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Original Article

Molecular analysis of isolated tumor glands from endometrial endometrioid adenocarcinomas

Running title: Molecular analysis of uterine cancer

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ABSTRACT

We studied the extensive molecular alterations of endometrial endometrioid adenocarcinoma (EEA) using a crypt isolation method. We analyzed copy number variation (CNV) using a single nucleotide polymorphism (SNP) array, genetic mutations (*KRAS*, *BRAF*, *p53*, *PIK3CA*), DNA methylation and microsatellite instability (MSI) status. In addition, loss of PTEN protein expression was examined. Increased chromosome copy numbers of 1q21.2-44 (22%) and 10q11.21-23.31 (28%) were seen relatively frequently in EEA, and copy-neutral loss of heterozygosity (LOH) was also observed in 10q22.1-26.3 (22%). The CNV patterns of EEA were classified into 4 groups through hierarchical cluster analysis. Cluster 1 had many CNVs of 10q, and cluster 2 was characterized by MSI status. In cluster 3, increased CNVs of 1q were often seen. In cluster 4, *p53* mutations were detected. *KRAS* and *PIK3CA* mutations and reduced PTEN protein expression were common to all groups. On the other hand, CpG island methylator phenotype (CIMP) was rare in all groups. The data indicated an association with chromosomal gain of 1q and 10q or 10q copy-neutral LOH in some cases. We suggest that EEA consists with 4 groups that are characterized with molecular alterations.

Key words: CIMP, CNV, endometrial endometrioid adenocarcinoma, MSI, PTEN, SNP array

INTRODUCTION

The frequency of cancer in female reproductive organs is increasing, with a rising trend in uterine cancer in particular.[1] The disease rate of uterine cancer has risen more than that of cervical cancer year-over-year. Its frequency is highest among menopausal and postmenopausal women, and the importance of diagnosis and treatment of uterine cancer is expected to increase. In recent years, progress has been made in studies of the mechanisms of tumor onset.[2-7]

From the viewpoint of molecular pathology, uterine cancer can be broadly classified into Types 1 and 2.[8] Type 1 includes well differentiated to moderately differentiated endometrioid adenocarcinoma that is hormone-dependent and has a good prognosis. A high frequency of *PTEN* genetic mutations has been observed in Type 1 and *KRAS* mutations are also relatively numerous. Type 1 also includes microsatellite instability (MSI).[9-11] In contrast, Type 2 includes poorly differentiated endometrioid adenocarcinoma, serous carcinoma and clear cell carcinoma, which have poor prognosis.[8] Type 2 is characterized by *p53* mutations or numerous instances of loss of heterozygosity (LOH).[9-11] Type 1 occurs nine times more frequently than Type 2.[4]

Endometrial endometrioid adenocarcinoma (EEA) is by far the most common of uterine cancers and its molecular abnormalities constitute a single molecular disease type (Type 1). However, MSI and microsatellite stable (MSS) cancers, which are contrasting molecular disease types, are included in Type 1, and the molecular pathological characteristics of MSS have not been studied in EEA. In the colon, MSS is considered in nearly the same molecular type as chromosomal instability (CIN).[11] However, the two are not strictly equivalent. Thus, there are three molecular disease types in cancer—MSI, CIN, and non-CIN—with the latter two being equivalent to the concept of MSS. In most EEAs, tumors appear diploid,[12] indicating that most EEAs are not accompanied by major changes at the chromosome level.

However, there have been few comprehensive detailed analyses of molecular abnormalities in EEA at the chromosome level. Moreover, there have been no reports of molecular abnormalities of cancer cells sampled exclusively from isolated cancerous crypts. Thus, in the present study, we extracted DNA from cancerous crypts of EEA and analyzed (1) abnormalities at the chromosome level (CNV), (2) *p53*, *KRAS*, *BRAF*, *PIK3CA*, and PTEN expression abnormalities, (3) MSI and (4) DNA methylation. The objective was to examine the interrelationships among the above molecular abnormalities.

MATERIALS AND METHODS

Patients

We enrolled 32 cases of EEA surgically resected from April, 2011 through August, 2013, in the Department of Obstetrics and Gynecology at Iwate Medical University Hospital. Patients who had undergone preoperative chemotherapy were excluded. The clinicopathological backgrounds of the 32 patients are shown in Table 1. Histological classification was performed according to General Rules for Management of Uterine Cancer, Rev. 3.[13] Degree of nuclear atypia was classified based on the pathology standards of our hospital. According to this classification, those exhibiting degree of nuclear atypia and nucleiform irregularity with clearly elevated nucleoli and nuclear chromatin were classified as high grade nuclear atypia. Those exhibiting uniform nuclear size without noticeable nucleiform irregularity or elevated nucleoli and nuclear chromatin were classified as low grade [Fig. 1 (A)].

The present study was conducted with the approval of the ethics committee of the Iwate Medical University School of Medicine (approval number H24-208).

Crypt Isolation Method

Fresh samples of tumor tissues and normal tissues were obtained from resected EEAs. The

tumor samples were obtained primarily from the central area of the tumors. Normal endometrial mucosa was taken from the most distal portion of endometrium. Crypt isolation from the tumor and normal mucosa was performed in accordance with a previously reported method.[14] Briefly, fresh mucosa and tumor were minced with a razor into minute pieces and then were incubated at 37℃ for 30 min in calcium- and magnesium-free Hanks' balanced salt solution (CMF) containing 30 mmol/L EDTA. Following this procedure, the tissue was then stirred in CMF for 30 - 40 min. Normal and tumor glands were separated from the endometrial mucosa or fibrous stroma. The isolated tumor glands were immediately fixed in 70% ethanol and stored at 4℃ until used for DNA extraction [Fig. 1 (B)].

The fixed isolated tumor glands were observed under a dissection microscope (SZ60, Olympus, Tokyo, Japan). The isolated tumor glands were processed routinely to confirm its nature using paraffin-embedded histological sections. Contamination by other materials such as interstitial cells was not evident in the samples that were examined, as shown in previous reports.[14]

Total Genome CNV/LOH Analysis by SNP Array

Total genome CNV/LOH analysis was performed using a single nucleotide polymorphism (SNP) array. Extracted DNA was adjusted to a concentration of > 50 μg/μL. CNV analysis was performed using an Illumina HumanCytoSNP-12 BeadChip (Illumina, Inc. San Diego, USA) with roughly 300,000 probes. In the analysis of chromosome CNVs, the CNV value was calculated from B allele frequency and log R ratio using the Illumina KaryoStudio software program,[15] and the chromosome CNVs were classified as described below. In the classification of chromosome copy number variations by CNV partition algorithms,[16] a copy number > 2 was defined as chromosomal gain and < 2 as LOH, and a region in which

homoalleles were in sequence with a copy number of 2 as copy-neutral LOH.

For those cases in which we observed CNV > 10 M base-pair (bp), they were defined as "large general changes" for each class, i.e., chromosomal gain, LOH and copy-neutral LOH. These were termed large general gain, large general LOH, and large general copy-neutral LOH, respectively. In addition, to evaluate the extent of the CNVs, the total length of bp in the abnormal regions of each subject was calculated and analyzed. Cluster analysis was performed on the CNV data obtained by SNP array using the ward's linkage algorithm (JMP version 10, SAS Institute, Cary, NC, USA).

p53, KRAS, BRAF and *PIK3CA* Mutation Analysis

Mutation analysis of each gene was performed by polymerase chain reaction (PCR). *KRAS* and *BRAF* were analyzed by pyrosequencing using respective *KRAS* and *BRAF* Pyro Kits (Qiagen, Hilden, Germany). When a total of 15% or more mutations in the tumor sample examined were observed, "mutation-positive" was termed.

