[Environmental Health and Preventive Medicine 10, 208–212, July 2005]

Short Communication

Effects of Hair Treatment on Hair Mercury—The Best Biomarker of Methylmercury Exposure?

Miwako DAKEISHI[1], Kunihiko NAKAI[2], Mineshi SAKAMOTO[3], Toyoto IWATA[1], Keita SUZUKI[2], Xiao-Jie LIU[3], Tomoko OHNO[1], Tomoko KUROSAWA[1], Hiroshi SATOH[2] and Katsuyuki MURATA[1]

[1]*Department of Environmental Health Sciences, Akita University School of Medicine, Akita, Japan*
[2]*Department of Environmental Health Sciences, Tohoku University Graduate School of Medicine, Sendai, Japan*
[3]*Department of Epidemiology, National Institute for Minamata Disease, Minamata, Japan*

Abstract

Objectives: Exposure misclassification is a major obstacle to obtain accurate dose-response relationships. In order to solve this problem, the impact of hair treatment on total mercury in hair was assessed in Japanese women.

Methods: A cross-sectional study was carried out among 327 women at age 24–49 years to determine hair mercury levels and estimate daily mercury intakes from seafood by using a food frequency questionnaire.

Results: Hair mercury levels in the women and daily mercury intake ranged from 0.11 to 6.86 (median 1.63) μg/g and from 0.77 to 144.9 (median 15.0) μg/day, respectively. The hair mercury was positively correlated with the daily mercury intake ($p<0.001$). When the women were divided into two subgroups based on artificial hair-waving, hair coloring/dyeing, residence (non-fishing and fishing areas), and working status, a significant difference in the hair mercury level was observed between the women with and without artificial hair-waving only ($p<0.001$). The multiple regression analysis showed that the log-transformed hair mercury level was significantly related to the log-transformed daily mercury intake (standardized regression coefficient $\beta s=0.307$) and artificial hair-waving ($\beta s=-0.276$); but not to hair coloring/dyeing, residence, working status or age. Permanent hair treatment was estimated to reduce total mercury in hair by approximately 30%, after adjusting for daily mercury intake and other possible factors.

Conclusions: These findings suggest that hair mercury is not the best biomarker of methylmercury exposure when a study population includes women with artificial hair-waving.

Key words: hair mercury, daily mercury intake, permanent hair treatment, exposure biomarker, Japanese women

Introduction

The total mercury concentration in hair has been reported to be affected by various preanalytical factors besides analytical imprecision, for instance, adhesion of environmental mercury vapor (1), permanent hair treatment (2–4), and hair color (5), although hair mercury is believed to reflect the average methylmercury concentrations circulating in the blood (6, 7) and it is frequently used as the biomarker of individual exposures to methylmercury. If any preanalytical factors exist in a study population, a dose response (or effect) relationship obtained from a study based on hair mercury may be overlooked or underestimated (8), because the effects of such factors on hair mercury do not seem to have been explored in detail. In fact, neither the Faroese birth cohort study nor the Seychelles child development study provided information on hair treatment in the Materials and Methods sections (9–11). The degree of exposure misclassification in hair mercury may be inferred from comparisons between exposure indicators. In this study, we determined hair mercury levels in Japanese mothers and estimated daily mercury intakes from seafood by using a food frequency questionnaire (FFQ), in order to evaluate the effects of preanalytical factors, especially hair

Received Feb. 25, 2005/Accepted Mar. 14, 2005
Reprint requests to: Katsuyuki MURATA
Department of Environmental Health Sciences, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
TEL: +81(18)884-6085, FAX: +81(18)836-2608
E-mail: winestem@med.akita-u.ac.jp

treatment of artificial waving and coloring/dyeing, on hair mercury.

Materials and Methods

Subjects

The nature of the procedures used in this study was explained to parents with a first grader (7-year-old child), of 28 different elementary schools in Akita and Tottori Prefectures, Japan, 14 of which were located near a fishing harbor (i.e., fishing areas). In Japan, there were many mines and smelters 30 years ago, and it is probable that soil or water was contaminated by lead, cadmium, mercury vapor, etc; therefore, the study population did not include people who came from such areas. Finally, 327 mothers participated (12). This study was carried out with the approval of the ethical review committee at the Akita University School of Medicine.

Methods

Hair samples were collected by cutting strands of hair close to the scalp from the occipital area in all mothers. The hair length ranged from 5 to 30 (mean 10) cm. Total mercury in aliquots of dried hair samples (15 to 20 mg), which were cut into small pieces (<2 mm) with scissors after being washed well with detergent and rinsed two times with acetone, was determined by the cold vapor atomic absorption spectrophotometry method at the National Institute for Minamata Disease (13, 14).

A detailed survey of the frequency and volume of seafood ingested in a year was conducted by a trained interviewer at the schools or civic centers, showing 25 kinds of full-scale pictures including fish, shellfish and seaweed items (e.g., tuna, swordfish, skipjack tuna, codfish, flatfish, mackerel, sardine, sea bream, whale, salmon, eel, crab, prawn, octopus, squid, oyster, sea urchin, fish paste, shellfish, seaweed, etc.) to each mother, based upon the FFQ (4, 15), i.e., a modified version of Date et al. (16). Then, the total mercury intake from seafood (μg/year) was estimated on the basis of the previous references on mercury concentrations in seafood (17, 18), and daily mercury intake (μg/day) was calculated dividing by 365 days. Moreover, questionnaires on artificial hair-waving and hair coloring/dyeing were collected from the mothers, and a medical doctor confirmed them, together with working status, using the interview method.

Statistical analysis

The relationships between the hair mercury level and daily mercury intake were analyzed by the Spearman rank correlation coefficient (r_s). The Mann-Whitney U test was used to compare the two subgroups divided on the basis of artificial hair-waving, hair coloring/dyeing, residence, and working status. Logarithmic transformation ($\log_{10}$) of the hair mercury concentration and daily mercury intake was used because of skewed distributions. The relation of the daily mercury intake, artificial hair-waving, hair coloring/dyeing, residence, working status, and age to the hair mercury level was examined by the multiple regression analysis. Artificial hair-waving, hair coloring/dyeing, and working status were scored as "absence"=0 and "presence"=1; also, residence was scored as "non-fishing area"=0 and "fishing area"=1. Also, the analysis of covariance was used to compare hair mercury concentrations in mothers with and without artificial hair-waving (or hair coloring/dyeing) after adjustment for daily mercury intake, residence, working status, age, and hair coloring/dyeing (or artificial hair-waving). All analyses, with two-sided p values, were performed using the Statistical Package for the Biosciences (19).

Results

The hair mercury concentrations in the 327 Japanese mothers at 24–49 (mean 36) years of age ranged from 0.11 to 6.86 (median 1.63) μg/g, and the daily mercury intakes, calculated from the FFQ data, were between 0.77 and 144.9 (median 15.0) μg/day. Among the mothers, there was a significant correlation between the hair mercury and daily mercury intake (Fig. 1). As the average value of body weight was 54.6 kg in 16,353 women aged 30–44 years, residing in Akita Prefecture (2002's data of the Akita Prefectural Center of Health Care), body weight of 55 kg for mothers was used to convert daily ingested dose (μg/day) to that per body weight (μg/kg body weight per day). Assuming the methylmercury content of 93% in seafood mercury (20), the mothers were suspected of having ingested methylmercury at a geometric mean of 0.25 μg/kg body weight per day, as shown in Table 1.

When the 327 mothers were divided into two subgroups based on artificial hair-waving, hair coloring/dyeing, residence, and working status (Table 2), there was only a significant differ-

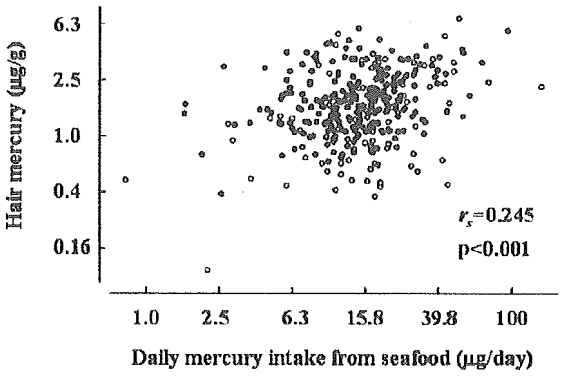

Fig. 1 Relationship between daily mercury intake and hair mercury level in 327 Japanese mothers. r_s, open circle, and closed circle indicate the Spearman rank correlation coefficient, and mothers with and without artificial hair-waving, respectively.

