岩手医科大学
審査学位論文
（博　士）

JIMA Vol. 66, No. 3 (Aug. 2014) pp. 85-100.

Original

Molecular analysis of endometrial endometrioid adenocarcinoma based on estrogen receptor, progesterone receptor, and HER2 expression: a proposal for novel classification of endometrioid adenocarcinomas

Hidetoshi TOMABECHI [1),2)], Tamotsu SUGAI [1)], Yasuko SUGA [1),2)], Takayuki NAGASAWA [2)], Noriyuki UESUGI [1)], Kazuyuki ISHIDA [1)], Satoshi TAKEUCHI [2)] and Toru SUGIYAMA [2)]

[1)] Division of Molecular Diagnostic Pathology, Department of Pathology, School of Medicine, Iwate Medical University, Morioka, Japan

[2)] Department of Obstetrics and Gynecology, School of Medicine, Iwate Medical University, Morioka, Japan

(Received on January 14, 2014 & Accepted on January 22, 2014)

Abstract

Molecular subtype of breast cancer based on estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) expressions plays a crucial role in treatment. Such classification has also been proposed for endometrioid adenocarcinoma (EA), which, like breast cancer, also forms estrogen-dependent tumors. The aim of this study was to examine molecular alterations in EA based on the expression of these biomarkers. Samples from 83 EAs were obtained. Using the crypt isolation method, we classified EAs based on expression of ER, PgR, and HER2. Microsatellite instability (MSI), DNA methylation status, loss of heterozygosity (LOH) and mutations in oncogenes were examined. Types were defined as follows: Type A (ER+ and/or PgR+, HER2-), Type B (ER+ and/or PgR+, HER2+), Type C (ER/PgR-, HER2+), and Type D (ER/PgR-, HER2-). The frequency of *PTEN* expression in Type C was lower than that in other types. *Ki-ras* mutations were more frequent in Type C, and *PIK3CA* mutations were more common in Type A and B. The frequency of LOH-high status in Type C and D was significantly higher than that in Type A and B. Subtypes based on these biomarkers in EA were characterized by each different molecular abnormality.

Key words : endometrioid adenocarcinoma, ER, PgR, HER2, molecular alteration

I. Introduction

Endometrial carcinoma (EC) comprises about 4% of cancers in women worldwide, with a higher incidence in developed countries. The American Cancer Society estimated that endometrial cancer was the fourth most common cancer diagnosed and the eighth leading cause of cancer deaths in women in 2010 [1)]. EC is thought to be one of the most important gynecological cancers.

Bockman first described the two main clinicopathological variants of EC [2)]. Type I tumors are low-grade and estrogen-sensitive endometrioid adenocarcinomas (esEAs) that

usually develop in perimenopausal women and coexist or are preceded by complex and atypical endometrial hyperplasia. In contrast, type II tumors, which mainly occur in older women, are non-endometrioid adenocarcinomas (NEAs), very aggressive, insensitive to estrogen stimulation, and arise occasionally in endometrial polyps or from precancerous lesions developing in atrophic endometrium. NEAs are usually serous or clear cell carcinomas. Moreover, the molecular alterations involved in the development of NEAs are different from those of esEAs. While esEAs show microsatellite instability (MSI) and mutations in the PTEN, k-RAS, PIK3CA, and ß-catenin genes, NEAs exhibit alterations in p53 expression, loss of heterozygosity (LOH) on several chromosomes, and other molecular alterations, including changes in the expression of STK15, p16, E-cadherin, and HER2 [3,4].

Sporadic colorectal cancer is a consequence of the accumulation of genetic and epigenetic alterations that result in the transformation of normal colonic epithelial cells into adenocarcinomas. The loss of genetic stability and subsequent genetic alterations in tumor suppressor genes and oncogenes initiate carcinogenesis and tumor progression. Toyoda et al. have shown that DNA hypermethylation in cancer-related genes plays a major role in colorectal carcinogenesis [5]. The aberrant methylation of promoter regions precedes genetic alterations and epigenetic event that are associated with early-stage disease. Therefore, DNA methylation is closely associated with carcinogenesis. However, it is not clearly known whether a similar mechanism is associated with the development of EC.

Endometrial endometrioid adenocarcinomas (EEAs) are also thought to comprise various tumor types in terms of prognosis, malignancy, and molecular mechanisms. However, the conventional hypothesis for tumorigenesis in endometrial carcinomas is that EEA accounts for many cases collectively classified into type I [2]. Thus, the diversity of EEA is not clearly understood. Like hormone-dependent breast cancers, EEA is known to develop in an estrogen-dominant environment. Hormone-dependent breast tumors have recently been classified into four types based on estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) expression [6], and this classification has revealed associations among histological characteristics, prognosis, malignancy, and therapeutic responsiveness in breast cancers [7-9]. However, few pathological or molecular pathological studies based on the expression of these aforementioned factors have examined EEA. Thus, development of an effective classification system may be important for further understanding of EEA and for developing effective treatment strategies against EEA.

Our aim in this study was to correlate the clinicopathological and molecular pathological findings for EEAs with their ER, PgR, and HER2 expression status in order to propose a comprehensive classification system for EEAs.

II. Materials and Methods

1. Tumor samples

Between April 2004 and September 2012, 83 EEAs were obtained surgically from 83 patients undergoing resection of the uterus at the Iwate Medical University Hospital after obtaining informed consent. The patients

Table 1. Clinicopathological findings for endometrial endometrioid carcinomas in this study

Total (endometrioid carcinoma)	n = 83
Age (means)	31 ~ 82 (57.0)
Grade	
1	44 (53.0)
2	28 (33.7)
3	11 (13.3)
FIGO stage	
I	55 (66.3)
II	8 (9.6)
III	18 (21.7)
IV	2 (2.4)
Lymphovascular invasion	
negative	66 (79.5)
positive	17 (20.5)

(%)

ranged in age from 31 to 82 years (mean, 57.3 years). Patients who underwent radiotherapy and chemotherapy before surgery were not included in the study. The study was approved by the Ethics Committee of Iwate Medical University School of Medicine (reg. no. H24-80), waiving the requirement for informed consent for this study. Tumor histological type and stage were classified according to the General Rules for Clinical and Pathological Management of Uterine Corpus Cancer [10]. Clinicopathological data for the 83 patients in our study are shown in Table 1.

