

TABLE 1 - CHARACTERISTICS<sup>1</sup> OF SUBJECTS ACCORDING TO QUINTILES OF VEGETABLE CONSUMPTION

	Men					Women				
	1 (low)	2	3	4	5 (high)	1 (low)	2	3	4	5 (high)
Age (years)	48.7	49.0	49.4	50.4	50.3	49.0	49.1	49.4	50.0	50.3
Body mass index (kg/m <sup>2</sup> )	23.6	23.4	23.7	23.4	23.4	23.7	23.5	23.6	23.4	23.5
History of peptic ulcer (%)	12.0	12.7	12.2	13.2	12.6	5.6	5.5	6.6	6.1	6.5
Family history of gastric cancer (%)	9.4	9.8	8.3	11.2	11.1	7.8	9.0	9.7	10.6	10.6
Educational level (%)										
Junior school	50.4	51.0	44.3	50.3	45.5	64.2	57.3	53.1	53.5	48.7
High school	38.4	38.0	39.4	39.0	38.5	28.3	33.3	35.6	36.5	37.6
College or higher	11.1	11.0	16.3	10.7	16.0	7.6	9.4	11.3	10.1	13.7
Smoking status (%)										
Never	21.1	22.9	27.9	23.2	26.6	90.3	92.1	92.3	94.4	94.2
Past	21.4	21.3	23.2	22.8	26.3	2.1	1.6	1.9	1.5	1.3
Current	57.5	55.7	48.9	54.0	47.2	7.6	6.3	5.7	4.1	4.5
Alcohol consumption (g/day)	32.1	30.7	22.9	31.2	23.9	2.6	1.8	1.2	1.4	1.1
Energy intake (KJ/day)	8.7	9.2	8.0	9.9	8.8	5.7	5.8	5.4	6.1	5.8
Highly salted food intake (g/day) <sup>2</sup>	5.4	5.7	6.0	6.4	6.4	3.4	3.7	3.5	4.3	3.8
Vegetable juice consumption (g/day)	18.3	17.6	21.1	16.1	26.8	12.0	14.0	16.4	11.0	16.1
Fruit juice consumption (g/day)	32.4	31.4	38.1	32.0	40.4	32.6	33.1	35.0	32.2	30.0
Use of vitamin A supplement (%)	1.3	1.8	1.7	2.7	2.1	2.4	2.0	2.0	2.1	2.1
Use of vitamin C supplement (%)	4.1	3.5	4.2	4.2	4.3	7.1	6.6	7.6	5.9	6.8
Use of vitamin E supplement (%)	3.4	2.8	4.0	3.4	3.9	7.2	8.1	9.5	7.0	8.7

<sup>1</sup>Data were obtained from food frequency questionnaire. -<sup>2</sup>Sum of salted fish roe and salted fish gut.

TABLE 2 - RELATIVE RISK (95% CONFIDENCE INTERVALS) OF GASTRIC CANCER BY FREQUENCY OF VEGETABLE AND FRUIT CONSUMPTION: JPHC STUDY 1990-1999

