Original Article

Alcohol Consumption and Lung Cancer Mortality in Japanese Men: Results from Japan Collaborative Cohort (JACC) Study

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BACKGROUND: The relationship between alcohol consumption and increased risk of lung cancer is controversial. This study was set up to investigate the association between alcohol consumption and death from lung cancer in a large Japanese cohort.
METHODS: The subjects comprised 28,536 males, aged 40–79 years, living throughout Japan. During 268,464 person-years of follow-up, 377 lung cancer deaths were recorded. The hazard ratio (HR) of alcohol consumption for lung cancer mortality was calculated using the Cox proportional hazards model after adjustment for age, smoking and family history of lung cancer.
RESULTS: There was no association between increased mortality from lung cancer and alcohol consumption among current drinkers. Compared with subjects who had never drunk alcohol, the HRs (95% confidence interval [CI]) of death from lung cancer for light (consuming <25.0 g ethanol per day), moderate (25.0–49.9 g per day) and heavy (≥50 g per day) drinkers were 0.81 (95% CI=0.61–1.07), 0.82 (0.61–1.11) and 0.97 (0.66–1.43), respectively. Further adjustment for fruit and vegetable intake did not change the results, and there was no change in HR materially after excluding those patients who died during the first 5 years of follow-up.
CONCLUSIONS: These findings indicate that alcohol consumption was not associated with increased lung cancer mortality in this population of Japanese men.
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Although alcohol is a risk factor for several sites of cancer,[1] its relationship with lung cancer is still controversial. Recently, Bandera reviewed epidemiologic studies on alcohol consumption and lung cancer that presented smoking-adjusted risk estimates, and concluded that there might be an increased risk of lung cancer associated with drinking alcohol.[2] On the other hand, a meta-analysis of the relationship between alcohol consumption and lung cancer risk by Korte presented a smoking-adjusted excess risk of lung cancer only in the very high alcohol consumption category (ethanol consumption was ≥2,000 g per month). [3] These authors concluded that the results should be interpreted with caution due to the possibility that there was residual confounding in this group of subjects, and few studies have presented data on subjects who consumed 2,000+ g ethanol per month. Many of the previous studies on alcohol and lung cancer had problems with the methodology, including insufficient control for potential confounding factors such as smoking and dietary variables, and inappropriate grouping of subjects who had never drunk alcohol and ex-drinkers into a single referent category. Furthermore, most previous studies were conducted in Western countries, where the type of alcohol consumed and other drinking habits are quite different to those in Japan. In addition, individuals with the atypical allele of the aldehyde dehydrogenase 2 (ALDH2) gene, which results in inactive ALDH2 activity, bringing about a high blood concentration of acetaldehyde, the initial metabolite of alcohol, are prevalent in the Japanese population.[4] Acetaldehyde has been shown to be carcinogenic in experimental animals,[5] and therefore Japanese people may have a different susceptibility to lung cancer than Westerners.

This study was undertaken to examine the association between alcohol consumption and lung cancer mortality in a large Japanese cohort, with control for confounding factors and the separation of subjects who had never consumed alcohol from ex-drinkers.

METHODS

Study Cohort

The methodology of the baseline survey and follow-up in the JACC study, Japan Collaborative Cohort Study for Evaluation of Cancer Risk Sponsored by Monbusho (the Ministry of Education, Science, Sports and Culture of Japan) has been described in detail elsewhere.[6] In brief, the established cohort members comprised 110,792 inhabitants (aged 40–79 years) of 45 study areas throughout Japan (46,465 men and 64,327 women). In most areas, individuals were selected from participants in municipal health check-ups, and in other areas from whole populations or voluntary groups. Between 1988 and 1990, the individuals completed a self-administered questionnaire containing questions on medical history and lifestyle factors such as smoking, alcohol consumption, diet, physical activity and reproductive history. This study was approved by the Ethical Board of Nagoya University School of Medicine.

Exposure Assessment

With regard to alcohol consumption, subjects were asked whether they were current or ex-drinkers, or had never drunk alcohol. Current drinkers were further asked about their drinking frequency, the type of beverage usually consumed, and the total amount drunk on a single occasion. The frequency of alcohol drinking was divided into four categories: less than once a week, once or twice a week, three or four times a week, and almost every day. For the beverage types usually consumed, subjects were asked to choose from five types (*sake*=rice wine, *shochu*=white spirits, beer, whisky and wine). However, the drinking frequency and amount consumed on a single occasion of each alcoholic beverage were not asked. The total amount consumed on a single occasion was converted by respondents into the corresponding equivalent of *sake* expressed as the traditional unit of *go* (1 *go* of *sake* = 180 mL, containing 22.8 g ethanol). In practice, the questionnaire presented the amount of alcoholic beverage which contained the same quantity of ethanol as 1 *go* of *sake*, and the subjects referred to this when recording the total amount consumed on a single occasion.

Average daily consumption of alcohol for each subject was calculated by multiplying the total amount consumed on a single occasion by the drinking frequency. Average daily consumption was then converted to the amount of ethanol in grams by multiplying the figure by 22.8.

The information on smoking habits was obtained by asking the subjects about their smoking status (current or former smoker, or never smoked), the age when they started smoking, and the average number of cigarettes smoked per day. Former smokers were also asked their age at cessation of smoking and/or length of time since they had stopped smoking. With regard to diet, the frequency of consumption of 33 common food items was asked (almost never, 1–2 times/month, 1–2 times/week, 3–4 times/week, and almost every day), in addition to the number of bowls of rice and miso-soup consumed per day.

Follow-up

The vital and residential status of the subjects was followed up to December 31, 1999 by collation with residential registration in the various municipalities. Causes of death were ascertained from death certificates, which were coded according to the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10).

Of the 46,465 original male cohort members, 1,557 subjects in two study areas were excluded because the questionnaire used in these districts did not include an item on drinking frequency. A further 4,412 subjects in four areas were also excluded because the questionnaire used there did not include an item on the amount of alcoholic beverage consumed on a single occasion, and a further 6,009 subjects in six areas were excluded because questions about past history of cancer and/or the frequency of consumption of green leafy vegetables, oranges, or fruit other than oranges were not included in the questionnaire used there.

Finally, 27 subjects who reported a history of lung cancer in the questionnaire and 5,924 subjects with incomplete information about their drinking or smoking habits were also excluded. Consequently, the number of subjects evaluated in this study was 28,536. No analysis was conducted of female cohort members because their prevalence of daily drinking was low (4.7%).

The number of person-years of follow-up was calculated for each subject from the month of entry into the study until the month of death from any cause, the month of emigration outside the study area, or December 1999, whichever occurred first. A total of 268,464 person-years were accumulated and 377 lung cancer deaths (ICD-10=C34) were documented. A total of 883 subjects (3.1%) moving out of the study area during the follow-up period were identified from the residential registration; these individuals have been treated as censored cases.

Statistical Analysis

Hazard ratios (HR) of lung cancer mortality in ex- and current drinkers compared with subjects who had never drunk alcohol was calculated, using the Cox proportional hazards regression model, employing the SAS® PHREG procedure.[7] HRs were adjusted for age, smoking and family history of lung cancer. Current drinkers were subdivided into three categories according to their daily ethanol consumption calculated from the baseline data, and the HR for each category was also assessed. In order to adjust for smoking status, current smokers were divided into six categories using the Brinkman Index (the number of cigarettes smoked per day multiplied by the number of years of smoking) as 1–399, 400–799, 800–1,199, 1,200–1,599, 1,600–1,999, and 2,000+, and ex-smokers into five categories according to how long they had stopped smoking: 0–4, 5–9, 10–14, 15–19 and 20+ years; these indices of smoking history were included in the model as dummy variables. The HR was also calculated with further adjustment for the frequency of consumption of green-leafy vegetables (1–2 times/week or less, 3–4 times/week, and almost every day), oranges and fruit other than oranges (1–2 times/month or less, 1–2 times/week, and 3–4 times/week or more), because a higher frequency of intake of green-leafy vegetables, oranges and fruit other than oranges was associated with a significant reduction in lung cancer mortality among males in this cohort[8]. Additionally, in order to examine the effect of including cases already diagnosed as lung cancer before the baseline survey, the HR was calculated after excluding cases that died in the first 5 years of follow-up. To assess effect modification by smoking, stratified analysis was conducted according to smoking status.

RESULTS

Table 1 shows the baseline characteristics of subjects according to the level of alcohol consumption. The relative proportions of current drinkers, ex-drinkers and subjects who had never drunk alcohol were 73.1%, 6.8% and 20.0%, respectively. As alcohol consumption increased, the prevalence of individuals who had never smoked and those who ate vegetables and fruit every day tended to decrease. Among ex-drinkers, the prevalence of a history of stroke, myocardial infarction, liver disease and diabetes was higher than in the other groups.

Table 1. Baseline chacteristics of subjects by level of alcohol consumption.

				Current drinkers (ethanol intake)			
	All Subjects	Never drinkers	Ever drinkers	≦24.9 g/day	25.0-49.9 g/day	≧50.0 g/day	Ex-drinkers
Number of subjects	28,536	5,716	22,820	10,244	7,511	3,112	1,953
Mean age (year)	57.3	59.2	56.8	57.2	56.1	53.8	62.7
Smoking (%)							
Current	52.5	47.7	53.7	47.4	59.1	68.2	43.1
Past	26.4	22.6	27.3	28.5	26.0	19.1	39.6
Never	21.1	29.7	19.0	24.1	15.0	12.8	17.3
Daily dietary consumption (%)							
Green leafy vegetables	29.5	30.0	29.4	29.2	29.6	26.9	33.5
Oranges	26.6	33.4	24.9	27.2	23.1	18.1	31.3
Fruits other than oranges	27.2	32.5	26.0	28.1	24.1	19.6	31.9
Family history of lung cancer (%)	2.0	2.1	2.0	2.0	2.1	2.2	1.5
Past history (%)							
Stroke	2.0	2.0	2.0	1.7	1.3	0.8	7.9
Myocardial infarction	2.8	3.2	2.7	2.6	2.2	2.3	5.9
Liver disease	7.8	6.4	8.2	7.3	7.1	8.9	16.3
Diabetes	6.4	6.1	6.5	6.3	5.4	6.0	12.5

Table 2 presents the HR of lung cancer for drinkers compared with subjects who had never drunk alcohol. There was no association between current alcohol consumption and risk of lung cancer. After adjustment for age, smoking and family history of lung cancer, the HRs (95% confidence interval [CI]) were 0.81 (0.61–1.07) for those drinking <25.0 g ethanol per day, 0.82 (0.61–1.11) for those drinking 25.0–49.9 g per day, and 0.97 (0.66–1.43) for those drinking 50.0 g or more per day. The risk of lung cancer was also calculated for those drinking 66.7+ g per day (2,000+ g per month), and there was no clear risk elevation (HR=1.09, 95% CI=0.73–1.62). In contrast, the HR of lung cancer increased for ex-drinkers (HR=1.39, 95% CI=0.98–1.96). After further adjustment for the frequency of consumption of green-leafy vegetables, oranges and fruit other than oranges, the result did not change materially. The analysis was repeated after excluding cases that died in the first 5 years of follow-up; the HR for current drinkers was slightly elevated, but alcohol consumption was still not related to increased lung cancer mortality.