Single strand conformational polymorphism (SSCP) analysis was used to screen PCR products derived from exons 5-8 of the *p53* gene and from exon 9 and 20 of the *PIK3CA* gene in patient tumor and normal serum samples. Direct sequencing was analyzed by an automatic ABI PRISM 310 Genetic Analyzer using an ABI PRISM Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).[17]

Immunohistochemical Analysis

Immunostaining was performed using an automatic immunostainer (Dako Autostainer) and the primary antibody was anti-PTEN (6H2.1, 1:100 dilution, Dako). Staining positivity of PTEN was determined by comparing interstitial cells. When staining was low or negative in at least 90% of the cancer cells, it was judged to be negative for PTEN expression.[18]

MSI Analysis

Analysis was performed by PCR using the markers *BAT25, BAT26, D5S346, D2S123*, and *D17S250* according to NCI criteria.[11] In MSI determinations, those in which MSI was seen in 2 or more markers were determined as MSI-high, and those with less than 2 were determined as MSI-low.

DNA Methylation Analysis

The DNA methylation rate in promoter regions of *MLH-1, MINT-1, MINT-2, MINT-31, p16* was analyzed by bisulfite pyrosequencing using the DNA obtained from the isolated tumor glands recovered as described above.[19] A DNA methylation rate of 30% or higher was considered DNA methylation-positive. Those in which 2 or more of the 5 markers were methylation-positive were termed CIMP-positive, and those in which 1 or no methylation-positive markers were termed CIMP-negative.

Statistical Analysis

Statistical analysis of data was performed using the statistical software Stat Mate III for Windows Ver. 3.07 (Atom, Tokyo, Japan) and PRISM6 (GraphPad Software, La Jolla, CA). A χ^2 test and Man-Whitney U-test was performed for comparing 2 groups. $P < 0.05$ was considered a significant difference.

RESULTS

SNP Array Analysis of EEA

Figure 2 (a) shows the total number of regions in which the chromosome copy number was abnormal according to SNP array analysis of the 32 patients diagnosed with EEA. In the

abnormal region of each chromosome, large general gains were seen in 1q21.2-44 (7/32, 21.9%), 8q22.2-24.3 (4/32, 12.5%), 10p15.3-11.1 (7/32, 12.5%), 10q11.21-23.31 (9/32, 28.1%) and 10q23.31-26.3 (5/32, 15.6%). In addition, large general copy-neutral LOH was seen in 10q22.1-26.3 (7/32, 21.9%). In large general LOH, there were no frequently appearing aberrations. On the other hand, chromosomal gain of < 10 Mbp was seen in 5q35.3 (3/32, 9.3%), 7q11.23 (3/32, 9.3%) and 16q23.1 (3/32, 9.3%) (Table 2).

The total length of alterations in chromosomal gain and copy-neutral LOH were significantly higher than the LOH (P < 0,01). No significant difference was seen between chromosomal gain and copy-neutral LOH [Fig. 2 (b)].

Mutation analysis and immunohistochemical examination

p53 mutations were seen in 4 of the 32 cases (12.5%). Mutations of *KRAS*, *BRAF*, and *PIK3CA* were detected in 5 cases (15.6%), only 1 case (3.1%) and 8 cases (25.0%), respectively (Table 3). Reduced PTEN expressions was seen in 21 cases (65.6%) (Table 3).

MSI Analysis

MSI-high was observed in 7 of the 32 cases (21.9%) (Table 3). For the total length of alterations in chromosomal gains, MSI-high tended to be more frequent than MSI-low (P = 0.063). The total length of alterations in overall CNVs was significantly smaller (P < 0.05) (Fig. 3).

DNA Methylation Analysis

In DNA methylation analysis, 4 of the 32 cases (12.5%) were CIMP-positive (Table 3). Methylation of *MLH-1* was found in 3 of the 32 cases (9.4%). Although the frequency of *MLH-1* methylation in MSI-high cases (2/7, 28.6%) tended to be higher than that in MSI-low

cases (1/25, 4.0%), no significant difference was found.

Cluster Analysis in Copy Number Variation

Cluster analysis was performed based on the results of the CNV analysis. Clusters were classified into 4 groups, and Figure 4 (A) shows a heat map of the data. In cluster 1, chromosomal gain and copy-neutral LOH were characteristically seen with high frequency in the chromosome 10q ($P < 0.01$), and in cluster 3, chromosomal gain and copy-neutral LOH were characteristically seen with high frequencies in the chromosome 1q ($P < 0.05$) (Table 3). Compared with clusters 1 and 3, cluster 2 was characterized by having a significantly smaller total length of alterations in chromosomal gain and overall CNV ($P < 0.01$) [Fig. 4 (B)]. On the other hand, in cluster 4, sporadic changes without any characteristic abnormalities were seen.

Cluster Analysis and Clinicopathological Examinations

We compared cluster classification and clinicopathological parameters. In cases classified in cluster 1, the frequency of cases exhibiting high grade nuclear atypia was high (6 of 7 cases, 85.7%). Conversely, in cluster 2, the frequency of low grade nuclear atypia was high (13 of 17 cases, 76.5%), and there was a significant difference between the 2 groups ($P < 0.05$) (Table 3).

Cluster Analysis and Clinical Molecular Biological Examination

No significant correlation was observed between cluster classification and cancer-associated gene mutation analysis or immunostaining results. With regard to possible associations between a cluster group and MSI, all cases MSI-positive were seen in cluster 2 with a frequency of 41.2% (7 of 17 cases) (Table 3). In a comparison of each cluster group and DNA

methylation analysis, cases with CIMP-positive were seen in cluster 1 (1/7, 14.3%) and 2 (3/17, 17.6%), but no significant difference in the frequencies of CIMP-positive cases was seen between clusters (Table 3). Although reduced expression of PTEN and mutation of *PIK3CA* were commonly observed in the 4 groups, a correlation between chromosomal gain of 10q and expression of PTEN were not found.

DISCUSSION

The molecular mechanisms underlying the onset of EEA are generally classified into Types 1 and 2. However, it is known that tumorigenesis of EEA that accounts for many Type 1 cases is not attributable to a single mechanism, but is made up of different molecular pathways. In particular, it is clear that MSI-type molecular abnormalities have a different tumorigenic mechanism than MSS-type, but they are collectively classified in EEA. We believe that the differences between the two must be clarified. The MSS-type molecular mechanism of EEA has not been sufficiently studied thus far. Since molecular abnormalities of MSS-type cancer cells are characterized by instability of chromosomal abnormalities,[20] DNA ploidy often also exhibits an aneuploid pattern. However, it has been reported that EEA often exhibits a diploid pattern.[12] In diploid cells, chromosome-level abnormalities are generally in a small region, and in MSI cancer, which is an abnormality of the microsatellite region, cells are known to be diploid.[21] The frequency of MSI in EEA is approximately 30%,[2, 22-24] and it is difficult to explain the fact that EEA often exhibits a diploid pattern by MSI alone. Therefore, to understand in depth the nature of EEA, it is important to analyze the association between chromosome-level abnormalities and the molecular and clinicopathological characteristics of Type 1 cancer.

The crypt isolation method was used in the present analysis to obtain DNA that originated exclusively from cancer cells. Crypt isolation is an excellent method that satisfies

the requirements for both high quantity and high quality DNA from cancer cells. To obtain highly reliable results in an SNP array, a certain quantity of unfragmented DNA must be used. The results in the present study were both highly reproducible and reliable. Isolated cancerous crypts are extremely effective for performing molecular analysis of cancer.