	Frequency/week				Test for trend p value	≥ 1 day/week (2-4)
	< 1 day (1) <sup>1</sup>	1-2 days (2)	3-4 days (3)	Almost daily (4)		
Green vegetables						
Cases	11	94	168	131		393
Person-years	8,019	90,501	157,323	123,195		371,019
RR <sup>2</sup>	1.00	0.76 (0.41-1.41)	0.77 (0.42-1.42)	0.73 (0.40-1.36)	0.55	0.76 (0.42-1.38)
RR <sup>3</sup>	1.00	0.79 (0.41-1.51)	0.80 (0.42-1.52)	0.77 (0.40-1.46)	0.62	0.79 (0.42-1.48)
RR <sup>4</sup>	1.00	0.78 (0.39-1.56)	0.76 (0.38-1.49)	0.75 (0.38-1.48)	0.57	0.76 (0.39-1.48)
Yellow vegetables						
Cases	39	174	131	60		365
Person-years	21,292	145,321	139,092	73,332		357,745
RR <sup>2</sup>	1.00	0.78 (0.55-1.10)	0.71 (0.49-1.02)	0.66 (0.44-1.00)	0.06	0.74 (0.53-1.03)
RR <sup>3</sup>	1.00	0.82 (0.57-1.18)	0.71 (0.49-1.04)	0.66 (0.43-1.01)	0.03	0.76 (0.54-1.07)
RR <sup>4</sup>	1.00	0.68 (0.47-0.98)	0.63 (0.43-0.92)	0.56 (0.36-0.88)	0.03	0.64 (0.45-0.92)
White vegetables						
Cases	12	71	135	186		392
Person-years	6,351	79,198	143,688	149,800		372,686
RR <sup>2</sup>	1.00	0.54 (0.29-0.99)	0.50 (0.28-0.90)	0.55 (0.31-0.99)	0.64	0.53 (0.30-0.94)
RR <sup>3</sup>	1.00	0.61 (0.31-1.18)	0.54 (0.28-1.03)	0.59 (0.31-1.12)	0.57	0.57 (0.31-1.07)
RR <sup>4</sup>	1.00	0.51 (0.26-1.00)	0.44 (0.23-0.85)	0.49 (0.26-0.93)	0.40	0.48 (0.25-0.89)
Pickled vegetables						
Cases	37	50	87	230		367
Person-years	61,449	68,368	64,031	185,190		317,588
RR <sup>2</sup>	1.00	0.89 (0.58-1.38)	1.20 (0.79-1.82)	0.99 (0.67-1.46)	0.99	1.00 (0.69-1.45)
RR <sup>3</sup>	1.00	0.79 (0.51-1.23)	1.05 (0.68-1.61)	0.86 (0.57-1.28)	0.57	0.88 (0.60-1.28)
RR <sup>4</sup>	1.00	0.92 (0.57-1.48)	1.12 (0.70-1.80)	0.97 (0.62-1.51)	0.92	0.98 (0.65-1.49)
Fruit						
Cases	44	102	122	136		360
Person-years	29,424	104,736	112,637	132,241		349,614
RR <sup>2</sup>	1.00	0.64 (0.45-0.91)	0.65 (0.46-0.92)	0.65 (0.46-0.93)	0.16	0.65 (0.47-0.89)
RR <sup>3</sup>	1.00	0.68 (0.47-0.98)	0.67 (0.47-0.97)	0.70 (0.48-1.01)	0.25	0.68 (0.49-0.95)
RR <sup>4</sup>	1.00	0.71 (0.48-1.05)	0.69 (0.47-1.03)	0.70 (0.46-1.04)	0.23	0.70 (0.49-1.00)

RR, rate ratio. -<sup>1</sup>Reference category. -<sup>2</sup>Adjusted for age, gender and area. -<sup>3</sup>Adjusted for age, gender, area, educational level, smoking status, BMI, alcohol intake, use of vitamin A, C, E supplement, total energy intake, highly salted food intake, history of peptic ulcer and family history of gastric cancer. -<sup>4</sup>Analyses with first-year and second-year cases excluded.

years, the risk reductions in yellow and white vegetables were clearer.

Table III presents RRs of total gastric cancer according to quintiles of total vegetable consumption. Pickled vegetables were excluded from total vegetables. Compared to subjects with the lowest quintiles of vegetable consumption, those with higher quintiles tended to have a lower risk of gastric cancer. The results were similar after the exclusion of gastric cancer cases diagnosed in first and second fol-

low-up years. However, the risk did not decline in a stepwise manner as consumption increased from the second through the highest quintiles. Consequently, tests for a linear trend were not statistically significant. When the second to highest quintiles of consumption were combined and compared to the lowest quintile of total vegetable consumption, a nonsignificantly lower risk was observed.

