It is generally accepted that Bathsheba was painted in 1654 modeled by Hendrickje Stoffels. In 1654, Hendrickje was 28 years old. She was Rembrandt's de facto wife as of 1649 and had a pregnancy in 1652. She became pregnant again and delivered their daughter Cornelia in 1654 October.

No records on health of Hendrickje until her premature death on 21 Jul 1663 (37 years old).

In 2000, Bourne proposed on alternative diagnosis of an infective process such as tuberculous mastitis or less likely chronic lactational breast abscess [6]. He discussed that if the body of model was Hendrickje, she could hardly have lived 9 years with advanced breast cancer without any effective treatment. Possibly she had a chronic inflammatory condition, either tuberculous mastitis and less likely lactational breast abscess.

Other works by Rembrandt modeled by Hendrickje

We can see other works by Rembrandt modeled by Hendrickje. Rembrandt left several oil paintings modeled by Hendrickje around 1654, including "Hendrickje Stoffels" (1655, Paris Musee Louvre) "Woman in a Doorway" (1656, Berlin Gemeldegalerie) and famous "Hendrickje bathing" (1655, London National Gallery). No paintings show the naked left breast of the model. But we can see a healthy slightly obese woman at 25–30 years, and observe no remarkable signs of cachexia or chronic consumption.

Further, we can see several female nude etchings (1658, New York, Pierpont Morgan Library) and a precise drawing "Hendrickje in the Artist's Studio" (1654, Oxford, Ashmolean museum). We can observe frontal view of the left breast in 3 etchings in New York and left posterior view in Oxford drawing. Neither etchings nor drawing show deformities or distortions such as dimpling or peau d' orange appearance and axillary lymph node swelling.

Discussion

Taken together, if the model of Bathsheba was Hendrickje and she suffered from left breast disease at the time of the painting, it must be a benign and possibly reversible change. Because Hendrickje delivered Rembrandt's daughter Cornelia in 1654 and survived for 9 more years after the delivery and painting of Bathsheba. If the left mammary changes were caused by breast cancer she would not have survived so long. As Bourne pointed out, the mean survival of breast cancer patients was 2–3 years before the application of modern surgical treatment, radiation and/or chemotherapy.

Diagnosis of tuberculous abscess looks less probable. Because breast involvement of tuberculosis is a rather rare complication. If the model had suffered from tuberculosis, she must show some signs of chronic consumption and hardly had a chance becoming pregnant. Further, untreated tuberculous abscesses persist for several years and result in permanent deformity of the breast. As we cannot see any changes in the Oxford drawing of 1654 and in the New York etchings of 1658, we need to abandon the possible diagnosis of tuberculous abscess and benign breast tumours including fibroma.

We present as a possible alternative, chronic lactational breast abscess and mastopathy. Even in the 21st century Japan and other developed countries, lactating women are often experience mastitis. However they can be effectively treated by the oral administration of antibiotics. In serious cases, they also can be treated by bromocriptin administration that inhibits lactation.

It is possible that lactating women in the mid 17th century had a higher prevalence of mastitis and subsequent breast abscess. In the acute phase of mastitis, redness and swelling are remarkable but they become less observable in the chronic phase and after abscess formation. Hendrickje delivered her second child Cornelia in October of 1654, she had a chance of breast-feeding and subsequent infection. However, if Bathsheba was painted in 1654, birth of Cornelia looks less probable as the cause of chronic breast abscess formation. Thus, we consider it was painted early 1654, before the conception of Cornelia or during the first trimester of pregnancy. As Hendrickje had been reported to become pregnant in 1652, she had the opportunity for lactation and related inflammation. The outcome of Hendrickje's first pregnancy is not known. As there are no record concerning Rembrandt's children except for Titus (borne in 1641, the only living son between Rembrandt's first wife Saskia) and Cornelia, Hendrickje's pregnancy in 1652 possibly resulted in miscarriage or neonatal death due to premature delivery.

Our hypothesis explains why Bathsheva shows no evidence of abdominal striae, which can often be observed in multiparous women. Premature labour or miscarriage even in the first trimester of pregnancy never leave abdominal striae but can induce lactation and lactation without feeding is a major

cause of subsequent breast milk retention and inflammation.

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The incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men

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Abstract

Recent data from Hiby (2004) have suggested that a combination of maternal killer immunoglobulin receptor (KIR) AA genotype and fetal HLA-C2 genotype increases the risk of pre-eclampsia. Different human populations have a reciprocal relationship between KIR AA frequency and HLA-C2 frequency. Japanese people have highest frequency of KIR-AA alleles and lowest frequency of HLA-C2 alleles. However, Caucasians have a moderate frequency of KIR-AA and HLA-C2 alleles. If this hypothesis is correct, the incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men should be higher than that among couples consisting of Japanese women and Japanese men. Therefore, we investigated the incidence of pre-eclampsia among 324 couples consisting of Japanese women and Caucasian men. The incidence of pre-eclampsia in these couples consisting of Japanese women and Caucasian men was similar to that in Japanese women and Japanese men. Our data do not support that of Hiby et al. [Hiby, S.E., Walker, J.J., O'Shaughnessy, K.M., Redman, C.W.G., Carrington, M., Trowsdale, I., Moffett, A., 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of pre-eclampsia and reproductive success. J. Exp. Med. 200, 957–965], although we did not check the haplotypes for HLA-C and KIR.

Keywords: Etiology; HLA-C; KIR; Pre-eclampsia; Human population

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1. Introduction

A number of hypotheses on the etiology of pre-eclampsia have been reported (Roberts et al., 1989; Arngrimsson et al., 1990; Perry and Martin, 1992; Meekins et al., 1994; Robillard et al., 1994; Zhou et al., 1997; Dekker et al., 1998; Redman et al., 1999; Koelman et al., 2000; Saito and Sakai, 2003). One commonly discussed hypothesis is the immunogenetic maladaption hypothesis (Dekker et al., 1998; Robillard et al., 2002; Koelman et al., 2000; Saito and Sakai, 2003; Chaouat et al., 2005). Immune recognition of fetal (paternal) antigens is suggested by the increased risk of pre-eclampsia in first pregnancies (MacGillivray, 1983; Skjaerven et al., 2002) and in multiparous women after changing partners (Robillard et al., 1999; Li and Wi, 2000; Trogstad et al., 2001). There is also an increased risk in women who have received donated gametes, such as artificial donor insemination (AID) (Hoy et al., 1999), oocyte donation and embryo donation (Soderstrom-Anttila et al., 1998). These findings suggest that maternal tolerance to paternal antigens is important for the maintenance of pregnancy, and immunogenetic maladaptation of tolerance system might induce pre-eclampsia. Extravillous trophoblasts (EVT) express four unique class I MHC molecules: HLA-G, HLA-E, HLA-F and HLA-C (Kovats et al., 1990; Ishitani et al., 2003; King et al., 1996). Only HLA-C is polymorphic, so paternal HLA-C on EVT can be recognized by killer immunoglobulin receptors (KIR) on maternal NK cells (Moffett-King, 2002). Recently, Hiby et al. (2004) reported interesting data showing that the combination of maternal KIR-AA, which has no activating receptors, and the fetal HLA-C2 group is associated with pre-eclampsia. They showed also that additional activating KIRs decrease the incidence of KIRs pre-eclampsia when the fetus has an HLA-C2 allele.

Recent data demonstrate that populations with a high KIR-AA genotype frequency have a low frequency of HLA-C2 alleles and vice versa (Williams et al., 2002; Yawata et al., 2002; Norman et al., 2001; Crum et al., 2000; Cook et al., 2003; Toneva et al., 2001; Rajalingam et al., 2002; Whang et al., 2003; Wang et al., 1997). Hiby et al. (2004) hypothesized that the KIR-AA/HLA-C2 combination in a given population would be selected against by deleterious effects such as pre-eclampsia. Japanese people have the highest frequency of KIR-AA genotype at around 60%, and the lowest frequency of HLA-C2 genotype at around 9% (Yawata et al., 2002). Conversely, Australian aborigines and New India have the lowest frequency of KIR-AA genotype and highest frequency of HLA-C2 genotype (Norman et al., 2001; Cook et al., 2003; Rajalingam et al., 2002). If the hypothesis of Hiby et al. (2004) is correct, the incidence of pre-eclampsia in couples consisting of Japanese women and Australian aborigine or New India men should be high. However, such couples are very rare. On the other hand, the number of couples consisting of Japanese women and Caucasian men has been increasing. Caucasians have moderately high frequencies of KIR-AA genotype and HLA-C2 genotype. The frequency ratio of the HLA-C2 genotype is 30–35%, which is three to four times higher than that in Japanese (Williams et al., 2002; Hiby et al., 2004). Therefore, we investigated the frequency of pre-eclampsia in couples consisting of Japanese women and Caucasian men.

2. Materials and methods

2.1. Study patients

Between January 2002 and October 2005, a total of 328 couples consisting of Japanese women and Caucasian men delivered infants at our hospital. There were 332 infants (324 singletons and four sets of twins). We selected the 324 singleton subjects, because twin pregnancy is one of the risk factors for pre-eclampsia. As a control, 36,829 singleton pregnant women were selected from the 2003 database of the Japan Society for Obstetrics and Gynecology. In this database, the nationalities of patients or husbands were not recorded, but the vast majority of cases (more than 98%) were couples consisting of Japanese women and Japanese men in Japan. Therefore, we used this database as the control.