Examining chromosomal regions containing copy number abnormalities provides important information for identifying genes that play key roles in oncogenesis. In the present study, chromosomal gains were seen with relatively high frequencies in 1q21.2-44 (21.9%), 8q22.2-24.3 (12.5%), 10p15.3-11.1 (12.5%), 10q11.21-23.31 (28.1%) and 10q23.31-26.3 (15.6%). Copy-neutral LOH was seen frequently in 10q22.1-26.3 (21.9%). On the other hand, the frequency of regions exhibiting LOH was low. Chromosomal gain has often been reported in 1q, 8q, 10p, 10q, and 19p,[25] and LOH has been reported in 4q, 16q, and 18q.[26, 27] Levan K *et al.* reported that chromosomal gain was often seen in 1q25-q42 (30%), 19pter-p13.1 (26%), 19q13.1-q13.3 (19%), 8q21-q22, 8q22-qter (17%), 10q21-q23 (14%) and 10p (13%). LOH was often seen in 4q22-qter (8%), 16q21-qter (8%), and 18q21-qter (8%).[28] Analysis of the cancer genome atlas (TCGA) data suggest that chromosomal gain was shown in 1p36-p31 (7.8%), 1q12-q44 (17.8%), 3q21-q29 (8.9%), 7q31-q36 (7.8%), 8q12-q24 (10.0%) and 11q11-q14 (5.6%), while copy-neutral LOH were low, and there was no evidence of LOH. Although reported loci of chromosomal abnormality in TCGA are very similar to those in the present study, the frequency of abnormalities, especially copy-neutral LOH and LOH were lower than in the present study. We suggest that crypt isolation method would be advantageous for molecular analysis including CNV analysis using SNP array. From the above results, we believe that chromosomal gains in 1q, 8q, 10p and 10q are characteristic abnormalities in EEA. On the other hand, copy-neutral LOH of 10q was seen in some cases in the present study. We believe this to be due to the fact that chromosomal abnormalities not observed in the past were detected because our analytic method used a SNP array with

isolated tumor glands. LOH, which is closely associated with tumorigenesis in EEA, has not been reported in previous studies, and results have been similar to those of the present study. While it is possible that an increased chromosome copy number has a role in the occurrence of EEA, it was also suggested that LOH or a reduced chromosome copy number has little association with the occurrence of EEA.

It has been demonstrated that chromosome-level abnormalities are important in the progression of many types of cancer. In a report by Yamamoto E *et al.*, it was shown that CNVs of chromosome alleles have a key role when colon adenoma becomes cancerous.[29] Furthermore, a comparison of gastric mucosal cancer and gastric submucosal cancer by Sugai T *et al.* also indicated that accumulated LOH was seen in the latter.[30] These findings suggest that chromosome-level abnormalities are important in the progression of cancer. There have been several reports on chromosome-level abnormalities in EEA.[2] Pere H *et al.* compared EEA, serous adenocarcinoma and clear cell carcinoma and found that the frequency of copy number aberrations was lower in the former.[31] This report suggests that the frequency of copy number aberrations is low in EEA. In the present study, copy number aberrations were expressed as total DNA length. A report by Sawada T *et al.* also determined copy number aberrations in colon cancer as total DNA length,[29] but it is clear that compared with these findings, the degree of copy number aberrations in EEA is small. This suggests the possibility that copy number abnormalities do not have an important role in the progression of EEA.

Chromosome CNVs contribute to the progression of many cancers. Suehiro Y *et al.* compared the number of copy number abnormalities in EEA classified by stages and reported that the number was small in early stage cancers.[32] This report suggests that genome-level abnormalities have an important role in infiltration in EEA. On the other hand, there was no correlation between increasing stage and CNVs in the present study (data not shown), and future examination of the association between increased CNVs and stages is required.

In this study, we performed hierarchical cluster analysis of 32 cases of EEA. The results were stratified into clusters 1, 2, 3 and 4. The features of cluster 1 were copy-neutral LOH and chromosomal gain in the long arm of chromosome 10, whereas in cluster 3, a high frequency of chromosomal gain was seen in the long arm of chromosome 1. There were fewer CNVs in cluster 2, and the frequency of MSI was high. Here, we found no indication of an association between clinicopathological findings and genetic mutations that appear commonly in EEA. This finding shows that the abnormalities in PTEN, *KRAS*, and *PIK3CA* in EEA [9-11] are characteristic of early stages of oncogenesis. That is, it demonstrates that they are genetic abnormalities common to EEA. However, the number of cases analyzed was small, and we believe that further study of these points is required.

The occurrence of MSI was higher in cluster 2 than in the other groups. The total length of copy number aberrations in MSI-high and MSI-low was examined in this study, but the total length of copy number aberrations in MSI-low was long. We believe that it is greatly affected by the total length of increased copy number; however, no statistically significant difference was seen between MSI-high and MSI-low in this regard. Although a close association between MSI and CIMP has been pointed out in colon cancer,[33] a similar association was not indicated in EEA. The marker of Toyota M *et al.*[33] used in the present study is considered to be an excellent CIMP marker in digestive tract cancers such as colon cancer, but we believe that it might not accurately detect CIMP in EEA that has a completely different onset mechanism. Many of the cancers in cluster 2 were high MSI, but the frequency of chromosome-level abnormalities was low. Cluster 2 included a high number of cases, and we believe that it is a typical type of EEA. In the present study, we found that infiltration occurred without chromosome-level abnormalities being acquired. There is also the possibility of a molecular disease type in which various epigenetic abnormalities have key roles in molecular tumorigenesis in EEA.

We examined chromosome copy number abnormalities in EEA using an SNP array. Chromosomal gains in EEA were seen with relatively high frequencies in 1q, 8q, 10p and 10q, but the frequency of LOH was low. Our data demonstrated that, the frequencies of chromosomal gains were greater than those of LOH in EEA, suggesting that chromosomal gains have an important role in this disease. In hierarchical cluster analysis, it was shown that patterns of chromosomal abnormalities could be stratified into 4 groups. In our study of chromosome copy number using SNP array analysis of isolated tumor crypts, we believe that the data are more reliable than those previously reported. Thus, this study provides valuable findings in the study of molecular abnormalities in EEA. Future additional study is required.

Conflict of interests

We declare that we have no conflicts of interest.

REFERENCES

1. Collins Y, Holcomb K, Chapman-Davis E, Khabele D, Farley JH. Gynecologic cancer disparities: a Report from the Health Disparities Taskforce of the Society of Gynecologic Oncology. *Gynecol Oncol* 2014; **133**: 353–61.
2. Matias-Guiu X, Prat J. Molecular pathology of endometial carcinoma. *Histopathology* 2013; **62**: 111–23.
3. Abal M, Planaguma J, Gil-Moreno A *et al.* Molecular pathology of endometrial carcinoma: transcriptional signature in endometrioid tumors. *Histol Histopathol* 2006; **21**: 197–204.
4. The Cancer Genome Atlas Research Network, Integrated genomic characterization of endometrial carcinoma. *Nature* 2013; **497**:67-73.
5. Muraki Y, Banno K, Yanokura M *et al.* Epigenetic DNA hypermethylation: Clinical applications in endometrial cancer. *Oncol Rep* 2009; **22**: 967–72.
6. Tao MH, Freudenheim JL. DNA methylation in endometrial cancer. *Epigenetics* 2010; **6**: 491–8.
7. Banno K, Yanokura M, Susumu N *et al.* Relationship of the aberrant DNA hypermethylation of cancer-related genes with carcinogenesis of endometrial cancer. *Oncol Rep* 2006; **16**: 1189–96.
8. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecologic oncology* 1983; **15**: 10–7.
9. Bansal N, Yendluri V, Wenham RM. The Molecular Biology of Endometrial Cancers and the Implications for Pathogenesis, Classification, and Targeted Therapies. *Cancer Control* 2009; **16**: 8–13.
10. Risinger JI, Maxwell GL, Berchuck A, Barrett JC. Promoter Hypermethylation as an Epigenetic Component in Type1 and Type2 Endometrial Cancers. *Ann N Y Acad Sci*

2003; **983**: 208–12.