We did not take vegetable juice consumption into account in our estimation of total vegetable consumption because of many miss-

TABLE III - RELATIVE RISK (95% CONFIDENCE INTERVALS) OF GASTRIC CANCER BY HISTOLOGIC CLASSIFICATION ACCORDING TO QUINTILES OF TOTAL VEGETABLE CONSUMPTION<sup>1</sup>: JPHC STUDY 1990-1999

	Intake in quintiles/category					Test for trend p value	Quintile 2-5
	1 (low) <sup>1</sup>	2	3	4	5 (high)		
Mean intake (g/day)	55.8	96.2	126.7	173.1	214.5		152.6
Total gastric cancer							
Cases	89	77	60	103	75		315
Person-years	75,406	75,908	75,541	76,427	75,756		303,632
RR <sup>2</sup>	1.00	0.83 (0.61-1.12)	0.75 (0.54-1.04)	0.92 (0.69-1.24)	0.74 (0.54-1.01)	0.18	0.81 (0.64-1.03)
RR <sup>3</sup>	1.00	0.86 (0.63-1.17)	0.75 (0.53-1.05)	0.90 (0.67-1.22)	0.75 (0.54-1.04)	0.17	0.82 (0.64-1.05)
RR <sup>4</sup>	1.00	0.75 (0.54-1.06)	0.70 (0.49-1.02)	0.85 (0.62-1.18)	0.73 (0.51-1.03)	0.19	0.76 (0.59-0.99)
Cardia cancer							
Cases	11	8	6	15	7		36
RR <sup>2</sup>	1.00	0.67 (0.27-1.66)	0.61 (0.22-1.65)	0.98 (0.45-2.17)	0.53 (0.20-1.37)	0.47	0.71 (0.36-1.41)
RR <sup>3</sup>	1.00	0.79 (0.30-2.06)	0.60 (0.20-1.82)	1.10 (0.46-2.60)	0.65 (0.24-1.79)	0.74	0.81 (0.38-1.71)
RR <sup>4</sup>	1.00	0.40 (0.12-1.31)	0.65 (0.21-1.96)	1.17 (0.49-2.79)	0.52 (0.17-1.57)	0.94	0.70 (0.33-1.51)
All noncardia							
Cases	58	52	45	76	58		231
RR <sup>2</sup>	1.00	0.86 (0.59-1.25)	0.85 (0.57-1.25)	1.05 (0.74-1.49)	0.88 (0.61-1.27)	0.93	0.91 (0.68-1.22)
RR <sup>3</sup>	1.00	0.87 (0.59-1.27)	0.82 (0.55-1.23)	1.04 (0.72-1.48)	0.84 (0.57-1.23)	0.74	0.90 (0.66-1.21)
RR <sup>4</sup>	1.00	0.82 (0.54-1.23)	0.76 (0.49-1.18)	1.00 (0.68-1.47)	0.90 (0.60-1.34)	0.98	0.87 (0.64-1.20)
Undifferentiated type							
Cases	22	13	18	30	29		90
RR <sup>2</sup>	1.00	0.55 (0.28-1.10)	0.84 (0.45-1.56)	1.04 (0.59-1.83)	1.11 (0.63-1.96)	0.22	0.89 (0.56-1.43)
RR <sup>3</sup>	1.00	0.58 (0.29-1.17)	0.87 (0.45-1.67)	1.06 (0.59-1.91)	1.22 (0.68-2.19)	0.14	0.93 (0.57-1.53)
RR <sup>4</sup>	1.00	0.54 (0.25-1.18)	0.81 (0.39-1.67)	0.93 (0.47-1.81)	1.39 (0.74-2.60)	0.10	0.90 (0.53-1.55)
Differentiated type							
Cases	36	36	26	40	26		128
RR <sup>2</sup>	1.00	0.96 (0.60-1.52)	0.84 (0.50-1.39)	0.91 (0.58-1.44)	0.65 (0.39-1.08)	0.12	0.84 (0.58-1.22)
RR <sup>3</sup>	1.00	0.96 (0.61-1.53)	0.78 (0.46-1.31)	0.83 (0.55-1.40)	0.53 (0.31-0.91)	0.03	0.79 (0.54-1.15)
RR <sup>4</sup>	1.00	0.88 (0.54-1.45)	0.72 (0.41-1.26)	0.88 (0.54-1.44)	0.54 (0.31-0.96)	0.07	0.76 (0.51-1.13)

RR, rate ratio.—<sup>1</sup>Reference category.—<sup>2</sup>Adjusted for age, gender and area.—<sup>3</sup>Adjusted for age, gender, area, educational level, smoking status, BMI, alcohol intake, use of vitamin A, C, E supplement, total energy intake, highly salted food intake, stomach disorders and family history of stomach carcinoma.—<sup>4</sup>Analyses with first-year and second-year cases excluded.—<sup>5</sup>Pickled vegetables excluded.