Table 1 Distribution of daily methylmercury intakes (μg/kg body weight per day), estimated from 327 Japanese mothers, under the assumption that body weight of mother was 55 kg, and methylmercury-mercury ratio in seafood was 0.93

Daily intake	Number of mothers	Proportion (%)
≤0.1	27	8.3
≤0.2	89	27.2
≤0.3	86	26.3
≤0.4	60	18.3
≤0.5	27	8.3
>0.5	38	11.6

Table 2 Mercury in hair and daily mercury intake in two subgroups divided according to artificial hair-waving, hair coloring/dyeing, residence, and working status in Japanese mothers

	Median (range)	Median (range)	p*
Artificial hair-waving:	*Absence* (N=219)	*Presence* (N=108)	
Hair mercury (μg/g)	1.81 (0.39~5.83)	1.31 (0.11~6.86)	<0.0001
Daily mercury intake (μg/day)	14.5 (1.61~94.1)	16.5 (0.77~144.9)	0.11
Hair coloring/dyeing:	*Absence* (N=69)	*Presence* (N=258)	
Hair mercury (μg/g)	1.74 (0.64~6.86)	1.58 (0.11~5.83)	0.08
Daily mercury intake (μg/day)	16.3 (2.65~74.5)	14.7 (0.77~144.9)	0.68
Residence:	*Non-fishing areas* (N=127)	*Fishing areas* (N=200)	
Hair mercury (μg/g)	1.84 (0.48~4.79)	1.55 (0.11~6.86)	0.08
Daily mercury intake (μg/day)	15.0 (0.77~74.5)	16.6 (1.63~144.9)	0.94
Working status:	*Without job* (N=120)	*With job* (N=207)	
Hair mercury (μg/g)	1.64 (0.42~4.86)	1.62 (0.11~6.86)	0.63
Daily mercury intake (μg/day)	14.9 (2.80~144.9)	15.0 (0.77~94.1)	0.83

* Mann-Whitney U test.

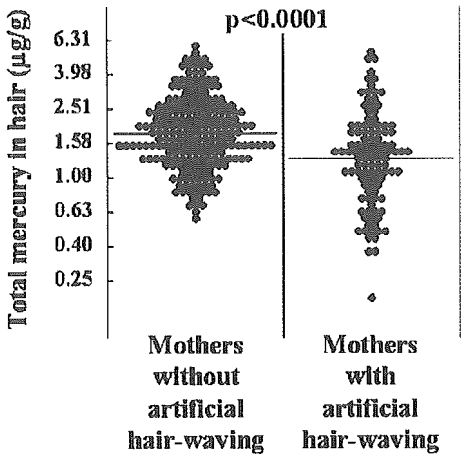

Fig. 2 Hair mercury concentrations in 219 mothers without and 108 mothers with artificial hair-waving after the adjustment for daily mercury intake, hair coloring/dyeing, residence, working status, and age: results of analysis of covariance.

ence in the hair mercury level between the mothers with and without artificial hair-waving. Using the multiple regression analysis, the log-transformed hair mercury level was significantly related to the log-transformed daily mercury intake (standardized regression coefficient βs=0.307, p<0.001) and artificial hair-waving (βs=−0.276, p<0.001); but, not to hair coloring/dyeing (βs=−0.065, p=0.21), residence (βs=−0.070, p=0.18), working status (βs=−0.012, p=0.81) or age (βs=0.046, p=0.37). The hair mercury concentrations adjusted by daily mercury intake and the above factors were significantly higher in the mothers without artificial hair-waving (geometric mean, 1.81 μg/g) than in the mothers with (1.29 μg/g), as shown in Fig. 2; but, no significant difference in the hair mercury was seen between the mothers without hair coloring/dyeing (1.74 μg/g) and with (1.59 μg/g) (p=0.21).

Discussion

In a previous study, we examined the accuracy of daily mercury intake estimated from the FFQ data of 154 mothers residing in Akita, Japan (4). Also, another study of the FFQ with 122 food items reported that the correlation coefficients between nutrients estimated by the first and second tests conducted at an interval of one week (i.e., reproducibility) ranged from 0.64 for vegetable protein to 0.78 for calcium (16). In this study using the FFQ, a large number of mothers including those who had different food habits in Japan were investigated, and they were suspected of ingesting mercury amounting to median 15 μg/day from seafood and freshwater fish, which was similar to the level of mothers residing in Akita (4). Since the daily mercury intake in our study was significantly correlated with hair mercury levels, daily mercury intake from seafood could reflect the individual methylmercury exposure to some extent.

The principal findings in 327 Japanese mothers of this study were that artificial hair-waving was associated with current total mercury levels in hair, and that permanent hair treatment reduced total mercury in hair by approximately 30% as a mean value, even after adjusting for daily mercury intake, hair coloring/dyeing, and other possible factors (Fig. 2). We used aliquots of hair samples corresponding to the duration of a mean of ten months for the determination of total mercury, whereas we did not have accurate information on when the mothers got their hair permed. In fact, the distributions of hair mercury in the mothers seemed to differ; i.e., open circles indicating a mother with artificial hair-waving, as shown in Fig. 1, were somewhat skewed to the downward direction. Yamamoto and Suzuki (2) explained that thioglycolate in artificial waving lotion removed hair mercury effectively. Likewise, one experiment by Yasutake et al. (3) reported that more than 30% of the hair mercury in four women was removed by a single treatment of the above lotions, and repeated treatments further removed the hair mercury. This error is unmeasurably bigger than analytical imprecisions in the laboratory; the latter has been estimated to be less than 5% (5, 8). For that reason, the myth that mercury concentration in hair reflects the methylmercury concentration circulating in the blood (6, 7) may collapse if a study population includes subjects with such hair treatment.

In the present study, hair coloring/dyeing was not signifi-

cantly associated with current hair mercury levels, and no significant difference in the hair mercury level was observed between the mothers with and without hair coloring/dyeing, although approximately 10% of hair mercury in the mothers with hair coloring/dyeing seemed to be removed when compared with the mothers with natural hair. On the contrary, a preliminary study has reported that the overall average mercury concentration in four subjects was 14.2 μg/g for white hair and 15.3 μg/g for pigmented hair (5); in another report, total and organic mercury concentrations in Japanese elderly men and women were significantly higher in naturally grey hair than in dark hair (21). Thus, it is likely that when women conceal grey hair by coloring or dyeing, the hair treatment may cause possible differences in the hair mercury level. Further research is necessary to explore whether mercury concentrations in naturally grey hair or in pigmented hair are higher, as well as whether the ratio of mercury concentrations in naturally grey hair and pigmented hair differs with respect to race.

Neither daily mercury intakes nor hair mercury levels in Japanese mothers differed significantly according to residence (Table 2). A similar finding has been observed both between fishing and non-fishing areas and between cities and towns in Akita Prefecture (4). Also, working status was not relevant to the daily mercury intake or hair mercury level. Since no mothers residing near the areas where a mine/smelter existed in the past were included, the possibility of environmental mercury vapor binding to the hair was minimal. In this way, the impacts of preanalytical factors except the above hair treatment on hair mercury levels appear to be omitted in the current study.

In environmental epidemiological research, it is essentially impossible to obtain an error-free measurement of exposure. When unavoidable measurement error, i.e. artificial hair-waving, is ignored, it is probable that the estimation of the exposure effects is biased toward the null hypothesis (22, 23). In interpreting epidemiological studies based on hair mercury only, attention should be directed toward the consequences of exposure misclassification. Rather, mercury in blood or methylmercury in cord tissue, together with hair mercury, may be recommended in such studies on risk assessment (9, 12, 24), because the usefulness of the mercury concentration in cord blood has been emphasized as a main risk indicator (25).

Acknowledgements

We thank Dr. Satoshi Terui, and the Akita and Tottori Prefectural Education Boards for their cooperation in assembling the study population. This study was supported by a grant from the Ministry of the Environment, Japan.