2. Crypt isolation technique for tumor cell isolation

Fresh tumor and normal tissue samples were obtained from surgical specimens removed during surgery for EEA. A sample of normal endometrium was removed from a site distant from the lesion. Crypt isolation from the tumor and normal endometrium was performed as described previously [11, 12]. This method can separate all crypts from intervening stroma. Briefly, fresh endometrium and tumor were minced with a razor then incubated at 37 °C for 30 min in calcium- and magnesium-free Hanks' balanced salt solution (CMF) containing 30 mmol/L ethylene-diaminetetraacetic acid (EDTA). Following this procedure, the tissue was stirred in CMF for 30–40 min. The isolated crypts were immediately fixed in 70% ethanol and stored at 4 °C until DNA extraction. The fixed, isolated crypts were examined under a dissection microscope (SZ60; Olympus, Tokyo, Japan). Normal crypts were clearly distinguishable from tumor crypts based on their characteristic features reported elsewhere [13]. The isolated crypts were processed routinely for histopathological analysis to morphologically confirm they were well isolated. No contamination (such as interstitial cells) was observed in any of the 83 samples. A representative sample is shown in Figure 1.

3. Immunohistochemical procedure

EEA specimens were fixed in buffered formalin and embedded in paraffin according to routine procedures. For this study, 3 μm-thick sections were prepared, dried, deparaffinized, and rehydrated before microwave treatment (H2500, Microwave Processor, Bio-Rad, Hercules, CA, USA) in Tris-(hydroxymethyl) aminomethane-EDTA buffer (pH 9.0) for 30 min. An automatic staining machine (DAKO Envision+ system) was used for the immunohistochemical procedure [14]. The slides were counterstained in hematoxylin, dehydrated, and mounted. For immunohistochemistry (IHC) analysis, samples from isolated crypts and tissue blocks of samples adjacent to samples isolated using the crypt isolation method were examined.

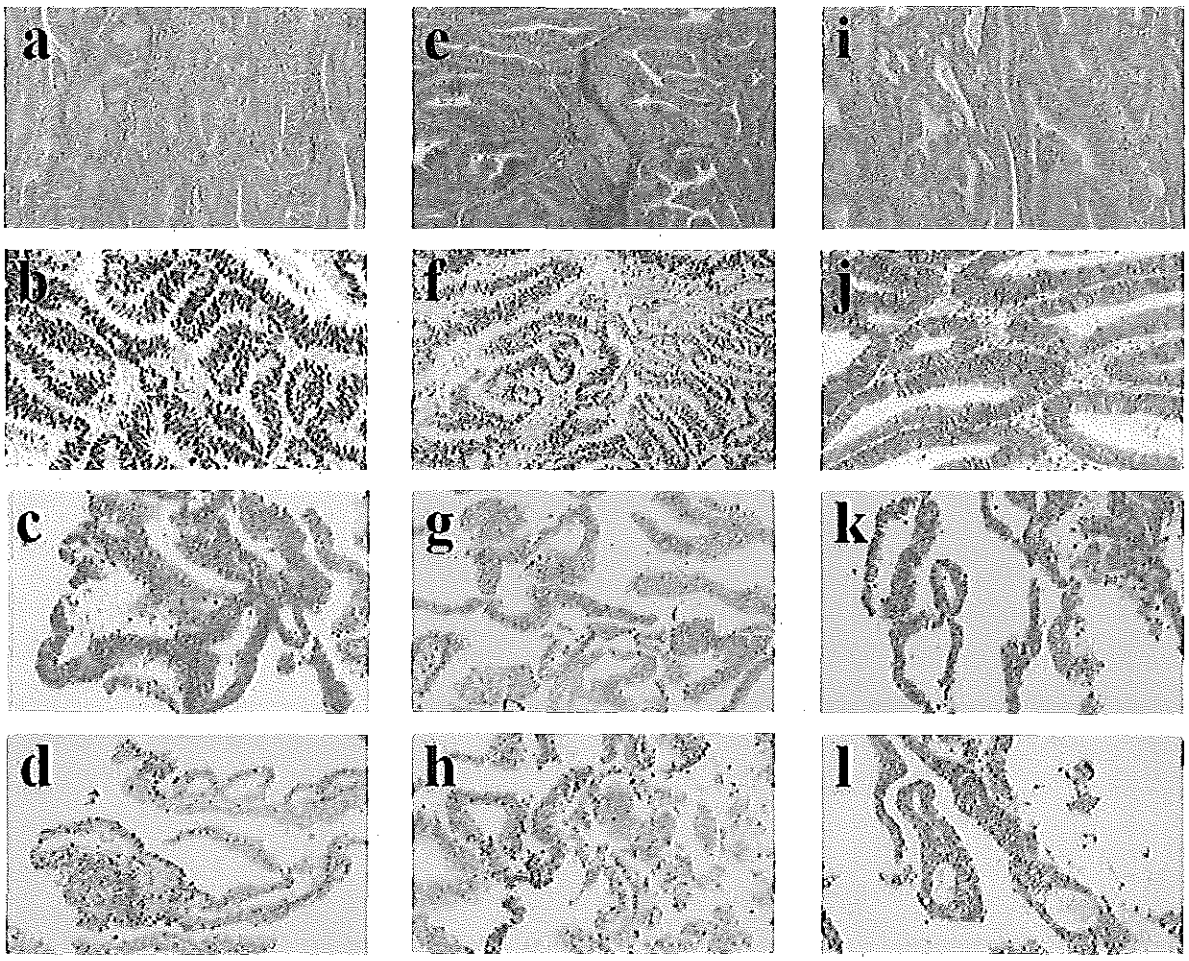

Fig 1. Case 1 (a) Histological section of endometrial endometrioid adenocarcinoma, Grade 1. (H&E, 100 ×). (b) Positive immunohistochemical staining for ER in specimen (100 ×). (c) Histology of crypts (H&E, 200 ×). (d) Positive immunohistochemical staining for ER in crypts (200 ×). Case 2 (e) Histological section of endometrial endometrioid adenocarcinoma, Grade 1. (H&E, 100 ×). (f) Positive immunohistochemical staining for PgR in specimen (100 ×). (g) Histology of crypts (H&E, 200 ×). (h) Positive immunohistochemical staining for PgR in crypts (200 ×). Case 3 (i) Histological section of endometrial endometrioid adenocarcinoma, Grade 1. (H&E, 100 ×). (j) Immunohistochemical staining (score 3) for HER2 (100 ×). (k) Histology of crypts (H&E, 200 ×). (l) Positive immunohistochemical staining for HER2 in crypt (200 ×). The definition of each score is described in the Materials and Methods section.