ing values and the possibility of misclassification from the different number of frequency categories compared to vegetable and fruit. We did not observe any association between vegetable or fruit juice intake and the risk of gastric cancer (data not shown). Nor did we find any substantial modification of the association between vegetables, fruit and the risk of gastric cancer in analyses stratified by alcohol consumption, smoking status, high and low consumption of nitrate- and nitrite-containing foods including processed meat and dried fish (data not shown). These findings did not differ when the analyses were limited to men (data not shown).

Table III also presents RRs of subsites and histologic type-specific gastric cancer according to the quintiles of total vegetable consumption. No striking differences in associations were seen according to subsite-specific gastric cancer, although apparent protective effects appeared in the differentiated type of gastric cancer.

#### DISCUSSION

In this population-based prospective study in Japan, both vegetables and fruits were associated with a lower risk of gastric cancer. This risk was fairly constant over the consumption of 1-2 or more days of vegetables and fruits per week and also across the second through the fifth quintiles of total vegetable intake. Furthermore, a comparison of less than 1 day per week to at least 1 day per week of vegetable and fruit consumption and a comparison of the lowest quintile to the combination of the other four quintiles of total vegetable consumption showed inverse associations with the risk of gastric cancer. Our results suggest that consumption of vegetables and fruit as low as 1 day per week may serve to protect against gastric cancer. We also found that the association between vegetable and fruit consumption and the risk of gastric cancer did not vary substantially according to the anatomical subsites or the histologic subtypes.

Our findings are in general agreement with those of 4 prospective studies, which show an inverse association between the risk of gastric cancer and the consumption of vegetables and fruits. In an early study of 122,261 Japanese men, even the rare or occasional consumption of green and yellow vegetables, compared to no consumption, was significantly associated with lower risk of gastric cancer.<sup>5</sup> In 2 studies among Japanese Hawaiians, 1 study found an inverse association with the consumption of vegetables and fruits,<sup>11</sup> while the other revealed an inverse association with the consumption of fruits and the combined intake of vegetables and fruits, but no association with regard to vegetable intake.<sup>12</sup> A study of Swedish twins showed a significant inverse association for the combined intake of vegetables and fruits.<sup>13</sup> In contrast, 7 prospective studies found no association of both vegetable and fruit consumption with the risk of gastric cancer.<sup>6-10,14,15</sup>

Higher consumption of vegetables and fruits has been associated with a reduced risk of gastric cancer in numerous case-control studies.<sup>3</sup> However, in such studies, reports of vegetable and fruit consumption are susceptible to biased recall of past diet.<sup>34</sup> In our study, we assessed the consumption of vegetables and fruits before the gastric cancer and other diseases were diagnosed, thus avoiding recall bias. Furthermore, we observed similar results in the analysis with the exclusion of gastric cancer cases diagnosed in the first 2 years of follow-up. These findings suggest that the protective effect of vegetables and fruits demonstrated in case-control studies is not entirely attributable to recall bias.

Because a large number of gastric cancer cases were identified in our cohort study, it was possible to analyze the association between vegetable and fruit consumption and the risk of gastric cancer by histologic subtypes and anatomic subsites. Some studies suggest that etiologic or environmental factors for cardia cancer were different from those for noncardia cancer.<sup>35</sup> However, in our study, no remarkable difference was observed in the association

between vegetable or fruit consumption and the risk of cardia or noncardia cancers. With regard to the relation between the histologic type of distal cancer and vegetables and fruits, 1 case-control study in the United States reported vegetables and fruits may play an important role in preventing the intestinal type in men and women combined,<sup>24</sup> but the other study in Japan reported the intake of vegetables and fruits was only associated with intestinal type in women and not in men.<sup>36</sup> In contrast, 2 other case-control studies reported the same pattern of association between vegetable and fruit consumption and both the intestinal and diffuse type.<sup>23,25</sup>