When stratified analysis according to smoking habits was performed, there was no significantly increased risk of lung cancer associated with current alcohol consumption, regardless of smoking status (Table 3). However, due to the small number of subjects in most categories, the statistical power of this analysis was limited.

DISCUSSION

No positive association between alcohol consumption and death from lung cancer was found in this large cohort of Japanese men, and the results were not modified by smoking status.

Alcohol is an established risk factor for several sites of cancer, and several potential biological mechanisms by which alcohol could increase cancer risk have been proposed, such as the carcinogenic effect of acetaldehyde and a reduction in the detoxification capacity of liver enzymes that metabolize carcinogens.[9] However, results from previous prospective studies[10-18] and case-control studies[19-27] investigating the relationship between alcohol consumption and risk of lung cancer with adjustment for smoking status were not consistent. Several prospective[10-12] and case-control[19-22] studies showed an increased risk of lung cancer with alcohol consumption, and one case-control study presented only the risk stratified by smoking status and indicated an increased risk in heavy smokers.[23] However, other studies failed to show any elevated risk of lung cancer with alcohol consumption.[13-18, 24-27] This inconsistency in results may be due to differences in the methodology used in the studies. Confounding by dietary variables and definition of reference category of alcohol consumption are important issues, in addition to residual confounding by smoking and misclassification of alcohol consumption. Certain dietary fac-

Table 2. Hazard ratio (HR)* of lung cancer by level of alcohol consumption.

	Person-years	No. of deaths	HR1	(95% CI)	HR2	(95% CI)	HR3	(95% CI)
Never drinkers	52,956	91	1.00	(reference)	1.00	(reference)	1.00	(reference)
Ever drinkers	215,508	286	0.90	(0.71-1.14)	0.96	(0.73-1.26)	1.03	(0.73-1.46)
Current drinkers (ethanol intake)								
≦24.9 g/day	97,334	113	0.81	(0.61-1.07)	0.81	(0.59-1.11)	0.90	(0.60-1.34)
25.0-49.9 g/day	71,863	85	0.82	(0.61-1.11)	0.90	(0.64-1.26)	0.99	(0.65-1.50)
≧50.0 g/day	29,679	38	0.97	(0.66-1.43)	0.98	(0.64-1.50)	1.08	(0.63-1.83)
Ex-drinkers	16,633	50	1.39	(0.98-1.96)	1.68	(1.16-2.45)	1.69	(1.03-2.76)
P for trend 1†			0.61		0.92		0.74	
P for trend 2‡			0.38		0.32		0.58	

* : HR1 is adjusted for age, smoking and family history of lung cancer. HR2 is further adjusted for intake of green-leafy vegetables, oranges and fruits other than oranges. HR3 means the relative risk with adjustment for the same covariates as those used for calculation of HR2 after cases who died in first five years of follow-up were excluded.

† : P for trend among current drinkers. The test for linear trends includes never drinkers.

‡ : P for trend among current drinkers. The test for linear trends excudes never drinkers.

CI: confidence interval

Table 3. Hazard raito (HR)* of lung cancer by level of alcohol consumption according to smoking status.

		Never drinkers	Ever drinkers	Current drinkers (ethanol intake)			Ex-drinkers	P for trend 1†	P for trend 2‡
				≤24.9 g/day	25.0-49.9 g/day	≥50.0 g/day			
Never smokers	Person-years	13,368	36,575	21,297	9,607	3,331	2,339		
	No. of deaths	5	13	7	1	1	4		
	HR	1.00	1.22 (0.43-3.45)	1.10 (0.35-3.51)	0.37 (0.04-3.18)	1.15 (0.13-9.98)	4.20 (1.12-15.72)	0.61	0.54
Ex-smokers	Person-years	10,035	50,374	24,004	16,089	4,993	5,288		
	No. of deaths	19	61	27	15	2	17		
	HR	1.00	0.74 (0.44-1.25)	0.64 (0.36-1.16)	0.67 (0.34-1.33)	0.34 (0.08-1.47)	1.37 (0.71-2.64)	0.13	0.53
Current smokers (Cigarretes/day)									
≤20	Person-years	15,227	72,388	30,626	26,672	10,579	4,510		
	No. of deaths	33	110	44	35	16	15		
	HR	1.00	0.86 (0.58-1.27)	0.76 (0.48-1.20)	0.78 (0.48-1.27)	1.09 (0.59-2.01)	1.32 (0.72-2.43)	0.99	0.20
>20	Person-years	6,509	28,142	9,425	10,140	7,084	1,493		
	No. of deaths	12	57	11	23	13	10		
	HR	1.00	1.31 (0.69-2.47)	0.74 (0.33-1.70)	1.49 (0.73-3.03)	1.31 (0.58-2.93)	2.64 (1.13-6.14)	0.20	0.15

* : Adjusted for age, family history of lung cancer, intake of green-leafy vegetables, oranges and fruits other than oranges.

† : P for trend among current drinkers. The test for linear trends includes never drinkers.

‡ : P for trend among current drinkers. The test for linear trends excudes never drinkers.

95% confidence intervals in parentheses.

tors, especially fruit and vegetables, represent one possible factor that has consistently been shown to protect against lung cancer,[1] and several investigations showed that the intake of fruit, vegetable, or related antioxidants was different according to drinking habits.[28-30] However, only five studies have examined the risk adjusted for consumption of these foods or related antioxidants.[15,16,20,23,26] In addition, ex-drinkers should be separated from those who have never drunk alcohol, and only the latter should be considered as the reference group, because ex-drinkers may quit drinking due to the effects of pre-clinical symptoms of lung cancer and thus show a higher incidence of lung cancer or mortality than those who have never drunk. Therefore, the use of non-drinkers, including both ex-drinkers and those who have never drunk alcohol, as the reference group may underestimate the effect of alcohol. However, only four of the previous studies separated ex-drinkers from subjects who had never drunk as the reference group. [14,20,22,25]

The present study had some merits compared with previous investigations that examined the relationship between alcohol and risk of lung cancer. This was a prospective study, so that various potential biases inherent in case-control studies were avoided. Adjustments were made for fruit and vegetable intake as potential confounding factors. Ex-drinkers were separated from subjects who had never drunk, and it was therefore possible to estimate the risk for drinkers compared with life-long abstainers. Adjustment for smoking history was performed not only for current smokers according to the amount and duration of smoking, but also for ex-smokers by recording the length of time they had stopped smoking.

The increase in lung cancer mortality in ex-drinkers found in this study may be due to inclusion of subjects who stopped drinking due to pre-clinical symptoms of lung cancer. However, after excluding cases who died in the first 5 years of follow-up, the overall results did not change. Ex-drinkers had different characteristics, such as a history of disease, from subjects who had never drunk, however, the HR for ex-drinkers did not change materially even after adjustment for a history of disease such as stroke, myocardial infarction, liver disease and diabetes (data not shown). Residual confounding by unknown factors could have contributed to the results. As no positive association between alcohol consumption and lung cancer mortality was observed in current drinkers, it is unlikely that a history of heavy drinking was the cause of an increase in lung cancer mortality in ex-drinkers.

The effect of alcohol consumption on lung cancer may differ according to the type of beverage consumed. Among previous studies investigating the effects of several different types of beverage, some reported an increased risk of lung cancer associated with the consumption of beer, [20,23] hard liquor,[11,26] or both.[12] The present study did not investigate the association between any particular type of alcoholic beverage and lung cancer mortality because the questionnaire asked subjects about all types of alcoholic beverage consumed by selection from five types, but did not allow quantification of the amount of each beverage consumed. In this cohort, 71.5% of current drinkers consumed *sake* (rice wine), and 52.1% consumed beer. To our knowledge, the effect of *sake* on lung cancer has not been investigated in previous studies, so that it is not clear whether the fact that approximately two-thirds of current drinkers in this cohort usually consumed *sake* affected the results of this study. There were relatively few drinkers who consumed beverages with a high ethanol concentration, such as whisky and *shochu*. The proportion of whisky drinkers and *shochu* drinkers was 17.8% and 19.0%, respectively. A recent prospective study suggested that wine has a protective effect against lung cancer,[12] although the proportion of wine drinkers in the present cohort was low (2.7%), and so the effect of wine consumption in this study would have been small.

Death from lung cancer was used as the end point, and this may have affected the results of this study for the following reasons: first, some of the subjects may have suffered from lung cancer at the baseline survey and changed their alcohol consumption due to the disease. However, the HR for current drinkers compared with those who had never drunk did not change materially after excluding cases who died in the first 5 years of follow-up. Second, lung cancer cases were only censored at the time of death, so that the impact of alcohol on the prognosis may have been reflected on the result. However, since the survival rate of patients with lung cancer was low,[31] the association between alcohol consumption and risk of lung cancer may not be distorted substantially.

Alcohol consumption at baseline was used as the marker of alcohol exposure, and past alcohol consumption was not considered. Therefore, evaluation of exposure at a single time point may have diluted the true effect of alcohol on lung cancer. However, duration of alcohol consumption, another important indicator of alcohol exposure, was not associated with lung cancer mortality (data not shown).

In conclusion, this JACC study found no association between alcohol intake and death from lung cancer. The results do not support the hypothesis that there is a relationship between alcohol intake and increased risk of lung cancer.

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Peroxisome Proliferator-Activated Receptor γ and Growth Inhibition by Its Ligands in Uterine Endometrial Carcinoma

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Abstract

Purpose: In this study, we evaluated the correlation between endometrial carcinoma and peroxisome proliferator-activated receptor γ (PPARγ) expression and assessed whether PPARγ ligands influence carcinoma growth.