11. 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* 1998; **58**: 5248–57.
12. Kildal W, Micci F, Risberg B *et al.* Genomic imbalances in endometrial adenocarcinomas - Comparison of DNA ploidy, karyotyping and comparative genomic hybridization. *Mol Oncol* 2012; **6**: 98–107.
13. Japan Society of Obstetrics and Gynecology, The Japanese Society of Pathology, Japan Radiological Society, Japanese Society for Therapeutic Radiology and Oncology. *The General Rules for Clinical and Pathological Management of Uterine Corpus Cancer, The 3rd Edition.* 2012, Tokyo, Kanehara.
14. 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* 1989; **39**: 725–30.
15. Talseth-Palmer BA, Holliday EG, Evans TJ *et al.* Continuing difficulties in interpreting CNV data: lessons from a genome-wide CNV association study of Australian HNPCC/lynch syndrome patients. *BMC Med Genomics* 2013; **26**: 6: 10.
16. Marenne G, Real FX, Rothman N *et al.* Genome-wide CNV analysis replicates the association between GSTM1 deletion and bladder cancer: a support for using continuous measurement from SNP-array data. *BMC Genomics* 2012; **20**: 13: 326.
17. Catasus L, Gallardo A, Cuatrecasas M, Prat J. Concomitant PI3K-AKT and p53 alterations in endometrial carcinomas are associated with poor prognosis. *Mod Pathol* 2009; **22**: 522–9.
18. Djordjevic B, Hennessy BT, Li J, Barkoh BA, Luthra R, Mills GB, Broaddus RR. Clinical

assessment of PTEN loss in endometrial carcinoma: immunohistochemistry outperforms gene sequencing. *Mod Pathol* 2012; **25**: 699–708.

19. Yamamoto E, Suzuki H, Yamano H *et al.* Molecular Dissection of Premalignant Colorectal Lesions Reveals Early Onset of the CpG Island Methylator Phenotype. *Am J Pathol* 2012; **181**: 1847–61.
20. Muresu R, Sini MC, Cossu A *et al.* Chromosomal abnormalities and microsatellite instability in sporadic endometrial cancer. *Eur J Cancer* 2002; **38**: 1802–9.
21. Atkin NB. Microsatellite instability. *Cytogenet Cell Genet* 2001; **92**: 177–81.
22. Lax SF, Kendall B, Tashiro H, Slebos RJ, Hedrick L. The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* 2000; **88**: 814–24.
23. Catasus L, Machin P, Matias-Guiu X, Prat J. Microsatellite instability in endometrial carcinomas: clinicopathologic correlations in a series of 42 cases. *Hum Pathol* 1998; **29**: 1160–4.
24. Dec G, Romanowicz-Makowska H, Smolarz B, Kulig A. Microsatellite instability (MSI) analysis in patients with endometrial cancer. *Pol J Pathol* 2005; **56**: 15–9.
25. Suzuki A, Fukushige S, Nagase S, Ohuchi N, Satomi S, Horii A. Frequent gains on chromosome arms 1q and/or 8q in human endometrial cancer. *Hum Genet* 1997; **100**: 629–36.
26. Sonoda G, du Manoir S, Godwin AK *et al.* Detection of DNA Gains and Losses in Primary Endometrial Carcinomas by Comparative Genomic Hybridization. *Genes Chromosomes Cancer* 1997; **18**: 115–25.
27. Murayama-Hosokawa S, Oda K, Nakagawa S *et al.* Genome-wide single-nucleotide polymorphism arrays in endometrial carcinomas associate extensive chromosomal instability with poor prognosis and unveil frequent chromosomal imbalances involved in

the PI3-kinase pathway. *Oncogene* 2010; **29**: 1897–908

28. Levan K, Partheen K, Osterberg L, Helou K, Horvath G. Chromosomal alterations in 98 endometrioid adenocarcinomas analyzed with comparative genomic hybridization. *Cytogenet Genome Res* 2006; **115**: 16–22.
29. Sawada T, Yamamoto E, Suzuki H *et al.* Association Between Genomic Alterations and Metastatic Behavior of Colorectal Cancer Identified by Array-Based Comparative Genomic Hybridization. *Genes Chromosomes Cancer* 2013; **52**: 140–9.
30. Sugai T, Habano W, Endoh M, *et al.* Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers. *Int J Cancer* 2010; **127**: 2500–9.
31. Pere H, Tapper J, Wahlström T, Knuutila S, Butzow R. Distinct Chromosomal Imbalances in Uterine Serous and Endometrioid Carcinomas. *Cancer Res* 1998; **58**: 892–5.
32. Suehiro Y, Umayahara K, Ogata H *et al.* Genetic Aberrations Detected by Comparative Genomic Hybridization Predict Outcome in Patients with Endometrioid Carcinoma. *Genes Chromosomes Cancer* 2000; **29**: 75–82.
33. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681–6.

Table 1 Clinicopathological findings for endometrial endometrioid adenocarcinomas in this study

Clinicopathological findings		
Total		32 (%)
Age (means)		29-89 (53.0)
Grade	1	18 (56.3)
	2	13 (40.6)
	3	1 (3.1)
Nuclear atypia	High	14 (43.7)
	Low	18 (56.3)
FIGO stage	I	22 (68.8)
	II	5 (15.6)
	III	5 (15.6)
	IV	0 (0.0)
Myometrium invasion	< 1/2	20 (62.5)
	≧1/2	12 (37.5)
Lymph node metastasis	positive	3 (9.4)

Table 2 Frequency of copy number variations in 32 cases of endometrioid adenocarcinoma

Chromosome region	Alteration	Frequency (%)
1q21.2-44	LG gain	7/32 (21.9)
5q35.3	Gain	3/32 (9.3)
7q11.23	Gain	3/32 (9.3)
8q22.2-24.3	LG gain	4/32 (12.5)
10p15.3-11.1	LG gain	7/32 (12.5)
10q11.21-23.31	LG gain	9/32 (28.1)
10q23.31-26.3	LG gain	5/32 (15.6)
10q22.1-26.3	LG copy-neutral LOH	7/32 (21.9)
16q23.1	Gain	3/32 (9.3)

Gain, Chromosomal gain; LG copy-neutral LOH, Large general copy-neutral LOH; LG gain, Large general gain;

Table 3 Clinicopathological findings and molecular biological analysis and 1q chromosomal gain, 10q chromosomal gain and copy-neutral LOH in each cluster group