The antibodies used in this study are shown in Table 2.

4. Immunohistochemical assessment of ER, PgR, and HER2

Immunoreactivity to anti-HER2 antibodies was observed in the cell membrane and was scored semiquantitatively using the Food and Drug Administration (FDA) approved scoring system as: 0, no immunostaining; 1+, incomplete membranous immunostaining of less than 10% of tumor cells; 2+, weak complete membranous immunostaining of greater than 10% of tumor cells; and 3+, strong complete membranous staining of greater than 10% of tumor cells. Scores of 0 or 1+ indicated a negative result, while scores

Table 2. List of antibodies used inthis study

Antibody	Clonarity	Clone	Supplier	Dilution
ER	Monoclonal	1D5	Dako	1 : 2
PgR	Monoclonal	PgR636	Dako	1 : 6
HER2	Polyclonal	–	Dako	1 : 300
p53	Monoclonal	DO-7	Novocastra	1 : 100
PTEN	Monoclonal	6H2.1	Dako	1 : 100

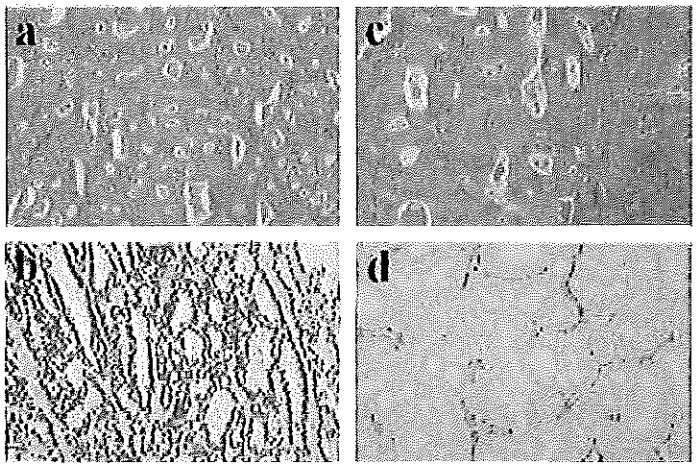

Fig 2. Case 1(a) Endometrial endometrioid adenocarcinoma, Grade 1 (H&E, 100 ×). (b) Positive immunohistochemical staining for p53 (100 ×). Case 2 (c) Endometrial endometrioid adenocarcinoma, Grade 1 (H&E, 200 ×). (d) negative immunohistochemical staining for PTEN (200 ×). The definition of each immunohistochemical staining score is described in the Materials and Methods section.

of 2+ and 3+ were regarded as positive for HER2 expression [15]. ER and PgR expression was quantified based on the percentage of stained cells and the intensity of nuclear staining. The percentage of positive cells was graded as follows: 1 = 0-25% of nuclei stained; 2 = 26-75% of nuclei stained; 3 = more than 76% of nuclei stained. The staining intensity was scored as follows: 1 = absent or weak, 2 = strong, 3 = very strong. The sum of both of the parameters determined the IHC score. IHC scores of 2 or less were considered negative, whereas IHC scores of 3 or more were considered positive [16]. These IHC scores were evaluated independently by 2 experienced pathologists. Representative tumors and crypts are shown in Figure 1.

5. Molecular subtypes of EEA using ER, PgR, and HER2

Subtype definitions of EEAs were as follows: Type A (ER+ and/or PR+, HER2-), Type B (ER+ and/or PR+, HER2+), Type C (ER-, PR-, HER2+), and Type D (ER-, PR-, HER2-).

6. Immunohistochemical assessment for p53 and PTEN expression

Immunostaining data for p53 were expressed as the percentages of positive epithelial cells in relation to the total number of cells counted in at least 5-10 representative

high-power fields (500–1,000 epithelial cells). Only nuclear staining was regarded as significant [17]. Tumor cells with weak staining were not considered positive. In the evaluation of p53 overexpression, samples showing greater than 30% staining were considered positive. Tumors considered positive showed diffuse positive cytoplasmic and nuclear staining in the majority (> 90%) of cells. Positive staining in tumor cells was comparable to that detected in normal stromal cells. Tumors with no staining or only rare staining (< 10%) were considered negative for PTEN [18]. Representative tumors are shown in Figure 2.

7. DNA extraction

DNA was extracted from normal and tumor samples isolated by the crypt isolation method (83 crypts were isolated from each sample), using standard sodium dodecylsulfate/proteinase K treatment. Samples were resuspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA [pH 8.0]) to the equivalent of 1000 cells/μL. DNA was extracted in a similar fashion.

8. Mutation analysis of the *Ki-ras*, *BRAF*, and *PIK3CA* genes

Mutations in codons 12 and 13 in the *Ki-ras* gene and the mutation cluster region of the *BRAF* gene were examined using bisulfate pyrosequencing as described previously [19]. Mutation in exons 9 and 20 in *PIK3CA* was examined by direct sequencing using the ABI PRISM Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing fragments were detected by capillary electrophoresis using an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems) [20].

9. Assessment of LOH by polymerase chain reaction

LOH studies were performed by polymerase chain reaction (PCR) amplification of 13 highly polymorphic microsatellite markers (D3S2402, D3S1234, D5S107, D5S346, D5S299, D5S82, D10S2491, D10S2492, D13S118, D13S153, TP53, D18S487, and DCC) located at 6 chromosomal loci (3p, 5q, 10q, 13q, 17p, and 18q) [21]. Seventeen microsatellite sequences were obtained from specific primers reported in the GDB Human Genome Database (http://gdbwww.gdb.org/gdb/). Sequences of the primers used are described elsewhere [14]. The data were collected automatically and analyzed by GeneScan 3.1 software (Applied Biosystems). LOH was determined by calculating the ratio of the peak areas of the constitutional alleles as described previously [22]. In this study, we defined LOH as less than 0.7 (q-value) in this ratio.

10. Scoring of LOH status

LOH status was scored according to the following criteria. A tumor sample was considered to be LOH-high if 4 or more of the markers showed allelic loss. When the data showed that less than 3 markers were lost, the tumor was designated as LOH-low.