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Tohoku J. Exp. Med., 2006, **208**, 109-115

Lactation and Risk of Endometrial Cancer in Japan: A Case-Control Study

CHIKAKO OKAMURA, YOSHITAKA TSUBONO,[1] KIYOSHI ITO, HITOSHI NIIKURA, TADAO TAKANO, SATORU NAGASE, KOHSUKE YOSHINAGA, YUKIHIRO TERADA, TAKASHI MURAKAMI, SHINJI SATO,[2] DAISUKE AOKI,[3] TOSHIKO JOBO,[4] KUNIHIRO OKAMURA and NOBUO YAEGASHI

Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai, Japan,
[1]Tohoku University School of Public Policy, Sendai, Japan,
[2]SS Ladies Clinic, Sendai, Japan,
[3]Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan, and
[4]Department of Obstetrics and Gynecology, Kitasato University School of Medicine, Kanagawa, Japan

OKAMURA, C., TSUBONO, Y., ITO, K., NIIKURA, H., TAKANO, T., NAGASE, S., YOSHINAGA, K., TERADA, Y., MURAKAMI, T., SATO, S., AOKI, D., JOBO, T., OKAMURA, K. and YAEGASHI, N. *Lactation and Risk of Endometrial Cancer in Japan: A Case-Control Study.* Tohoku J. Exp. Med., 2006, **208** (2), 109-115 —— The incidence of endometrial cancer is rapidly increasing in Japan. Although the risk factors in European populations have been well described, there are few epidemiologic studies regarding risk factors for endometrial cancer in Japanese women. This hospital-based case-control study among Japanese women was carried out from 1998 to 2000. The cases were selected from women with endometrial cancer (n =155), and the controls selected from women attending the university gynecological outpatient clinic for cervical cancer screening (n = 96). Subjects were interviewed to ascertain breast feeding practices, contraceptive usage, as well as potential risk factors for endometrial cancer. We observed a lower risk of endometrial cancer associated with oral contraceptive (OC) and a higher risk associated with higher body mass index (BMI), and older ages at first and last delivery. Gravidity reduced odds ratio (OR) for endometerial cancer to 0.34 (95% confidence interval [CI] 0.13-0.92). Compared with parous women who had never breastfed, the multivariate OR for women with a history of breastfeeding was 0.37 (95% CI, 0.17-0.82). Additionally, a greater lapse of time since breastfeeding increased OR for endometrial cancer by over three times. In conclusion, the present study has indicated that breastfeeding reduces the risk of endometrial cancer in Japanese women. ——— endometrial cancer; breastfeeding; risk factor; case-control study

Received September 12, 2005; revision accepted for publication November 30, 2005.
Correspondence: Nobuo Yaegashi, Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan.
e-mail: yaegashi@mail.tains.tohoku.ac.jp

Age-adjusted incidence rates for endometrial cancer have doubled during the past two decades among Japanese women. The rising incidence possibly may be due to changes in lifestyle, or changes in reproductive factors such as childbearing and contraception, as these characteristics have been associated with endometrial cancer risk in Western populations. In Western countries, there is considerable evidence that reproductive factors play a role in the etiology of endometrial cancer. Nulliparity and obesity have been associated with a higher risk, whereas oral contraceptive (OC) use has been associated with a lower risk (Kirschner et al. 1981; Kelsey et al. 1982; Zumoff 1982; Austin et al. 1991; Schapira et al. 1991; Brinton et al. 1992; Shu et al. 1992; Kalandidi et al. 1996; McPherson et al. 1996; Iemura et al. 2000; Herrinton et al. 2001). A few studies have examined the association between breastfeeding and endometrial cancer risk (Rosenblatt and Thomas 1995; Salazar-Martinez et al. 1999; Newcomb and Trentham-Dietz 2000); however, the findings from these studies are inconsistent.

The reproductive characteristics of Japanese women, however, are different from those of Western populations. For instance, 15%, 36%, and 59% of contraceptive-using women choose OCs in the United States, France, and Germany, respectively, whereas the prevalence of OC use is only 1.5% among Japanese women who use contraception. Only 1.8% of Japanese women older than 50 years have used hormone replacement therapy (HRT), whereas the prevalence of HRT usage is 53% among US women aged 50-59 years. These differences make it difficult to generalize findings obtained in Western studies to Japanese women. There have, however, been a few studies evaluating risk factors for endometrial cancer in Japanese women (Inoue et al. 1994; Hirose et al. 1996, 1999). Therefore, this study was undertaken to further characterize endometrial cancer risk factors in Japanese population.

Subjects and Methods

This case-control study was a collaborative investigation in three areas of Japan (Tokyo, Kanagawa, and Miyagi). Cases were accrued from three university hospitals from January 1, 1998, through December 31, 2000. Eligible cases included Japanese women between 20 and 80 years of age who underwent surgery for a diagnosis of endometrioid endometrial cancer confirmed by histology. The cases resided in defined geographic catchment areas, and had not received treatment previously. One hundred sixty seven cases were eligible for the study and 12 subjects refused to participate. Thus, 155 (93%) of the eligible cases participated. Stage distribution of the cases was as follows: stage I, n = 104; stage II, n = 14; stage III, n = 33; and stage IV, n = 4.

The controls were selected from women who attended gynecologic outpatient clinics in the university hospitals for cervical cancer screening. Controls included only women with intact uteri. Ninety six women were included as controls; however, 9 women refused participation (participation rate, 91%). Cases and controls were not matched in terms of age or other variables.

The protocol for this study was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan).

Gynecologists interviewed the cases and controls using a standard questionnaire asking about demographic information, medical history, cigarette use, and reproductive history (parity, gravidity, and ages at first pregnancy, last delivery, menarche, menopause, and lactation). Body mass index (BMI) was calculated based on self-reports of weight (kg)/height $(m)^2$. The distribution of continuous variables was examined among cases and controls and divided into two or three categories.

To estimate the risk of endometrial cancer associated with various factors, we calculated age-adjusted and multivariate odds ratio (ORs) along with 95% confidence interval (CI) using unconditional logistic regression analysis. Statistical Analysis System (SAS Institute, Cary, NC, USA) software was used for all statistical analyses.

Results

The mean ages of cases and controls were 56.1 years and 49.6 years, respectively. Table 1 presents age-adjusted ORs and 95% CIs of the selected variables for the risk of endometrial cancer. Higher BMI was associated with higher risk (p = 0.01). OC use was associated with a lower risk of disease (OR, 0.16; 95% CI, 0.04-0.66), although only three cases and ten controls used OCs. Intra-uterine device use, history of HRT, smoking, sterility, hypertension, diabetes mellitus,

TABLE 1. *Baseline characteristics of cases and controls*

Caracteristics	Cases	%	Controls	%	Age-adjusted OR	95% CI	*p* value
Age (years)							
< 45	15	9.7	39	40.6			
45-55	52	33.6	23	24			
55-65	55	35.4	24	25			
≧ 65	33	21.3	10	10.4			
BMI (kg/m^2)							
< 20.04	36	23.3	26	27.1	1.00		
20.04-21.63	27	17.4	35	36.5	0.47	0.22-0.99	
21-64-23.92	45	29.0	20	20.8	1.24	0.58-2.67	
≧ 23.93	47	30.3	15	15.6	1.92	0.86-4.30	0.01
Oral contraceptive use							
Never	152	98.1	86	89.6	1.00		
Ever	3	1.9	10	10.4	0.16	0.04-0.66	0.01
IUD use							
Never	148	95.5	90	93.8	1.00		
Ever	7	4.5	6	6.2	0.54	0.17-1.71	0.29
HRT use							
Never	132	85.16	85	88.5	1.00		
Ever	23	14.84	11	11.5	1.4	0.63-3.14	0.41
Cigarette smoking							
Never	126	81.3	77	80.2	1.00		
Ever	29	18.7	19	19.8	1.30	0.65-2.61	0.52
Sterility							
Never	143	92.3	87	90.6	1.00		
Ever	12	7.7	9	9.4	0.81	0.31-2.11	0.66
Hypertension							
Never	115	74.2	87	90.6	1.00		
Ever	40	25.8	9	9.4	2.15	0.95-4.86	0.45
Diabetes mellitus							
Never	139	89.7	92	95.8	1.00		
Ever	16	10.3	4	4.2	1.82	0.56-5.92	0,32
Personal cancer history							
Never	139	89.7	92	96.8	1.00		
Ever	16	10.3	4	4.2	1.78	0.55-5.73	0.33

and personal cancer history were not associated with risk. There were 20 persons who had personal cancer history. Among them 11 persons had breast cancer and the remaining nine persons had cancer history at various sites, such as colon cancer, rectal cancer, thyroid cancer, gastric cancer, lung cancer, and ovarian cancer. Four of the 20 persons had hormone therapy.

Table 2 shows the ORs for the association of endometrial cancer with reproductive factors.