Gestational hypertension was diagnosed by the following definitions: blood pressure (BP) levels >140/90 mmHg after 20 weeks of gestation. Gestational hypertension with proteinuria as indicated by a single albumin reading at least 30 mg/dl (a dipstick reading of 1+) after 20 weeks of gestation was diagnosed as pre-eclampsia. Statistical analysis was performed by Student's *t*-test.

3. Results

Table 1 summarizes the characteristics of couples consisting of Japanese women and Caucasian men, and the controls. There were no significant differences in age, parity, gestational weeks at delivery, maternal body weight at delivery, and treatment for sterility between the test couples and controls.

We observed gestational hypertension in 2.16% of couples consisting of Japanese women and Caucasian men, and in 3.84% of controls (Table 2). The frequencies of gestational hypertension among nulliparous and muliparous test couples were the same as that among controls. The relative risks of the incidence of gestational hypertension among test samples were 0.68 in nulliparous, 0.27 in multiparous, and 0.56 in total couples. The 95% confidence intervals (CI) were 0.3–1.55 in nulliparous, 0.04–1.91 in multiparous, and 0.27–1.19 in total couples, respectively. The frequency of pre-eclampsia among test couples (1.54%) was slightly lower compared to that among controls (2.67%) (Table 2), although there was

Table 1
Characteristics of couples consisting of Japanese women and Caucasian men and controls

	Couples consisting of Japanese women and Caucasian men (n=324)	Control (n=36,829)	p-Value
Age (years)	32.2 ± 4.3	31.8 ± 4.1	0.743
Primipara	199 (61.4%)	21816 (59.2%)	0.426
Maternal body weight at delivery (kg)	62.8 ± 6.9	62.1 ± 7.2	0.825
Neonatal body weight (g)	3133.0 ± 519.6	3026.2 ± 502.5	0.693
Therapy for sterility	17 (5.2 %)	1593 (4.3%)	0.417

The data were shown as mean ± S.D.

Table 2
Frequency of gestational hypertention and pre-eclampsia of couples consisting of Japanese women and Caucasian men and controls

	Couples consisting of Japanese women and Caucasian men ($n=324$)	Control ($n=36,829$)	p-Value	RR (95% CI)
Gestational hypertension				
Nulliparous	6/199 (3.02%)	961/21816 (4.41%)	0.341	0.68 (0.3–1.55)
Multiparous	1/125 (0.8%)	452/15013 (3.01%)	0.149	0.27 (0.04–1.91)
Total	7/324 (2.16%)	1413/36829 (3.84%)	0.117	0.56 (0.27–1.19)
Pre-eclampsia				
Nulliparous	4/199 (2.01%)	681/21816 (3.12%)	0.369	0.64 (0.24–1.74)
Multiparous	1/125 (0.8%)	303/15013 (2.02%)	0.334	0.40 (0.06–2.85)
Total	5/324 (1.54%)	984/36829 (2.67%)	0.209	0.58 (0.24–1.4)

RR, relative risk; CI, confidence interval.

no significant difference between the two groups. We observed pre-eclampsia in 3.12% of nulliparous women and 2.02% of multiparous women in the control group. The frequencies of pre-eclampsia among nulliparous women and multiparous women in the test group were 2.01% and 0.8%, respectively. There were no significant differences in the frequency of pre-eclampsia between couples consisting of Japanese women and Caucasian men, and the control group (Table 2). The relative risks and 95% CI of the incidence of pre-eclampsia among test samples were 0.64 (0.24–1.74) in nulliparous, 0.40 (0.06–2.85) in multiparous, and 0.58 (0.24–1.40) in total couples.

4. Discussion

The report by Hiby et al. (2004) has had significant impact on consideration of the pathophysiology of pre-eclampsia. They pointed out that the combination of maternal KIR-AA genotype and fetal HLA-C2 genes is at increased risk for pre-eclampsia, and showed that different human populations have a reciprocal relationship between KIR-AA frequency and HLA-C2 frequency. Their hypothesis is attractive, but further studies are needed for verification. As one method of proving their hypothesis, an epidemiological study could be valid. A population with a high frequency of KIR-AA genotype and a low frequency of HLA-C2 would result in a low frequency (3–5%) of pre-eclampsia but, if women from a population with a high frequency of HLA-AA marry men from a population with a high frequency of HLA-C2, the prevalence of pre-eclampsia should be increased. The Japanese population has the highest frequency of KIR-AA (~60%) (Yawata et al., 2002; Hiby et al., 2004), while, Caucasian populations have a 3.5 times higher frequency of HLA-C2 genotype than Japanese. If hypothesis of Hiby et al. (2004) is correct, the prevalence rate of pre-eclampsia among such couples should show three- to four-fold increase.

However, our data have now shown that the incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men did not differ significantly from that

among couples consisting of Japanese women and Japanese men. In this cohort, considering the nulliparae as pre-clampsia is often a disease of first pregnancies, the prevalence of gestational hypertensive disorders of pregnancy among nulliparae was 5.03% in cases versus 7.53% in controls. The relative risk and 95% CI of pre-eclampsia among the test group were 0.64 (0.24–1.74) in nulliparous, 0.40 (0.06–2.85) in multiparous, and 0.58 (0.24–1.4) in total couples. They did not reach to three- to four-fold increase in prevalence which was calculated by the hypothesis of Hiby et al. (2004). Thus, our epidemiological study does not support that hypothesis, although we did not investigate individual KIR and HLA-C genotypes. Robillard et al. (1994) reported that the duration of sexual cohabitation effects the risk of gestational hypertension. Unfortunately, we could not obtain the information about the length of sexual cohabitation. The present findings suggest that further investigations of maternal KIR genotype and fetal HLA-C genotype, or duration of sexual cohabitation, are needed to confirm this intriguing hypothesisin pre-eclampsia.

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IDO expression on decidual and peripheral blood dendritic cells and monocytes/macrophages after treatment with CTLA-4 or interferon-γ increase in normal pregnancy but decrease in spontaneous abortion

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Recent data demonstrated that $CD4^+CD25^+$ regulatory T (Treg) cells and an enzyme called indoleamine 2,3-dioxygenase (IDO) mediate maternal tolerance to the fetus. Interestingly, Treg cells express the CTLA-4 molecule on their surface, and B7 (CD80/86) ligation by CTLA-4 enhanced IDO activity of dendritic cells (DCs) and monocytes by the induction of interferon gamma (IFN-γ) production. In this study, we studied the IDO expression on peripheral blood monocytes and decidual monocytes or DCs after treatment with CTLA-4/Fc fusion protein or IFN-γ using flow cytometry. IDO expressions on both peripheral blood DC and decidual DC and monocytes were up-regulated during normal pregnancy. On the other hand, both IDO expression on DC and monocytes after IFN-γ treatment or CTLA-4 treatment were decreased in spontaneous abortion cases. The expression of CD86 on peripheral blood and decidual monocytes and DC in spontaneous abortion cases was lower compared with those in normal pregnancy subjects. Also, IFN-γ production by decidual and peripheral blood mononuclear cells after CTLA-4/Fc treatment in spontaneous abortion cases was significantly lower than those in normal pregnancy subjects. These data suggest that CTLA-4 on Treg cells up-regulates IDO expression on decidual and peripheral blood DC and monocytes by the induction of IFN-γ production.

Key words: CTLA-4/dendritic cell/IDO/pregnancy/regulatory T cell

Introduction

A fetus is a semi-allograft to the maternal host, and T cells are aware of fetal alloantigens. Using T-cell receptor (TCR) transgenic mice, Tafuri *et al.* (1995) demonstrated that maternal H-$2K^b$-specific $CD8^+$ T cells were functionally tolerized by fetal H-$2K^b$ alloantigen, but this state lasted only briefly after parturition. Supporting evidence was obtained from studies in which pregnant mice carrying syngeneic or allogeneic fetuses were treated with a pharmacologic inhibitor of an enzyme called indoleamine 2,3-dioxygenase (IDO) (Munn *et al.*, 1998). IDO is expressed in specific populations of macrophages (Mφ) and dendritic cells (DCs), giant trophoblasts in mice and extravillous trophoblasts and villous trophoblasts in humans (Munn *et al.*, 1999, 2002; Hwu *et al.*, 2000; Sedlmayr *et al.*, 2002; Baban *et al.*, 2004; Honig *et al.*, 2004; Kudo *et al.*, 2004). These findings suggest that immunosuppressive Mφ and DC in decidua prevent maternal T-cell activation by depriving T cells of tryptophan. Serum tryptophan levels decrease from the first trimester of human pregnancy (Schrocksnadel *et al.*, 1996), suggesting that tryptophan metabolism protects the allogenenic fetus in humans by inducing maternal tolerance, although IDO-deficient mice produce litters of normal sizes at normal rates compared with wild mice (Baban *et al.*, 2004).

