Molecular Characteristics of Metastatic Lesions Have Superior Prognostic Impact on Endometrial Cancer

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ABSTRACT

Background/Aim: In endometrial cancer (EC), lymph node (LN) metastasis significantly impacts prognosis. Thus far, no studies have reported the molecular genetics of each metastatic lesion. This study aimed to investigate the molecular characteristics of primary and metastatic LNs and their association with clinical outcomes. Patients and Methods: The clinicopathological and molecular characteristics of 33 patients with EC with regional LN metastasis (FIGO stage IIIC) were investigated; we evaluated the mutational status of p53 and DNA mismatch repair (MMR) proteins in the primary lesion, all the positive LNs (102 lesions), mutational variation between primary and paired metastatic lesions, inter-lesion heterogeneity, and their association with clinical outcomes. Results: Immunohistochemically, 12 patients (36.4%) displayed aberrant p53 expression in metastatic lesions, and a concordant rate of 93.4% was observed between primary and metastatic lesions. Inter-lesion heterogeneity was observed in 20 cases (60.6%). In Kaplan–Meier analysis, patients with aberrant p53 expression in metastatic LNs exhibited worse progression-free survival (PFS) than those with wild-type p53 expression (p=0.008). Wild-type p53 expression in primary lesion with inter-lesion heterogeneity had a significantly worse PFS (p=0.049) than those without heterogeneity. In the Cox univariate analysis, p53 expression in metastatic LNs was significantly associated with recurrence (p=0.013). Genetic diversity between primary and metastatic lesions and among metastases was validated by evaluating p53 and MMR proteins by using immunohistochemistry (IHC) analysis. Conclusion: The molecular characteristics of metastatic lesions in addition to those of primary lesions could provide beneficial prognostic information in patients with EC with regional LN metastasis.

The incidence of endometrial cancer (EC), which is the most common gynecological malignancy worldwide, is increasing. Japan exhibits the same tendency with >12,000 new cases annually (1-6). Conventionally, EC is classified into two groups according to clinical and endocrine features (7-9). Type I EC is characterized by low-grade tumors, mostly endometrioid adenocarcinomas associated with hyperestrogenism. Moreover, type I EC often contains atypical hyperplasia, which is considered to indicate precursor lesions, and its prognosis is favorable. Type II EC is characterized by high-grade tumors other than endometrioid G1 and G2, such as serous and clear cell carcinoma. Type II EC does not correlate with hyperestrogenism; it arises from the atrophic endometrium and carries a poor prognosis. The Cancer Genome Atlas (TCGA) classifies EC into four distinct categories based on molecular genetics, while the clinical behavior varies according to the four subtypes (7). These findings highlight the growing importance of molecular characteristics for an overall understanding of tumors and their management strategies. These findings have recently shifted the paradigm of EC classification; molecular characteristics were first mentioned concerning on endometrioid carcinoma, in the revised 5th edition of the WHO classification of female genital tumors (10).

Approximately 90% of cancer-related deaths are thought to be attributed on failure to control metastases rather than the primary tumor (11); thus, the molecular characteristics of metastatic lesions might have a more critical role in prognosis than those of primary tumors. Intra-tumor heterogeneity is thought to be present in the earliest days of carcinogenesis (12, 13), and metastatic lesions may have heterogeneous characteristics even at the start of treatment. Moreover, lymph node (LN) metastases are perceived to contain high levels of genetic diversity compared to distant metastases (14).

Most previous studies, including those based on the TCGA data, have focused on primary lesions without the mention of metastatic lesions. To the best of our knowledge, no study has previously investigated the molecular genetics of each metastatic lesion.

The current study focused on the analysis of p53 and mismatch repair (MMR) protein expression in primary and metastatic LNs, their clinicopathological characteristics, and their association with clinical outcomes.

Patients and Methods

Patients. This retrospective study enrolled 33 patients with stage IIIC EC who were treated at the Okayama University Hospital between January 2011 and December 2019. Patients who received preoperative chemotherapy and/or did not undergo systematic lymph node dissection were excluded from the study. This study was approved by the ethics committee of the Okayama University (approval number: 1901-022). Informed consent was obtained from all the participants. All the procedures were performed according to the relevant ethical standards and institutional ethics committee regulations.