Experimental Design: We examined the presence and cellular distribution of PPARγ protein in 42 normal endometria, 32 endometria with hyperplasia, and 103 endometria with endometrial carcinoma by immunohistochemistry. We then compared PPARγ mRNA expression in endometrial carcinoma with that in normal endometria using real-time reverse transcription-PCR. We subsequently confirmed expression of PPARγ mRNA by real-time reverse transcription-PCR and PPARγ protein by immunoblotting in endometrial carcinoma cell lines (Ishikawa, Sawano, and RL95-2 cells). We further examined the effects of PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a naturally occurring PPARγ ligand, to these endometrial carcinoma cell lines. We also examined the status of apoptosis and p21 mRNA expression of these endometrial carcinoma cell lines following addition of 15d-PGJ_2.

Results: PPARγ immunoreactivity was detected in 11 of 23 (48%) of proliferative-phase endometrium, 14 of 19 (74%) of secretory-phase endometrium, 27 of 32 (84%) of endometrial hyperplasia, and 67 of 103 (65%) of carcinoma cases. PPARγ immunoreactivity was significantly lower in endometrial carcinoma than in secretory-phase endometrium ($P = 0.012$) and endometrial hyperplasia ($P = 0.006$). There was a significant positive association between the status of PPARγ and p21 expression in endometrial carcinoma ($P < 0.0001$). There was a significant negative association between the body mass index and PPARγ labeling index of carcinoma tissue in the patients with endometrial carcinoma ($P < 0.0001$). PPARγ mRNA was expressed abundantly in normal endometria but not in endometrial carcinoma. We showed that PPARγ agonist 15d-PGJ_2 inhibited cell proliferation and induced p21 mRNA of endometrial carcinoma cell lines.

Conclusion: We showed the expression of PPARγ in human endometrial carcinoma and the effects of PPARγ ligand in endometrial carcinoma cells. These findings suggest that a PPARγ ligand, 15d-PGJ_2, has antiproliferative activity against endometrial carcinoma.

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Peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor superfamily of transcription factors. PPARs function as transactivation factors following heterodimerization with retinoid X receptors (RXR) and bind to its specific response elements, termed peroxisome proliferator-responsive elements, of various target genes (1). PPARs have a subfamily of three different isoforms: PPARα, PPARβ/δ, and PPARγ. All isoforms play important roles in the regulation of several metabolic pathways, including lipid biosynthesis and glucose metabolism. There are distinctive differences in the distribution of these receptor subtypes in humans. PPARα has been detected mainly in the myocardium, kidney, and liver; PPARβ/δ is expressed in most tissues; and PPARγ has been found primary in adipocytes and immune cells (2, 3).

PPARγ plays important roles in the regulation of lipid homeostasis, adipogenesis, insulin resistance, and development of various organs (4–6). Naturally occurring PPARγ ligand, a

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), activates PPARγ at micromolar concentrations in human *in vivo* (7–9). Synthetic PPARγ ligands are known as thiazolidinediones, including troglitazone, rosiglitazone, and pioglitazone. These synthetic ligands have been used for the treatment of insulin resistance in type II diabetes mellitus. In addition, thiazolidinediones have been proposed in differentiation-mediated therapy of various human carcinomas associated with high levels of PPARγ (10).

Various *in vitro* studies showed that PPARγ ligands have a potent antiproliferative activity for a wide variety of neoplastic cells (11). PPARγ agonist was reported to inhibit the proliferation of carcinoma cells, and phase II clinical trials using PPARγ ligands have recently been done as a novel therapy for advanced patients with breast carcinoma, histologically confirmed prostate carcinoma, liposarcoma, and metastatic colon carcinoma (12). The outcomes of these trials are, however, still controversial. Mueller et al. reported that one advanced prostate carcinoma patient had a dramatic decrease in serum prostate-specific antigen as a result of troglitazone treatment (13), but Burstein et al. reported troglitazone had little apparent clinical value among patients with treatment-refractory metastatic breast carcinoma (14). In addition, Debrock et al. reported no significant changes in the histologic appearance of the liposarcomas following treatment with rosiglitazone (15).

The expression and effectiveness of PPARγ has been extensively studied in breast, prostate, and colon carcinoma, but little is known about PPARγ in uterine endometrial carcinoma. In addition, obesity, excess estrogen, type II diabetes, and hypertension are some of the important risk factors of endometrial carcinoma (16–19), but the effects of PPARγ agonists to endometrial carcinoma are largely unknown. Therefore, in this study, we first examined the expression of PPARγ in endometrial carcinoma, correlated the findings, including the status of progesterone receptor, estrogen receptor α (ERα), ERβ, Ki-67, and p21 and the body mass index (BMI) of the patients, and examined the growth inhibition of the carcinomas by PPARγ agonists *in vitro* to clarify the possible biological and clinical roles of PPARγ in human endometrial malignancy.

Materials and Methods

Patients and tissues. All patients were informed of the purpose of this study, and informed consent was obtained before participation. The research protocol was approved by the Ethics Committee of Tohoku University Graduate School of Medicine. Uterine endometrial carcinoma tissues (49 well differentiated, 32 moderately differentiated, and 22 poorly differentiated; 66 stage I, 12 stage II, 22 stage III, and 3 stage IV) were obtained from 103 patients who underwent surgery at the Department of Obstetrics and Gynecology, Tohoku University Hospital (Sendai, Japan) from 1993 to 2004. All the subjects were Japanese. All endometrial carcinoma specimens were obtained after 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. Information regarding age, BMI, stage, grade, primary surgery, postoperative therapy and recurrence, and complications were all retrieved by a review of the chart. BMI was calculated by dividing weight in kilograms by the height in meters squared. We defined obesity in these patients as a BMI ≥ 25 (20). A standard primary treatment for endometrial carcinoma at Tohoku University Hospital from 1993 to 2004 was 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 the 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. None of the patients used oral contraceptives. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO and staged according to the International Federation of Gynecology and Obstetrics system (21, 22). About 60% of 103 patients received pelvic radiation therapy (50 Gy) or three to six courses of chemotherapy consisting of the cisplatin-based combination regimen CAP (cisplatin 60-70 mg/m^2, doxorubicin 40 mg/m^2, and cyclophosphamide 500 mg/body weight) 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 of the archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital.

Normal endometrial tissues were obtained from unaffected endometrium of normally menstruating females who underwent hysterectomy for cervical carcinoma *in situ*. Biopsy specimens from 23 cases were in the proliferative phase, and 19 cases were in the secretory phase. Fourteen had simple hyperplasia, 9 had complex hyperplasia with atypia, and 8 had complex hyperplasia without atypia. All specimens were routinely processed (i.e., 10% formalin fixed for 24-48 hours), paraffin embedded, and thin sectioned (3 μm).

Antibodies. Monoclonal antibodies for PPARγ were purchased from Perseus Proteomics Co. Ltd. (Tokyo, Japan). This antibody recognizes both human PPARγ1 and PPARγ2. Monoclonal antibody for β-actin was purchased from Sigma-Aldrich Co. (St. Louis, MO), monoclonal antibody for progesterone receptor was purchased from Chemicon International, Inc. (Temecula, CA), monoclonal antibodies for ERα and Ki-67 were purchased from Dako Cytomation Co. Ltd. (Glostrup, Denmark), monoclonal antibody was purchased for ERβ from Gene Tex, Inc. (San Antonio, TX), and monoclonal antibody was purchased for p21 from Pharmingen (San Diego, CA).

Immunohistochemistry. A Histofine kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used in this study. Antigen retrieval for PPARγ immunostaining was done by heating the slides in an autoclave at 120°C for 5 minutes (p21; 15 minutes) in citric acid buffer [2 mmol/L citric acid, 9 mmol/L trisodium citrate dehydrate (pH 6.0)]. Dilution of antibodies used in this study was as follows: 1:300 for PPARγ, 1:40 for progesterone receptor, 1:50 for ERα, 1:1,500 for ERβ, 1:50 for Ki-67, and 1:250 for p21. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl (pH 7.6), 0.006% H_2O_2] and counterstained with hematoxylin. As a positive control for immunohistochemistry of PPARγ, normal mammary glands and omentum tissues were employed in this study. Immunohistochemical staining was evaluated by two independent observers (K.O. and T.S.) without knowledge of clinical outcomes. PPARγ immunoreactivity was detected in the nucleus, and the immunoreactivity was quantitated as the labeling index (LI). Five hundred carcinoma cells in each field were selected and the LI was determined as the percentage of positive cells per 500 carcinoma cells. For each tissue section, at least three fields were photographed under light microscopy at ×200. Cases with a PPARγ LI of >10% were considered PPARγ-positive endometrial carcinoma in this study. Interobserver and intraobserver differences were <5% in our present study.

Table 1. Complications in endometrial carcinoma patients

	Endometrial carcinoma ($n = 101$), n (%)
Obesity (BMI ≥ 25)	
+	45 (44.6)
–	56 (55.4)
Hypertension	
+	56 (55.4)
–	45 (44.6)
Diabetes mellitus (type II)	
+	24 (23.8)
–	77 (76.2)
Hyperlipidemia	
+	14 (13.9)
–	87 (86.1)
Breast carcinoma	
+	5 (5.0)
–	96 (95.0)

Cell lines and media. Uterine endometrial carcinoma cell lines, Ishikawa 3-H-12, Sawano, and RL95-2, were cultured in phenol red–free RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 1% penicillin/streptomycin. Ishikawa cells were originally established from well-differentiated human endometrial carcinoma (23). Sawano cells were naturally raised cisplatin-resistant cells (24). RL95-2 cells are ER-positive endometrial carcinoma cell line.

Real-time PCR. Total RNA from tissues, Ishikawa, Sawano, and RL95-2, were extracted using TRIzol reagent (Invitrogen Life Technologies, Inc., Gaithersburg, MD), and reverse transcription kit [SuperScript II Preamplification System (Gibco-BRL Invitrogen Co., Carlsbad, CA)] was used in the synthesis of cDNA. The LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany) was used to semiquantify the mRNA expression levels using real-time PCR. The primer sequences used in this study were as follows: PPARγ 5′-ATGCTGGCCTCCTTGATGAATAA-3′ and 5′-AGGGCTTGTAGCAGGTTGTCTTG-3′ (266 bp), RXRα 5′-TTCGCTATGCTCTCAG-3′ and 5′-ATAAGGAAGGTGTCAATGGG-3′ (113 bp), RXRβ 5′-GAAGCTCAGGCAAACACTAC-3′ and 5′-TCTTTGTTGTCCC-3′ (111 bp), RXRγ 5′-GCAGTTCAGAGGACATCAAGCC-3′ and 5′-GCCTCACTCTCAGCTCGCTCTC-3′ (352 bp), ribosomal protein L 13a 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ and 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (125 bp), and p21 5′-GGAAGACCATGTGGACCTGT-3′ and 5′-GGATTAGGGCTTCCTCTTGG-3′ (178 bp). Settings for the PCR thermal profile were initial denaturation at 95°C for 1 minute followed by 40 amplification cycles of 95°C for 1 second, annealing at 58°C (RXRα), 62°C (PPARγ), 60°C (RXRβ and p21), 68°C (ribosomal protein L 13a and RXRγ) for 15 seconds, and extension at 72°C for 15 seconds. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. Fat tissue cDNA was used as a positive control. The mRNA levels were summarized as the ratio of ribosomal protein L 13a and subsequently evaluated as a ratio (%) compared with that of controls. The PCR products were separated electrophoretically on a 2% agarose gel and stained with ethidium bromide.