11. Analysis of MSI

The primers proposed by the National Cancer Institute Workshop on Microsatellite Instability (BAT25, BAT26, D5S346, D2S123, and D17S250) were used in this study [23]. The PCR conditions have been described elsewhere [24]. Products were run on an ABI PRISM 377 fluorescent DNA sequencer. MSI was defined as the presence of an additional peak. A tumor sample was considered to be MSI-high (MSI-H) when

Table 3. Clinicopathological findings for endometrial endometrioid carcinomas in each subtype

Histological subtype	Type A (%)	Type B (%)	Type C (%)	Type D (%)
Endometrioid carcinoma	15 (19.2)	52 (62.7)	10 (12.8)	6 (7.7)
Age (means)	33-81 (58.5)	40-80 (55.7)	50-77 (61.8)	46-71 (57.6)
Grade				
Gradel	8 (53.3)	32 (61.5)	3 (30.0)	2 (33.3)
Grade2	5 (33.3)	16 (30.8)	5 (50.0)	1 (16.6)
Grade3	2 (13.3)	4 (7.7)	2 (20.0)	3 (50.0)
FIGO stage				
I / II	14 (93.3)	43 (82.7)	4 (40.0)	2 (33.3)
III / IV	1 (6.7)	9 (17.3)	6 (60.0)	4 (66.7)
Lymphovascular invasion				
negative	13 (86.7)	44 (84.6)	6 (60.0)	3 (50.0)
positive	2 (13.3)	8 (15.4)	4 (40.0)	3 (50.0)

2 or more of the markers demonstrated instability and MSI-low when only 1 marker was unstable. However, tumors showing one alteration using the above criteria and categorized as MSI-low were considered MSS in this analysis.

12. Confirmation of DNA methylation status

Bisulfite treatment of genomic DNA was carried out as described previously [25]. For examination of methylation status, we used a bisulfate pyrosequencing restriction analysis as described previously [26]. *SFRP1, SFRP2, SFRP5, DKK-2, DKK-3, MLH-1, p16, HOXA9, mir-34b/c,* and *MINT-1* genes were analyzed using primers, restriction enzymes, and conditions as previously described [17, 26]. Tumors were classified as methylation-negative/low (methylation-low) if 1 or 2 loci were methylated and methylation-high if 3 or more were methylated.

13. Statistical analysis

The data were analyzed using the χ^2 test with the aid of StatView-IV software (Abacus Concepts, Berkeley, CA, USA). Samples were determined to be significantly different when p-values were 0.05 or less.

III. Results

1. IHC subtype associations with clinicopathological findings

The characteristics of each subtype are given in detail in Table 3. The frequencies of FIGO stage I/II in Type A or Type B tumors were significantly higher than those of Type C or Type D tumors, respectively ($p<0.01$). There were no significant differences in the frequencies of tumor grade and lymphovascular invasion between each type.

2. Immunohistochemical findings

The results for overexpression of p53 and loss of PTEN expression are displayed in Figure 3. The frequency of p53 overexpression in Type C was significantly higher than that in Type B ($p<0.05$) and loss of PTEN expression in Type C was significantly lower than in Type A and B ($p<0.05$).

3. Analysis of *Ki-ras, BRAF,* and *PIK3CA* mutations

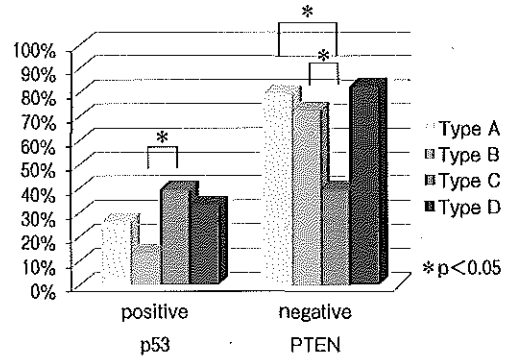

Fig. 3. Frequencies of p53 overexpression and loss of PTEN expression in each subtype.

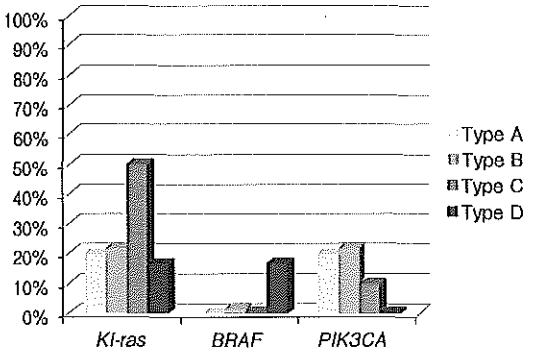

Fig. 4. Frequencies of Ki-ras, BRAF, and PIK3CA mutations in each subtype.

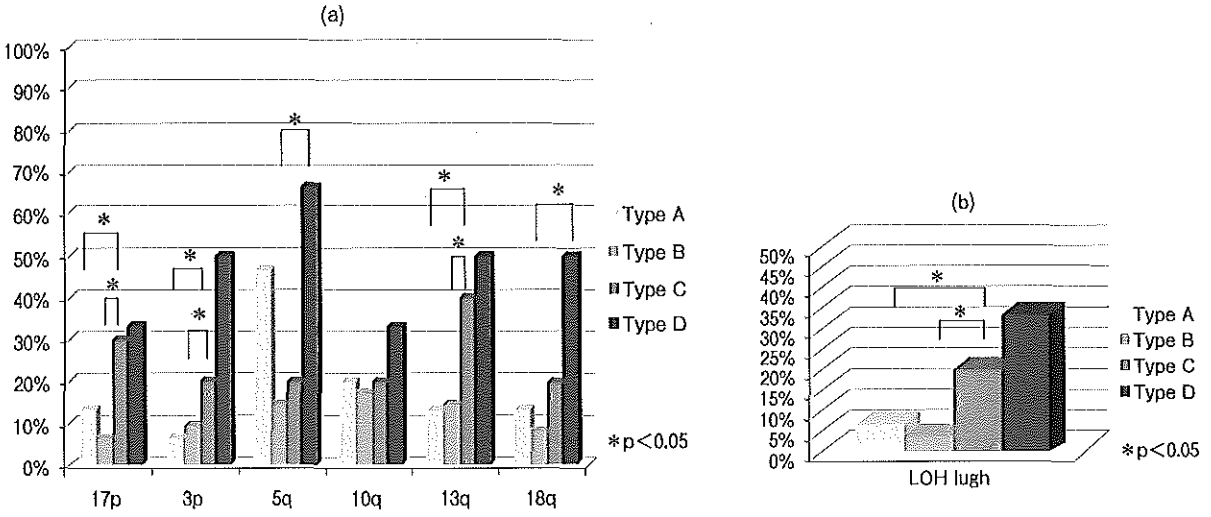

Fig. 5. (a) Frequencies of LOH at multiple cancer-related chromosomal loci in each subtype.
(b) Frequencies of LOH-high status in each subtype.