		Total (%)	Cluster1 (%)	Cluster2 (%)	Cluster3 (%)	Cluster4 (%)	*P*-value
Total		32 (100)	7 (21.9)	17 (53.1)	6 (18.8)	2 (6.2)	
Age (means)		29-89 (53.0)	38-72 (52.6)	29-63 (50.7)	39-89 (57.3)	60-63 (61.5)	
Grade	1	18 (56.3)	2 (28.6)	12 (70.6)	4 (66.7)	0 (0)	
	2	13 (40.6)	4 (57.1)	5 (29.4)	2 (33.3)	2 (100)	
	3	1 (3.1)	1 (14.3)	0 (0)	0 (0)	0 (0)	
Nuclear atypia †	High	12 (37.5)	6 (85.7)	4 (23.5)	1 (16.7)	1 (50.0)	†P<0.05
	Low	20 (62.5)	1 (14.3)	13 (76.5)	5 (83.3)	1 (50.0)	
FIGO stage	I / II	27 (84.4)	6 (85.7)	13 (76.5)	6 (100)	2 (100)	
	III/IV	5 (15.6)	1 (14.3)	4 (23.5)	0 (0)	0 (0)	
Lymph node metastasis	+	3 (9.4)	1 (14.3)	2 (11.8)	0 (0)	0 (0)	
1q gain ‡		7 (21.9)	0 (0)	0 (0)	6 (100)	1 (50.0)	‡P<0.01
10q gain, copy-neutral LOH §		13 (40.6)	7 (100)	2 (11.8)	2 (33.3)	2 (100)	§P<0.05
p53		4 (12.5)	1 (14.3)	2 (11.8)	0 (0)	1 (50.0)	
KRAS		5 (15.6)	2 (28.6)	2 (11.8)	0 (0)	1 (50.0)	
BRAF		1 (3.1)	0 (0)	0 (0)	1 (16.7)	0 (0)	
PIK3CA		8 (25.0)	3 (42.9)	4 (23.5)	1 (16.7)	0 (0)	
PTEN		21 (65.6)	6 (85.7)	11 (64.7)	3 (50.0)	1 (50.0)	
MSI-high		7 (21.9)	0 (0)	7 (41.2)	0 (0)	0 (0)	
CIMP-positive		4 (12.5)	1 (14.3)	3 (17.6)	0 (0)	0 (0)	

† In cluster 1, high nuclear atypia was seen significantly more often. Conversely, in cluster 2, the frequency of low grade nuclear atypia was high, and there was significant difference between the 2 groups ($P < 0.05$). ‡ In cluster 3, chromosomal gain in 1q was seen significantly more often ($P < 0.01$). § In cluster 1, chromosomal gain and copy-neutral LOH in 10q was seen significantly more often ($P < 0.05$).

FIGURE LEGENDS

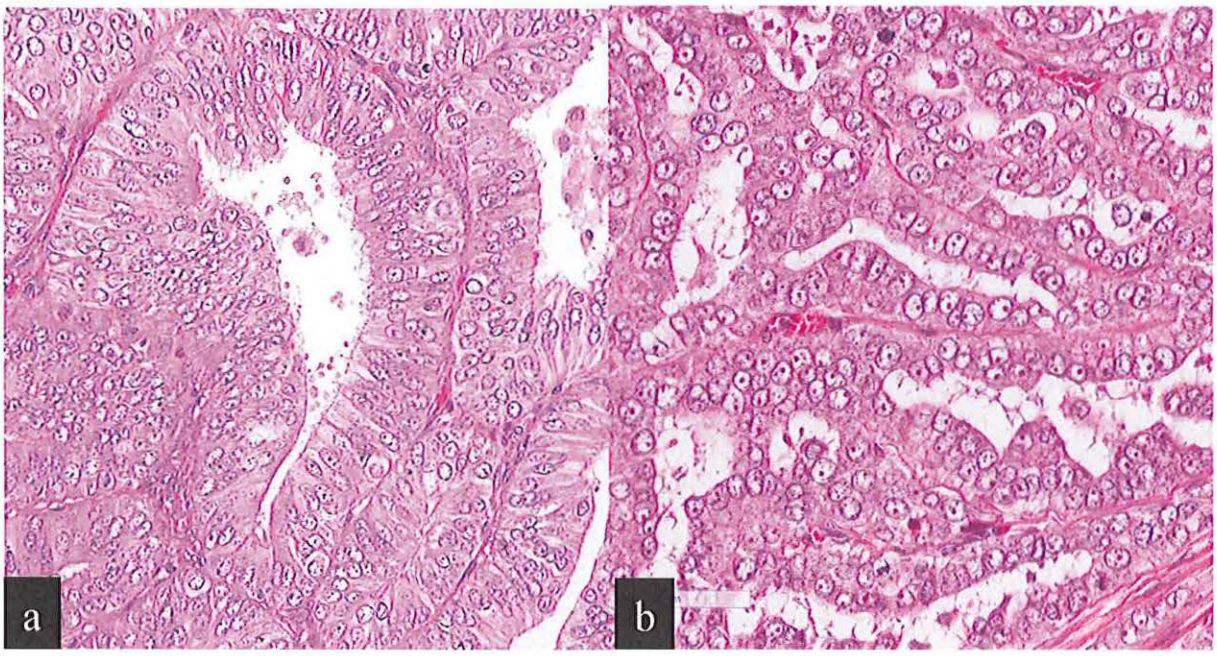
Figure 1 (**A**) Representative histological features of nuclear atypia in endometrial endometrioid adenocarcinoma (EEA). (**a**) Nuclear atypia, low (H&E, ×200). (**b**) Nuclear atypia, high (H&E, ×200). (**B**) (**a**) Histological section EEA (H&E, x40). (**b**) Isolated cancer crypts under a dissecting microscope. (**c**) Histological feature of isolated cancer glands (H&E, ×40). (**d**) Histological feature of isolated cancer glands (H&E, ×100).

Figure 2 (**a**) Overall copy number variations (CNVs) for each chromosome derived from 32 cases of endometrial endometrioid adenocarcinoma (EEA). Color coding indicates changes on each chromosome. Blue: loss of heterozygosity (LOH) (0, 1), orange: copy-neutral LOH (2), red: chromosomal gain (3, 4). (**b**) Total copy number variation regions in 32 cases of EEA. The total number of regions in which chromosome CNVs were seen in each subject are plotted as dots. The median value is represented by a horizontal line. The median values were 9.3×10^7 base pair (bp) for overall CNV, 5.8×10^7 bp for chromosomal gain, 5.3×10^6 bp for copy-neutral LOH, and 9.8×10^4 bp for LOH.

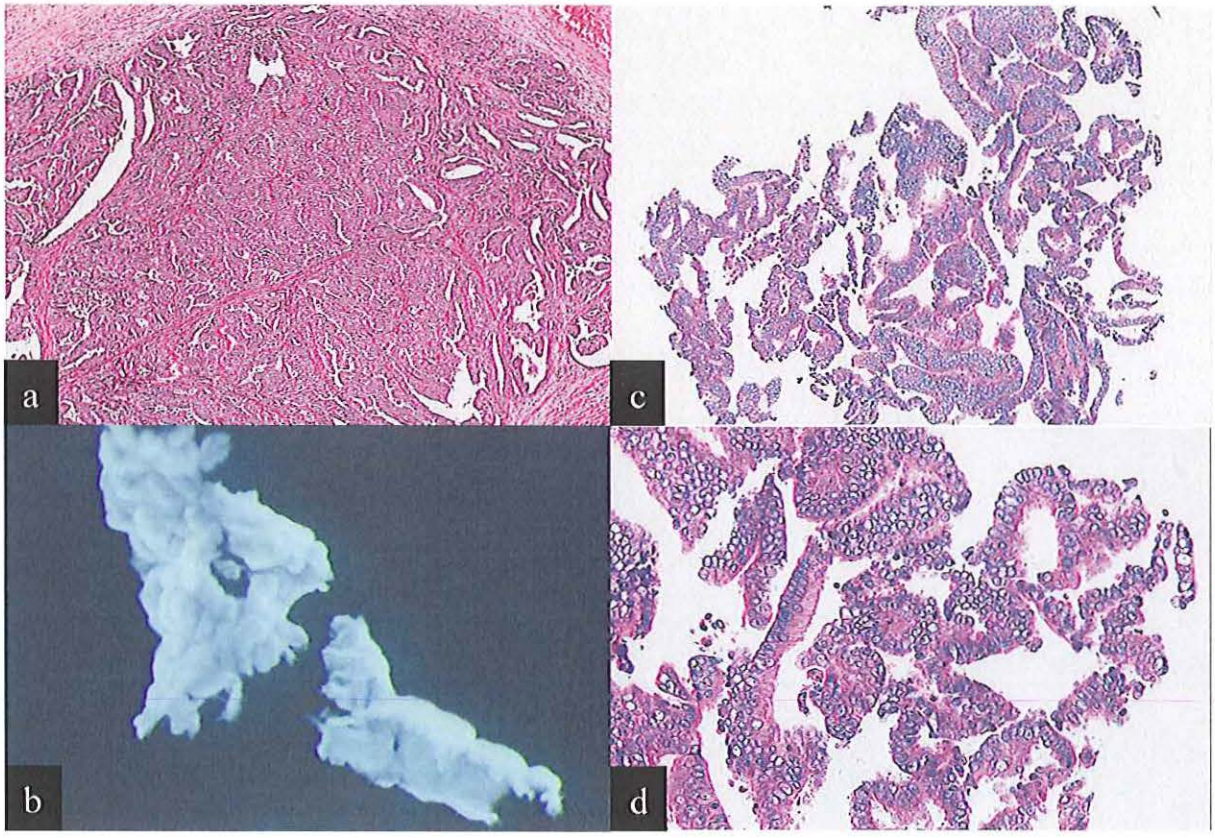
Figure 3 Overall chromosome copy number variations (CNVs) in microsatellite instability (MSI)-high and low. Each dot represents the total length of abnormal copy number regions in each case. The horizontal line represents the median value. (**a**) Overall CNV: median values were 6.30×10^6 base pair (bp) for MSI-high and 1.49×10^8 bp for MSI-low ($P < 0.05$). (**b**) Chromosomal gain: median values were 2.11×10^6 bp for MSI-high and 1.05×10^8 bp for MSI-low. (**c**) Copy-neutral loss of heterozygosity (LOH): median values were 5.18×10^6 bp for MSI-high and 5.48×10^6 bp for MSI-low. (**d**) LOH: median values were 8.51×10^5 bp for MSI-high and 9.60×10^4 bp for MSI-low.