Immunoblotting. Cells were grown to 70% confluence in 10-cm plates, and after removal of culture medium with PBS, nuclear protein was extracted by conventional method. The protein concentration was measured by a Model 680 microplate reader (Bio-Rad, Hercules, CA) using Bradford reagent (Bio-Rad). In all, 60 μg nuclear protein of each sample was mixed with an equal volume of 2× concentrated SDS-PAGE sample buffer, boiled, and then transferred to nitrocellulose membrane (Hybond polyvinylidene difluoride, Bio-Rad). The membranes were incubated in blocking solution (PBS containing 3% nonfat milk and 0.1% Tween 20) and then incubated in 1:500 dilution of PPARγ antibody in PBS (containing 0.1% Tween 20) overnight at 4°C. After incubation with horseradish peroxidase–labeled anti-mouse IgM (Santa Cruz Biotechnology, Santa Cruz, CA), the antigen-antibody complex was visualized with enhanced chemiluminescence system (Amersham, Freiburg, Germany). β-Actin (Sigma-Aldrich) was used as internal positive control, and the dilution was 1:1,000.

Cell proliferation assay and apoptosis analysis. Cell proliferation was assessed by the Cell Counting Kit-8 (Wako Pure Chemical Industries, Osaka, Japan). We also examined the status of apoptosis in Ishikawa, Sawano, and RL95-2 cells using an apoptosis screening kit (Wako), which employed a modified terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method (25). Ishikawa, Sawano, and RL95-2 cells were plated at 5×10^3 per well in 96-well plates and cultured with phenol red–free RPMI 1640 containing 10% dextran-coated, charcoal-stripped fetal bovine serum at 37°C for 2 days in a CO_2 incubator. 15d-PGJ_2 was purchased from Biomol Research Laboratories, Inc. (Butler Pike, PA), dissolved in ethanol, and added to the wells (day 0), and the assay was done on days 0, 1, 3, and 5. Control cells were treated with vehicle only. For cell proliferation assay, WST-1 solution was added to each well of the plates and incubated at 37°C for 2 hours. The absorbance at 450 nm was measured in a microplate reader SpectraMax 190 (Molecular Devices Corp., Osaka, Japan). The cell number and apoptosis index were calculated according to the following equation: (cell absorbance value after test materials treated / vehicle control cell absorbance value) and were subsequently evaluated as a ratio (%) compared with that of controls.

Statistical analysis. The statistical analysis was done using StatView 5.0 (SAS Institute, Inc.) software. An association between immunoreactivity for PPARγ and clinicopathologic factors, results of cell proliferation assay, and results of real-time PCR were evaluated using Bonferroni/Dunn test, Mann-Whitney U test, or Wilcoxon test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method and the statistical significance was calculated using the log-rank test. Results were considered significant when the $P < 0.05$.

Results

Complications of endometrial carcinoma patients. The median BMI for carcinoma patients was 24.6 (range, 17.3-39.6). BMI ≥ 25 was 45 (44.6%) of endometrial carcinoma patients examined in our present study (Table 1). The number of

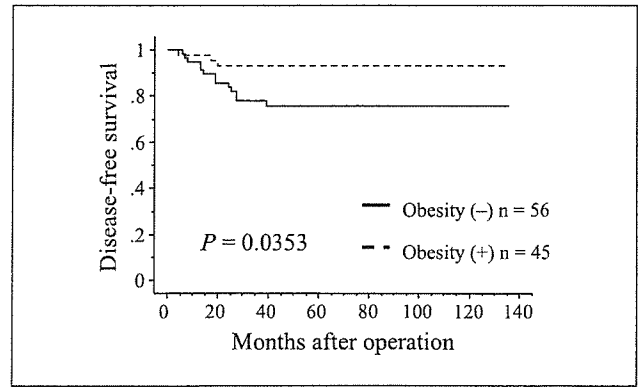

Fig. 1. Disease-free survival in endometrial carcinoma patients. Patients with obesity had longer disease-free survival.

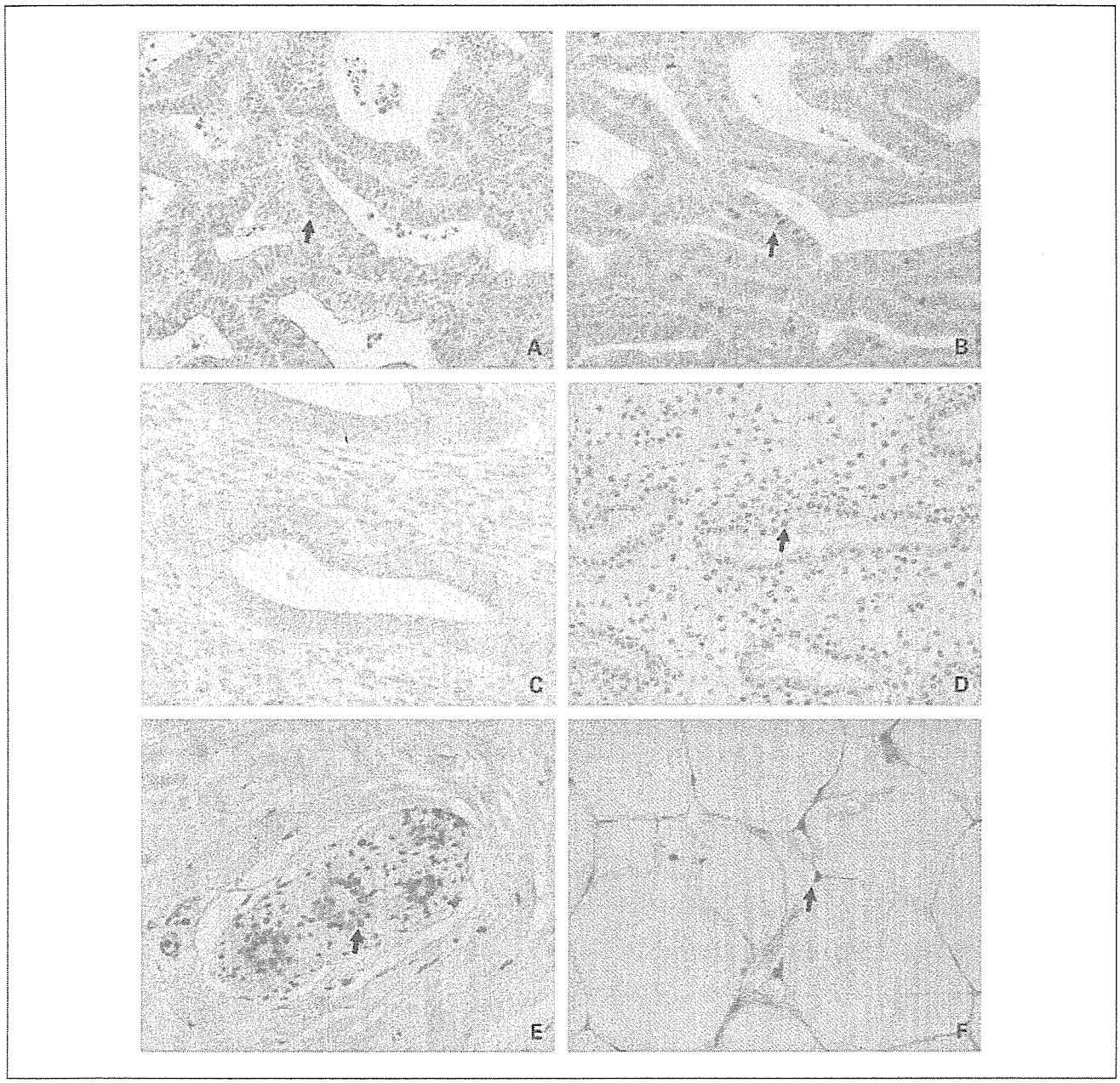

Fig. 2. Immunohistochemistry for PPARγ and p21 (×200). *Arrows,* immunoreactive cells. *A,* PPARγ immunoreactivity was detected in the nuclei of the endometrial carcinoma cells. *B,* p21 immunoreactivity was also detected in the nuclei of the endometrial carcinoma cells. *C,* a case of negative immunoreactivity for PPARγ in the normal proliferative-phase endometrium. *D,* immunoreactivity for PPARγ was detected in the normal secretory-phase endometrium. *E,* immunoreactivity for PPARγ was detected in the nuclei of epithelial cells in normal mammary glands. *F,* PPARγ immunoreactivity was positive in the nuclei of adipocytes.

patients with hypertension was 56 (55.4%) and the number of patients with type II diabetes was 24 (23.8%). A significant association was detected between obesity and disease-free survival (P = 0.0353; Fig. 1), but there were no significant correlations between obesity and overall survival of the patients (P = 0.0844; data not shown).

Immunohistochemistry and correlation with clinicopathologic variables. Positive immunoreactivity for PPARγ was detected in the nuclei of carcinoma cells, normal endometrial gland cells, and hyperplastic cells (Fig. 2A and C-E). PPARγ immunoreactivity was detected in 67 of 103 (65%) of carcinoma cases, 11 of 23 (48%) of proliferative-phase endometrium, 14 of 19 (74%) of secretory-phase endometrium, and 27 of 32 (84%) of endometrial hyperplasia. Immunoreactivity of PPARγ was significantly lower in endometrial carcinoma than in secretory-phase endometrium (P < 0.0001) and endometrial hyperplasia (P = 0.0015). Lower immunoreactivity was also detected in proliferative endometrium than in secretory-phase endometrium (P = 0.0163) and endometrial hyperplasia (P = 0.0008; Fig. 3A). Results of immunohistochemistry for PPARγ and their correlation with clinicopathologic variables, such as age, stage, and grade of the patients, are summarized in Table 2.