Findings for *Ki-ras, BRAF,* and *PIK3CA* mutations are summarized in Figure 4. Mutations in the *Ki-ras* gene were more frequent in Type C (50.0%) than in other types of tumors. Mutations in the *PIK3CA* gene were more common in Type A (20.0%) and Type B (21.6%). Additionally, mutations in the *BRAF* gene were found only in Type D (16.7%). The differences were not significant.

4. Analysis of LOH

The LOH data are summarized in Figure 5. Allelic loss of 10q was a common alteration in all subtypes. Allelic losses of 17p and 13q were more frequent in Type C (30% and 40%, respectively) and D (33.3% and 50%, respectively) than in Type B (6.7% and 14.6%, respectively; $p<0.05$). The frequencies of 3p and 18q allelic losses were significantly higher in Type D (50% and 50%, respectively) than in Type B (9.6% and 8.3%, respectively; $p<0.01$). Finally, allelic loss at 5q was more frequently found in Type A (46.7%) and D (27.3%) than in Type B (14.6%; $p<0.05$).

The frequency of LOH-high tumors was

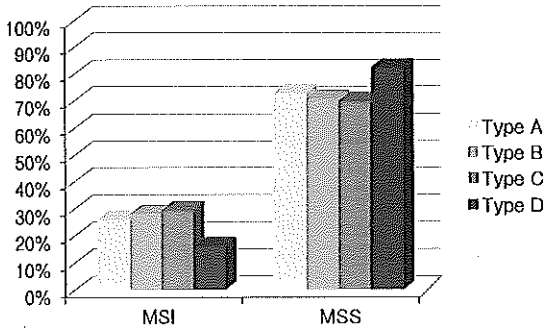

Fig. 6. Frequencies of MSI and MSS tumors in each subtype.

significantly higher in Type C (20%) and D (33.3%) than in Type B (3.8%; $p<0.05$).

5. Analysis of MSI

The frequency of MSIs in each subtype is shown Figure 6. There was no difference in the frequency of MSIs among each subtype. MLH-1 methylation status differed significantly between MSIs (56.5%) and MSSs (6.7%; $p<0.001$).

6. Analysis of methylation

The methylation frequencies at several loci for each tumor type are shown in Figure 7. A high frequency of *SFRP1* methylation was observed in all subtypes (73.3%, 56.3%, 60.0%, and 83.3%, respectively). Although methylation of *SFRP2, HOXA9*, and *mir34b/c* was more frequent in Type A, B, and D (40.0%, 48.1%, and 50.0%, respectively, for *SFRP2*; 86.7%, 80.8%, and 100.0% for *HOXA9*; and 46.7%, 48.1%, and 66.7% for *mir34b/c*) compared with Type C (20.0% for *SFRP2*; 40.0% for *HOXA9*; and 20.0% for mir34b/c), differences were not significant. Only difference in *HOXA9* methylation was significant ($p<0.05$).

7. Analysis of molecular status

The molecular status of each subtype is displayed in Figure 8. LOH-L/methylation-H status was more frequently found in Types A, B, and D (26.7%, 36.5%, and 50.0%, respectively) than in Type C (10.0%). The frequency of LOH-L/methylation-H in Type D was significantly higher than in Type C (p

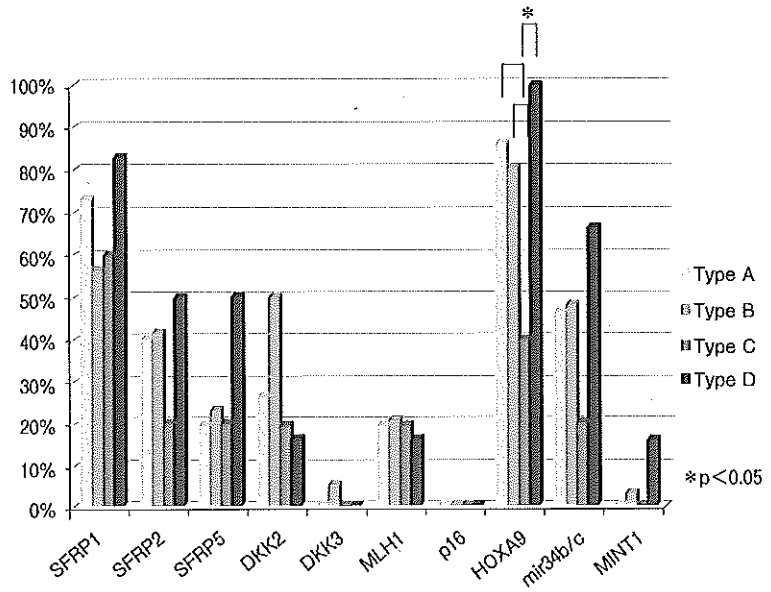

Fig. 7. Frequencies of multiple gene promoter methylations in each subtype.

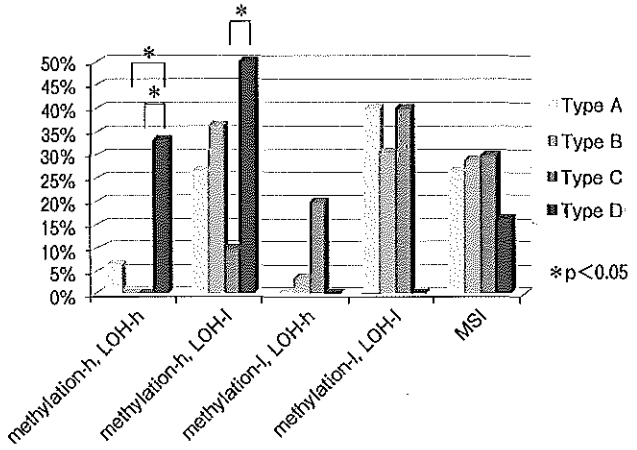

Fig. 8. Frequencies of LOH and methylation status in each subtype.