Recently, it has been reported that T-cell responses are regulated by $CD4^+CD25^+$ regulatory T (Treg) cells, and these Treg cells play a very important role in immunotolerance. $CD4^+CD25^+$ Treg cells express the CTLA-4 molecule on their surface, and CTLA-4 can enhance the IDO activity of DC and Mφ (Grohmann *et al.*, 2002; Fallarino *et al.*, 2003). IDO induction in Mφ or DC is one mechanism by which $CD4^+CD25^+$ Treg cells induce tolerance. Interestingly, recent data showed that $CD4^+CD25^+$ Treg cells are essential for the maintenance of allogeneic pregnancy in mice by the induction of maternal tolerance to fetal antigens (Aluvihare *et al.*, 2004; Zenclussen *et al.*, 2005). In human pregnancy, regulatory T cells increase in peripheral blood and decidua, and decidual $CD4^+CD25^+$ Treg cells express CTLA-4 on their surfaces (Heikkinen *et al.*, 2004; Sasaki *et al.*, 2004; Somerset *et al.*, 2004). However, it has not been reported whether CTLA-4 can induce IDO protein expression in DC or monocytes during pregnancy.

IDO expression is also up-regulated by interferon gamma (IFN-γ) treatment (Taylor and Feng, 1991; Munn *et al.*, 1999, 2004). IFN-γ is produced by decidual T cells and natural killer (NK) cells (Saito *et al.*, 1993; Jokhi *et al.*, 1994) and plays important roles in angiogenesis (Ashkar *et al.*, 2000). In this study, we checked the IDO expression in peripheral blood and decidual monocytes or DC after treatment with CTLA-4 or IFN-γ in normal pregnancy subjects. We further compared these effects in spontaneous abortion cases with those in normal pregnant subjects. Our data showed that the IDO expression on DC and

monocytes was up-regulated by IFN-γ or a soluble fusion protein of CTLA-4 and immunoglobulin Fc (CTLA-4/Fc) treatment during normal pregnancy, but both IDO expression on DC and monocytes with IFN-γ or CTLA-4/Fc treatment were decreased in spontaneous abortion cases, suggesting that $CD4^+CD25^+$ Treg cells and IFN-γ play important roles in the maintenance of pregnancy by the up-regulation of IDO expression.

Materials and methods

Blood and tissue samples

Heparinized venous blood samples and decidual samples were obtained from induced abortion cases (n = 14; age 30.2 ± 4.5 years; gestational age at sampling 7.0 ± 1.3 weeks) and spontaneous abortion cases (n = 10) in the first trimester (age 31.5 ± 4.1 years; gestational age at sampling 7.2 ± 1.8 weeks). We obtained decidual samples and peripheral blood samples from the same patients. Blood samples were obtained from non-pregnant healthy women (n = 10; age 30.5 ± 3.1 years). Informed consent was obtained from all patients. Peripheral blood mononuclear cells were isolated by the standard Ficoll–Hypaque method. The decidual tissues were macroscopically separated from chorionic villi and then cut into small pieces using a razor blade and vigorously shaken for 2 min. These samples were then filtered through a 32 μm nylon mesh, and decidual mononuclear cells (leucocytes) were purified by the Ficoll–Hypaque method (Saito *et al.*, 1992; Tsuda *et al.*, 2002). Decidual tissues were not enzymatically digested to prevent enzymatic treatment from affecting the fluorescence intensity of surface antigens. The yield of decidual leukocytes was 8.5 ± 3.0 × 10^6 cells. All of the sampling and use of tissues for this study were approved by the Toyama Medical and Pharmaceutical University Ethics Committees.

Culture system and IDO expression by flow cytometry

Isolated mononuclear cells (1 × 10^6/ml) were cultured using Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum in 12 well flat-bottomed plates for 24 h in the presence or absence of IFN-γ (R&D Systems, Minneapolis, MN, USA) or recombinant human CTLA-4/IgG Fc chimera (CTLA-4/Fc) (R&D Systems). For flow cytometric analysis, non-adherent and adherent cells were collected with a cell scraper after 24 h. Cells were first stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD14 mouse monoclonal antibody (mAb) (Becton Dickinson, San Diego, CA, USA) or FITC-conjugated lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56; Becton Dickinson) mAbs and PE-conjugated anti-HLA-DR mAb (Becton Dickinson). Then, to stain intracellular molecules, cells were treated with permeabilizing solution (Becton Dickinson). These cells were secondarily stained with a biotin-labelled anti-human IDO mAb (Takikawa *et al.*, 1988), followed by streptavidin-PC5 (Beckman Coulter, Marseille, France). Isotype-matched mouse IgGs were used as a negative control. Flow cytometric analysis was performed on a fluorescence-activated cell sorter (FACS) Calibur using CellQuest software (Becton Dickinson). To analyse macrophages, a gate was set around the monocytes based on forward and side scatter (Figure 1, upper left) and then a gate was set on $CD14^+$ cells (Figure 1, upper middle). After that, the population of intracellular IDO^+ cells in $CD14^+$ cells was calculated (Figure 1, upper right). To analyse DCs, a gate was set around the lymphocytes avoiding granulocytes (Figure 1, lower left) and a gate was set on lineage $markers^-$/$HLA\text{-}DR^+$ (Figure 1, lower middle). After that, the population of intracellular IDO^+ cells in lineage $markers^-$/$HLA\text{-}DR^+$ cells was calculated (Figure 1, lower right).

Cytokine quantitation

Culture supernatants were collected and analysed by the enzyme-linked immunosorbent assay (ELISA) method. An ELISA kit (ENDOGEN, Rockford, IL, USA) was used to quantify human IFN-γ in supernatants.

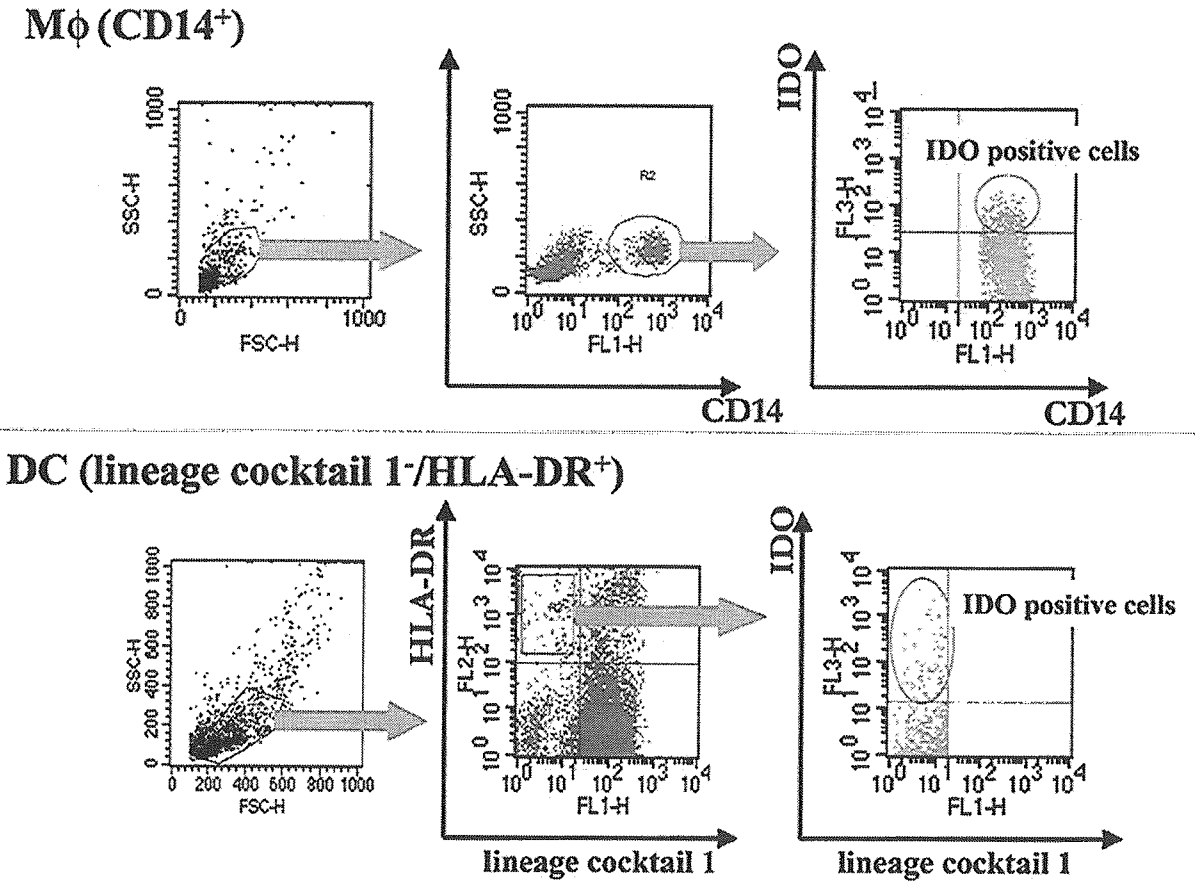

Figure 1. Indoleamine 2,3-dioxygenase (IDO)-positive cells in $CD14^+$ monocytes and lineage $markers^-$/$HLA\text{-}DR^+$ dendritic cells (DCs) analysed by flow cytometry.

B7 molecule expression by flow cytometry

Isolated mononuclear cells were stained with FITC-conjugated anti-CD14 mAb or FITC-conjugated lineage cocktail mAbs and APC-conjugated anti-HLA-DR mAb (Becton Dickinson). Then, to stain B7 molecules, cells were also stained with a PE-conjugated anti-CD80 mAb (Becton Dickinson) and biotin-labelled anti-CD86 mAb (Becton Dickinson), followed by streptavidin-PC5.