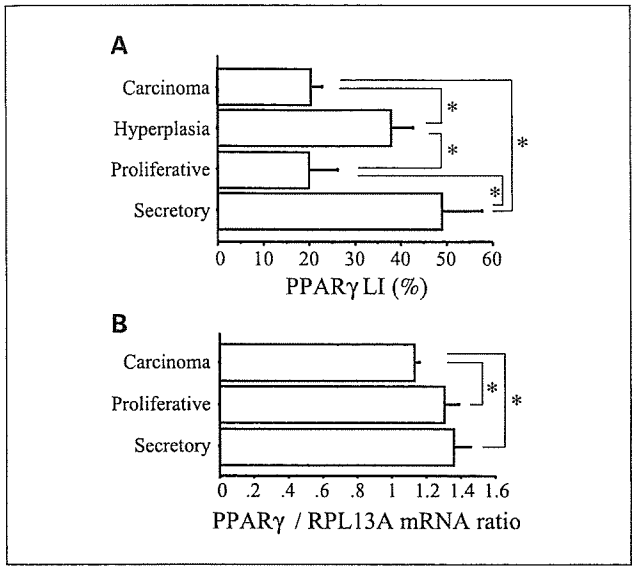

Fig. 3. *A*, LI of PPARγ. Immunoreactivity of PPARγ was significantly higher in endometrial hyperplasia and normal endometrium in secretory phase (*, $P < 0.05$) than in endometrial carcinoma and normal endometrium in proliferative phase. *Bars*, SE. *B*, semiquantitative real-time reverse transcription-PCR for PPARγ in the endometrial carcinoma and normal endometrial tissue. Expression of PPARγ mRNA was significantly higher in proliferative-phase and secretory-phase endometrium than in endometrial carcinoma. *, $P < 0.05$. *Bars*, SD of triplicate samples.

PPARγ LI was significantly correlated with p21 LI ($P < 0.0001$). BMI in endometrial carcinoma patients was significantly associated with PPARγ LI of their carcinoma cells ($P < 0.0001$). No significant associations were detected between PPARγ immunoreactivity and overall survival ($P = 0.6367$) or disease-free survival of the patients ($P = 0.1168$; data not shown).

Real-time PCR analysis. We examined the expression of PPARγ mRNA in 28 endometrial carcinoma patients and 14 normal controls. PPARγ mRNA was abundantly expressed in secretory endometrium. There were significant differences between endometrial carcinoma and proliferative-phase tissues ($P = 0.0115$) or secretory-phase tissues ($P = 0.0217$; Fig. 3B), but no significant differences were detected between proliferative-phase and secretory-phase endometrium. In carcinoma cell lines, PPARγ mRNA was also expressed in Ishikawa, Sawano, and RL95-2 cells (Fig. 4A). It is well known that PPARγ heterodimerizes with RXRs, and we therefore examined the expression of RXRs-RXRα, β, and γ-mRNAs in these cells. These mRNAs were also expressed in Ishikawa, Sawano, and RL95-2 cells (data not shown).

Immunoblotting. Immunoblotting analysis was done using Ishikawa, Sawano, RL95-2, and MCF-7 cells to detect the presence of PPARγ protein. MCF-7 cells were used as a positive control. Immunoreactive bands corresponding to PPARγ were detected in all carcinoma cell lines (Fig. 4B).

Cell proliferation assay and apoptosis analysis. We examined the effects of naturally occurring PPARγ ligand on the cell growth *in vitro* (Fig. 5A-C). 15d-PGJ_2 markedly suppressed cell growth in Ishikawa, Sawano, and RL95-2 cells in both dose- and time-dependent manners. In all three cell lines,

Table 2. Correlation between PPARγ immunoreactivity and clinical variables in endometrial carcinomas

Factor	*N* = 103	PPARγ immunoreactivity		*P*
		+	−	
Age				
<50	20	14	6	0.5806
≥50	83	53	30	
Stage				
I + II	78	48	30	0.1869
III + IV	25	19	6	
Grade				
1 + 2	81	49	32	0.0629
3	22	18	4	
BMI* (median)	24.6	24.2	24.9	<0.0001†
p21 (median)	7.0	9.0	5.0	<0.0001†
ERα (median)	23.0	20.5	26.0	0.757†
ERβ (median)	5.0	10.5	4.0	0.1495†
PR (median)	25.0	26.5	21.0	0.2313†
Ki-67 (median)	32.0	30.0	36.0	0.969†

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.
*$N = 101$.
†Compared with PPARγ LI.

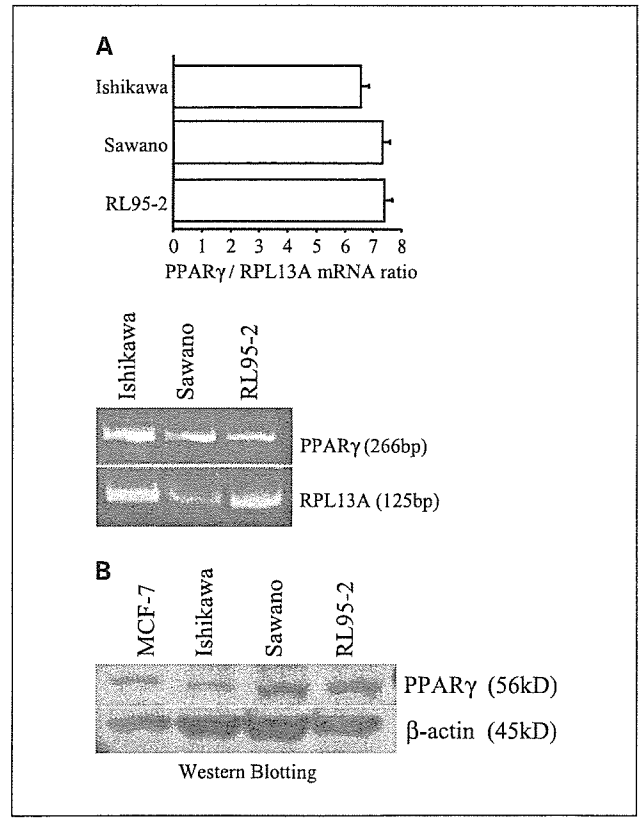

Fig. 4. *A*, semiquantitative real-time reverse transcription-PCR analysis of PPARγ in endometrial carcinoma cell lines. *Bars*, SD of triplicate samples. Agarose gel pictures show PPARγ mRNA. *B*, results of Western blotting of carcinoma cell lines. MCF-7 was used as a positive control and β-actin was used as an internal positive control.

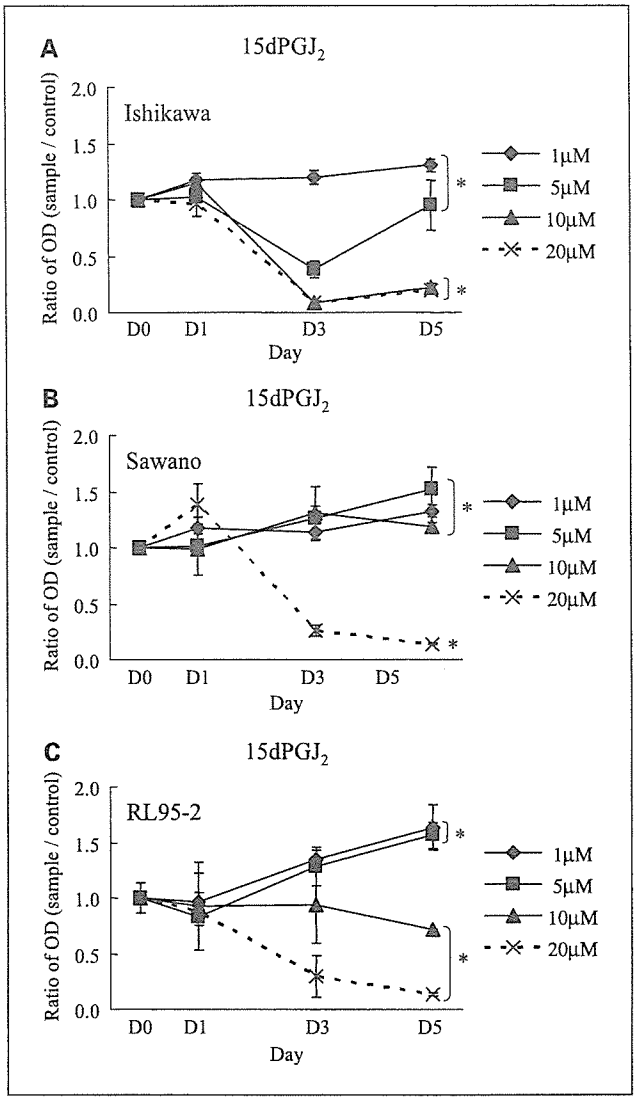

Fig. 5. Effect of 15d-PGJ_2 on the proliferation of (*A*) Ishikawa, (*B*) Sawano, and (*C*) RL95-2 cells. The concentrations of ligand are 1, 5, 10, and 20 μmol/L. Values are fold of the control absorbance. *Points*, mean of triplicate wells; *bars*, SD. *, $P < 0.05$. OD, absorbance.

growth suppression was detected after 3 days of the 15d-PGJ_2 addition.

We also examined the status of apoptosis after adding 15d-PGJ_2. Apoptosis indexes of Ishikawa, Sawano, RL95-2 cells were not significantly altered under 10 and 20 μmol/L 15d-PGJ_2 treatments for 5 days (Fig. 6A-C).

The expression of p21 mRNA after 15d-PGJ_2 treatment was summarized in Fig. 7A to C. The p21 mRNA expression increased in both dose- and time-dependent manners.