<0.05). There were significant differences of LOH-H/methylation-H status between Type D (33.3%) and Types B and C (0% and 0%, respectively).

IV. Discussion

In this study, DNA samples obtained by the crypt isolation technique were used instead of those obtained from fresh tissues, as would usually be used. Recent studies have detected various molecular abnormalities not only in cancer cells but also in the intervening stromal cells. In ulcerative colitis, the presence of molecular abnormalities has been reported in the stromal cells of inflammatory mucosa [27, 28]. It is also known that mutations in *PTEN* and *DPC4* occur in interstitial cells of juvenile polyposis [29-31]. Therefore, in order to analyze molecular abnormalities specific to tumor cells, it is essential to collect only tumor cells. Crypt isolation allows a large amount of high quality DNA to be obtained from tumor cells, thereby providing a useful method for identifying abnormalities specific to tumor cells. Microdissection has also been employed for specifically analyzing tumor cells, but this method has been recognized as being inappropriate for obtaining large amounts of high quality DNA. However, few molecular analyses of EEAs have used isolated tumor crypt cells; instead, most clinical specimens being analyzed have used samples that also contain intervening stroma. In our study, crypt specimens were isolated from EAs without stromal contamination. The crypt isolation technique is expected to provide a useful molecular analysis method for EEA.

In this study, we analyzed the clinicopathological characteristics of each tumor type, classified according to ER, PgR, and HER2 expression. We found that most Type A and B tumors, which were hormone receptor-positive, were at stage I/II, with highly differentiated profiles showing no vascular invasion. In contrast, Type C and D tumors, which were hormone receptor-negative, were at stage III/IV, and most were moderately to poorly differentiated. Previous reports have also shown correlations among disease stage, differentiation status, and prognosis in patients with hormone receptor-positive tumors [32-35], consistent with our results. However,

contradictory reports have described HER2 overexpression as a poor prognostic factor [36] or as unrelated to prognosis [37]. Further analysis of HER2 overexpression as a prognostic factor is needed. The results of the present study, like those of others reported to date, suggest that hormone receptor-positive tumors are generally found at a relatively early stage and are highly differentiated. In contrast, there is no tendency for increases in the frequency of HER2 overexpression in poorly differentiated, advanced cancer, according to the classification employed in the present study.

p53 overexpression was observed at a low frequency in all tumor types, but the frequency tended to be higher in Type C and D tumors than in Type A and B tumors. According to reports published to date, the frequency of p53 overexpression is high in type 2 and low in type 1 cancers (10–20%) [38, 39]. Our results suggest that p53 overexpression is associated with Type C tumorigenesis. As discussed above, the extent of p53 overexpression is generally low in EA, which raises the possibility that cancers with HER2 overexpression have molecular abnormalities different from those of cancers that express other hormone receptors. In the present study, it was unclear at which stage p53 was overexpressed in Type C tumors. Thus, future research is required to determine the possible role of p53 overexpression in Type C tumors at earlier stages. Furthermore, the present study showed that the frequency of p53 overexpression was high in Type D tumors, as well, although the difference did not reach statistical significance. Although further studies are necessary because of the limited number of Type D tumors in this study, p53 overexpression has been reported to be associated with a poor prognosis in breast cancer, warranting further studies of p53 overexpression in EEA.

PTEN mutations have been observed in 50–80% of cases with Type I EC and are a major molecular abnormality in this cancer [40]. Loss of *PTEN* activity has also been observed at an earlier stage, characterized by atypical endometrial hyperplasia, indicating that *PTEN* inactivation is an important early abnormality in EC [41]. In the present study, loss of or reduced *PTEN* expression was observed in all tumor types other than Type C. This raises the possibility that Type C tumors develop through a tumorigenic mechanism different from that of the other types. Although the cause of this difference is unclear, the development of tumors for Type C tumors may resemble that of so-called Type 2 EC, which progresses from atrophic endometrium to poorly differentiated EA by bypassing endometrial hyperplasia. Further studies focusing on this possibility are necessary.

A previous study has shown that *K-ras* mutation is a common alteration in EC [4]. The frequencies of point mutations in codons 12 and 13 are approximately 6% to 16% in endometrial hyperplasia [42, 43]. In the present study, *K-ras* mutations were more frequent in Type C. This finding indicates that *K-ras* mutation plays an important role in the development of Type C tumors. The present results highlight the importance of *K-ras* mutation in the development of some specific types of EC.

In colorectal cancer, *BRAF* mutations have been shown to occur frequently in

patients with CIMP or MSI [44], showing a characteristic clinicopathological pattern. In EEA, MSIs are reported to occur at a high frequency [45], but a relationship between MSI and *BRAF* mutations has not been reported. In the present study, *BRAF* mutations were rare, except in Type D tumors. No correlation of this mutation with MSIs or CIMPs was detected. These results suggest that, unlike *BRAF* mutations in colorectal cancer, *BRAF* mutations in EEA are not associated with a clinicopathologically or molecular pathologically characteristic disease type.

PIK3CA mutations have been shown to occur in some colorectal cancers and are regarded as an important driver mutation in this type of cancer [46]. Oncogenic mutation of *PIK3CA* is reportedly present in EC, as well, with a frequency of approximately 40% in EEA in particular [4, 40]. This mutation is thus considered to play an important role in the development of EEA. In the present study, *PIK3CA* mutations were observed in approximately 20% of Type A and B tumors. The observed frequency was lower than those reported previously, and the reason for this discrepancy is unclear. However, our results suggest that the frequency of *PIK3CA* mutations varied among subtypes, warranting further elucidation of the role of this mutation. In this study, we also investigated LOH at each cancer-related chromosomal location. The frequency of LOH was found to be clearly higher in Type C and D tumors than in Type A and B tumors. In colorectal cancer, two types of representative molecular abnormalities – chromosomal instability (CIN) and MSI – have been demonstrated [47]. The former is associated with relatively large chromosomal abnormalities, accompanied by common abnormalities, such as LOH and *p53* mutations [47, 48]. In contrast, the latter is associated with few chromosomal abnormalities, with only low accumulation of LOH and mostly without *p53* mutations [48, 49]. A CIN-type mechanism may play a major role in some specific type of EC, especially Type D. MSI-positive cancer was observed in all tumor types, showing no differences in frequency among different types. In colorectal cancer, MSI is thought to be caused by DNA methylation in the promoter region of the *MLH-1* gene [47, 49]. MSI is reported to occur at a frequency of approximately 10% in colorectal and gastric cancers, while MSI is observed in 9–30% of EEAs [4, 40], giving EEAs the highest frequency of MSI among cancers of all organs. MSI is a common alteration in all types of EC.