Statistical analysis

The data were analysed by Friedmann test for paired samples or Mann–Whitney *U*-test for unpaired samples. $P < 0.05$ was considered significant.

Results

IDO expression in peripheral blood and decidual monocytes and DCs

First, we examined the dose dependency of IFN-γ or CTLA-4/Fc in the expression of IDO on peripheral blood monocytes and DCs of normal early pregnant women ($n = 3$). Treatment with IFN-γ or CTLA-4/Fc both increased IDO expression dose dependently and IDO expression reached a plateau at 1000 U/ml of IFN-γ and 10 μg/ml of CTLA-4/Fc (Figure 2). From these results, we decided to use 1000 U/ml of IFN-γ and 10 μg/ml of CTLA-4/Fc.

As shown in Figure 3, under non-stimulated conditions, the expression of IDO in peripheral blood monocytes and DCs of non-pregnant subjects was very low. The expression rates in both peripheral blood monocytes and DCs of normal pregnancy subjects were significantly higher ($P = 0.0077$ and $P = 0.0077$, respectively) than those of non-pregnant subjects. Both IFN-γ and CTLA-4/Fc treatment enhanced the population of IDO-expressing peripheral blood monocytes and DCs of non-pregnant subjects. IFN-γ induced higher IDO expression in monocytes compared with that by CTLA-4/Fc, and CTLA-4/Fc induced higher IDO expression in DCs compared with that by IFN-γ. In spontaneous abortion cases, IDO expression of monocytes with IFN-γ treatment was significantly lower ($P = 0.0139$) compared with that in normal pregnancy subjects. CTLA-4/Fc-induced IDO expression in peripheral blood DCs of spontaneous abortion cases was significantly lower ($P = 0.0192$) than that in normal pregnancy subjects.

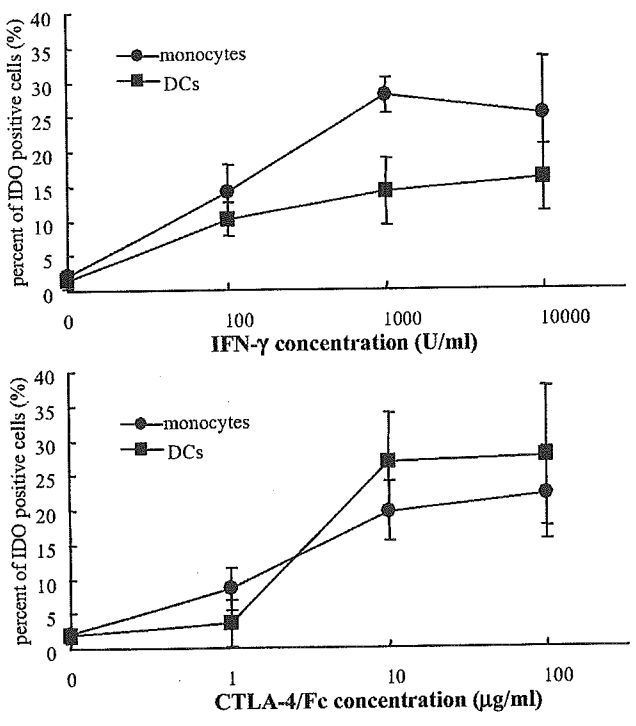

Figure 2. Indoleamine 2,3-dioxygenase (IDO) expression in monocytes and dendritic cells (DCs) stimulated by a range of doses of interferon gamma (IFN-γ) or CTLA-4/Fc. Peripheral blood mononuclear cells were treated for 24 h with IFN-γ (at concentrations of 0, 100, 1000 and 10 000 U/ml) or CTLA-4/Fc (at concentrations of 0, 1, 10 and 100 μg/ml). The percent of IDO-positive cells of monocytes or DCs were analysed by flow cytometry.

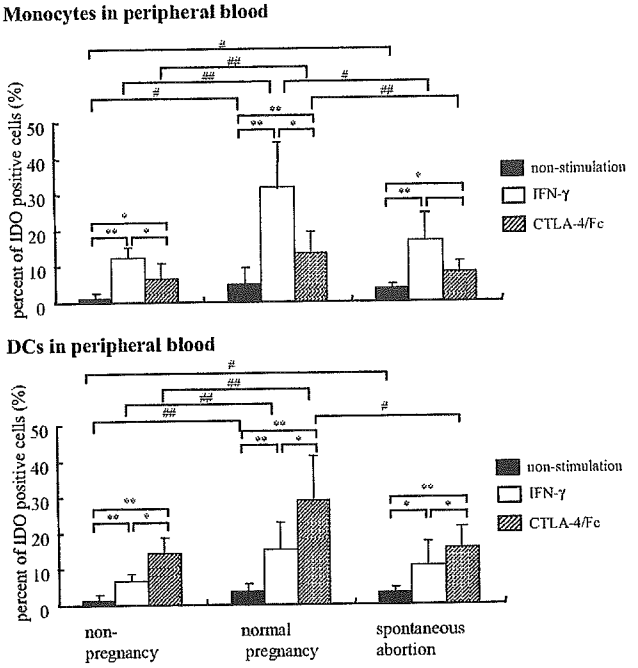

Figure 3. Comparison of indoleamine 2,3-dioxygenase (IDO) expression between normal pregnancy and spontaneous abortion in peripheral blood monocytes and dendritic cells (DCs). *, $P < 0.05$ and **, $P < 0.01$, respectively, when the data were analysed by Friedmann test. #, $P < 0.05$ and ##, $P < 0.01$, respectively, when the data were analysed by Mann–Whitney *U*-test.

IDO expression in non-stimulated decidual monocytes and DCs of spontaneous abortion cases were significantly lower ($P = 0.0002$ and $P = 0.0029$, respectively) than those in normal pregnant women (Figure 4). IDO expression in both decidual monocytes and DCs were significantly elevated by both IFN-γ and CTLA-4/Fc treatments. IFN-γ mainly augmented the expression of IDO in monocytes, whereas CTLA-4/Fc mainly augmented the expression in DCs. The response levels of decidual monocytes in spontaneous abortion cases with both IFN-γ and CTLA-4/Fc treatments were significantly lower ($P = 0.0028$ and $P = 0.010$, respectively) compared with those in normal pregnant subjects. The response levels of decidual DCs in spontaneous abortion cases by CTLA-4/Fc treatment were significantly lower ($P = 0.0032$) compared with those in normal pregnant subjects.

IFN-γ production induced by CTLA-4/Fc

It has been reported that CTLA-4 induces IDO expression in DC through the induction of IFN-γ production (Grohmann *et al.*, 2002; Munn *et al.*, 2004). To investigate whether IDO expression by CTLA-4/Fc was through IFN-γ production, we checked the IFN-γ secretion by mononuclear cells treated by CTLA-4/Fc for 24 h.

As shown in Figures 5 and 6, CTLA-4/Fc induced IFN-γ secretion by both peripheral blood mononuclear cells and decidual leucocytes.

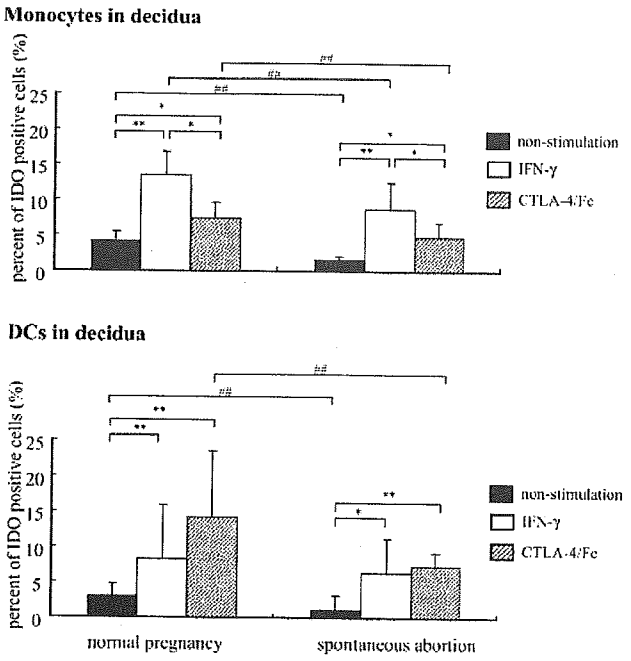

Figure 4. Comparison of indoleamine 2,3-dioxygenase (IDO) expression between normal pregnancy and spontaneous abortion in decidual monocytes and dendritic cells (DCs). *, $P < 0.05$ and **, $P < 0.01$, respectively, when the data were analysed by Friedmann test. #, $P < 0.05$ and ##, $P < 0.01$, respectively, when the data were analysed by Mann–Whitney *U*-test.

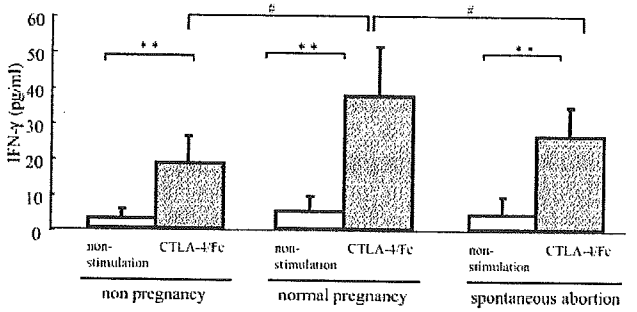

Figure 5. Interferon gamma (IFN-γ) concentration in the supernatant of peripheral blood mononuclear cells stimulated by CTLA-4/Fc. **, $P < 0.01$ when the data were analysed by Friedmann test. #, $P < 0.05$ when the data were analysed by Mann–Whitney *U*-test.