Figure 4 (**A**) A heat map of clustering classification by a single nucleotide polymorphism (SNP) array data: Hierarchical cluster analysis was performed based on SNP array data of the 32 cases of endometrial endometrioid adenocarcinoma, and they were classified into 4 clusters. The results of mutation, immunohistochemical, microsatellite instability (MSI) and methylation analysis are shown at left. (**B**) Comparison of the total length of alteration in copy number variations (CNVs) between cluster groups: Each dot indicates the total length of the alteration in CNVs of each subject. The horizontal line indicates median value (*$P < 0.05$, **$P < 0.01$). (**a**) Overall CNV: median values were 2.39×10^8 base pair (bp) in cluster 1, 6.30×10^6 bp in cluster 2, 2.31×10^8 bp in cluster 3 and 1.19×10^9 bp in cluster 4. (**b**) Chromosomal gain: median values were 1.49×10^8 bp in cluster 1, 1.12×10^6 bp in cluster 2, 2.11×10^8 bp in cluster 3 and 6.46×10^8 bp in cluster 4. (**c**) Copy-neutral loss of heterozygosity (LOH): median values were 5.03×10^7 bp in cluster 1, 3.88×10^6 bp in cluster 2, 4.54×10^6 bp in cluster 3 and 2.27×10^7bp in cluster 4. (**d**) LOH: median values were 9.60×10^4 bp in cluster 1, 1.26×10^5 bp in cluster 2, 0 bp in cluster 3 and 5.22×10^8 bp in cluster 4.

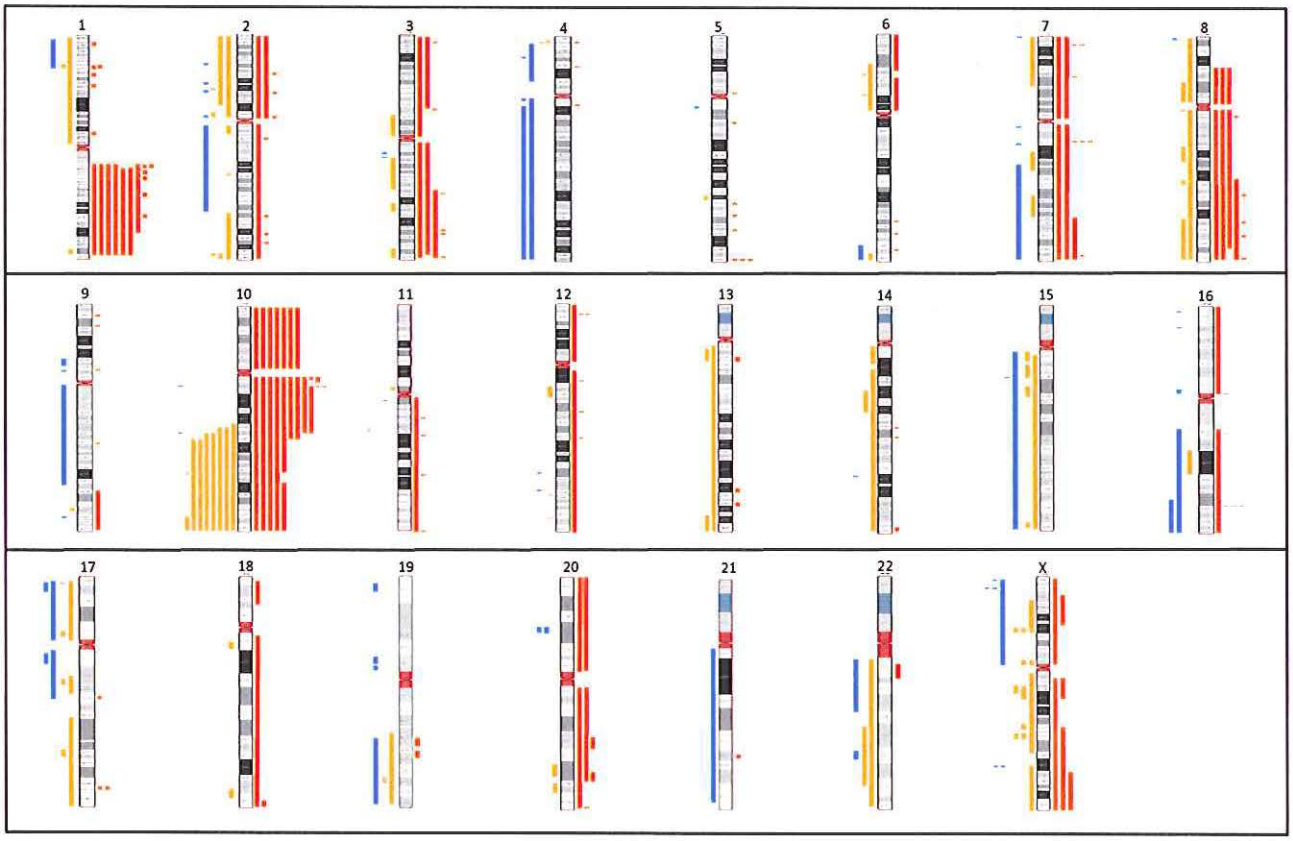
(A)



