In addition, estrogen sulfotransferase activities were reported to be highest during the secretory phase of the menstrual cycle (40, 41). In our present study, steroid sulfatase was absent through the menstrual cycle, and estrogen sulfotransferase was highly expressed in normal endometrium of secretory phase, which were consistent with the results of previous studies. On the other hand, steroid sulfatase was strongly expressed in 86% of the endometrial carcinoma cases, but estrogen sulfotransferase was expressed only in 29% of the cases. Decreased expression of estrogen sulfotransferase and 17β-hydroxysteroid dehydrogenase type 2 in endometrial carcinoma may be related to abundant *in situ* availability of 17β-estradiol. Therefore, *in situ* estrogen activity in endometrial carcinoma is much higher than that in normal endometrium. However, there are no correlations between steroid sulfatase or estrogen sulfotransferase and clinicopathological parameters and clinical outcomes of the patients. Therefore, these data indicated that not only steroid sulfatase and estrogen sulfotransferase but also Aromatase and 17β-hydroxysteroid dehydrogenase type 2 may independently contribute to the regulation of *in situ* estrogen availability and/or activity in human endometrial carcinoma. In addition, there was a significant positive correlation between steroid sulfatase/estrogen sulfotransferase ratio and clinical outcomes of the patients. Therefore, the results of the present study demonstrated that increased steroid sulfatase and decreased estrogen sulfotransferase expression in human endometrial carcinomas result in increased *in situ* availability of biologically active estrogens and may be related to various estrogen-dependent features of carcinoma.

In endometrial carcinoma, 17β-hydroxysteroid dehydrogenase type 1 expression was not detected (21). This is in contrast to the study of 17β-hydroxysteroid dehydrogenase type 1 in breast cancer in which nearly half of the cases demonstrated 17β-hydroxysteroid dehydrogenase type 1 immunoreactivity in carcinoma cells, and 17β-hydroxysteroid dehydrogenase type 2 was not expressed (20, 44). We have reported recently that estrogen sulfotransferase is an independent prognostic factor in human breast carcinoma (45). However, there is no correlation between estrogen sulfotransferase and clinical outcomes of the patients in this study. These results indicate that intratumoral estrogen metabolism is different between human breast and endometrial carcinoma, although both of them are sex steroid-dependent malignancies.

In conclusion, the results from our study also suggest that induction of estrogen sulfotransferase and 17β-hydroxysteroid dehydrogenase type 2 and/or inhibition of steroid sulfatase and aromatase may also have possible important therapeutic potential as an endocrine therapy for endometrial carcinoma. Utilization of selective agents designed with the intent to block the expression of enzyme related to intratumor estrogen production can result in a more specific inhibition of estrogen actions in endometrial carcinoma, which may, in turn, eventually lead to an improvement in the prognosis in some of the patients, but additional investigations are required for clarification.

REFERENCES

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics. CA-Cancer J Clin 1996;46:5–27.

2. Silverberg SG, Mullen D, Faraci JA, Makowski EL, Miller A, Finch JL, Sutherland JV. Clinical-pathologic comparison of cases in postmenopausal women receiving exogenous estrogens. Cancer 1980;45: 3018–26.

3. Lippman ME, Swain SA. Endocrine-responsive cancers of humans. In: Wilson JD, Foster DW, editors. Williams textbook of endocrinology. Philadelphia: Saunders, 1992. pp. 1577–97.

4. Miller WR, Hawkins RA, Forrest AM. Significance of aromatase activity in human breast cancer. Cancer Res 1982;42:3365–8.

5. Miller WR, Forrest AP. Oestradiol synthesis by a human breast carcinoma. Lancet 1974;2:866–8.

6. Peltoketo H, Isomaa V, Maentausta O, Vihko R. Complete amino acid sequence of human placental 17β-hydroxysteroid dehydrogenase deduced from cDNA. FEBS Lett 1988;239:73–7.