CTLA-4/Fc-induced IFN-γ secretion by peripheral blood mononuclear cells in normal pregnancy subjects was significantly higher compared with those in non-pregnancy subjects (37.8 ± 13.8 pg/ml versus 18.0 ± 7.6 pg/ml, $P = 0.0126$). IFN-γ levels in spontaneous abortion cases were significantly lower compared with those in normal pregnancy subjects in both peripheral blood and decidua (26.0 ± 8.2 pg/ml versus 37.8 ± 13.8 pg/ml; $P = 0.048$ and 307.9 ± 237.6 versus 663.9 ± 277.4; $P = 0.0052$, respectively).

The expression of B7 molecules in peripheral blood and decidual monocytes and DCs

It has been reported that engagement of B7-1/B7-2 (CD80/CD86) molecules on monocytes and DCs by CTLA-4/Fc activates a signalling pathway leading to the induction of IDO (Fallarino *et al.*, 2003; Munn *et al.*, 2004). We investigated whether the expression of IDO correlated with the expression of B7 molecules in peripheral and decidual monocytes and DCs.

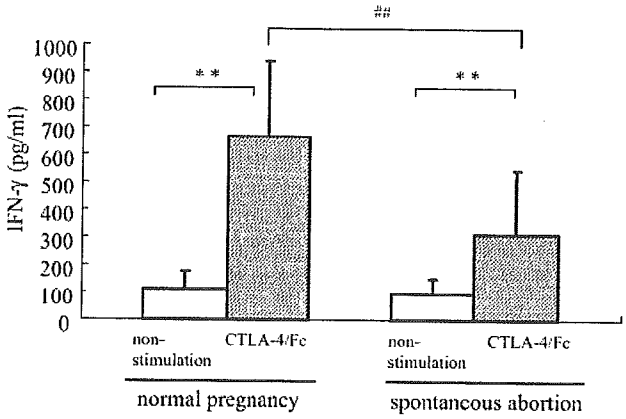

Figure 6. Interferon gamma (IFN-γ) concentration in the supernatant of decidual leucoctyes stimulated by CTLA-4/Fc. **, $P < 0.01$ when the data were analysed by Friedmann test. ##, $P < 0.01$ when the data were analysed by Mann–Whitney *U*-test.

As summarized in Table I, CD86 expression on peripheral blood monocytes and DCs in normal pregnancy subjects was significantly higher than those in non-pregnant subjects. CD86 expressions on peripheral blood monocytes, decidual monocytes and decidual DCs in spontaneous abortion cases were significantly lower compared with those in normal pregnancy subjects. On the other hand, CD80 expression on both peripheral blood and decidual monocytes and DCs in normal pregnancy subjects was the same as those in spontaneous abortion cases.

Discussion

During pregnancy, the fetus is prevented from maternal immune rejection. Several specialized mechanisms have evolved to protect the semi-allograftic fetus from maternal immune attack. Studies in mice have suggested that the fetus is protected from immune rejection by maternal T cells by means of IDO-dependent depletion of tryptophan (Munn *et al.*, 1998). Recent data suggest that $CD4^+CD25^+$ Treg cells expand during pregnancy (Aluvihare *et al.*, 2004; Heikkinen *et al.*, 2004; Sasaki *et al.*, 2004; Somerset *et al.*, 2004; Zenclussen *et al.*, 2005), and Treg cells mediate maternal tolerance to the fetus (Aluvihare *et al.*, 2004). Treg cells constitutively express CTLA-4, and recently it was proposed that CTLA-4 induces IFN-γ production by DCs, which leads to the expression of IDO in DCs (Fallarino *et al.*, 2003; Munn *et al.*, 2004). Thus, the two mechanisms that induce maternal tolerance to the fetus have been linked.

We studied IDO expression on decidual and peripheral blood DCs and monocytes by CTLA-4/Fc using flow cytometry. CTLA-4/Fc treatment induced IDO expression in both decidual and peripheral blood DC and monocytes. The amplitude of IDO expression in DCs by CTLA-4/Fc was rather high compared with monocytes. On the other hand, the amplitude of IDO expression by IFN-γ in monocytes was rather high compared with DCs. Heikkinen *et al.* (2004) reported that IFN-γ induced the expression of IDO mRNA in peripheral blood $CD14^+$ monocytes from pregnant women, but CTLA-4/Fc did not induce IDO mRNA in peripheral blood monocytes. Their data are inconsistent with our data. Our data showed that CTLA-4/Fc induced IDO expression in both peripheral blood and decidual $CD14^+$ monocytes, although the expression rate of IDO was rather low compared to

Table I. Comparison of the expression of B7 molecules between normal pregnancy and spontaneous abortion

	Monocytes [Mean ± SD (%)]			Dendritic cells [Mean ± SD (%)]		
	Non-pregnancy (n = 10)	Normal pregnancy (n = 10)	Spontaneous abortion (n = 8)	Non-pregnancy (n = 10)	Normal pregnancy (n = 10)	Spontaneous abortion (n = 8)
Peripheral blood						
CD80	2.35 ± 2.32	3.72 ± 4.30	4.11 ± 5.10	5.67 ± 1.83	5.38 ± 2.25	3.64 ± 3.36
CD86	33.40 ± 12.88*	60.75 ± 26.54	35.45 ± 14.08*	8.55 ± 2.23*	14.15 ± 5.70	10.80 ± 8.70
Decidua						
CD80	Not tested	7.06 ± 5.81	7.17 ± 6.03	Not tested	4.14 ± 1.68	5.26 ± 2.42
CD86	Not tested	54.29 ± 21.88	19.07 ± 9.60†	Not tested	17.83 ± 4.92	8.20 ± 4.72†

The data were analysed by Mann–Whitney U-test.
*$P < 0.05$ versus normal pregnancy.
†$P < 0.01$ versus normal pregnancy.

that with IFN-γ treatment. Post-transcriptional regulation of IDO may induce IDO protein expression in monocytes with CTLA-4/Fc treatment.

Binding of CTLA-4 and CD80/86 (B7 complex), followed by IFN-γ production by DCs, is required for the induction of IDO by Treg cells (Fallarino *et al.*, 2003; Munn *et al.*, 2004). These data showed that CD86 expression on peripheral blood monocytes and DCs and decidual DCs of spontaneous abortion cases was significantly decreased compared with normal pregnant subjects. These findings might explain why the CTLA-4/Fc-mediated IFN-γ secretion by decidual mononuclear cells in spontaneous abortion cases was significantly lower than that in normal pregnancy subjects (Figure 6). To clarify the mechanism of IDO expression in DCs and monocytes by CTLA-4/Fc, we examined the IFN-γ secretion level by CTLA-4/Fc-stimulated peripheral blood mononuclear cells and decidual mononuclear cells. The results showed that IFN-γ production by peripheral and decidual mononuclear cells after CTLA-4/Fc treatment in spontaneous abortion cases was significantly lower than those in normal pregnancy subjects, suggesting that IFN-γ production by the binding of CTLA-4 on Treg cells and CD80/86 expressing DC and monocytes could induce IDO expression in normal pregnancy subjects. However, in spontaneous abortion cases, suppressed IFN-γ production by the binding of CTLA-4 and decreased CD80/86 expressing DC and monocytes might reduce the IDO expression in DC and monocytes. It is well known that cytokine profiles in decidual T cells are in the Th2-dominant state (Piccinni *et al.*, 1998; Saito *et al.*, 1998; Tsuda *et al.*, 2002; Kwak-Kim *et al.*, 2003; Michimata *et al.*, 2003), so the finding that CTLA-4/Fc treatment induces a large amount of IFN-γ secretion by decidual mononuclear cells is interesting. Decidual mononuclear cells contain IFN-γ-producing NK cells, monocytes, T cells, NKT cells and DCs. Further study is needed to clarify which cells in the decidua produce IFN-γ after CTLA-4/Fc treatment.

Another important finding is that IDO expression in unstimulated decidual DCs and monocytes was up-regulated compared with those in peripheral blood. Human pregnancy decidua contains an abundance of Treg cells which express CTLA-4 at a high level (Heikkinen *et al.*, 2004; Sasaki *et al.*, 2004). These findings suggest that surface CTLA-4 on Treg in the decidua induces IDO expression in decidual DCs and monocytes preventing the maternal lymphocyte activation against the fetal allograft. It has been reported that the population of decidual $CD4^+CD25^+$ Treg cells and the surface CTLA expression on Treg cells are significantly lower in decidua from spontaneous abortion compared with those from specimens from induced abortion (Sasaki *et al.*, 2004). In this study, IDO expressions in both decidual DCs and monocytes of spontaneous abortion cases were suppressed compared with those of normal pregnant subjects. Decreased CTLA-4-expressing Treg cells and suppressed IFN-γ production by CTLA-4 in the decidua of spontaneous abortion cases might induce the low expression of IDO in DCs and monocytes. Munn *et al.* (2002, 2004) reported that specific populations of DCs only express IDO with IFN-γ treatment. Specific populations of DCs and monocytes which could express the IDO enzyme with IFN-γ or CTLA-4-/Fc treatment might increase in the pregnancy decidua, and the population of these DCs and monocytes might decrease in spontaneous abortion.