Some studies reported that current smokers showed a strong increase in risk for gastric cancer<sup>37</sup> and that vegetable and fruit consumption is more protective among smokers than nonsmokers.<sup>38</sup> This is because of the relative deficit of vegetable and fruit consumption in smokers who then experience a marked risk-reducing effect when their intake increases. In our study, the percentage of current smokers was relatively high even in the highest quintile of vegetable consumption. Hence, the difference in risk reduction between current smokers and nonsmokers was not readily apparent. Results were inconclusive for nitrate- and nitrite-containing foods and gastric cancer in an epidemiologic study,<sup>3</sup> although many N-nitroso compounds are known to be carcinogenic from animal experiments.<sup>39</sup> In our study, no association was observed between pickled vegetable consumption and the risk of gastric cancer. The intake frequency of pickled vegetables was relatively common, being categorized as almost daily in 48.6%. The consumption of pickled vegetables might have been more varied in subjects consuming almost daily. The consumption of processed meat and dried fish had no substantial effect on the association between vegetable and fruit consumption and the risk of gastric cancer.

We did not measure *H. pylori* infection, a strong risk factor for gastric cancer.<sup>40</sup> Our previous ecologic study showed a prevalence of *H. pylori* seropositivity from 63–76% among subjects 40–49 years of age in these 4 study areas. However, it was not associated with vegetable and fruit intake in a cross-sectional analysis.<sup>41</sup> But a synergistic interaction between *H. pylori* infection and dietary factors in gastric cancer has been suggested.<sup>42</sup> On the other hand, the study in Sweden found that ascorbic acid and beta carotene were protective in both cardia and noncardia cancer only in *H. pylori*-infected subjects.<sup>25</sup>

In our study, the histologic classification of gastric cancer cases was based on the available data from pathology records, but the

original specimens were neither collected nor reclassified. This limitation may have led to histologic misclassification to some extent and contributed to the absence of differences in the findings according to the histologic subtypes.

We used a relatively crude food frequency questionnaire, which includes only 44 food items (only 5 questions for fruits and vegetables), uses rough frequency scales (4 or 6 categories), does not ask or specify portion size information and asks about the recent consumption (rather than the average consumption during a previous year as usual). This is because we had not originally intended, at the outset of our study, to estimate the absolute amount of consumption of foods and nutrients based on this questionnaire. Nevertheless, we observed moderate correlations between many of the food and nutrient intakes estimated from this questionnaire with the consumption measured by 28- or 14-day diet records collected from a subsample of participants in the main cohort study over 4 or 2 seasons of the year.<sup>29</sup> The results of this validation study indicate that our questionnaire has practical utility in assessing the relative consumption of various nutrients and foods including fruits and vegetables. The nondifferential misclassification of fruit and vegetable consumption would have led to the underestimation of the inverse association between these foods and the risk of gastric cancer. Since vegetable intake information was gathered using only 3 questions, it was difficult to classify the details beyond the quintile level, suggesting that total vegetable intake was underestimated from the FFQ. In our validation study, the mean daily total vegetable intake of 271.8 g was estimated from diet records, against only 132.9 g from the FFQ in the same subjects. This underestimation makes it difficult to determine the exact weight to assign vegetable intake for reducing the risk of gastric cancer.

In conclusion, our investigation supported that vegetable and fruit intake, even in low amounts, is associated with a lower risk of gastric cancer. Although no striking differences in the association were seen between cardia and noncardia cancer, an inverse association was higher in differentiated rather than in undifferentiated types of gastric cancer.

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

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ABE, Y., ITO, K., OKAMURA, C., NIKURA, 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

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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

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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  $29.0 \pm 4.88$  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  $\chi^2$  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 screening 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 321 subjects 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)		
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)	8,605	96 (1.12)
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 IIb.

### 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 re-

port 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.

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## Steroid Sulfatase and Estrogen Sulfotransferase in Human Endometrial Carcinoma

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### 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, 17 $\beta$ -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 sig-

nificantly 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* 17 $\beta$ -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 17 $\beta$ -estradiol, respectively (4, 5). The enzyme 17 $\beta$ -hydroxysteroid dehydrogenase catalyzes the reversible interconversion of E1 and 17 $\beta$ -estradiol. 17 $\beta$ -Hydroxysteroid dehydrogenase type 1 catalyzes the 17 $\beta$ -reduction of biologically weak E1 to strong 17 $\beta$ -estradiol (6–8), whereas 17 $\beta$ -hydroxysteroid dehydrogenase type 2 preferentially catalyzes the oxidation of 17 $\beta$ -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 17 $\beta$ -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-

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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, 17 $\beta$ -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 Obstétristes 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  $\mu$ m).