7. Luu-The V, Labrie C, Zhao HF, et al. Characterization of cDNAs for human estradiol 17β-dehydrogenase and assignment of the gene to chromosome 17: evidence of two mRNA species with distinct 5′-termini in human placenta. Mol Endocrinol 1989;3:1301–9.

8. Gast MJ, Sims HF, Murdock GL, Gast PM, Strauss AW. Isolation and sequencing of a cDNA clone encoding human placental 17β-estradiol dehydrogenase: identification of the putative cofactor binding site. Am J Obstet Gynecol 1989;161:1726–31.

9. Wu L, Einstein M, Geissler WM, Chan HK, Elliston KO, Andersson S. Expression cloning and characterization of human 17β-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20α-hydroxysteroid dehydrogenase activity. J Biol Chem 1993;268: 12964–9.

10. Ruder HJ, Loriaux L, Lipsett MB. Estrone sulfate: production rate and metabolism in man. J Clin Invest 1972;51:1020–33.

11. Samojlik E, Santen RJ, Worgul TJ. Plasma estrone-sulfate: assessment of reduced estrogen production during treatment of metastatic breast carcinoma. Steroids 1982;39:497–507.

12. Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR. Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. J Steroid Biochem Mol Biol 2000;72:23–7.

13. Pasqualini JR, Chetrite GS. Estrone sulfatase versus estrone sulfotransferase in human breast cancer: potential clinical applications. J Steroid Biochem Mol Biol 1999;69:287–92.

14. Aksoy IA, Wood TC, Weinshilboum R. Human liver estrogen sulfotransferase: identification by cDNA cloning and expression. Biochem Biophys Res Commun 1994;200:1621–9.

15. Falany CN, Krasnykh V, Falany JL. Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. J Steroid Biochem Mol Biol 1995;52:529–39.

16. Dooley TP, Haldeman-Cahill R, Joiner J, Wilborn TW. Expression profiling of human sulfotransferase and sulfatase gene superfamilies in epithelial tissues and cultured cells. Biochem Biophys Res Commun 2000;277:236–45.

17. Yamamoto T, Kitawaki J, Urabe M, et al. Estrogen productivity of endometrium and endometrial cancer tissue; influence of aromatase on proliferation of endometrial cancer cells. J Steroid Biochem Mol Biol 1993;44:463–8.

18. FIGO Stages-1988 revision. Gynecol Oncol 1989;35:125–7.

19. Saeki T, Takashima S, Sasaki H, Hanai N, Salomon DS. Localization of estrone sulfatase in human breast carcinomas. Breast Cancer 1999;6:331–7.

20. Sasano H, Frost AR, Saitoh R, et al. Aromatase and 17β-hydroxysteriod dehydrogenase type 1 in human breast carcinoma. J Clin Endocrinol Metab 1996;81:4042–6.

21. Utsunomiya H, Suzuki T, Chika C, et al. The analyses of 17β-hydroxysteroid dehydrogenase isozymes in human endometrial hyperplasia and carcinoma. J Clin Endocrinol Metab 2001;86:3436–43.

22. Utaaker E, Stoa KF. Oestrone sulphatase activity of the rat uterus in different hormonal states. Horm Res 1980;13:180–5.

23. Utsumi T, Yoshimura N, Takeuchi S, et al. Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. Cancer Res 1999;59:377–81.

24. Tokunaga K, Nakamura Y, Sakata K, et al. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 1987;47:5616–9.

25. Salido EC, Yen PH, Barajas L, Shapiro LJ. Steroid sulfatase expression in human placenta: immunocytochemistry and in situ hybridization study. J Clin Endocrinol Metab 1990;70:1564–7.

26. Dumoulin FL, Nischalke HD, Leifeld L, et al. Semi-quantification of human C-C chemokine mRNAs with reverse transcription/real-time PCR using multi-specific standards. J Immunol Methods 2000;241: 109–19.

27. Read SJ. Recovery efficiencies on nucleic acid extraction kits as measured by quantitative LightCycler PCR. Mol Pathol 2001;54:86–90.