In this study, we studied the expression of IDO as determined by flow cytometry. We should check whether these results are correlated with IDO activity or not. We studied the concentration of kynurenine, the major IDO degradation byproduct [using high-performance liquid chromatography (HPLC) analysis], in the conditioned media following *in vitro* culture of the peripheral blood mononuclear cells and decidual leukocytes. IDO-mediated tryptophan degradation in both peripheral and decidual leukocytes were up-regulated during pregnancy. On the other hand, both kynurenine concentration after IFN-γ treatment or CTLA-4 treatment were decreased in spontaneous abortion cases suggesting that IDO expression determined by flow cytometry is well correlated with the IDO enzyme activity.

In conclusion, our data showed that IDO expression in decidual monocytes and DCs by CTLA-4/Fc or IFN-γ treatment were increased in therapeutic abortion decidua but were decreased in spontaneous abortion. CTLA-4 on Treg cells might play a role in the maintenance of pregnancy by the induction of IDO in DCs and monocytes.

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PTEN and p53 abnormalities are indicative and predictive factors for endometrial carcinoma

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Abstract. PTEN (phosphatase and tensin homologue deleted on chromosome 10) and p53 alterations were expected to be diversely involved in endometrial carcinogenesis. Patients (n=92) with endometrial carcinoma (EC) were analyzed, and PTEN and p53 were immunostained in the tissue sections. Tumor histology, grade of differentiation, presence of endometrial hyperplasia, staining status of PTEN and p53 and clinical information were examined. There were 37 cases (40%) negative for PTEN staining, which suggests lost or reduced PTEN function. Loss of PTEN staining was significantly related to the advanced staging in the grade 1 (G1) and grade 2 (G2) endometrioid adenocarcinoma group (p=0.026). Also, 18 cases (20%) showed positive staining for p53. p53 staining was largely found in grade 3 (G3) endometrioid adenocarcinoma and other phenotypes of EC. In the G1 and G2 group, all 29 cases with reduced PTEN staining showed p53-negative staining (p=0.025). In the G3 and others group, 6 of 8 cases with reduced PTEN staining showed p53-positive staining. p53-positive staining was associated with a high probability of tumor recurrence in the G1 and G2 group (p=0.0234). In contrast, in the G3 and others group, p53-positive cases had a low probability of tumor recurrence (p=0.0473). Both PTEN and p53 staining may be good indicators of clinical stage and probability of tumor recurrence in EC. Reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at the a late phase of carcinogenesis. Thus, immunohistochemistry for PTEN and p53 in biopsy specimens of EC can provide supportive information for determining a treatment plan.

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Introduction

Uterine endometrial carcinoma is the fourth most frequent malignancy in females (1). Several genetic abnormalities were reported in endometrial carcinoma (2). Mutation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was one of the molecular abnormalities in endometrial carcinoma. A K-*ras* mutation was reported to be approximately 10-30% in endometrial carcinoma (3). A frequency of mutations in the ß-catenin gene was shown to be 14-44% in endometrial carcinoma (4). p53 abnormality, the most critical event leading to cancer in general, was also observed in endometrial carcinoma at 10-25% (5-7). It was reported that insulin-like growth factors (IGFs) played a role in mediating estrogen-induced endometrial proliferation, and therefore IGF signaling was a risk factor for endometrial carcinoma (8,9).

The hyperplasia-carcinoma sequence has been suggested in endometrial carcinogenesis. In accordance with the general classification of endometrial cancers, tumors with endometrial hyperplasia were categorized as type I, which mostly contains grade 1 (G1) and grade 2 (G2) endometrioid adenocarcinoma. Tumors without endometrial hyperplasia were categorized as type II, which contains mostly grade 3 (G3) endometrioid adenocarcinoma and other histological types, such as adenosquamous carcinoma, and serous, clear cell and mucinous adenocarcinoma (10). Type I tumors are known to be caused by excess hormonal stimulants, such as estrogen and/or progesterone relatives (11,12). Type II tumors are generally recognized as developing from atrophic endometrial tissue in older women and are independent of hormonal stimulation (11,13,14). Risk factor(s) for type II tumors remain unknown. Type I tumors are associated with mutations in the K-*ras* as well as the PTEN gene (11). They often have microsatellite instability, but do not usually possess mutations in the p53 gene (11). In contrast, type II tumors mostly have p53 mutations, but seldom have microsatellite instability or K-*ras* or PTEN mutations (11).

PTEN was first identified as a tumor suppressor gene located in 10q23, and the mutations were widely distributed in cancers ranging from brain to prostate (15). It was soon revealed that PTEN was responsible for Cowden's disease, a cancer predisposition syndrome (16). Although PTEN mutations were found predominantly in advanced cancers in

Table I. Clinicopathological characteristics of patients with endometrial carcinoma.

Characteristics	No. of patients	Type I[a]	Type II[a]	p-value
Total analyzed	92	49	43	
Histology group[b]				0.043
G1 and G2	67	40	27	
G3 and others	25	9	16	
FIGO[c] stage				0.019
I	56	37	19	
II	12	5	7	
III	20	6	14	
IV	4	1	3	
Age (years)				0.046
<60	61	37	24	
≥60	31	12	19	
Menopause				<0.001
(-)	30	24	6	
(+)	62	25	37	
Pregnancy				0.964
(-)	13	7	6	
(+)	79	42	37	
Prognosis				0.566
Dead	7	3	4	
Alive	85	46	39	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II: endometrial carcinoma without endometrial hyperplasia. [b]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma. [c]International Federation of Gynecology and Obstetrics.

Table II. Relationship between histological characteristics and clinical stage.

Histological characteristics	FIGO[a] stage I	II	III	IV	p-value
Differentiation[b]					0.085
G1	24	4	4	0	
G2	23	5	6	1	
G3	5	1	6	2	
Adenosquamous	3	2	4	0	
Serous	1	0	0	1	
Histology group[c]					0.004
G1 and G2	47	9	10	1	
G3 and others	9	3	10	3	

[a]International Federation of Gynecology and Obstetrics. [b]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [c]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others: endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

general, it was reported that mutations occurred as an early event in endometrial carcinogenesis (2,17-19). Endometrial hyperplasia as well as endometrial carcinoma have been shown to have PTEN mutations in 20-30% and 30-80% of cases, respectively (2,17-23). The significance of PTEN mutations in endometrial carcinoma is interpreted in two opposite and conflicting ways. One interpretation is that PTEN alterations are related to a better prognosis (24,25), and the other is that the mutations result in a poor survival rate (22,23,26). Thus, clinical significance of the PTEN abnormality in endometrial carcinoma is not fully understood.

The tumor suppressor protein p53 plays an important role in mediating a response to stress, such as that induced by DNA damage or hyperproliferative signals resulting in either growth arrest or apoptosis (27,28). It was reported that a p53 abnormality relates to a later stage in endometrial carcinogenesis (24,25,29,30). Singh *et al* reported that simultaneous abnormality in PTEN and p53 were rare in head and neck squamous cell carcinoma (31). They also suggested that activation of phosphatidylinositol-3-kinase (PI3K) and mutation of p53 were mutually exclusive events, and either event is able to promote a malignant phenotype of the tumor (31). However, in the endometrial carcinoma, the examination of abnormalities in both PTEN and p53 pathways at the same time, using clinical materials, has not previously been performed.

In this study, we investigated abnormalities of PTEN and p53 in human endometrial carcinoma by immunohistochemistry, and examined the relationship of the abnormality of PTEN with that of p53 in endometrial carcinoma. Moreover, we analyzed the clinical significance of PTEN and p53 abnormalities in endometrial carcinoma.

Materials and methods

Cases and tissue samples. Tissue specimens of 92 patients who underwent surgery for endometrial carcinoma at Dokkyo University School of Medicine were analyzed. The clinical stage of the cancer progression was estimated according to the International Federation of Gynecology and Obstetrics (FIGO) 1988 criteria (32). Surgically-resected tissues were used for hematoxylin and eosin staining. Histological diagnosis, differentiated grade, depth of cancer invasion, and presence or absence of hyperplasia of the adjacent endometrium were evaluated based on the Armed Forces Institutes of Pathology (AFIP) classification (10).