Discussion

In our present study, weak PPARγ immunoreactivity was detected in endometrial carcinoma tissues. PPARγ mRNA expression in carcinoma tissues was also lower than that in normal tissues. Similar results have also been reported in esophageal (26) and lung (27) carcinomas. Normal human ureter also expressed PPARγ protein, but there was a significant loss of PPARγ expression in high-grade transitional cell carcinomas (28). Therefore, the results of our study are also consistent with these results, which support the hypothesis that the PPARγ gene is a tumor suppressor gene, and dysfunction of PPARγ contributes to tumorigenesis (11). However, several studies also showed that PPARγ expression was more marked in carcinoma tissues than in normal tissues in several types of human malignancies. For instance, Zhang et al. analyzed 56 specimens of normal ovary and neoplasm using immunohistochemistry (29). Immunoreactive PPARγ was not detected in normal ovaries. However, PPARγ immunoreactivity in ovarian tumor tissues was significantly higher than in normal ovaries and benign ovarian tumors (29). Ikezoe et al. examined the expression of PPARγ in 339 clinical samples and 71 various cancer cell lines, including colon cancer, breast cancer, prostate cancer, lung cancer, osteosarcoma, glioblastoma, and leukemia. All of the cell lines and clinical samples expressed PPARγ as detected by real-time PCR and/or Western blot, but their expression levels varied widely among samples (30). Therefore, the results above indicated that the expression of PPARγ is dependent on tissue specificity

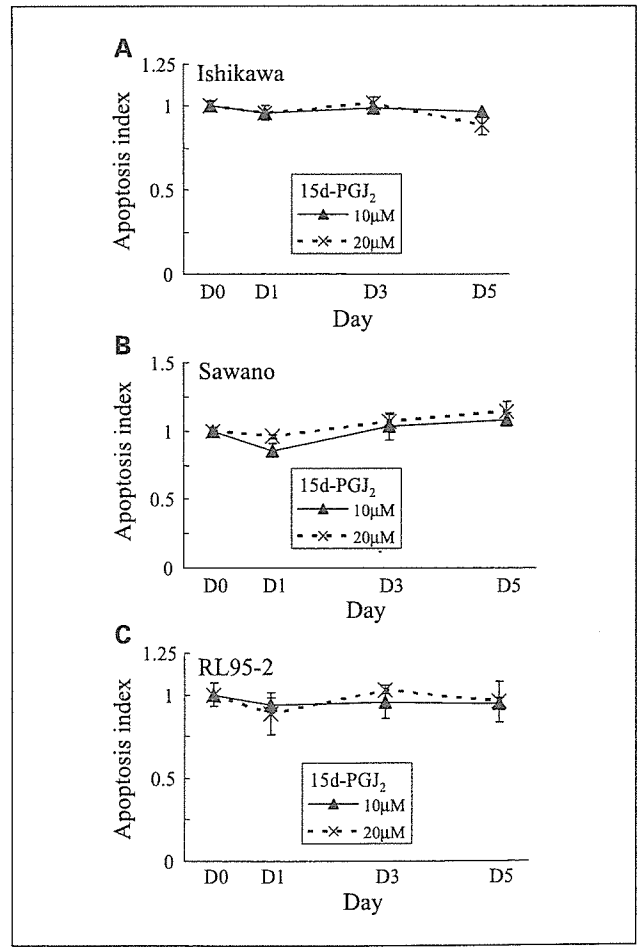

Fig. 6. Apoptosis index of (*A*) Ishikawa, (*B*) Sawano, and (*C*) RL95-2 cells under 15d-PGJ_2 treatments. The apoptosis index was calculated as the fold of the control absorbance. *Points*, mean of triplicate wells; *bars*, SD.

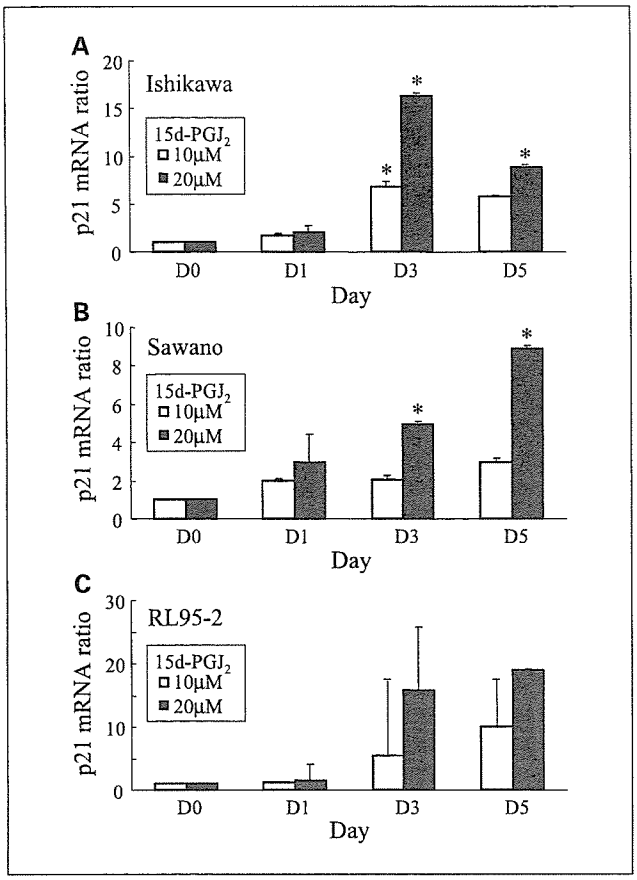

Fig. 7. Semiquantitative real-time reverse transcription-PCR analysis of p21 under 15d-PGJ_2 treatments in endometrial carcinoma cell lines. (*A*) Ishikawa, (*B*) Sawano, and (*C*) RL95-2 cells. Values are fold of the control value. *, $P < 0.05$ versus day 0. *Bars*, SD of duplicate samples.

and/or the mutational events that are required for cancer development.

We showed that PPARγ LI was higher in endometrium in the secretory phase than the proliferative phase. We examined previously the expression of RXRs in normal endometrium, endometrial hyperplasia, and endometrial carcinoma (31). RXRγ immunoreactivity was detected in the nuclei of epithelial cells of the secretory-phase endometrium but not of the proliferative phase. Loughney et al. also reported that intracellular concentration of all-*trans* retinoic acid, a ligand of RXRs, was elevated during the secretory phase because of a marked reduction of cellular retinoic acid protein type II mRNA (32). PPARγ was shown to heterodimerize RXRs, which is also consistent with the results of our study. PPARγ may also have antiproliferative effects in secretory-phase endometrium.

In *in vitro* experiments, 15d-PGJ_2 markedly inhibited cell proliferation of the endometrial carcinoma cell lines at 10 and 20 μmol/L. 15d-PGJ_2 is both an endogenous PPARγ ligand and a direct inhibitor of several other signal transduction pathways (33). 15d-PGJ_2 has been considered an endogenous ligand; Forman et al. reported that prostaglandin D_2 is the major prostaglandin in most tissues and PGJ_2 derivatives may be produced at several of these sites (8). Nosjean et al. also showed that prostaglandins were cyclooxygenase products, and a final product of this pathway, PGJ_2, is nonenzymatically converted into 15d-PGJ_2 (7). Parikh et al. reported the following three findings. (*a*) 15d-PGJ_2 activated PPARγ-dependent signaling systems more potently than other fatty acids that have been studied. (*b*) A representative synthetic cyclopentenone-prostaglandin, Δ^{12}-PGJ_2, was actively transported into the nucleus in a time- and temperature-dependent manner with Michaelis-Menten kinetics, suggestive of carrier-mediated active transport. Finally, formation of immunoreactive 15d-PGJ_2 has been detected during the propagation and resolution of inflammation in association with PPARγ activation (34). Nakashiro et al. reported that low-dose 15d-PGJ_2 (0.5 μmol/L) almost completely inhibited the growth of nonneoplastic human urothelial cell line and 10 μmol/L 15d-PGJ_2 suppressed the growth of neoplastic urothelial cells (28). Several investigators also showed that 15d-PGJ_2 inhibited the growth of pancreatic carcinoma cells (35), lung carcinoma cells (36, 37), and gastric carcinoma cells (38). These antiproliferative effects of 15d-PGJ_2 discussed above are all considered to be mediated by several pathways: PPARγ dependent and PPARγ independent. The lipopolysaccharide-induced transcription responses of activator protein-1, nuclear factor-κB, and STAT1 can be repressed by 15d-PGJ_2 only in the presence of PPARγ (39). Two identified candidates have been proposed to mediate PPARγ-independent actions of 15d-PGJ_2: the nuclear factor-κB system and the extracellular signal-regulated kinase signaling pathway. We showed that 15d-PGJ_2 suppressed cell proliferation of the endometrial carcinoma cell lines. If 15d-PGJ_2 decreased cell proliferation through PPARγ-dependent pathways, 15d-PGJ_2 may represent a new therapy for human PPARγ-positive endometrial carcinoma.

In this study, PPARγ immunoreactivity was significantly correlated with that of p21 in endometrial carcinoma tissues, and expression of p21 was induced by 15d-PGJ_2 at mRNA levels in Ishikawa, Sawano, and RL95-2 cells. Apoptosis indexes of cell lines were not altered under 10 and 20 μmol/L 15d-PGJ_2 treatment for 5 days. Several studies showed that PPARγ ligands induced cyclin-dependent kinase inhibitors, such as p21, in various types of carcinoma cells (25, 40, 41). The p21 protein inhibits cyclin-dependent kinases and mediates cell cycle arrest and cell differentiation. A potential conserved consensus peroxisome proliferator-responsive element was detected in the promoter region of p21 gene (25, 40). Suzuki et al. reported that the expression of p21 was significantly induced by 15d-PGJ_2 at both mRNA and/or protein levels in MCF-7 breast carcinoma cell line and apoptosis index of MCF-7 cells was not significantly altered under the 15d-PGJ_2 for 3 days (25). Jung et al. also showed that a large portion of human cervical carcinoma cell line C-4II cells showed growth arrest at G_1 phase with the induction of p21 following ciglitazone treatment (41). They also reported that PPARγ ligands suppressed cervical cancer cell proliferation by inhibiting cell growth, not by triggering apoptosis at least in this cell line examined (41). Shen et al. also reported that 15d-PGJ_2 induced the expression of cyclin-dependent kinase inhibitor p21 protein in human chondrosarcoma cells in a p53-independent manner, which seems to be involved in the mechanism of inhibition of cell proliferation (40). Results of our present study are consistent with all of these studies reported previously and suggest that PPARγ also regulates the expression of p21 in endometrial carcinoma tissues.