28. Thomas DB. Do hormones cause cancer? Cancer 1984;53:595–604.

29. Vihko R, Apter D. Endogenous steroids in the pathophysiology of breast cancer. CRC Crit. Rev Oncol/Hematol 1989;9:1–16.

30. Dao TL, Hayes C, Libby PR. Steroid sulfatase activities in human breast tumors. Proc Soc Exp Biol Med 1974;146:381–4.

31. Pasqualini JR, Gelly C, Lecerf F. Estrogen sulfates: biological and ultrastructural responses and metabolism in MCF-7 human breast cancer cells. Breast Cancer Res. Treat 1986;8:233–40.

32. Pasqualini JR, Chetrite G, Blacker C, et al. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab 1996;81:1460–4.

33. Santner SJ, Feil PD, Santen RJ. In situ estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. J Clin Endocrinol Metab 1984;59:29–33.

34. Burns GR. Purification and partial characterization of arylsulphatase C from human placental microsomes. Biochim Biophys Acta 1983;759:199–204.

35. Dibbelt L, Kuss E. Human placental steryl-sulfatase. Enzyme purification, production of antisera, and immunoblotting reactions with normal and sulfatase- deficient placentas. Biol Chem Hoppe Seyler 1986;367:1223–9.

36. Recchione C, Venturelli E, Manzari A, Cavalleri A, Martinetti A, Secreto G. Testosterone dihydrotestosterone and oestradiol levels in postmenopausal breast cancer tissues. J Steroid Biochem Mol Biol 1995;52:541–6.

37. Suzuki T, Darnel AD, Akahira JI, et al. 5alpha-reductases in human breast carcinoma: possible modulator of in situ androgenic actions. J Clin Endocrinol Metab 2001;86:2250–7.

38. Isola JJ. Immunohistochemical demonstration of androgen receptor in breast cancer and its relationship to other prognostic factors. J Pathol 1993;170:31–5.

39. Poulin R, Baker D, Labrie F. Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75–1 human breast cancer cell line. Breast Cancer Res Treat 1988;12:213–25.

40. Tseng L, Liu HC. Stimulation of arylsulfotransferase activity by progestins in human endometrium in vitro. J Clin Endocrinol Metab 1981;53:418–21.

41. Clarke CL, Adams JB, Wren BG. Induction of estrogen sulfotransferase in the human endometrium by progesterone in organ culture. J Clin Endocrinol Metab 1982;55:70–5.

42. Falany JL, Falany CN. Regulation of estrogen sulfotransferase in human endometrial adenocarcinoma cells by progesterone. Endocrinology 1996;137:1395–401.

43. Brooks SC, Rozhin J, Pack BA, et al. Role of sulfate conjugation in estrogen metabolism and activity. J Toxicol Environ Health 1978;4: 283–300.

44. Poutanen M, Isomaa V, Lehto VP, Vihko R. Immunohistochemical analysis of 17β- hydroxysteroid dehydrogenase in benign and malignant human breast tissue. Int J Cancer 1992;50:386–90.

45. Suzuki T, Nakata T, Miki Y, et al. Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. Cancer Res 2003;63: 2762–70.

DTD 5

ARTICLE IN PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Gynecologic Oncology xx (2004) xxx–xxx

Gynecologic Oncology

www.elsevier.com/locate/ygyno

Expression of copper-transporting P-type adenosine triphosphatase (ATP7B) as a prognostic factor in human endometrial carcinoma

Takeshi Aida[a,b], Yuji Takebayashi[a,*], Takao Shimizu[b], Chikako Okamura[b], Masashi Higasimoto[a], Atsuko Kanzaki[a], Kentaro Nakayama[c], Kunihiko Terada[d], Toshihiro Sugiyama[d], Kohji Miyazaki[c], Kiyoshi Ito[b], Seiichi Takenoshita[a], Nobuo Yaegashi[b]

[a]*Department of Surgery II, Fukushima Medical University, Fukushima, Hikarigaoka 1, 960-1295, Japan*
[b]*Department of Obstetrics and Gynecology, Tohoku University School of Medicine, Seiryou-Machi 1-1, Aoba-ku, Sendai 980-8570, Japan*
[c]*Department of Obstetrics and Gynecology, Shimane Medical University, Izumo 693-8501, Japan*
[d]*Department of Biochemistry, Akita University School of Medicine, Akita 010-8543, Japan*