TABLE 2. *Multivariate Odds Ratio and 95%Confidence Intervals for Endometrial Cancers-According to Reproductive Factors*

Variables	Cases	%	Controls	%	OR*	95% CI	*p* value
Menopausal status							
Pre	51	32.9	55	57.3	1.00		
Post	104	67.1	41	42.7	0.91	0.39-2.14	0.82
Gravidity							
Never	20	12.9	9	9.4	1.00		
Ever	135	87.1	87	90.6	0.34	0.13-0.92	0.03
No. of pregnancies							
0	20	12.9	9	9.4	1.00		
1	27	17.4	16	16.7	0.43	0.14-1.33	
2	42	27.1	32	33.3	0.34	0.12-0.97	
≧ 3	66	42.6	39	40.6	0.29	0.10-0.85	0.04
Parity							
Never	36	23.2	21	21.9	1.00		
Ever	119	76.8	75	78.1	0.46	0.22-0.96	0.04
No. of deliveries							
0	36	23.2	21	21.9	1.00		
1	29	18.7	18	18.8	0.45	0.18-1.12	
2	68	43.9	44	45.8	0.47	0.21-1.04	
≧ 3	22	14.2	13	13.5	0.44	0.16-1.20	0.1
Age at first delivery**							
≦ 24	43	36	11	14.7	1.00		
25-26	36	30.3	23	30.7	0.45	0.18-1.10	
27-29	21	17.7	23	30.7	0.30	0.12-0.78	
≧ 30	19	16	18	24	0.35	0.13-0.96	0.05
Age at last delivery**							
≦ 25	23	19.3	6	8	1.00		
26-30	40	33.6	25	33.3	0.48	0.16-1.45	
31-33	39	32.8	26	34.7	0.45	0.15-1.36	
≧ 34	17	14.3	18	24	0.28	0.08-0.94	0.02

* OR adjusted for age, BMI, and oral contraceptive use.
** Parous women only.

The ORs were adjusted for age, BMI, and OC use. Gravidity was inversely associated with endometrial cancer risk. Women who reported ever being pregnant had only one third the risk of endometrial cancer compared with women who had never been pregnant (OR, 0.34; 95% CI, 0.13-0.92, $p = 0.03$). Women who reported three or more pregnancies had about one third the risk of women with no pregnancies (OR, 0.29; 95% CI, 0.10-0.85).

Parity was also inversely associated with endometrial cancer risk. Women who reported ever having delivery had about one half the risk of endometrial cancer compared with women who had never delivered (OR, 0.46; 95% CI, 0.22-0.96, $p = 0.04$). Higher age at the first or last deliveries was associated with a lower risk for endometrial cancer ($p = 0.05$, $p = 0.02$). Age at menarche, menopausal status, age at menopause, history of dysmenorrhea, and history of abortion were not associated with risk (data not shown).

Only parous women, representing 119 cases and 75 controls, were included in the analysis of the association between breastfeeding and endometrial cancer risk presented in Table 3. Table 3 also showed the age distribution of both cases and control and that of the lapse of the last breast feeding. The ORs were adjusted for age, BMI, and OC use as shown in Table 3. Compared with parous women who had never breastfed, the multivariate odds ratio for women who had ever breastfed was 0.37 (95% CI: 0.17-0.82, $p = 0.013$). A greater lapse of time since breastfeeding concluded was directly associated with an increased risk of endometrial cancer (OR of 20-29 years, 3.10, 95% CI: 1.14-8.48, and OR of 30 or longer, 3.85, 95% CI: 1.00-14.84, $p = 0.045$). Then, we analyzed the association between frequency or duration of breastfeeding and endometrial cancer risk, but did not find any significant association (data not shown).

Discussion

In this hospital-based case-control study among Japanese women, we observed a lower risk of endometrial cancer associated with OC use and gravidity, and a higher risk associated with higher BMI, older ages at first and last delivery and number of pregnancies. These findings were consistent with data obtained in prior Japanese studies (Inoue et al. 1994; Hirose et al. 1996, 1999). In contrast to the study by Inoue et al. (1994), our study failed to demonstrate an association between a history of hypertension, diabetes mellitus, or cancer.

Our study also demonstrated a reduction in the risk of endometrial cancer associated with breastfeeding. The proportion of never breastfeeding (35.3%) in endometrial cancer cases was larger than that in control, but the risk was signifi-

Table 3. *Multivariate Odds Ratio for Endometrial Cancers in relation to breastfeeding and age among parous women*

Variables	Cases (n)					Controls (n)					OR*	95% CI	p value
	Total	< 45	45-55	55-65	65 ≦	Total	< 45	45-55	55-65	65 ≦			
breastfeeding													
Never	42	1	10	24	7	11	3	3	2	3	1.00		
Ever	77	1	31	26	19	64	25	14	18	7	0.37	0.17-0.82	0.013
Years since last breastfed**													
1-19	12	1	9	1	1	33	24	7	2	0	1.00		
20-29	31	0	17	12	2	20	1	7	11	1	3.10	1.14-8.48	
≧ 30	34	0	5	13	16	11	0	0	5	6	3.85	1.00-14.84	0.045

* Adjusted for age, BMI and oral contraceptive use.
** Ever breastfed women only.

cant even after been adjusted for age, BMI, and contraceptive use. The risk reduction of endometrial cancer was associated not only with breastfeeding itself but also with time since the last breastfeeding. From 1982 to 2000, seven case-control studies conducted in six countries, including four developing countries, examined the relationship between breastfeeding and the risk of endometrial cancer. Four early studies, two of which were Japanese, failed to support an association (Kelsey et al. 1982; Brinton et al. 1992; Hirose et al. 1996, 1999). Three recent Western studies, however, suggested a protective effect of breastfeeding (Rosenblatt and Thomas 1995; Salazar-Martinez et al. 1999; Newcomb and Trentham-Dietz 2000). This effect was more pronounced with recent breastfeeding, diminishing as the history of breastfeeding became more remote (Rosenblatt and Thomas 1995; Newcomb and Trentham-Dietz 2000). Our findings were consistent with those of the latter studies, making this the first report that notes an inverse association between breastfeeding and the risk of endometrial cancer among Japanese women.

Exposure of the endometrium to estrogen in the absence of progesterone is thought to increase the risk of endometrial cancer (Key and Pike 1988- see comment for citation). In lactating women, the ovarian cycle is suppressed and blood estrogen levels are reduced (Baird et al. 1979). In the case of oral contraceptives, progesterone continually opposes estrogen, minimizing the duration of time the endometrium is exposed to unopposed estrogen. Thus, suppression of circulating estrogen levels, or opposition of estrogen by progesterone, may represent a biological mechanism accounting for the protective effects of pregnancy, oral contraceptives, and breastfeeding against carcinogenesis of endometrial tissue.

Among Japanese women, the birth rate decreased 28.1 to 9.3 per 1,000 during 1950-2000, and the proportion of women who exclusively breastfed decreased from 70.5% to 44.8% during the same period (Kaneda 2003). In our study, the proportion of women who breastfed for 13 months was 52.4%. The observed lower risk associated with breastfeeding in this study suggests that the recent increase in incidence of endometrial cancer in Japan may be in part attributed to a decrease in both the number of pregnancies and the prevalence of breastfeeding.

A limitation of this study was its lack of age matching. This resulted in a mean age of cases that was 6 years older than that of controls. It is unlikely that the lack of age matching resulted in serious distortion of our observations because all analyses were adjusted for continuous age. Furthermore, the findings in these studies were consistent with data obtained in several previous studies. Another limitation of the study was the small number of control. To overcome these limitations, in progress is our new case control study which matched ages of cases and controls and included two times more subjects of control. These data will confirm the present observations.

Acknowledgments

This study was supported by a grant for Scientific Research in a priority area from the Ministry of Education, Science, Sports and Culture, Japan, a grant from the Ministry of Education, Science, Sports and Culture, Japan, a grant from the Ministry of Health, Labor and Welfare, Japan, and the 21st Century COE Program Special Research Grant (Tohoku University) from the Ministry of Education Science, Sports and Culture, Japan.

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14-3-3σ in Endometrial Cancer – A Possible Prognostic Marker in Early-Stage Cancer

Kiyoshi Ito,[1] Takashi Suzuki,[2] Jun-ichi Akahira,[1,2] Michiko Sakuma,[1] Sumika Saitou,[1] Satoshi Okamoto,[1] Hitoshi Niikura,[1] Kunihiro Okamura,[1] Nobuo Yaegashi,[1] Hironobu Sasano,[2] and Satoshi Inoue[3,4]

Abstract

Purpose: We examined expression of 14-3-3σ, a regulator of cell proliferation, and evaluated its clinical significance in endometrioid endometrial carcinoma.

Experimental Design: One hundred three endometrioid endometrial adenocarcinoma cases were examined using immunohistochemistry with archival specimens. We correlated this finding with various clinicopathologic variables, including the status of estrogen receptor, progesterone receptor, and MIB-1 (Ki-57).