**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<sub>2</sub>-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. 17 $\beta$ -Hydroxysteroid dehydrogenase type 1 antibody (polyclonal) and 17 $\beta$ -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 pro-

vided 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, 17 $\beta$ -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 17 $\beta$ -hydroxysteroid dehydrogenase type 1, 1:5 17 $\beta$ -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<sub>2</sub>O<sub>2</sub>], 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 17 $\beta$ -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<sub>2</sub>PO<sub>4</sub>, 10 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, and 1 mmol/L EDTA (pH 7.5)], and centrifuged for 15 minutes at 1000  $\times$  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<sub>2</sub>, and E1 contained [<sup>3</sup>H]E1 at 20 nM. Reactions were started with the addition of 3'-phosphoadenosine 5'-phosphosulfate to final concentration of 20  $\mu$ mol/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 alkalize the solution. The reaction mixtures were centrifuged at 600  $\times$  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 EIS containing [6,7-<sup>3</sup>H]EIS ( $1.6 \times 10^5$  dpm, 0.5 pmol/L) at 20  $\mu$ mol/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 EIS (aqueous phase) from E1 (organic phase). The toluene layer was collected, and [<sup>3</sup>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  $\mu$ g) using 25 ng/ $\mu$ L oligo(dT)<sub>12-18</sub> 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<sup>-</sup> 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'-GGCGA-CAATTTCTGGTTCAT-3'] (14), steroid sulfatase [M16505; FWD 5'-AGGGTCTGGGTGTGTCTGTC-3' and REV 5'-AC-TGCAACGCCTACTTAAATG-3'] (23), and glyceraldehyde-3-phosphate dehydrogenase [M33197; FWD 5'-TGAACGGG-AAGCTCACTGG-3' and REV 5'-TCCACCACCCTGTTGCT-GTA-3'] (24). PCR was set up using 2 mmol/L MgCl<sub>2</sub>, 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  $\pm$  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  $\chi^2$  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. 1A). Estrogen sulfotransferase immunoreactivity was not detected in either epithelium or stromal cells of proliferative phase endometrium (Fig. 1B). 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 ( $3.93 \pm 2.95$ ) versus dead ( $8.35 \pm 4.53$ )]. There were

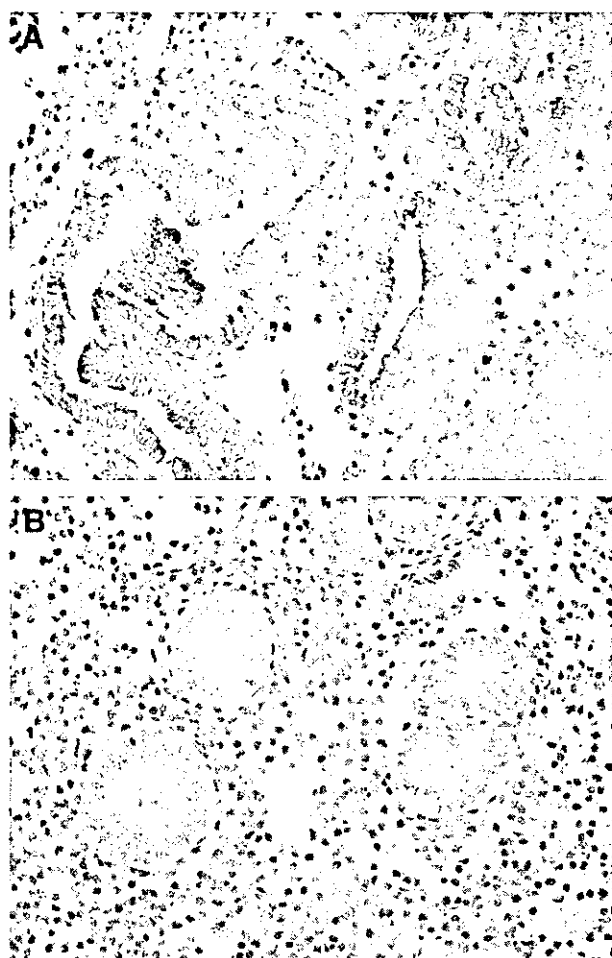


Fig. 1 Immunohistochemistry for estrogen sulfotransferase in normal endometrium obtained during secretory phase (A) and proliferative phase (B). Original magnification,  $\times 200$ .