Cell culture. Ishikawa cells (3-H-12-No107) were kindly provided by Dr M. Nishida (Kasumigaura National Hospital, Tsuchiura, Ibaraki, Japan) and HEC-1-A cells were purchased from American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Sigma) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 200 mmol/l

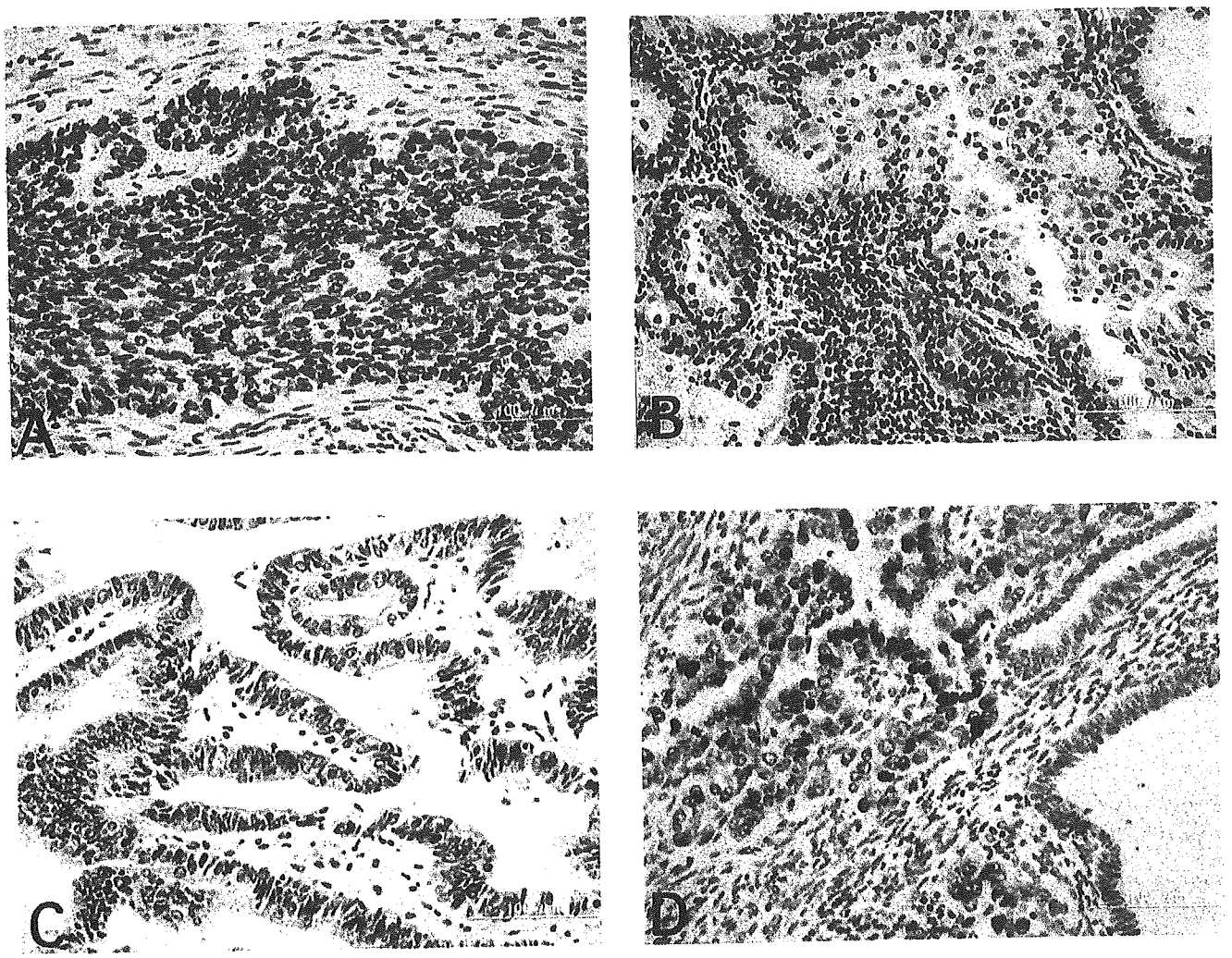

Figure 1. PTEN or p53 expression in endometrial carcinoma. (A) Abundant expression of PTEN. (B) Loss of PTEN expression. (C) Reduced PTEN expression. These three cases represent grade 3 (G3) endometrioid adenocarcinoma (A), G2 endometrioid adenocarcinoma (B), and G1 endometrioid adenocarcinoma (C). (D) p53-positive staining in G2 endometrioid adenocarcinoma; p53 positive-staining in the nuclei of the cancer cells are observed, in contrast to negative staining of the hyperplastic glands (lower right).

L-glutamine, and penicillin/streptomycin at 37°C in 95% air-5% CO_2. For immunohistochemical staining, cell pellets were fixed by 10% neutralized formaldehyde and embedded in paraffin as proceeded for tissue samples.

Immunohistochemical staining. Sections (4 μm-thick) were mounted on poly-L-lysine coated slides and deparaffinized in xylene and rehydrated through a series of graded alcohol. Antigen retrieval was performed for 10 min at 95°C in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Sections were also incubated with Protein Block Serum-Free solution (Dako, Carpenteria, CA) in order to block non-specific staining, according to the manufacturer's protocol. Anti-PTEN antibody (28H6; Novocastra, Balliol Business Park West, UK) and anti-p53 antibody (CM1; Novocastra) were used at 1:100 dilution for 60 min at room temperature, respectively. As a negative control, pre-immune serum was used instead of the specific antibodies to verify the specificity. The sections were washed with phosphate-buffered saline (PBS) 3 times each for 5 min. The Dako LSAB2 kit was used, based on the manufacturer's protocol (Dako), followed by PBS washing 3 times. Visualization was performed by immersing 3-3'-diaminobenzidine in chromogen substrate for 1 min. The stained slides were counterstained with hematoxylin and cover-slipped with EUKITT (O. Kindler, Freiburg, Germany).

Evaluation of the immunohistochemical staining. The status of PTEN staining was evaluated based on the staining intensity and distribution. Intensity was judged as strong, moderate, or weak. Distribution was scored as diffuse (<50% tumor staining), regional (15-50% tumor staining), and focal (<15% tumor staining). Tumors with intense to diffuse, intense to regional, intense to focal, and moderate to diffuse staining were considered positive for PTEN expression. Whereas tumors with moderate to regional, moderate to focal, or weak staining with any distribution were considered negative for PTEN expression.

For p53 staining, cases were defined as positive when >10% tumor cells showed strong nuclear staining.

Statistical analysis. Using Pearson's Chi-square test, the abnormalities of PTEN and/or p53 were assessed for their

Table III. Implication of the abnormality of PTEN with clinicopathological characteristics of the patients.

Clinicopathological characteristics	No. of patients	Expression of PTEN		p-value
		Reduced	Normal	
Total analyzed	92	37	55	
Tumor type[a]				0.114
Type I	49	16	33	
Type II	43	21	22	
Age (years)				0.255
<60	61	22	39	
≥60	31	15	16	
FIGO[b] stage				0.206
I	56	19	37	
II	12	8	4	
III	20	8	12	
IV	4	2	2	
Differentiation[c]				0.151
G1	32	13	19	
G2	35	16	19	
G3	14	2	12	
Adenosquamous	9	4	5	
Serous	2	2	0	
Histology group[d]				0.326
G1 and G2	67	29	38	
G3 and others	25	8	17	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II: endometrial carcinoma without endometrial hyperplasia. [b]International Federation of Gynecology and Obstetrics. [c]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [d]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

association with different clinical and pathologic parameters including clinical stage, tumor histological grade, and recurrence-free probability (RFP). The RFP was estimated using the Kaplan-Meier method, and compared using the log-rank test. All statistical analyses were performed using SPSS II software (version 11.0.1 for Windows; SPSS, Inc., Chicago, IL). $p<0.05$ was considered statistically significant.

Results

Clinicopathological characteristics of the patients. A total of 92 patients with endometrial carcinoma (age range, 31-82; mean age, 57) were examined. In our study, there were 49 cases of type I tumors, and 43 cases of type II tumors. Type I was largely composed of G1 and G2 endometrioid adenocarcinoma, and type II composed of G3 endometrioid adenocarcinoma and others (p=0.043, Table I). Type II tumors were found mostly in advanced clinical stages, and type I tumors were less advanced (p=0.019, Table I). Type I tumors occurred predominantly in young women with menstrual cycles (p=0.046 and p<0.001, respectively, Table I). Degree of cancer differentiation had a tendency towards a progressed clinical stage, although its relationship did not reach a significant level (p=0.085, Table II). However, when combining the G1 and G2 groups, and the G3 and others groups, the latter histological group was frequently found in an advanced clinical stage of the disease (p=0.004, Table II).

Table IV. Implication of the abnormality of PTEN with clinicopathological characteristics in the G1 and G2 histology group.

Clinicopathological characteristics	No. of patients	Expression of PTEN		p-value
		Reduced	Normal	
FIGO[a] stage				0.026
I	47	15	32	
II	9	7	2	
III	10	6	4	
IV	1	1	0	
Total	67	29	38	

[a]International Federation of Gynecology and Obstetrics.

Implication of the abnormality of PTEN with clinicopathological characteristics of the patients. We first tested the specificity of the 28H6 antibody for staining PTEN protein in formalin-fixed, paraffin-embedded samples. Ishikawa cells are reported to have two-point mutations in the PTEN gene, and both mutations produced the stop codon (33). On the other hand, HEC-1-A cells are reported to have the wild-type PTEN gene (33). The 28H6 antibody showed a negative result for Ishikawa cells and a positive result for HEC-1-A cells (data not shown). Therefore, we used the 28H6 antibody for further immunohistochemical study.

As shown in Fig. 1A, there were cancer cells possessing abundant PTEN expression in the nuclei. In contrast, there were cells in which staining for PTEN was dramatically reduced (Fig. 1B) or moderately decreased (Fig. 1C). After evaluating the staining status according to its area and intensity (see Materials and methods), 37 cases (40%) were judged as negative for PTEN, which suggests lost or reduced PTEN function in the cells. No significant relationship was observed between PTEN abnormalities and endometrial hyperplasia, age, clinical stage, or histology and degree of cancer differentiation (Table III). Moreover, the expression of PTEN, in other words PTEN function, was not related to the histological group, G1 and G2, and G3 and others (Table III). However, PTEN expression was significantly reduced in the G1 and G2 group at an advanced stage (p=0.026, Table IV).