Results: 14-3-3σ Immunoreactivity was detected in 78 of 103 (75.3%) of carcinoma cases. No statistically significant correlation was detected between status of 14-3-3σ and any of clinicopathologic variables examined. There was, however, a statistically significant correlation between loss of 14-3-3σ expression and adverse clinical outcome of the patients ($P = 0.0007$). In the early stages of cancer (stages I and II), 14-3-3σ immunoreactivity was absent in 5 of 10 (50.0%) patients who showed recurrence during follow-up, whereas its absence was detected in only 13 of 68 (19.1%) disease-free patients in the same period. In addition, 14-3-3σ immunoreactivity was absent in 4 of 5 (80.0%) patients who died, whereas its absence was detected in only 14 of 73 (19.2%) patients who had lived during the same period. Patients whose tumors were negative for 14-3-3σ were at much greater risk to develop recurrent and/or mortal disease ($P = 0.0372$ and 0.0067). In multivariate analysis using the Cox proportional hazards model, absence of 14-3-3σ turned out to be statistically independent risk factor in disease-free survival and overall survival even in patients with early-stage disease ($P = 0.0321$ and 0.0191).

Conclusions: Results of our study showed that loss or absence of 14-3-3σ determined by immunohistochemistry may be an important tool to identify endometrial carcinoma cases at high risk of recurrence and/or death, who are otherwise not detected by current clinical and pathologic evaluation, especially in the early stages of the disease. In addition, results of 14-3-3σ immunohistochemistry in the early stage of endometrial carcinoma could contribute to planning postoperative follow-up and adjuvant therapy.

14-3-3 Proteins have been found to play important roles in the regulation of various cellular processes, such as cell cycle progression, cell growth, apoptosis, and signal transduction (1, 2). In humans, seven different 14-3-3 isoforms have been identified. 14-3-3σ, a member of this family, is induced by DNA damage and is required for a stable G_2 cell cycle arrest in epithelial cells. Loss of *14-3-3σ* expression results in malignant transformation *in vitro* and supports tumor formation *in vivo*, which suggests that this gene has tumor-suppressive properties. The *14-3-3σ* gene was originally identified as a p53-inducible gene responsive to DNA-damaging agents (3). In response to DNA damage, 14-3-3σ is induced in a p53-dependent manner and prevents the cdc2/cyclin B1 complex from entering the nucleus. We showed previously that 14-3-3σ undergoes proteolysis mediated by estrogen finger protein, which is a target of the estrogen receptor (ER) acting as an ubiquitin ligase of 14-3-3σ in breast carcinoma cells (4). In addition, 14-3-3σ is silenced by CpG methylation in a large proportion of human carcinomas (1, 2). The expression of 14-3-3σ is shown to be frequently lost in human epithelial carcinoma, breast, gastric, lung (5–7), etc. We also reported recently that decreased expression of 14-3-3σ was significantly associated with poor prognosis in epithelial ovarian cancer (8).

Endometrial carcinoma is the most common malignancy of the female genital tract, and its incidence has recently increased (9). In normal endometrium, 14-3-3σ protein was expressed weakly in epithelial glandular cells (10). However, the status of 14-3-3σ protein and its possible roles have never been examined in endometrial carcinoma. We reported previously

Authors' Affiliations: Departments of [1]Obstetrics and Gynecology and [2]Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan; [3]Research Center for Genomic Medicine and Department of Molecular Biology, Saitama Medical School, Saitama, Japan; and [4]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Received 1/26/05; revised 6/23/05; accepted 7/14/05.

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Requests for reprints: Kiyoshi Ito, Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-Machi, Aoba-Ku, Sendai 980-8574, Japan. Phone: 81-22-717-7254; Fax: 81-22-717-7258; E-mail: kito@mail.tains.tohoku.ac.jp.

doi:10.1158/1078-0432.CCR-05-0187

Table 1. Summary of primary antibodies used in this study

Antibody	Source	Optimal dilution	Antibody retrieval
14-3-3σ (polyclonal)	Santa Cruz Biotechnology	1:100	Autoclave*
ER (monoclonal)	Immunotech (Marseilles, France)	1:2	Autoclave*
PR (monoclonal)	Chemicon (Temecula, CA)	1:30	Autoclave*
Ki-67 (monoclonal)	Immunotech	1:50	Autoclave*
p53 (monoclonal)	Biomeda (Foster City, CA)	1:40	Autoclave*

*Heat in an autoclave for 5 minutes in citric acid buffer [2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate (pH 6.0)].

the prognostic significance of p53 overexpression in endometrial cancer (11, 12). Therefore, decreased expression of 14-3-3σ may possibly have an important role in the development of endometrial cancer, because 14-3-3σ is directly regulated by p53.

To study the possible correlation between status of 14-3-3σ protein and prognosis of the patients, we examined its immunoreactivity in 103 cases of endometrioid endometrial cancer and correlated the findings with clinical outcome of the patients.

Materials and Methods

Patients and tissues. Twenty-five normal cycling human endometria (15 proliferative phase and 10 secretory phase) and 103

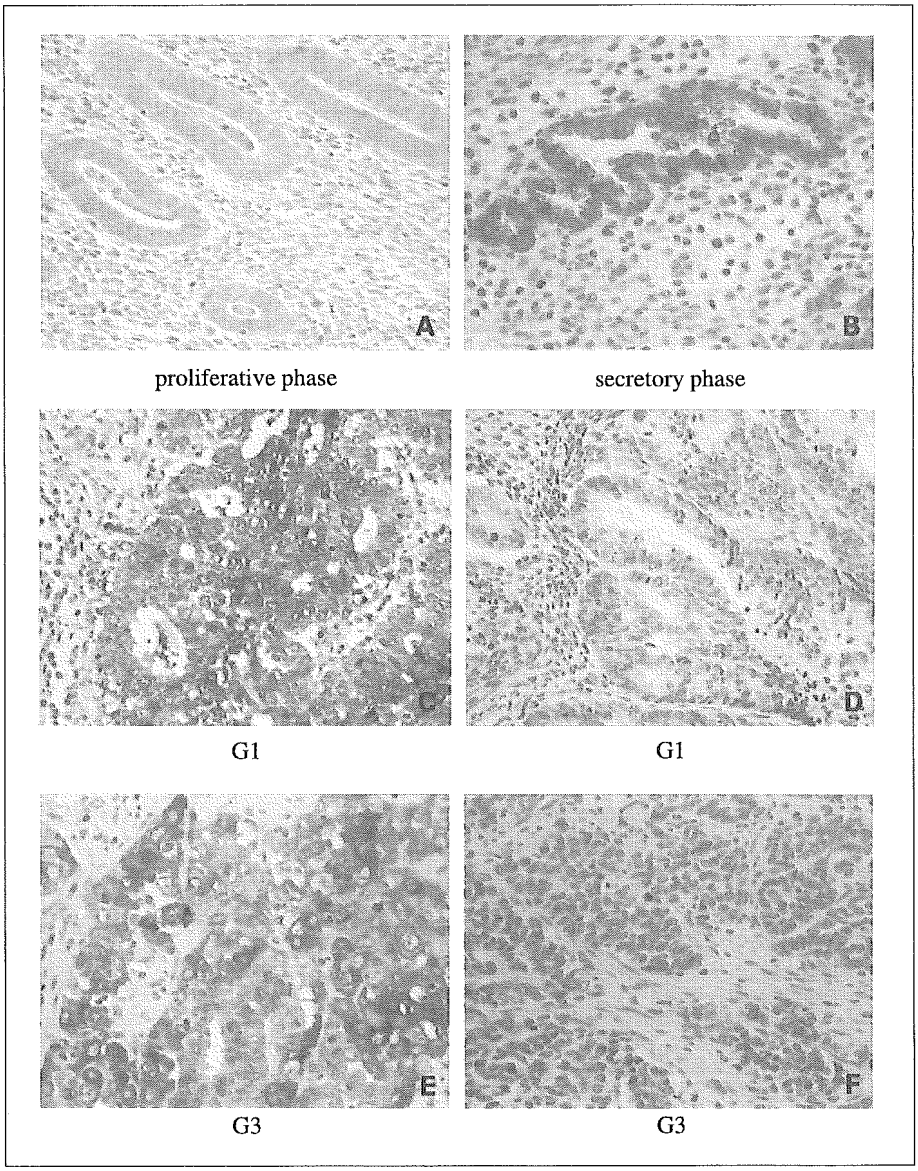

Fig. 1. Immunohistochemistry of 14-3-3σ expression: *A*, proliferative phase; *B*, secretory phase; *C* and *D*, endometrioid endometrial adenocarcinoma (G1); *E* and *F*, endometrioid endometrial adenocarcinoma (G3). 14-3-3σ Immunoreactivity was detected in the cytoplasm of glandular cells. 14-3-3σ Immunoreactivity was also detected in the cytoplasm of carcinoma cells. *C* and *E*, positive cases; *D* and *F*, negative cases. Original magnification, ×400.