Finally, we searched for methylation of tumor suppressor genes, a phenomenon postulated to be associated with the development of EEA. Important signal transduction pathways in oncogenesis include the Wnt and p53 pathways. *SFRP1*, *SFRP2*, and *DKK2* are related to the former pathway, while *mir-34b/c* is related to the latter. SFRP1 methylation was common to all tumor types, whereas methylation of *SFRP2* and *DKK2* varied depending on the tumor type. In contrast, *mir-34b/c* methylation was frequent in all tumor types except Type C. Previous studies have suggested that *mir-34b/c* and *p53* expression are interrelated [50]; given the high frequency of *p53* mutations in Type C and D tumors, *mir-34b/c* may be a promising target for therapy for Type D tumors. These findings suggest the importance of

DNA methylation of various genes in the development of EEA.

In the present study, we classified EEA into four types based on ER, PgR, and HER2 expression status and analyzed the molecular abnormalities in each type. The molecular abnormalities in EEA were related to the above four phenotypes, suggesting that these phenotypes, as proposed herein, may be useful for classifying EEAs. Conventionally, EEA is classified as a Type I cancer. However, this study demonstrated that type I cancer actually consists of tumor groups that are different both clinicopathologically and in terms of molecular abnormalities. In addition, it is possible that the optimal treatment approaches for EEA vary depending on the tumor types defined in this work. Further studies are warranted.

Acknowledgments

We thank Miss Erika Sugawara and Mr Toshiyuki Kasai for technical assistance. We also thank members of the Division of Molecular Diagnostic Pathology, Department of Pathology and the Department of Obstetrics and Gynecology, Iwate Medical University, for their support.

Conflict of interest: The authors have no conflict of interest to declare.

References

1) **Jemal A, Siegel R, Xu J, et al.**: Cancer statistics. CA Cancer J Clin **60**, 277-300, 2010.
2) **Bokhman JV**: Two pathogenetic types of endometrial carcinoma. Gynecol Oncol **15**, 10-17, 1987
3) **Lax SF** and **Kurman RJ**: A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses. Verhandlungen der Deutschen Gesellschaft fur Pathologie **81**, 228-232, 1997.
4) **Llobet D, Pallares J, Yeramian A, et al.**: Molecular pathology of endometrial carcinoma: practical aspects from the diagnostic and therapeutic viewpoints. J Clin Pathol **62**, 777-785, 2009.
5) **Toyoda M**: CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA **96**, 8681-8686, 1996.
6) **Bast RC Jr, Ravdin P, Hayes DF, et al.**: 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol **19**, 1865-1878, 2001.
7) **Lund MJ, Butler EN, Hair BY, et al.**: Age/race differences in HER2 testing and in incidence rates for breast cancer triple subtypes: a population-based study and first report. Cancer 116, 2549-2559, 2010.
8) **Sorlie T, Perou CM, Tibshirani R, et al.**: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA **98**, 10869-10874, 2001.
9) **Carey LA, Perou CM, Livasy CA, et al.**: Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA **295**, 2492-2502, 2006.
10) **Tsukamoto N**: General rules for clinical and pathological management of uterine corpus cancer. Nihon Rinsho 62, 285-289, 2004.
11) **Nakamura S, Goto J, Kitayama M, et al.**: Application of the crypt-isolation technique to flow-cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology **106**, 100-107, 1994.
12) **Arai T** and **Kino I**: Morphometrical and cell kinetic studies of normal human colorectal mucosa. Comparison between the proximal and the distal large intestine. Acta Pathol Jpn **39**, 725-730 1989.
13) **Nakamura S, Goto J, Kitayama M, et al.**: Application of the crypt isolation technique to flow-cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology **106**, 100-107, 1994.
14) **Sugai T, Habano W, Uesugi N, et al.**: Three independent genetic profiles based on mucin

expression in early differentiated-type gastric cancers--a new concept of genetic carcinogenesis of early differentiated-type adenocarcinomas. Mod Pathol **17**, 1223-1234, 2004.

15) **Grushko TA, Filiaci VL, Mundt AJ, et al.**: An exploratory analysis of HER-2 amplification and overexpression in advanced endometrial carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol **108**, 3-9, 2008.

16) **Gul AE, Keser SH, Barisik NO, et al.**: The relationship of cerb B 2 expression with estrogen receptor and progesterone receptor and prognostic parameters in endometrial carcinomas. Diagnostic Pathol **5**, 13, 2010

17) **Sugai T, Habano W, Endoh M, et al.**: Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers. Int J Cancer **127**, 2500-2509, 2010.

18) **Djordjevic B, Hennessy BT, Li J, et al.**: Clinical assessment of PTEN loss in endometrial carcinoma: immunohistochemistry outperforms gene sequencing. Mod Pathol **25**, 699-708, 2012.

19) **Kusano M, Toyota M, Suzuki H, et al.**: Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. Cancer **106**, 1467-1479, 2006.

20) **Catasus L, Gallardo A, Cuatrecasas M, et al.**: PI3K-AKT and p53 alterations in endometrial carcinomas are associated with poor prognosis. Mod Pathol **22**, 522-529, 2009.

21) **Sugai T, Habano W, Uesugi N, et al.**: Molecular validation of the modified Vienna classification of colorectal tumors. J.Mole Diagn **4**, 191-200, 2002.

22) **Sugai T, Habano W, Jiao YF, et al.**: Analysis of molecular alterations in left- and right-sided colorectal carcinomas reveals distinct pathways of carcinogenesis: proposal for new molecular profile of colorectal carcinomas. J Mol Diagn **8**, 193-201, 2006.

23) **Boland CR, Thibodeau SN, Hamilton SR, et al.**: A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res **58**, 5248-5257, 1998.

24) **Sugai T, Habano W, Nakamura S, et al.**: Use of crypt isolation to determine loss of heterozygosity of multiple tumor suppressor genes in colorectal carcinoma. Pathol Res Pract **196**, 145-150, 2000.