Received 8 July 2004

Abstract

Objective. Copper-transporting P-type adenosine triphosphate (ATP7B) has been reported to be associated with cisplatin resistance in vitro. However, the clinical significance of this transporter has not previously been addressed in endometrial carcinoma. Our goal was to investigate if ATP7B is expressed in endometrial carcinoma and whether its expression correlates with prognosis.

Methods. We performed immunohistochemical analysis of ATP7B using a monoclonal antibody against ATP7B in 51 endometrial endometrioid adenocarcinomas. 27 of 51 patients were treated with cisplatin-based chemotherapy after surgery.

Results. Cytoplasmic staining of tumor cells was observed in 37.3% (19/51 cases) of the analyzed carcinomas and no staining was observed in adjacent non-neoplastic cells. ATP7B positivity in the degree of differentiation of G2 and G3 carcinoma was significantly higher than that of G1 carcinoma ($P = 0.019$). The patients with ATP7B-positive tumors had a worse prognosis than that with ATP7B-negative tumors in overall survival and disease-free survival, respectively ($P < 0.01$).

Conclusions. These findings suggest that overexpression of ATP7B expression in endometrial carcinoma is correlated with unfavorable clinical outcome in patients treated with cisplatin-based chemotherapy. ATP7B expression could be considered as a prognostic factor in patients with endometrial carcinoma.

Keywords: ATP7B; Cisplatin; Endometrial carcinoma; Chemoresistance

Introduction

Endometrial carcinoma is one of the most common gynecological malignancies [1] and the incidence has increased in Japan. Although the treatment with cisplatin-based malignancy after reductive surgery has improved prognosis of patients with this carcinoma, one of the most important clinical problems in its treatment is the intrinsic/acquired resistance to cisplatin-based chemotherapy. Knowledge of the active mechanism of drug resistance may lead to new treatment strategies and may allow selection of those patients for specific treatment modalities.

Resistance to cisplatin includes decreased drug accumulation, enhanced detoxification, and increased DNA repair efficiency. Multidrug resistance (MDR) has been noted to be an important mechanism of drug resistance. Several genes including *MDR1*, *MRP*, and *LRP* have been identified [2–5]. *MDR1* and *MRP1* function as a drug efflux pump and are classified in the ABC transporter gene family [4,5] and are expressed in both human solid tumors

* Corresponding author. Fax: +81 24 548 3249.
E-mail address: tyuji@fmu.ac.jp (Y. Takebayashi).

0090-8258/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygyno.2004.12.042

and hematological malignancies [6,7]. The 110-kd LRP, the major vault protein, is frequently overexpressed in multidrug resistance cells and plays an important role in transport of drugs from nuclei to cytoplasm and confers to multidrug resistance in vitro [3]. *BCRP* (*MXR/ABCP*) gene, another member of the ABC transporter family, has been examined in breast, colon, gastric, and fibrosarcoma cell lines [8–11]. However, evidence that this molecule is involved in cisplatin resistance in vitro and in vivo has not been found. To sum up, the mechanism of cisplatin transport and its significance to drug resistance.

ATP7B is member of a class of heavy metal-transporting P-type ATPases that pump copper, cadmium, zinc, silver, or lead [13–17]. Copper is an essential trace element that is integrated into many enzymatic reactions. Excess copper is transported to the extracellular environment by an energy-dependent system [13], and alteration in copper homeostasis can cause severe problems. For example, Wilson disease (WND), an autosomal recessive disorder in copper transport, is characterized by chronic liver and/or neurological disorder, sometimes accompanied by kidney damage [18]. Detailed understanding of ATP7B is therefore crucial in several diseases including cancer. The fact that this transporter can also transport small molecule drugs is intriguing, and could potentially have significant value in the clinic.