endometrioid endometrial adenocarcinoma (49 well differentiated, 32 moderately differentiated, and 22 poorly differentiated; 66 stage I, 12 stage II, 22 stage III, and 3 stage IV) were retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan). The protocol for this study was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan). All carcinoma specimens were obtained from surgery. We obtained nonpathologic endometria from hysterectomy specimens performed due to carcinoma *in situ* of the uterine cervix at Tohoku University Hospital. All endometrial carcinoma specimens were obtained from hysterectomy. Median follow-up time of the patients examined in this study was 60 months (range, 2-148 months). Disease-free survival and overall survival were calculated from the time of initial surgery to recurrence and/or death or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in December 2004. Clinicopathologic findings of these patients, including age, histology, stage, grade, and preoperative therapy, were retrieved by review of patient charts. A standard primary treatment for endometrial carcinoma at Tohoku University Hospital was surgery consisting of total abdominal hysterectomy, salpingo-oopholectomy, pelvic and/or para-aortic lymphadenectomy, and peritoneal washing cytology. A total of 85 of 103 (83%) patients underwent complete surgery. Six of 85 patients had lymph node metastasis. The remaining 18 (17%) patients underwent total abdominal hysterectomy and salpingo-oopholectomy without lymphadenectomy because of obesity and/or poor performance status. None of these patients had received preoperative chemotherapy and/or hormonal therapy or pelvic irradiation. No patient had used oral contraceptives. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the WHO and staged according to the International Federation of Gynecology and Obstetrics system (13, 14). Sixty-eight of 103 patients received pelvic radiation therapy (50 Gy) or three to six courses of chemotherapy consisting of the cisplatin-based combination regimen CAP (60-70 mg/m^2 cisplatin, 40 mg/m^2 doxorubicin, and 500 mg/body cyclophosphamide) after operation. Patients who had early-stage and low-grade disease (stage Ia, grade 1; stage Ia, grade 2; and stage Ib, grade 1) and patients who were associated with poor performance status did not receive any adjuvant therapy. All specimens were routinely processed (i.e., 10% formalin fixed for 24-48 hours), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemistry. Immunohistochemical analysis was done employing the streptavidin-biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan) as described previously in detail by the authors (15). Polyclonal antibody for 14-3-3σ (N-14) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The characteristics of the primary antibodies employed in this study are summarized in Table 1. For immunostaining of 14-3-3σ, p53, ERα, progesterone receptor (PR), and Ki-67, the slides were heated in an autoclave at 121°C for 5 minutes in citric acid buffer [2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate (pH 6.0)] following deparaffinization for antigen retrieval. The dilutions of the primary antibodies used for our studies were as follows: 14-3-3σ, 1:100; p53, 1:40; ERα, 1:2; PR, 1:30; and Ki-67, 1:50. 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 nonneoplastic breast epithelial tissue were used as positive controls for 14-3-3σ, and breast cancer was also used as positive control for ERα. As a negative control, normal rabbit or mouse IgG was used instead of primary antibodies.

Semiquantitative analysis of immunohistochemical staining. For evaluation of ERα, PR, and Ki-67 immunoreactivity, labeling index was obtained in glandular or carcinoma cells as described by Utsunomiya et al. (16) with some modifications. In cases immunopositive for ERα, PR, and Ki-67, >1,000 glandular or carcinoma cells were counted in each case by two of the authors (K.I. and T.S.) independently after reviewing the slides and determining the areas of evaluation simultaneously with a double-headed microscope. The percentage of immunoreactivity (i.e., labeling index) was subsequently determined. Cases with interobserver differences of >5%, which occurred in 3% to 7% of the cases examined, were reevaluated together by two of the authors above using double-headed light microscopy. Intraobserver differences were <5% when examining the same selected fields of representative cases. The mean value was obtained in cases with interobserver differences of <5%. As immunoreactivities of 14-3-3σ and p53 were relatively homogeneous and clearly distinguishable as positive or negative, carcinoma cells were classified into the two groups without much differently (+, carcinoma cells with positive immunoreactivity; −, carcinoma cells with no immunoreactivity) by two of the same authors above.

Statistical analyses. Statistical analysis was done using SAS software version 5.0 (StatView, Cary, NC). The statistical difference between 14-3-3σ and characteristics of the patients was evaluated in a cross-table using the χ^2 test. Correlation between 14-3-3σ and p53, ERα, PR, and Ki-67 immunoreactivity was also assessed using Mann-Whitney *U* test.

Table 2. Correlation between 14-3-3σ immunoreactivity and clinicopathologic variables in endometrial cancer

	Total (n = 103)	14-3-3σ immunoreactivity		P
		Positive (n = 78)	Negative (n = 25)	
Age (median)	57.0	57.0	60.0	0.348
Grade, n (%)				
1	49 (47.6)	35	14	0.386
2	32 (31.0)	27	5	
3	22 (21.4)	16	6	
Stage				
I/II	78 (75.7)	60	18	0.815
III/IV	25 (24.3)	18	7	
p53 immunoreactivity				
Positive	15 (14.6)	11	4	0.055
Negative	88 (85.4)	67	21	
ER labeling index (median)	23.0	26.0	15.0	0.393
PR labeling index (median)	25.0	30.0	20.0	0.154
Ki-67 labeling index (median)	32.0	33.5	30.0	0.324

Table 3. Univariate analyses of predictors of disease-free survival and overall survival for 103 patients with endometrial cancer

Variable	Disease-free survival *P*	Overall survival *P*
14-3-3σ (positive vs negative)	0.0382	0.0041
Age (≤50 vs >50)	0.1159	0.0854
Stage (I/II vs III/IV)	0.2029	0.1163
Histologic grade (1-3)	0.0276	0.0063
p53 immunoreactivity (negative vs positive)	0.1601	0.0248
ER (positive vs negative)	0.0426	0.2643
PR (positive vs negative)	0.0004	0.0076
Ki-67 (positive vs negative)	0.4722	0.3449

Overall and disease-free survival curves were generated according to the Kaplan-Meier method, and the statistical significance was calculated using a log-rank test. Univariate and multivariate analyses were evaluated with Cox proportional hazards model. A result was considered significant when the $P < 0.05$.

Results

Normal cycling endometrium. 14-3-3σ Immunoreactivity was detected in the cytoplasm of glandular cells but not in the stromal cells of all the cases examined. Marked 14-3-3σ immunoreactivity was detected in the glandular cells of secretory phase mucosa compared with those of proliferative phase mucosa (Fig. 1A and B).

Association of 14-3-3σ expression with clinicopathologic variables and estrogen receptor α, progesterone receptor, Ki-67, and p53 immunoreactivity in patients with endometrial cancer. 14-3-3σ Immunoreactivity was detected in the cytoplasm of epithelial cancer cells, although ERα, PR, Ki-67, and p53 were confined exclusively to the nuclei of epithelial cells (Fig. 1C-F). 14-3-3σ Immunoreactivity was present in 78 of 103 (75.3%) cases of endometrioid endometrial carcinoma. The correlation between 14-3-3σ immunoreactivity and clinicopathologic variables, including ERα, PR, Ki-67, and p53 immunoreactivity, was examined. As seen in Table 2, no statistically significant correlation was detected between status of 14-3-3σ immunoreactivity and any of the variables examined in this study. The status of 14-3-3σ immunoreactivity tended to be inversely correlated with that of p53, but the correlation did not reach statistical significance. There were no statistically significant correlations between status of lymph node metastasis and 14-3-3σ expression ($P = 0.1456$).