25) **Kim YH, Kakar S, Cun L, et al.**: Distinct CpG island methylation profiles and BRAF mutation status in serrated and adenomatous colorectal polyps. Int J Cancer **123**, 2587-2593, 2008.

26) **Kamimae S, Yamamoto E, Yamano HO, et al.**: Epigenetic alteration of DNA in mucosal wash fluid predicts invasiveness of colorectal tumors. Cancer Prev Res (Phila) **4**, 674-683, 2011.

27) **Yagishita H, Yoshida T, Ishiguro K, et al.**: Epithelial and stromal genetic instability linked to tumor suppressor genes in ulcerative colitis-associated tumorigenesis. Scand J Gastroenterol **43**, 559-566, 2008.

28) **Matsumoto N, Yoshida T** and **Okayasu I**: High epithelial and stromal genetic instability of chromosome 17 in ulcerative colitis-associated carcinogenesis. Cancer Res **63**, 6158-6161, 2003.

29) **Marsh DJ, Roth S, Lunetta KL, et al.**: Exclusion of PTEN and 10q22-24 as the susceptibility locus for juvenile polyposis syndrome. Cancer Res **57**, 5017-5021, 1997.

30) **Houlston R, Bevan S, Williams A, et al.**: Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. Hum Mol Genet **7**, 1907-1912, 1998.

31) **Howe JR, Roth S, Ringold JC, et al.**: Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science **280**, 1086-1088, 1998.

32) **Pilka R, Mickova I, Lubusky M, et al.**: Expression of p53, Ki-67, bcl-2, c-erb-2, estrogen, and progesterone receptors in endometrial cancer. Ceska Gynekol **73**, 222–227, 2008.

33) **Creasman WT**: Prognostic significance of hormone receptors in endometrial cancer. Cancer **71**, 1467-1470, 1993.

34) **Fukuda K, Mori M, Uchiyama M, et al.**: Prognostic significance of progesterone receptor immunohistochemistry in endometrial carcinoma. Gynecol Oncol **69**, 220-225, 1998.

35) **Ehrlich CE, Young PC, Stehman FB, et al.**: Steroid receptors and clinical outcome in patients with adenocarcinoma of the endometrium. Am J Obstet Gynecol **158**, 796-807, 1988.

36) **Berchuck A, Rodriguez G, Kinney RB, et al.**: Overexpression of HER-2/neu in endometrial cancer is associated with advanced stage disease. Am J Obstet Gynecol **164**, 15-21, 1991.

37) **Backe J, Gassel AM, Krebs S, et al.**: Immunohistochemically detected HER-2/neu-expression and prognosis in endometrial

carcinoma. Arch Gynecol Obstet **259**, 189-195, 1997.

38) **Berchuck A**: Biomarkers in the endometrium. J Cell Biochem Suppl 23, 174-178, 1995.

39) **Lax SF, Kendall B, Tashiro H, et al.**: The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. Cancer **88**, 814-824, 2000.

40) **Bansal N, Yendluri V** and **Wenham RM**: The molecular biology of endometrial cancers and the implications for pathogenesis, classification, and targeted therapies. Cancer Control **16**, 8-13, 2009.

41) **Lax SF**: Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. Virchows Arch **444**, 213-223, 2004.

42) **Sasaki H, Nishii H, Takahashi H, et al.**: Mutation of the Ki-ras protooncogene in human endometrial hyperplasia and carcinoma. Cancer Res **53**, 1906-1910, 1993.

43) **Caduff RF, Johnston CM, Frank TS**: Mutations of the Ki-ras oncogene in carcinoma of the endometrium. Am J Pathol **146**, 182-188, 1995.

44) **Brim H, Mokarram P, Naghibalhossaini F, et al.**: Impact of BRAF, MLH1 on the incidence of microsatellite instability high colorectal cancer in populations based study. Mol Cancer **7**, 68, 2008.

45) **Basil JB, Goodfellow PJ, Rader JS, et al.**: Clinical significance of microsatellite instability in endometrial carcinoma. Cancer **89**, 1758-1764, 2000.

46) **Liao X, Morikawa T, Lochhead P, et al.**: Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. Clin Cancer Res **18**, 2257-2268, 2012.

47) **Lengauer C, Kinzler KW** and **Vogelstein B**: Genetic instability in colorectal cancers. Nature **386**, 623-627, 1997.

48) **Boland CR** and **Goel A**: Microsatellite instability in colorectal cancer. Gastroenterology **138**, 2073-2087, 2010.

49) **Jass JR, Whitehall VL, Young J, et al.**: Emerging concepts in colorectal neoplasia. Gastroenterology **123**, 862-876, 2002.

50) **Suzuki H, Yamamoto E, Nojima M, et al.**: Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis **31**, 2066-2073, 2010.

岩手医誌 66 巻, 3 号（平成 26 年 8 月）85-100 頁.

エストロゲンレセプター, プロゲステロンレセプター, HER2 発現に基づいた子宮類内膜腺癌の分子病理学的解析: 子宮類内膜腺癌の新しい分類の提唱

苫米地英俊[1,2], 菅井　有[1], 菅安寿子[1,2], 永沢崇幸[2], 上杉憲幸[1], 石田和之[1], 竹内　聡[2], 杉山　徹[2]

[1]岩手医科大学医学部, 病理学講座：分子診断病理学分野
[2]岩手医科大学医学部, 産婦人科学講座

(Received on January 14, 2014 & Accepted on January 22, 2014)

要旨

子宮内膜癌の分子腫瘍発生はこれまでホルモン依存性のタイプ 1（子宮内膜癌の 90%）と非依存性のタイプ 2 (子宮内膜癌の 10%) に分類して解析されてきた. 一方, 近年ホルモン依存性腫瘍の乳癌ではエストロゲンレセプター（ER）, プロゲステロンレセプター（PgR）, HER2 の発現を基に 4 つの形質に分類することが行われており, 組織学的特徴や予後, 悪性度, 治療の反応性などと良く関連することが明らかになっている. 本論文では, ER, PgR, HER2 の発現を基に, タイプ I 子宮類内膜癌を 4 つのタイプ（タイプ A: ER(+) もしくは PgR (+) かつ HER2 (-)、タイプ B: ER (+) もしくは PgR (+) かつ HER2 (+)、タイプ C: ER(-) かつ PgR (-) かつ HER2 (+)、タイプ D: すべて (-)）に分けて臨床病理学的, 分子病理学的に解析を行い, これらの分類の妥当性について検証することを目的とした.