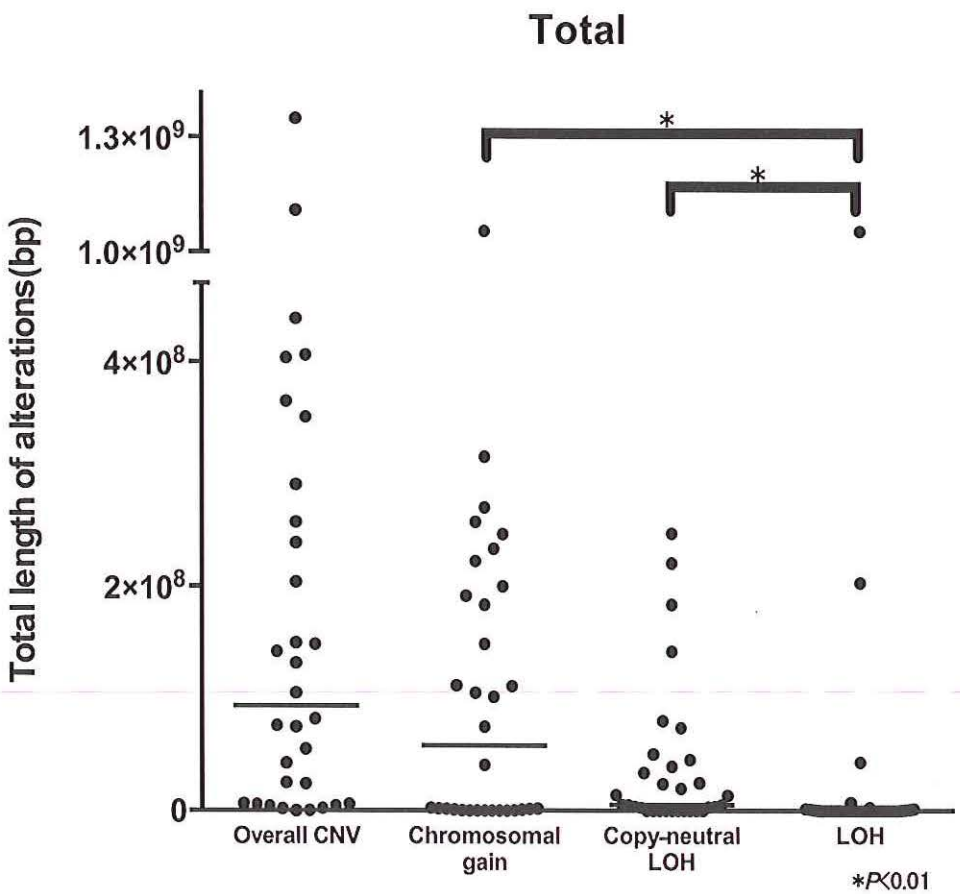
(B)

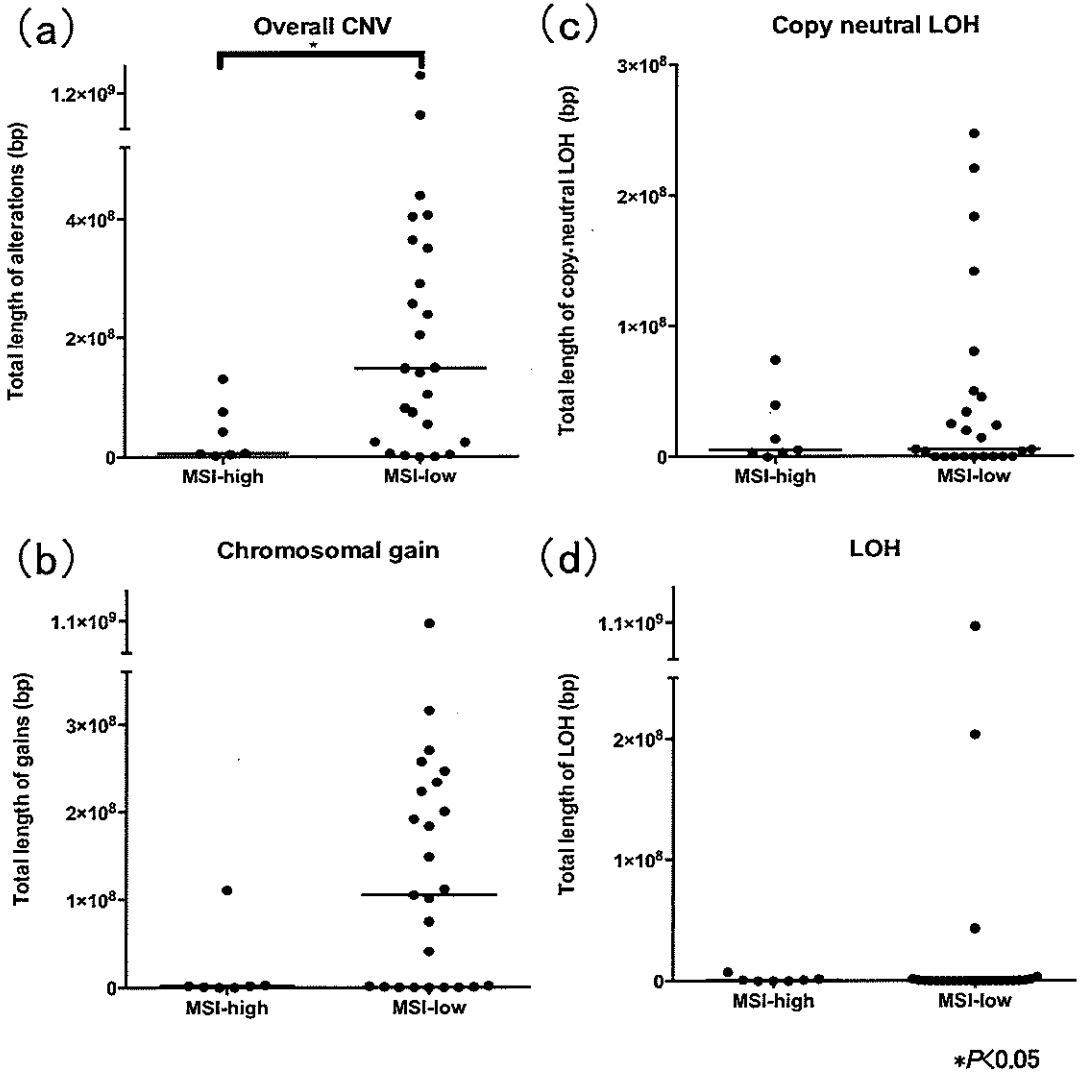


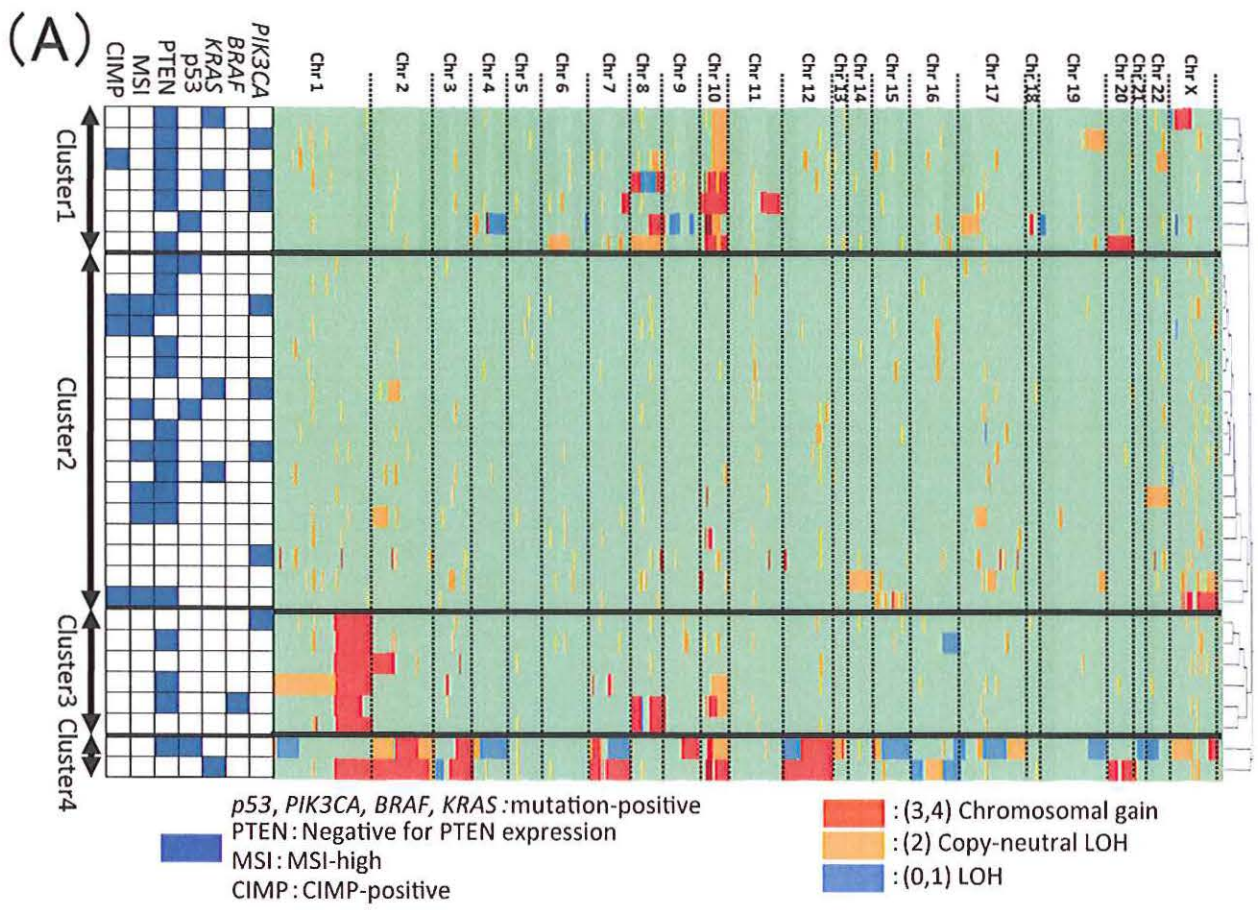
(a)