Association of 14-3-3σ expression with disease-free survival and overall survival in patients with endometrial cancer. 14-3-3σ Immunoreactivity was evaluated as a prognostic variable in the 103 cases using univariate analysis (Table 3; Cox proportional hazards model). In 103 cases, 14-3-3σ immunoreactivity was absent in 7 of 16 patients (43.8%) who showed recurrence during follow-up, whereas loss of its immunoreactivity was detected only in 18 of 87 (20.7%) disease-free patients for the same clinical follow-up period. 14-3-3σ Immunoreactivity was also absent in 6 of 9 (66.7%) patients who died, whereas loss of its immunoreactivity was detected only in 19 of 94 (20.2%) patients who had lived during the same period. Patients whose tumors were associated with absence of 14-3-3σ expression were at much greater risk to develop recurrent and/or mortal disease ($P = 0.0382$ and 0.0041). Indicators of clinical outcome of the patients, including ER, PR, and p53 status and histologic grade, were likewise significantly associated with poor outcome. Patients whose tumors were negative for 14-3-3σ

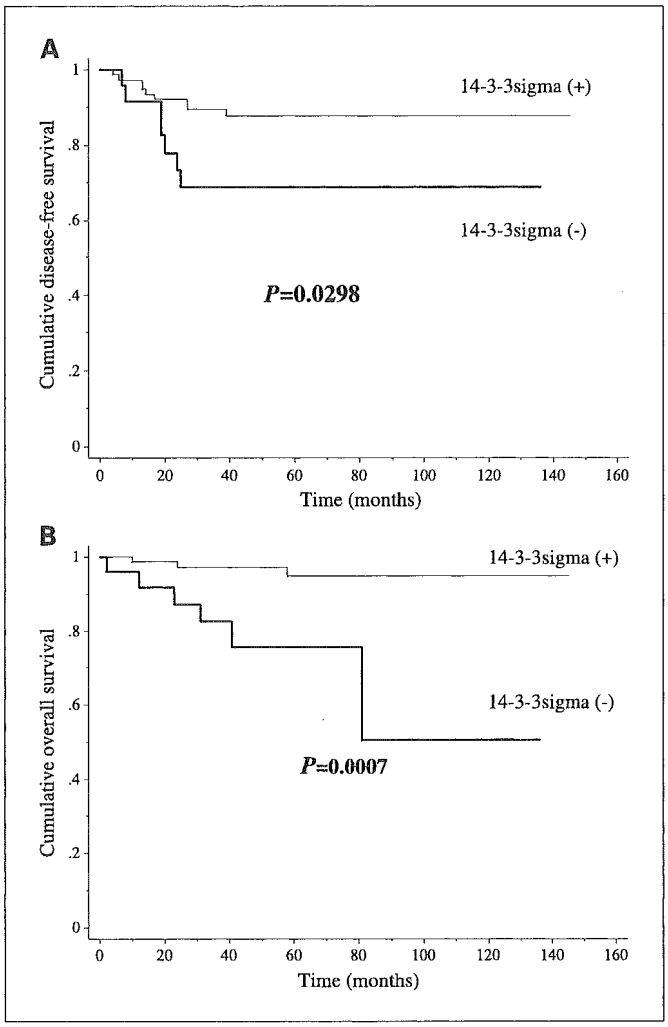

Fig. 2. *A*, correlation between 14-3-3σ immunoreactivity and recurrence for patients with endometrial cancer. *B*, correlation between 14-3-3σ immunoreactivity and survival for patients with endometrial cancer.

Table 4. Multivariate analyses of predictors of disease-free survival and overall survival for 103 patients with endometrial cancer

Variable	Disease-free survival		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
14-3-3σ (positive vs negative)	0.320 (0.114-0.894)	0.0297	0.185 (0.048-0.719)	0.0148
Stage (I/II vs III/IV)	0.687 (0.236-2.003)	0.4920	0.750 (0.190-2.964)	0.6810
Histologic grade (1-3)	1.473 (0.796-2.726)	0.2170	2.543 (1.030-6.281)	0.0431
p53 (negative vs positive)	0.587 (0.170-2.029)	0.3997	0.335 (0.089-1.261)	0.1058
ER (positive vs negative)	0.463 (0.168-1.273)	0.1356	1.134 (0.280-4.590)	0.8602
PR (positive vs negative)	0.142 (0.039-0.517)	0.0031	0.432 (0.091-2.049)	0.2904

NOTE: HR, hazard ratio; 95% CI, 95% confidence interval.

had also significantly worse disease-free survival and overall survival rates than 14-3-3σ-positive ones using log-rank tests (Fig. 2A and B; $P = 0.0298$ and 0.0007).

To determine whether the prognostic value of 14-3-3σ expression was independent of other risk factors associated with clinical outcome, we examined the data using multivariate analysis. The prognostic factors examined were 14-3-3σ, ER, PR, and p53 status, stage, and histologic grade. The findings are summarized in Table 4. Absence of 14-3-3σ expression was independently statistically significant as risk factor in disease-free survival and overall survival of the patients ($P = 0.0297$ and 0.0148). PR status was an independent risk factor only in disease-free survival, and histologic grade was an independent risk factor only in overall survival. ER status turned out not to be independent prognostic indicator in both disease-free survival and overall survival. Disease-free survival and overall survival were not significantly different between the two groups who received radiation therapy or chemotherapy (data not shown).

Significance of 14-3-3σ status in patients with early-stage disease of endometrial cancer. 14-3-3σ Expression was then evaluated as a prognostic variable in 78 cases with early-stage disease (stage I and II) using univariate analysis (Table 5; Cox proportional hazards model). In the total of 78 cases, 14-3-3σ immunoreactivity was absent in 5 of 10 (50.0%) patients who showed recurrence during follow-up, whereas loss of its immunoreactivity was detected only in 13 of 68 (19.1%) disease-free patients for the same period. 14-3-3σ Immunoreactivity was also not detected in 4 of 5 (80.0%) patients who died, whereas absence of its immunoreactivity was detected only in 14 of 73 (19.2%) patients who had lived during the same period. Patients whose tumors did not show 14-3-3σ immunoreactivity were at much greater risk to develop recurrent and/or mortal disease ($P = 0.0372$ and 0.0067). Patients whose tumors were negative for 14-3-3σ also had significantly worse disease-free survival and overall survival rates than 14-3-3σ-positive ones using log-rank tests (Fig. 3A and B; $P = 0.0251$ and 0.0002). In advanced-stage disease (stage III and IV), there was a trend for 14-3-3σ-negative cases to undergo aggressive biological behavior than 14-3-3σ-positive ones, although the differences did not reach statistical significance (data not shown). Multivariate analysis was done and summarized in Table 6. Absence of 14-3-3σ immunoreactivity was independently statistically significant as risk factor in disease-free survival and overall survival in patients with early-stage disease of endometrial carcinoma ($P = 0.0317$ and 0.0229).

Therefore, we examined the subgroup with completely surgically staged node-negative (International Federation of Gynecology and Obstetrics stage I and II) endometrial adenocarcinoma (Table 7). Absence of 14-3-3σ immunoreactivity still turned out to be independently statistically significant as risk factor in disease-free survival ($P = 0.0245$) although not significant in overall survival ($P = 0.0646$) of the patients.

Discussion

This is the first study that examined the status of 14-3-3σ protein and its possible roles in conjunction with clinical outcome of the patients in endometrial carcinoma. The *14-3-3σ* gene is well-known to be induced after DNA damage in a

Table 5. Univariate analyses of predictors of disease-free survival and overall survival for 78 patients with stage I and II endometrial cancer

Variable	Disease-free survival *P*	Overall survival *P*
14-3-3σ (positive vs negative)	0.0372	0.0067
Age (≦50 vs >50)	0.0856	0.2085
Histologic grade (1-3)	0.1527	0.1040
p53 immunoreactivity (negative vs positive)	0.6539	0.0870
ER (positive vs negative)	0.1368	0.9561
PR (positive vs negative)	0.0028	0.0001
Ki-67 (positive vs negative)	0.6605	0.8310

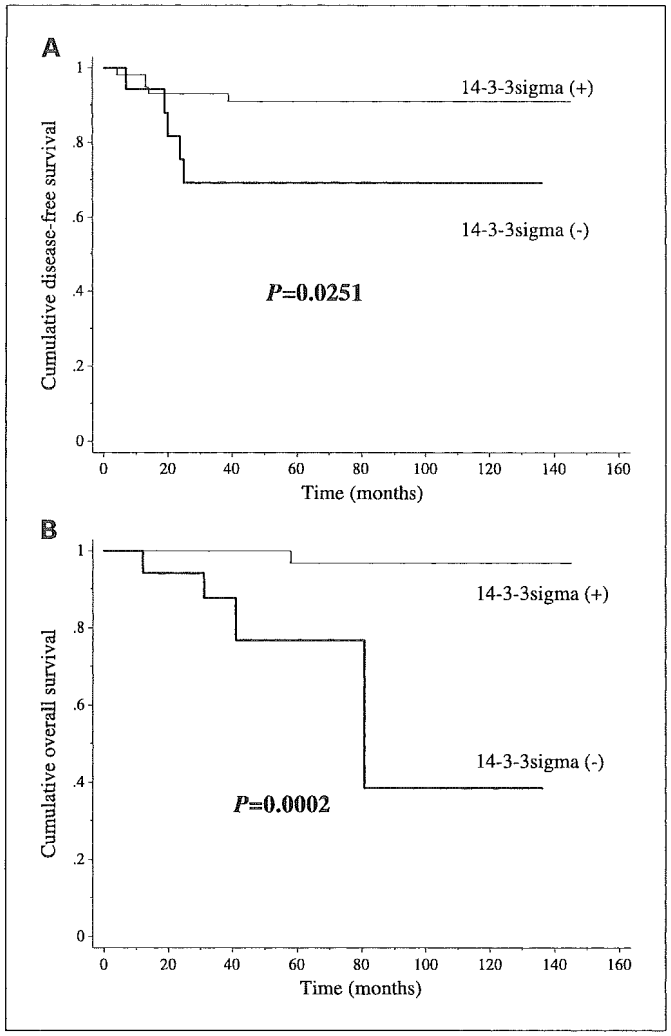

Fig. 3. *A*, correlation between 14-3-3σ immunoreactivity and recurrence for patients with early-stage (stage I and II) endometrial cancer. *B*, correlation between 14-3-3σ immunoreactivity and survival for patients with early-stage (stage I and II) endometrial cancer.