Table V. Implication of the abnormality of p53 with clinicopathological characteristics of the patients.

Clinicopathological characteristics	No. of patients	Nuclear accumulation p53		p-value
		Positive	Negative	
Total analyzed	92	18	74	
Tumor type[a]				0.403
Type I	49	8	41	
Type II	43	10	33	
Age (years)				0.103
<60	61	9	52	
≥60	31	9	22	
FIGO[b] stage				0.345
I	56	11	45	
II	12	1	11	
III	20	4	16	
IV	4	2	2	
Differentiation[c]				<0.001
G1	32	2	30	
G2	35	4	31	
G3	14	5	9	
Adenosquamous	9	5	4	
Serous	2	2	0	
Histology group[d]				<0.001
G1 and G2	67	6	61	
G3 and others	25	12	13	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II, endometrial carcinoma without endometrial hyperplasia. [b]International Federation of Gynecology and Obstetrics. [c]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [d]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

Table VI. Implication of the abnormality of p53 with clinicopathological characteristics in the G1 and G2 histology group.

Clinicopathological characteristics	No. of patients	Nuclear accumulation p53		p-value
		Positive	Negative	
FIGO[a] stage				0.423
I	47	6	41	
II	9	0	9	
III	10	0	10	
IV	1	0	1	
Total	67	6	61	

[a]International Federation of Gynecology and Obstetrics.

Table VII. Relationship between PTEN and p53 staining.

	Nuclear accumulation p53		p-value
	Positive	Negative	
Expression of PTEN in total cases (92 cases)			0.507
Reduced	6	31	
Normal	12	43	
Expression of PTEN in the G1 and G2 group[a] (67 cases)			0.025
Reduced	0	29	
Normal	6	32	
Expression of PTEN in the G3 and others group[a] (25 cases)			0.064
Reduced	6	2	
Normal	6	11	

[a]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

Implication of the abnormality of p53 with clinicopathological characteristics of the patients. As shown in Fig. 1D, nuclear p53 staining was confirmed in the cancer cells. There were 18 cases (20%) that showed positive staining for p53, while the remaining 74 cases (80%) were negative. There was no significant relationship between p53-positive staining and the presence or absence of endometrial hyperplasia, age distribution, or clinical stage (Table V). On the other hand, p53-positive staining was largely found in the G3 and others group ($p<0.001$, Table V). In contrast, there was no relationship between p53-positive staining and clinical stage in the G1 and G2 group ($p=0.423$, Table VI).

Relationship of the PTEN abnormality with the p53 abnormality. There was no significant relationship between the PTEN and p53 staining patterns in 92 cases of endometrial carcinoma ($p=0.507$, Table VII). However, in G1 and G2 group, there was significant relationship between the PTEN and p53 staining patterns ($p=0.025$, Table VII). All 29 cases with reduced PTEN staining pattern showed p53-negative staining (Table VII). All 6 cases with p53-positive staining pattern showed reduced PTEN staining (Table VII). In the G3 and others group, 6 of 8 cases with reduced PTEN staining pattern showed p53-positive staining, although it did not reach a significant level ($p=0.064$, Table VII).

Recurrence-free probability (RFP). In the G1 and G2 group, PTEN abnormality was not associated with tumor recurrence

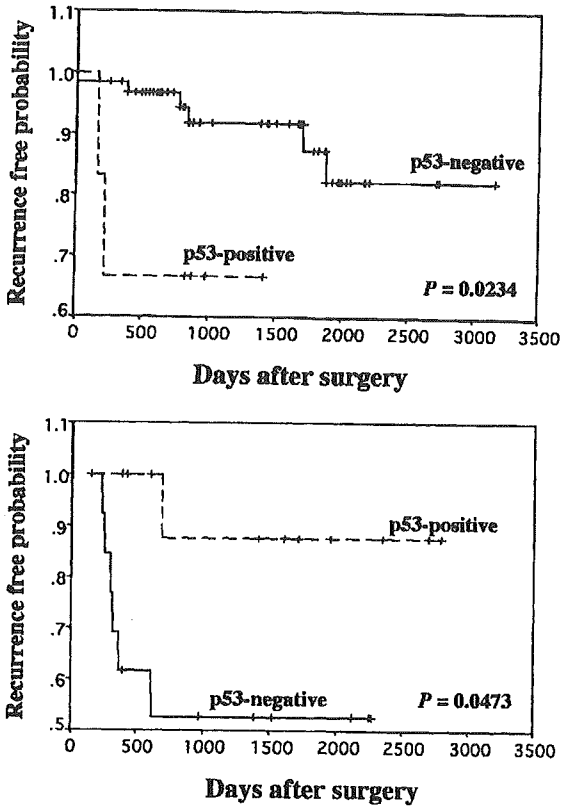

Figure 2. (A) Recurrence-free probability (RFP) for patients who had G1 and G2 endometrioid adenocarcinoma (G1 and G2 group) with or without a p53 abnormality. (B) RFP for patients who had G3 endometrioid adenocarcinoma and other phenotypes (G3 and others group) with or without a p53 abnormality.

(p=0.3149, data not shown), whereas patients with p53-positive staining showed a lower RFP than those without p53 staining (p=0.0234, Fig. 2A). In contrast, in the G3 and others group, patients with p53-positive staining had a higher RFP than those without p53 staining (p=0.0473, Fig. 2B). When we compared the PTEN abnormality or p53 abnormality and RFP in all histological types, no significant relationship was observed (data not shown).

Discussion

In this study, we demonstrated that loss of PTEN staining was significantly related to the advancement of disease staging in the G1 and G2 group, in which most tumors were categorized as type I tumors. Moreover, p53-positive staining was largely observed in the G3 and others group, in which most tumors were categorized as type II tumors. Furthermore, in endometrial carcinogenesis, reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at a late phase of carcinogenesis. p53-positive staining was associated with a high probability of tumor recurrence in the G1 and G2 group. In contrast, in the G3 and others group, cases with p53-positive staining had a lower probability of recurrence than those without p53 abnormalities.

In this experimental condition, PTEN was stained in the nucleus. PTEN does not have a nuclear localization signal (15), however several studies concerning PTEN immunohistochemistry showed nuclear localization of the PTEN protein (34-36). Although it was reported that phosphorylation of the PTEN protein would decrease PTEN activity and the phosphorylated-PTEN localized in the nucleus, most of the investigators evaluated the nuclear staining of PTEN as evidence of the normal function of PTEN protein. Gimm *et al* (37) reported that their monoclonal antibody 6H2.1 specifically recognized the 55 kDa protein only in cells with a normal PTEN gene, and the monoclonal antibody detected nuclear localization of the PTEN protein in several cells. They also confirmed that an absorption test using PTEN peptides completely abolished immunostaining with this antibody (37). Although an antibody we used was different from that of Gimm *et al*, clear nuclear staining of PTEN appeared to reflect the normal function of the PTEN protein.

The status of PTEN staining was evaluated based on the staining intensity and distribution. Tumors with intense to diffuse, intense to regional, intense to focal, and moderate to diffuse staining were considered positive for PTEN expression (normal PTEN function). Whereas tumors with moderate to regional, moderate to focal, or weak staining with any distribution were considered negative (dysfunction of PTEN: genetic deletion, truncation protein producing mutation, and down-regulation of gene expression). Some of the PTEN-negative cases were confirmed to have a genetic deletion or truncation protein-producing mutation in the PTEN gene (unpublished data).

For p53 staining, cases were defined as positive when >10% tumor cells showed strong nuclear staining. In our previous experiments (38-41), p53-positive staining tumor cells in other organs, such as colon, esophagus, gallbladder, and head and neck were confirmed to have a p53 missense mutation.

It was reported that AKT enhances MDM2-mediated ubiquitilation and degradation of wild-type p53 (42) and, more recently, that PTEN and PI3K inhibitor up-regulate p53 and block tumor-induced angiogenesis in glioma cells (43). Thus, PTEN activity up-regulates the wild-type p53 function via an inhibition of AKT-mediated MDM2 activation. In contrast, Stambolic *et al* (44) have reported that wild-type p53 directly binds to the promoter sequence, and enhances expression of the PTEN gene. Thus, the p53 and PTEN pathways have a cross-talk in their signaling pathway. In head and neck squamous cell carcinoma, Singh *et al* (31) reported that activation of PI3K (down-regulation of PTEN function) and mutation of p53 were mutually-exclusive events.

In our study, there was a significant relationship between PTEN and p53 staining patterns in the G1 and G2 group. Interestingly, all 29 cases with reduced PTEN staining pattern in the G1 and G2 group showed p53-negative staining. Furthermore, all 6 cases with p53-positive staining pattern in the G1 and G2 group showed normal PTEN staining. However, in contrast to the G1 and G2 group, 6 of 8 cases with reduced PTEN staining pattern in the G3 and others group showed p53-positive staining. In endometrial carcinogenesis, reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at a late phase of carcinogenesis.

In the G1 and G2 group, 32 of 67 cases (48%) did not show either PTEN or p53 abnormalities in our experiment. These tumors may have other genetic abnormalities, such as