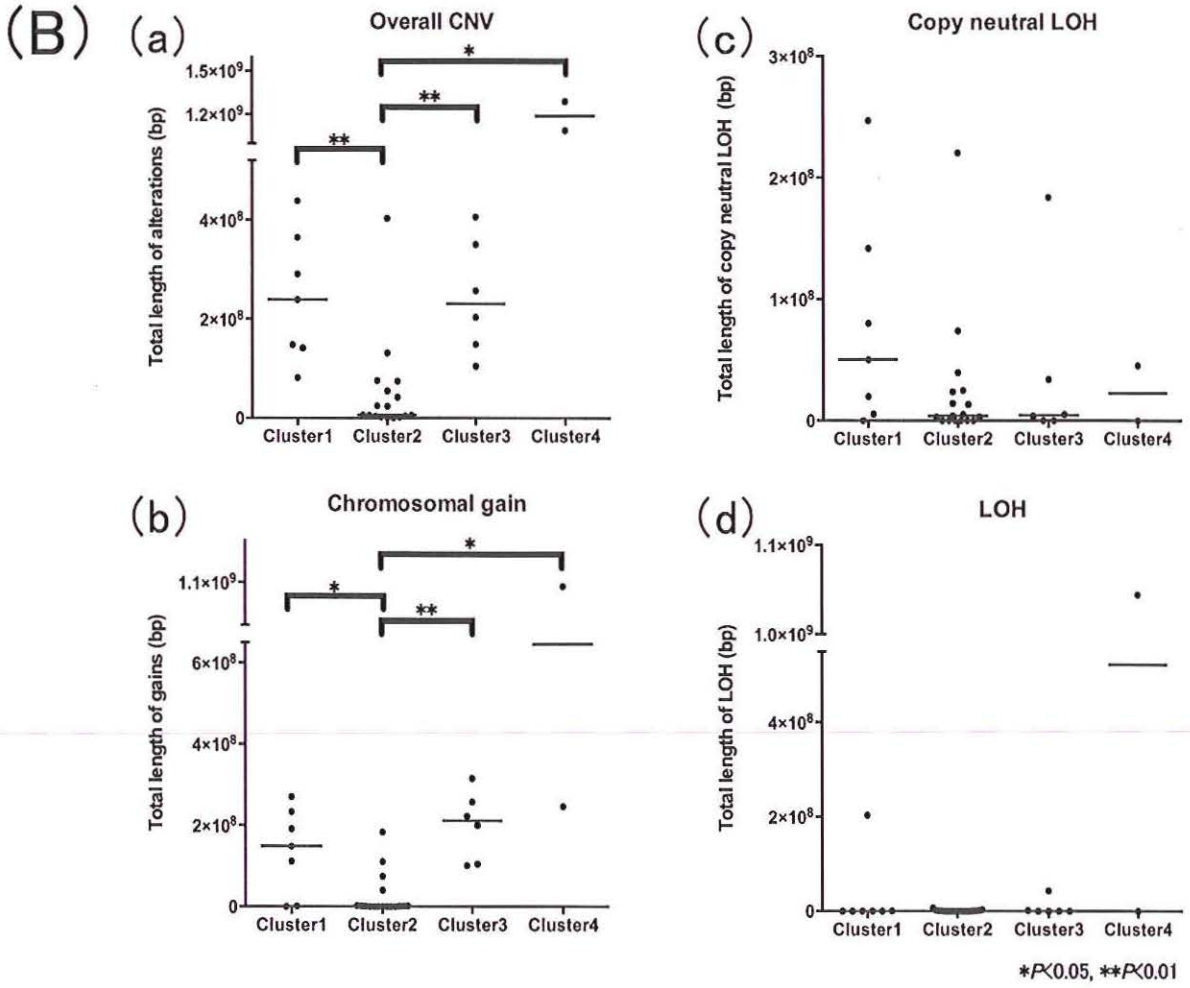

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X

(b)


Total
Total length of alterations(bp)
1.3×10^9
1.0×10^9
4×10^8
2×10^8
0
*
*
Overall CNV
Chromosomal gain
Copy-neutral LOH
LOH
*P<0.01


(a)
Overall CNV
*
Total length of alterations (bp)
1.2×10^9
4×10^8
2×10^8
0
MSI-high
MSI-low
(c)
Copy neutral LOH
Total length of copy.neutral LOH (bp)
3×10^8
2×10^8
1×10^8
0
MSI-high
MSI-low
(b)
Chromosomal gain
Total length of gains (bp)
1.1×10^9
3×10^8
2×10^8
1×10^8
0
MSI-high
MSI-low
(d)
LOH
Total length of LOH (bp)
1.1×10^9
2×10^8
1×10^8
0
MSI-high
MSI-low
*P<0.05


(A)
CIMP
MSI
PTEN
p53
KRAS
BRAF
PIK3CA
Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
Chr 16
Chr 17
Chr 18
Chr 19
Chr 20
Chr 21
Chr 22
Chr X
Cluster1
Cluster2
Cluster3
Cluster4
p53, PIK3CA, BRAF, KRAS : mutation-positive
PTEN : Negative for PTEN expression
MSI : MSI-high
CIMP : CIMP-positive
: (3,4) Chromosomal gain
: (2) Copy-neutral LOH
: (0,1) LOH


(B)
(a)
Overall CNV
Total length of alterations (bp)
1.5×10⁹
1.2×10⁹
4×10⁸
2×10⁸
0
Cluster1
Cluster2
Cluster3
Cluster4
(b)
Chromosomal gain
Total length of gains (bp)
1.1×10⁹
6×10⁸
4×10⁸
2×10⁸
0
(c)
Copy neutral LOH
Total length of copy neutral LOH (bp)
3×10⁸
2×10⁸
1×10⁸
0
(d)
LOH
Total length of LOH (bp)
1.1×10⁹
1.0×10⁹
4×10⁸
2×10⁸
0
*P<0.05, **P<0.01