p53-dependent manner and to play an important role in the G_2 checkpoint by sequestering the cdc2/cyclin B1 complex (1, 2). An inactivation of 14-3-3σ is also currently considered to play an important role in tumor development and/or progression. However, it is also true that 14-3-3σ may play a different role in tumor development and/or progression among different human organs. In urinary bladder carcinoma, for example, 14-3-3σ is highly up-regulated in pure squamous cell carcinoma, whereas it is down-regulated in invasive bladder urothelial cell carcinoma (17). In breast carcinoma, loss of 14-3-3σ expression becomes marked in the progression from atypical hyperplastic lesions to ductal carcinoma *in situ* (18, 19). Loss of 14-3-3σ protein was also reported in prostate carcinoma and its precursors (20–22). Therefore, loss or absence of 14-3-3σ expression is generally considered an early event during carcinogenesis in both breast and prostate carcinoma (18–22). Ostergaard et al. (23) reported that less differentiated bladder squamous cell carcinoma was associated with decreased expression of 14-3-3σ. We showed recently that loss of 14-3-3σ expression was correlated with advanced disease and/or high-grade tumor and significantly associated with poor prognosis in epithelial ovarian carcinoma (8). In our present study, the frequency of absence of 14-3-3σ immunoreactivity in clinically early disease and/or low-grade tumor was similar with that in advanced-stage and/or high-grade tumor, although decreased status of 14-3-3σ immunoreactivity was significantly associated with poor prognosis in endometrioid endometrial cancer. These results suggest that the loss of 14-3-3σ expression in endometrioid endometrial cancer may be associated with an aggressive biological characteristics, which play an important role in prognosis and/or recurrence, although it could be a relatively early event during their carcinogenesis.

In our present study, there were no significant differences of the findings between cases of early-stage and advanced-stage cancer, although advanced-stage cancer cases tended to be associated with worse prognosis than early-stage cases. These findings are considered to be due to the following reasons: the relatively small number of advanced-stage cancer cases, especially only 3 stage IV cases, and the fact that 15 of 22 (70%) cases of stage III examined were stage IIIa. Cases of stage IIIa, especially cytologic stage IIIa (positive peritoneal cytology alone), has been shown to be associated with much better prognosis than those of stage IIIc (24, 25). However, it awaits further investigation for clarifying the possible role of decreased status of 14-3-3σ immunoreactivity in advanced-stage endometrial carcinoma cases.

Endometrial carcinoma is the most common pelvic gynecologic carcinoma, and 80% to 90% of all cases are in clinically early stage (26). Five-year survival data of the patients revealed ~10% to 20% mortality in early-stage disease (26). There have been many controversies on the possible use of adjuvant therapy in patients of early-stage endometrial carcinoma (27–31). Results of large randomized trial (the Post-Operative Radiation Therapy in Endometrial Carcinoma) showed no

Table 6. Multivariate analyses of predictors of disease-free survival and overall survival for 78 patients with stage I and II endometrial cancer

Variable	Disease-free survival		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
14-3-3σ (positive vs negative)	0.241 (0.066-0.883)	0.0317	0.127 (0.022-0.752)	0.0229
Histologic grade (1-3)	1.469 (0.683-3.159)	0.3243	2.227 (0.749-6.619)	0.1496
p53 (negative vs positive)	1.182 (0.236-5.906)	0.8387	0.858 (0.149-4.931)	0.8635
ER (positive vs negative)	0.578 (0.161-2.077)	0.4011	2.272 (0.244-21.156)	0.4710
PR (positive vs negative)	0.115 (0.024-0.556)	0.0071	0.123 (0.012-1.280)	0.0795

Table 7. Multivariate analyses of predictors of disease-free survival and overall survival for 64 patients with completely surgically staged node-negative (FIGO stage I and II) endometrial cancer

Variable	Disease-free survival		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
14-3-3σ (positive vs negative)	0.189 (0.044-0.807)	0.0245	0.176 (0.028-1.111)	0.0646
Histologic grade (1-3)	1.708 (0.762-3.828)	0.1932	2.465 (0.774-7.853)	0.1270
p53 (negative vs positive)	1.007 (0.192-5.290)	0.9931	1.280 (0.172-9.554)	0.8095
ER (positive vs negative)	0.492 (0.121-2.005)	0.3223	2.326 (0.235-22.975)	0.4701
PR (positive vs negative)	0.060 (0.007-0.494)	0.0089	0.140 (0.013-1.509)	0.1050

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

significant differences between survivals of the patients with or without adjuvant therapy in stage I endometrial adenocarcinoma. However, analysis of their study was limited because complete surgical staging was not a requirement for entry of the patients into the protocol (29). Very recently, Keys et al. showed no significant differences between survival of the patients with or without adjuvant therapy in completely surgically staged node-negative intermediate risk (International Federation of Gynecology and Obstetrics stage Ib, Ic, II occult) endometrial adenocarcinoma (Gynecologic Oncology Group study). The estimated 4-year survival was 86% in the group with no additional therapy arm and 92% for whole radiation therapy arm, with no statistical difference between these two groups (31). Therefore, identification of additional prognostic markers could provide the information to avoid unnecessary adjuvant therapy and to plan effective systemic treatment. Several studies have attempted to identify prognostic factors of the patients with early-stage endometrial cancer. However, none of them have provided satisfactory results. Fiumicino et al. (32) showed that microsatellite instability was an independent indicator of recurrence in early-stage endometrial adenocarcinoma, but Maxwell et al. (33) reported that microsatellite instability is rather a favorable prognostic factor. In addition, MacDonald et al. (34) and Basil et al. (35) both independently reported the lack of any correlation between microsatellite instability and clinical outcome in endometrial cancer. Recently, Powell et al. (36) examined the prognostic significance of rDNA methylation and showed that tumor rDNA level turned out to be significant prognostic factor for both disease-free survival and overall survival in early-stage endometrial cancer. Powell et al. therefore identified the prognostic indicator of early-stage endometrial carcinoma. However, their methods require sufficient quantity of frozen specimens, and patients with small tumors often did not have adequate tumor tissue for examination in clinical early stage of endometrial carcinoma, which may limit the clinical value of this interesting prognostic marker.

In our study, we studied archival or surgical pathology materials and analyzed a remarkable number of the cases with follow-up data to show possible correlation between absence of 14-3-3σ and adverse clinical outcome using immunohistochemistry, which is a simple and useful method in surgical pathology specimens. In early-stage endometrial cancer, 14-3-3σ immunoreactivity was not detected in 5 of 10 (50.0%) patients who had recurrence during clinical follow-up, whereas absence of its immunoreactivity was detected only in 13 of 68 (19.1%) disease-free patients during the same period. 14-3-3σ Immunoreactivity was not detected in 4 of 5 (80.0%) patients who died, whereas loss of its immunoreactivity was detected only in 14 of 73 (19.2%) patients who had lived for the same period of clinical follow-up. Absence of 14-3-3σ expression was independently statistically significant as risk factor in disease-free survival and overall survival in patients in the early stage of the disease. Additionally, even in the subgroup with completely surgically staged node-negative (International Federation of Gynecology and Obstetrics surgical stage I and II) endometrial adenocarcinoma, absence of 14-3-3σ immunoreactivity still turned out to be independently statistically significant as risk factor in disease-free survival although not significant in overall survival of the patients.

These findings all indicate that absence of 14-3-3σ protein determined by immunohistochemistry could be a very important tool to identify the patients at high risk of recurrence and/or death, who are otherwise not detected by current clinical and pathologic evaluation, especially in the early stage of endometrial carcinoma. In addition, results of 14-3-3σ immunohistochemistry in early stage of endometrial carcinoma could contribute to planning postoperative follow-up and adjuvant therapy.

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