Approximately 50% of endometrial carcinoma patients had obesity and hypertension and 24% of the patients had type II diabetes mellitus in our present study. These results are also consistent with reported results of previous studies (16, 18, 42, 43). The NIH defines a normal BMI as 18.5 to 24.9. Overweight is defined as a BMI between 25.0 and 29.9. Class I obesity is a BMI between 30 and 34.9, class II obesity between 35.0 and 39.9, and class III obesity as >40 (44). However, among Japanese women, there are fewer class I obese people than in Western countries (19). Therefore, the Japan Society for the Study of Obesity originally defined class 1 obesity as BMI between 25 and 29.9, class 2 obesity as between 30 and 34.9, class 3 obesity as between 35 and 39.9, and class 4 obesity as ≥40 (19). Therefore, in this study, which examined Japanese patients with endometrial carcinoma, we defined obesity as a BMI ≥ 25. In our study of Japanese women with endometrial carcinoma, patients with endometrial carcinoma had high BMI, but paradoxically obese women with endometrial carcinoma had longer disease-free survival than non-obese women with carcinoma. Everett et al. reported that, in 396 endometrial carcinoma women, women with BMI > 40 had a lower recurrence rate compared with those with BMI < 30 (4.7% versus 13%), but the difference did not reach statistical significance ($P = 0.065$; ref. 42). Anderson et al. also reported that disease-free survival increased significantly ($P = 0.014$), and recurrence rates decreased as BMI increased (45). Therefore, an inverse correlation between biological behavior and obesity seems to be observed in both Japanese and Western patients with endometrial carcinoma. Further investigation is needed to clarify the mechanisms.

There was a significant negative correlation between PPARγ expression and BMI in women with endometrial carcinoma ($P < 0.0001$). To the best of our knowledge, this is the first study that analyzed the correlation between PPARγ expression and BMI in endometrial carcinoma patients. Kadowaki et al. reported that PPARγ was a thrifty gene mediating high-fat diet-induced obesity, adipose hypertrophy, and insulin resistance (46). They also reported that genetic or environmental factors causing obesity might interact with the PPARγ gene, leading to differences in insulin sensitivity between subjects with and without this substitution in overweight and obese subjects. Trujillo et al. reported that circulating adiponectin, a hormone produced exclusively by the adipocyte, was a biomarker of the metabolic syndrome (47). Adiponectin functions as an insulin sensitizer by decreasing hepatic glucose output and thereby contributing to the regulation of whole-body insulin homeostasis. Combs et al. reported induction of adipose tissue adiponectin expression and subsequent increases in circulating adiponectin levels represented a novel potential mechanism for PPARγ-mediated enhancement of whole-body insulin sensitivity (48). PPARγ agonists are known to raise circulating adiponectin levels (48). Maso et al. proposed that the combined effects of low plasma adiponectin and high BMI contributed to a >6-fold excess risk (49). These results may also explain an inverse correlation between BMI and PPARγ expression in endometrial carcinoma detected in our present study, but further investigations are awaited for clarification.

In our present study, we showed the expression of PPARγ in human endometrial carcinoma and the effects of PPARγ ligand in endometrial carcinoma cells. These findings suggest that a PPARγ ligand, 15d-PGJ_2, has antiproliferative activity against endometrial carcinoma.

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Progesterone receptor isoforms as a prognostic marker in human endometrial carcinoma

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The possible role of specific progesterone receptor (PR) isoforms (PRA and PRB) as predictive factors in endometrial carcinoma is unclear. The present study was undertaken to evaluate the clinical significance of intratumoral PR isoform status in patients with endometrioid endometrial carcinoma. We studied 103 cases of endometrioid endometrial carcinoma using immunohistochemistry. We correlated the findings with various clinicopathological parameters of the patients. PRA and PRB immunoreactivity was detected in 51/103 (48.5%) and 79/103 (76.7%) of carcinoma cases, respectively. A significant positive correlation was detected between the status of PRB immunoreactivity and the amount of PRB mRNA by real-time reverse transcription–polymerase chain reaction ($P = 0.012$). PR isoform expression was significantly lower in the cases with higher histological grade ($P = 0.0001$ and $P = 0.002$, for PRA and PRB, respectively). Cases that were negative for either one or both PR isoforms were significantly associated with shorter disease-free and overall survival of the patients. The absence of either one or both of these two PR isoforms was detected in all nine patients who died (100.0%), whereas the absence of these immunoreactivities was detected only in 43 of 94 (45.7%) patients who had lived during the same period. In addition, multivariate analysis demonstrated that an absence of PRA immunoreactivity was an independent risk factor in disease-free survival of the patients ($P = 0.0258$). The results of our study demonstrated that loss or absence of PR isoform expression determined by immunohistochemistry could become an important prognostic indicator in patients with endometrioid endometrial carcinoma. (*Cancer Sci* 2006; 97: 1308–1314)

Endometrial carcinoma is one of the most common malignancies of the female genital tract and its incidence, especially that of endometrioid endometrial carcinoma, has increased recently.[1] It is well known that uterine endometrial proliferation is under the control of both estrogen and progesterone. One of the physiological roles of progesterone in the regulation of glandular epithelium of the endometrium is to induce cellular differentiation and to antagonize estrogen-mediated cell proliferation.[2] Endometrial carcinogenesis is strongly associated with continued estrogen exposure without progesterone influence.[3,4] Progesterone has clinically been demonstrated to provide some protection against stimulatory effects of estrogenic agents. In addition, hormone replacement therapy using combinations of estrogens and progesterones yields a lower risk of endometrial carcinoma, despite increasing the incidence of breast carcinoma.[5,6] A number of the patients who wished to preserve their fertility were treated with progestin as a primary endocrine therapy for atypical hyperplasia and well-differentiated adenocarcinoma, although the effects of this treatment on the clinical outcome of patients have not always been satisfactory.[7–9]

Both estrogen and progesterone act through intranuclear receptors, estrogen receptors (ER) and progesterone receptors (PR), which belong to the superfamily of steroid hormone receptors.[10] The expression of ER and PR is generally considered to be coordinated because transcription of the PR gene is induced by estrogen and inhibited by progesterones in the great majority of estrogen-responsive cells.[11] In normal cycling human endometrium, PR is expressed abundantly in glandular epithelium during the proliferative phase of the cycle.[12] PR is present in two isoforms, termed PRA and PRB.[13] PRA is the truncated form of PRB, lacking 164 amino acids at the NH_2 terminus. These isoforms are translated from the same gene, but transcription is initiated from different promoters.[14] Studies addressing the individual effects of PR isoforms have been reported. Vegeto *et al.* reported that PRA could repress PRB activity in cells in which PRA was not transcriptionally active, and that PRA might be associated with a cell- and promotor-specific repressor of PRB.[15] Giangrande *et al.* also reported that differential cofactor binding resulted in the opposing transcriptional activities of PRA and PRB.[16] In addition, microarray analyses of human breast cancer cells expressing either PRA or PRB have confirmed that each PR isoform has a unique set of target genes, with little overlap.[17] These functional and transcriptional differences suggest that the development, invasiveness and metastatic potential of carcinoma cells can be influenced by the PR status of the tumor cells. We previously reported that loss of PRB was a significant prognostic factor in epithelial ovarian cancer.[18,19] In addition, breast carcinoma patients with PRA-rich tumors are in general associated with poorer disease-free survival rates.[20] In endometrial carcinoma, several studies demonstrated the PR isoform status of carcinoma cells.[21–23] Arnett-Mansfield *et al.* reported a reduced expression of either one or both of the PR isoforms in the great majority of endometrial tumors, compared with hyperplastic or normal endometrium.[21] De Vivo *et al.* demonstrated a polymorphism in the PRB promoter, which results in increased transcription of the PRB isotype. In a population-based study, this polymorphism was reported to be associated with increased risk for endometrial carcinoma.[22] In addition, hypermethylation of PRB alleles was detected in endometrial carcinoma.[23]

Results of previous studies demonstrated that high levels of ER and PR were directly correlated with a lower tumor grade, less myometrial invasion, and a lower incidence of lymph node metastases in the patients with endometrioid endometrial carcinoma.[24–27] In addition, the status of ER and PR in these carcinomas has been reported as an independent prognostic factor of the patients.[28] However, it is also true that there are many controversies regarding the possible roles of specific PR isoforms as predictive factors in endometrial carcinoma.[21,29–32] Fujimoto *et al.* reported that PRA could not be detected in advanced endometrial tumors.[29] In accordance with this, they later reported that PRB was expressed predominantly in distant metastases of endometrial carcinoma.[30] In contrast, Kumar *et al.* reported that downregulation of PRB may be associated with poorly differentiated endometrial carcinoma.[31] Sakaguchi *et al.*

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also proposed that the drastic decrement of PRB but not of PRA resulted in poor prognosis in endometrial carcinoma, although histological type was not described in their study.[32]

Therefore, in the present study, we carried out immunohistochemical analysis of 103 cases of endometrioid endometrial carcinoma, and correlated the findings with the clinicopathological features of the patients, including their clinical outcome, in order to study the possible roles and correlation between PR isoforms and prognosis of the patients.

Materials and Methods

Endometrial carcinoma patients and tissue preparation. One hundred and three endometrioid endometrial carcinomas (49 well differentiated, 32 moderately differentiated, 22 poorly differentiated; 66 stage I, 12 stage II, 22 stage III, 3 stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). None of the patients examined had received irradiation, hormonal therapy or chemotherapy prior to surgery. The median follow-up time of the patients examined in this study was 60 months (range, 2–148 months). The disease-free and overall survival times of the patients were calculated from the time of initial surgery to recurrence or death, or the date of last contact. The survival times of patients still alive or lost to follow-up were censored in December 2004. The clinicopathological findings of the patients, including age, histology, stage, grade and preoperative therapy, were retrieved by extensive review of the 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. Eighty-five out of 103 patients (83%) in this study underwent complete surgery as above. Six out of 85 patients had lymph node metastasis. The remaining 18 patients (17%) underwent total abdominal hysterectomy and salpingo-oopholectomy without lymphadenectomy because of obesity or their poor performance status. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO and staged according to the International Federation of Gynecology and Obstetrics system.[33,34] Sixty-eight out 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, G1, stage IA, G2 and stage IB, G1) and patients who were associated with poor performance status did not receive any adjuvant therapy. None of the patients received hormone therapy after operation. All specimens were processed routinely (i.e. 10% formalin fixed for 24–48 h, paraffin embedded, and thin sectioned [3 μM]).

Antibodies. Monoclonal antibodies for PRA (hPRa7) and PRB (hPRa2) were purchased from NeoMarkers (Fremont, CA, USA). The PRA (hPRa7) antibody used in this study recognized both PRA and PRB in immunoblot analysis.[35] However, Mote *et al.* reported that hPRa7 did not recognize PRB on immunohistochemistry in fixed tissues even after antigen retrieval, as evidenced by the absence of immunostaining by this antibody of the PRB-expressing MDA-MB-231/PRB cell line.[36] This was considered to be due to the inaccessibility of the epitope on PRB recognized by hPRa7 in 10% formalin-fixed and paraffin-embedded tissue specimens, possibly due to alteration of the conformation of the molecule in which the hPRa7 epitope is located in such a way to reduce its accessibility in immunohistochemistry. hPRa2 recognizes PRB exclusively.[35,37] Monoclonal antibodies for ERα, ERβ and Ki67 were purchased from Novocastra (Benton, NC, UK), Genetex (San Antonio, TX, USA) and DAKO Cytomation (Carpinteria, CA, USA), respectively.