Recently, copper-transporting P-type adenosine triphosphatase (ATP7B) was found to be associated with cisplatin resistance in vitro [12]. The *ATP7B* gene is induced by exposure to cisplatin in human prostate cells, and the *ATP7B*-transfected cells showed dramatic decrease of cisplatin concentration in cytoplasms [12]. Although an active efflux pump for cisplatin has yet to be identified, it is likely that ATP7B functions as efflux for cisplatin from some carcinoma cells. Furthermore, the expression of ATP7B was demonstrated as a cisplatin-based chemoresistance marker in ovarian cancer [19]. The aim of this retrospective study was to investigate the expression of ATP7B and to determine whether its expression was predictive of survival of patients with endometrial carcinoma.

Materials and methods

Patients and samples

Specimens of endometrial adenocarcinoma were collected from patients who underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, and pelvic and paraaortic lymphadenectomy at Tohoku University Hospital and Shimane Medical University Hospital between 1994 and 2001. Diagnostic verification and tumor subtyping and grading were conducted by gynecological pathologist using permanent pathologic specimens. All samples were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and immediately stored at −80°C until use. Surgical staging for primary endometrial carcinoma was performed according to guidelines of the International Federation of Obstetrics and Gynecology. The clinicopathologic variables such as age, degree of differentiation, and clinical stages are shown in Table 1. The patients with other histological variants of endometrial carcinoma were excluded—i.e., only pure endometrioid adenocarcinoma specimens were selected for this study. However, samplings from surgical specimens were avoided if the lesion in the uterine cavity was small in order to give priority to accurate pathological diagnosis. Among the 51 patients selected, 27 patients with risk factors were primarily treated with reductive surgery and three to six courses of postoperative chemotherapy, consisting of the cisplatin-based combination regimen CAP (cisplatin 60–70 mg/m^2, doxorubicin 40 mg/m^2, and cyclophosphamide 500 mg/body). The remaining 24 without clinicopathologic risk factors did not undergo the adjuvant chemotherapy. A signed informed consent, approved by the Institutional Review Board of Tohoku University Hospital and Shimane Medical University Hospital, was obtained from each patient before the surgery. After examining histopathological features of the sections stained with hematoxylin and eosin, the sections including more than 60% carcinoma cells were used.

Tissue staining and evaluation of stained sections

A 5-μm section of each submitted frozen block was stained with hematoxylin and eosin to verify the histopathologic diagnosis and the quality of fixation for immunohistochemical analysis. Immunostaining was performed on cryostat sections using immunoperoxidase procedure (Vectastain Elite ABC kit, Vector, Burlingame, CA). After recovering from O.C.T. compound, the sections were fixed in 10% neutral buffered formalin, incubated in 0.03% H2O2 in absolute methanol for 30 min at room temperature, and blocked in 3% skim milk in PBS for 30 min at room temperature. The sections were then incubated with 100-fold diluted monoclonal antibody against the

Table 1
Relationship between ATP7B expression and clinicopathologic variables

	ATP7B expression		
	Negative	Positive	
Total	32	19	
Age[a]	57.4 (26–79)	59.2 (37–76)	n.s.[b]
Grade			
G1	24	8	
G2/G3	8	11	$P = 0.019$[c]
Stage			
I	25	11	
II	2	3	
III	4	5	
IV	1	0	n.s.[c]

n.s.: not significant.
[a] Median (ranges).
[b] Mann–Whitney *U* test.
[c] Chi-square test.

NH2-terminal region of ATP7B, which included six copper-binding domains (amino acid number from 21 to 623; see Ref. [20]) for 15 h at 4°C. After rinsing with PBS, the sections were incubated in biotinylated horse anti-mouse IgG at 1:200 in 1.5% normal horse serum for 40 min at room temperature. Sections were then rinsed in PBS and incubated for 50 min at room temperature in the avidin–biotin horseradish peroxidase macromolecular complex. After rinsing with PBS, the sections were incubated for 7 min in 0.03% diaminobenzidine in PBS with 0.003% H2O2. The slides were counterstained with hematoxylin, dehydrated, and mounted. The serial sections were routinely incubated with irrelevant mouse IgG as negative control.