no associations among the expression of steroid sulfatase, estrogen sulfotransferase, Aromatase,  $17\beta$ -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 [ $P < 0.01$ ; + ( $0.48 \pm 0.22$  nmol/mg/hour) versus - ( $0.18 \pm 0.10$  nmol/mg/hour)] and estrogen sulfotransferase [ $P < 0.05$ ; + ( $0.41 \pm 0.46$  nmol/mg/hour) versus - ( $0.09 \pm 0.03$  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) [ $P < 0.05$ ; + ( $42.2 \pm 22.3$ ) versus - ( $20.2 \pm 9.3$ )]. There was also significant positive correlation between estrogen sulfotransferase immunoreactivity and results of estrogen sulfotransferase RT-PCR analysis [ $P < 0.05$ ; + ( $35.0 \pm 28.1$ ) versus - ( $15.9 \pm 7.4$ )].

## DISCUSSION

Estrogens, especially  $17\beta$ -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  $17\beta$ -estradiol formation in humans. One is through the aromatization of androstenedione or testosterone to E1 or  $17\beta$ -estradiol, which was considered a major pathway for peripheral estrogen production (4, 5). However, conversion of E1S and E2S into E1 and  $17\beta$ -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

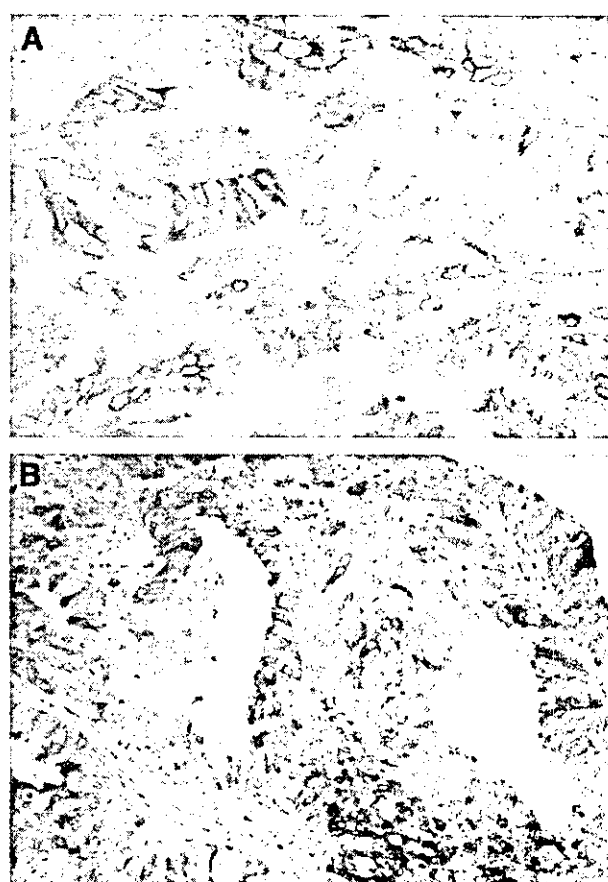


Fig. 2 Immunohistochemistry for steroid sulfatase (A) and estrogen sulfotransferase (B) in endometrial endometrioid adenocarcinoma. Original magnification,  $\times 200$ .



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

	STS expression		<i>P</i>
	+( <i>n</i> = 65)	-( <i>n</i> = 11)	
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		<i>P</i>
	+( <i>n</i> = 22)	-( <i>n</i> = 54)	
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 5 $\alpha$ -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 5 $\alpha$ -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 17 $\beta$ -hydroxysteroid dehydrogenase type 2 was also present in the endometrial glandular epithelium of secretory phase, but 17 $\beta$ -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 17 $\beta$ -hydroxysteroid dehydrogenase type 2 expression

**Table 3** Correlation between immunoreactivity of the enzymes

	STS LI	EST LI	17 $\beta$ HSD 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.
17 $\beta$ HSD 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; 17 $\beta$ HSD, 17 $\beta$ -hydroxysteroid dehydrogenase.