Immunohistochemistry. Immunostaining was carried out by the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). Antigen retrieval was carried out using an autoclave treatment for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilutions of the primary antibodies used in our study were as follows: PRA, 1/100; PRB, 1/100; ERα, 1/50; ERβ, 1/1500; and Ki67, 1/50. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6] and 0.006% H_2O_2), and counterstained with hematoxylin. Proliferative-phase endometrial glands were used as positive controls for immunohistochemistry of PR isoforms[26] and breast cancers were used as positive controls for ERα and ERβ. As a negative immunostaining control, normal rabbit or mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity. Evaluation of PRA, PRB, ERα, ERβ and Ki-67 was carried out in high-power fields (×400) using a standard light microscope. Two of the authors (SS and KI) searched all of the tissue sections simultaneously and determined the most representative areas using a double-headed light microscope. In all of the cases examined, a total of more than 500 tumor cells from three different representative fields were counted independently by the two authors, and the percentage of immunoreactivity (i.e. the labeling index [LI]) was determined. After completely reviewing the immunostained sections of each lesion, two of the authors (SS and KI) independently divided the cases into the following two groups: +, >10% positive cells; and –, <10% positive cells. Layfield *et al.* proposed the separation of ER- and PR-positive cases using LI cut-off points of 10% in the immunohistochemical analysis of human breast cancer.[38] The eighth St Gallen meeting also recommended that approximately 10% positive staining of cells for either ER or PR might be considered as a reasonable threshold for definite endocrine responsiveness.[39] Therefore, in the present study, we used the same cut-off point of 10% between positive and negative PR isoforms, based on the results of the studies above. Cases with discordant results (interobserver differences of >5%) were reevaluated simultaneously the two authors above using a double-headed light microscope. Consequently, the interobserver differences were less than 5% in this study.

Reverse transcription–polymerase chain reaction. Thirty-three specimens of fresh frozen tissues of endometrial carcinoma (i.e. specimens frozen immediately in liquid nitrogen and stored at −80°C) were available for the present study. Total RNA was extracted by homogenizing frozen tissue samples in 1 mL TRIzol reagent (Life Technologies, Gaithersburg, Grand Island, NY, USA), followed by phenol–chloroform extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at −80°C until processing for reverse transcription (RT). Total RNA (4 μg) was denatured at 70°C for 10 min and was reverse transcribed in the presence of 50 ng/μL Oligo (deoxythymidine) primer (Invitrogen, Carlsbad, CA, USA), 2.5 mmol/L $MgCl_2$, 0.5 mmol/L deoxy-NTPs, 10 mmol/L dithiothreitol and 10 IU ribonuclease H-reversed transcriptase (Superscript II RT, Invitrogen) for 60 min at 42°C and 15 min at 70°C on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Watertown, MA, USA). RT–polymerase chain reaction (PCR) analysis was carried out in order to examine the presence or absence of genomic DNA contamination. The RT step was performed in the absence of Superscript II RNase H-reverse transcriptase, followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium-bromide-stained 2% agarose gel. No bands were detected in these samples (data not shown). After an initial

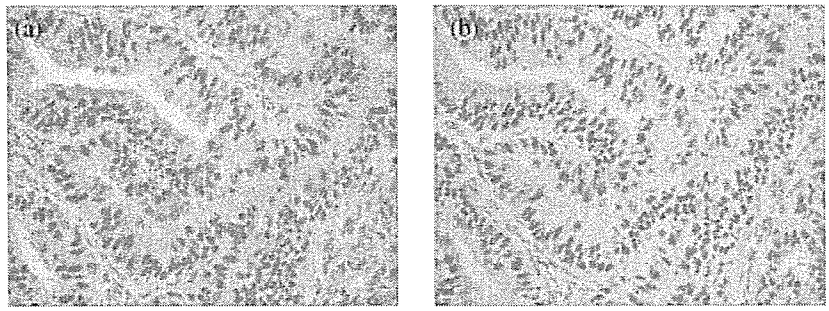

Fig. 1. Immunohistochemical staining for (a) progesterone receptor A (PRA) and (b) progesterone receptor B (PRB) in endometrioid endometrial carcinoma. PRA and PRB immunoreactive proteins were detected in the nuclei of carcinoma cells of G1 adenocarcinoma. Original magnification, ×400.

1 min denaturation step at 96°C, 35 cycles of PCR were carried out on thermal cycle under the following conditions: 45 s denaturation at 94°C, 30 s annealing at 58°C, and a 1.5 min extension at 72°C. In addition, cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche Diagnostics, Mannheim, Germany) using the DNA-binding dye SYBER Green I (Roche Diagnostics). The 20-μL reaction mixture contained 3 mM $MgCl_2$ for PRB and β-actin primer, 10 pmol/L of each primer and DNA-binding dye LightCycler-Fast Start DNA Master SYBR Green I. β-Actin expression was used to verify the integrity of RNA from each specimen. Human gene-specific primers used to amplify PRB and β-actin were as follows: PRB 5′ sense, ACACCTTGCCTGAAGTTTCG and PRB 3′ antisense, CTGTCCTTTTCTGGGGGACT (196 bp); β-actin 5′ sense, CCAACCGCGAGAAGATGAC and β-actin 3′ antisense, GGAAGGAAGGCTGGAAGAGT (459 bp). An initial denaturing step at 95°C for 10 min was followed by 35 cycles of 95°C for 15 s, 10 s annealing at 58°C (PRB) and 63°C (β-actin), and extension for 13 s at 72°C. The fluorescence intensity of the double-strand-specific SYBER Green I, which reflects the amount of specific PCR products formed, was read by the LightCycler at 85°C after the end of each extension step.[40] Using automated programs of the LightCycler software, the amount of PRB and β-actin template in each sample was calculated so as to dilute the standard cDNA equally. The actual values of PRB were corrected by the value of the β-actin template. Although conventional quantitative PCR requires the use of purified plasma cDNA in the construction of a standard curve, it was possible to semiquantify the PCR products with the LightCycler using purified cDNA of known concentrations.[41,42] 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 PE Applied Biosystems, Foster City, CA, USA) to verify amplification of the correct sequences. Frozen breast cancer tissue was used as a positive control. Negative control experiments did not contain cDNA substrate to study the presence of exogenous contamination of DNA. No amplified products were detected under these conditions.

Statistical analyses. Statistical analysis was carried out using SAS software (StatView, Version 5.0; SAS, Cary, NC, USA). The statistical significance of the association between PRA and PRB immunoreactivity and other parameters (grade, stage, age, ERα LI, ERβ LI and Ki-67 LI) was evaluated using the Mann–Whitney *U*-test and the χ^2-test. The statistical significance between PRA and PRB immunoreactivity was calculated using a correlation coefficient (r) and regression equation. The statistical significance between PRB immunoreactivity determined by immunohistochemistry and the status of mRNA determined by RT-PCR was evaluated using Fisher's exact probability test, and the statistical significance between PRB immunoreactivity and amounts of PRB mRNA determined by real time RT-PCR was evaluated using the Mann–Whitney *U*-test. The Kaplan–Meier method and statistical significance was calculated using a log-rank test. Univariate and multivariate analyses were evaluated using Cox's proportional hazards model. *P*-values less than 0.05 were considered significant.

Results

Immunohistochemistry and RT-PCR. Immunoreactivity for PRA and PRB was detected in the nuclei of carcinoma cells (Fig. 1). ERα, ERβ and Ki-67 were also confined exclusively to the nuclei of epithelial cells (data not shown). RT-PCR was carried out to confirm the expression of PRB using 33 cases in this study (Figs 2,3), because PRA has no specific sequence to distinguish it from PRB mRNA by RT-PCR. Twenty-five of these 33 cases were PRB positive and eight cases were PRB negative, as determined by immunohistochemistry. PRB mRNA was detected in 21 out of these 25 PR-positive cases (84%) and was not detected in five out of eight PRB-negative cases (Fig. 2). There was a statistically significant positive correlation between PRB immunoreactivity and mRNA expression examined by RT-PCR analysis ($P = 0.02$). In addition, amounts of PRB mRNA determined by real time RT-PCR were 8.89 (median values) in these PRB-positive and 0.41 (median values) in PRB-negative cases. A significant positive correlation was detected between PRB immunoreactivity and the amounts of PRB mRNA ($P = 0.012$) (Fig. 3). Eighty out of 103 cases (77.7%) demonstrated either or both PR isoforms in immunohistochemistry. Fifty-one out of 103 cases (48.5%) were PRA positive. Among these 51 PRA-positive cases only one case (1.9%) was PRA positive and PRB negative. However, PRB-positive cases were 76.7% (79/103), and 29 of these 79 PRB-positive cases (36.7%) were both PRB positive and PRA negative. The proportion of cases positive for both PRA and PRB was 48.5% (50/103), whereas the proportion of cases negative for both PRA and PRB was 22.3% (23/103). There was a significant positive correlation between PRA and PRB expression in endometrial carcinoma ($P = 0.004$). Results of the associations between clinicopathological parameters and immunoreactivity of PRA and PRB are summarized in Table 1. The status of PRA in G1, G2 and G3 endometrial carcinoma was 67.3% (33/49), 46.6% (15/32) and 13.6% (3/22), respectively, and the status of PRB was 87.8% (43/49), 78.1% (25/32) and 50.0% (11/22), respectively. PR immunoreactivity was significantly lower for carcinoma with higher histological grade ($P = 0.0001$ and $P = 0.002$, for PRA and PRB, respectively), whereas there were no correlation among the clinical stages of the cases. PRA and PRB expression was significantly positively correlated with ERα LI, and inversely with Ki-67 LI.

Relationship between PR isoform expression and prognosis. Progesterone receptor isoform status was evaluated as a prognostic variable in the patients with endometrioid endometrial carcinoma using univariate analysis. Results of univariate analysis are summarized in Table 2. The following variables were significantly associated with poorer disease-free survival and overall survival of the patients at the $P < 0.05$ levels: absence of PRA immunoreactivity; absence of PRB immunoreactivity;

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