The slides were examined and scored independently by two observers (T.A. and Y. T.) without knowledge of clinical information of the patients. If more than 10% of the tumor cells were stained, the samples were considered to be ATP7B-positive carcinomas. The 10% cutoff level was specified for the following reasons: (1) a 10% positive cells was considered the lowest level of expression that could be most consistently detected in cryostat sections; (2) Chan et al. demonstrated that a small percentage of cells positive for multidrug-resistance-related proteins (i.e., P-gp) could have clinical significance [21]; and (3) we evaluated validation of this cutoff in our previous studies, which examined ATP7B expression in ovarian carcinoma [19,22]. When the two observers' reports differed from each other, they evaluated together the images of stained sections on a TV-captured station.

Statistical analysis

Data analysis was performed using Statview Version 5 statistical software package. Continuous variables were analyzed with Mann–Whitney *U* test, and categorical variables were analyzed with chi-square test. Overall survival and disease-free survival were determined with the Kaplan–Meier method, and differences in survival between subgroups were compared with log-rank test. We estimated relative risks and 95% confidence interval of survivals, using Cox's proportional-hazards model with adjustment for risk factors. Less than 0.05 of *P* values was hypothesized to be significant. All reported *P* values were two sided.

Results

Expression of ATP7B protein in human endometrial carcinoma

We used 51 primary endometrial carcinoma tissues for the detection of ATP7B by immunohistochemistry using ATP7B monoclonal antibody. This antibody specifically reacted with ATP7B by immunoblotting analysis [19]. A granular staining of ATP7B was observed in cytoplasm of endometrial carcinoma cells (Fig. 1A) and some of carcinoma had no staining of ATP7B (Fig. 1B). In adjacent non-neoplastic cells, ATP7B expression was not detected (Fig. 1C). The immunostaining results were summarized in

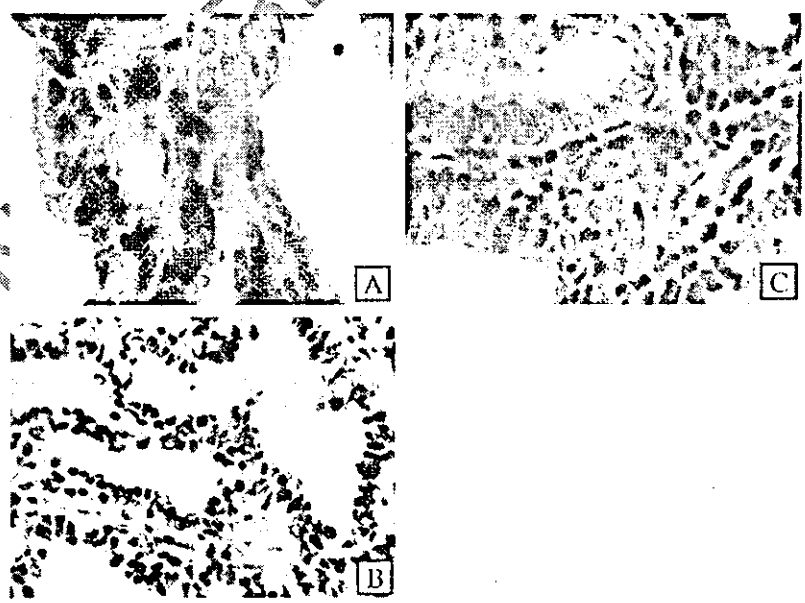

Fig. 1. Immunohistochemical staining of endometrial carcinoma specimens using antibodies to ATP7B in cryostat sections. (A) ATP7B-positive tumor stained with anti-ATP7B monoclonal antibody. Note the distinct cytoplasmic staining in endometrial carcinoma cells (immunoperoxidase stain, original magnification, ×400). (B) ATP7B-negative tumor (immunoperoxidase stain, original magnification, ×400). (C) Normal epithelium cells in the serial section (magnification, ×400).