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

Case	Enzyme Assay (nmol/mg protein/hr)		mRNA level (%)		Immunoreactivity	
	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 17 $\beta$ -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 17 $\beta$ -hydroxysteroid dehydrogenase type 2 in endometrial carcinoma may be related to abundant *in situ* availability of 17 $\beta$ -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 17 $\beta$ -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, 17 $\beta$ -hydroxysteroid dehydrogenase type 1 expression was not detected (21). This is in contrast to the study of 17 $\beta$ -hydroxysteroid dehydrogenase type 1 in breast cancer in which nearly half of the cases demonstrated 17 $\beta$ -hydroxysteroid dehydrogenase type 1 immunoreactivity in carcinoma cells, and 17 $\beta$ -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 17 $\beta$ -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.

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## Sentinel lymph node detection in patients with endometrial cancer

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### Abstract

**Objective.** The purpose of this study was to examine the feasibility of sentinel lymph node (SLN) detection in patients with endometrial cancer using preoperative lymphoscintigraphy and an intraoperative gamma probe.

**Patients and methods.** Between June 2001 and January 2003, 28 consecutive patients with endometrial cancer who were scheduled for total abdominal hysterectomy, bilateral salpingo-oophorectomy, total pelvic lymphadenectomy, and paraaortic lymphadenectomy at Tohoku University School of Medicine underwent sentinel lymph node detection. On the day before surgery, preoperative lymphoscintigraphy was performed by injection of 99m-Tc-phytate into the endometrium during hysteroscopy. At the time of surgery, a gamma-detecting probe was used to locate radioactive lymph nodes.

**Results.** At least one sentinel node was detected in each of 23 of the 28 patients (82%). The mean number of sentinel nodes detected was 3.1 (range, 1–9). Sentinel nodes could be identified in 21 of 22 patients (95%) whose tumor did not invade more than halfway into the myometrium. Eighteen patients had radioactive nodes in the paraaortic area. Most patients had a sentinel node in one of the following three sites: paraaortic, external iliac, and obturator. The sensitivity and specificity for detecting lymph node metastases were both 100%.

**Conclusion.** The combination of preoperative lymphoscintigraphy with intraoperative gamma probe detection may be useful in identifying sentinel nodes in early-stage endometrial cancer.

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**Keywords:** Sentinel lymph node; Endometrial carcinoma; <sup>99m</sup>Tc-phytate

### Introduction

Endometrial cancer is the most common type of gynecological malignancy in Western countries [1]. The primary surgical procedures for patients with endometrial cancer include total abdominal hysterectomy and bilateral salpingo-oophorectomy with clinical or pathologic assessment of the regional lymph nodes. The question of whether or not to perform systematic pelvic lymphadenectomy and paraaortic lymphadenectomy remains controversial in many countries because of the inability to predict those patients who would benefit from node resection. The incidence of lymph node metastasis in patients with clinical stage I and occult stage II endometrial carcinoma is approximately 10%

[2]. Among patients with lymph node metastasis, approximately 50% had lymph node metastasis in the pelvic area, 30% in both the pelvic and paraaortic areas, and 20% in the paraaortic area alone. Complete pelvic and paraaortic lymphadenectomy may produce severe surgical sequelae such as lymph cyst, lymphedema, massive bleeding, ileus, and urologic or vascular injury. To minimize these sequelae, some institutions perform retroperitoneal lymphadenectomy on only high-risk patients who have risk factors such as deep myometrial invasion, isthmus-cervix extension, extrauterine spread, special histological types, high grade, enlarged lymph nodes, etc. However, patient selection according to these factors is far from ideal, because over half of these high-risk patients are negative for lymph node metastasis.

In an effort to avoid complete systematic lymphadenectomy whenever possible, the sentinel lymph node (SLN) concept has been applied to the treatment of malignancies of various organs [3]. In the case of gynecological malignancies, the feasibility of this concept has been examined

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