All the patients were treated in accordance with the current Japan Society of Gynecologic Oncology (JSGO) guidelines (15); operative procedures included total hysterectomy, bilateral salpingo-oophorectomy, pelvic and/or para-aortic lymphadenectomy with or without omentectomy. Stage IIIC Patients with EC are categorized as high-risk for relapse, and adjuvant treatment is strongly recommended. Thus, all the patients in this study underwent systemic platinum-based combination chemotherapy after surgery.

Clinicopathologigal characteristics. The following data were extracted from the medical records: age, FIGO stage (IIIC1: positive pelvic LNs and IIIC2: positive para-aortic LNs with or without positive pelvic LNs), TNM classification, pathological findings such as histology, tumor grade, lymphovascular space invasion (LVSI), tumor volume (calculation: height × width × length / 2), tumor size (the largest diameter of the three dimensions), tumor volume and size were confirmed by

imaging findings, location and number of positive LN metastases, lymph node ratio (LNR: defined as the percentage of positive LNs to total dissected LNs), and clinical outcomes.

Immunohistochemistry (IHC) of p53 and MMR proteins. Formalin-fixed paraffin-embedded (FFPE) blocks of all positive metastatic LNs and representative primary tumors that contained large amounts of tumor tissue were prepared. Histological slides were carefully reviewed by the authors jointly (KO and KN), and no apparent specimen-specific morphological differences were confirmed. All the FFPE specimens were cut into 4-μm thick slices. Thereafter, the FFPE sections were deparaffinized with xylene and rehydrated using an ethanol gradient. Endogenous peroxidase activity was blocked with H_2O_2, followed by antigen retrieval using citrate buffer for p53 or ethylenediaminetetraacetic acid buffer for MMR proteins at 98°C for 20 min. Next, the slides were incubated at 15–25 °C with primary antibodies against p53 from Ventana Medical Systems (mouse, monoclonal, DO-7, prediluted, 60 min), MLH1 (mouse, monoclonal, M1, prediluted, 120 min), PMS2 (mouse, monoclonal, A16-4, prediluted, 60 min), MSH2 (mouse, monoclonal, G219-1129, prediluted, 60 min), and MSH6 (rabbit, monoclonal, SP93, prediluted, 30 min). The sections were then incubated with biotinylated secondary antibodies (Vectastain ABC mouse or rabbit IgG kit, PK-4001 or PK-4002; Vector Laboratories). Specific antigen-antibody reactions were visualized with diaminobenzidine tetrahydrochloride, and hematoxylin was used for nuclear counterstaining.

IHC evaluation of p53 and MMR proteins. The expression status of p53 was evaluated as described previously (16). In order to identify heterogeneous p53 expression, we divided wild-type expression into two subgroups according to the percentage of positive tumor cells: <10% and 10–80%. In the involved LNs, when at least one LN displayed aberrant expression, the overall p53 expression was classified as aberrant.

The expression status of MMR proteins was also evaluated as described previously (16). Tumors with >10% of its area displaying subclonal loss of these proteins were identified as heterogeneous and considered as dMMR (16). In the involved LNs, a mixed case of MMR-proficient (pMMR) and dMMR was identified as inter-LN heterogeneity and considered as dMMR.

Staining pattern was carefully evaluated by the authors jointly (KO and KN).

Statistical analysis. Analyses were performed using the SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p<0.05$. Comparisons between the two groups

were performed using the Mann–Whitney U test. Pearson's χ^2 test was used to test for independence. Survival curves were calculated using the Kaplan–Meier method with the log-rank test. A univariate Cox regression model was used to evaluate the prognostic factors for progression-free survival (PFS).

Results

Clinicopathological characteristics. The clinicopathological findings and outcomes are summarized in Figure 1 and Table I. The age at diagnosis ranged from 32–77 years (median 57 years). Histologically, 14 patients (42.4%) had low-grade tumors (endometrioid G1 or G2) and 19 (57.6%) had high-grade tumors (any histology besides endometrioid G1 or G2). The number of resected and positive LNs ranged from 20–84 (median 41) and 1–20 (median 5), respectively. The LNR ranged from 1% (1/72) to 100% (20/20). The median tumor volume was 42.0 cm^3 (2.7–963.1 cm^3) and the median largest tumor size was 5.6 cm (2.0–16.3 cm). Furthermore, the median duration of observation was 62 months (10–136 months); 14 patients (42.4%) relapsed, and four patients died. The median PFS was 47 months.

Expression status of p53 in primary and metastatic lesions. We performed IHC analysis of p53 expression and identified 21 (63.6%) and 12 (36.3%) cases as wild and aberrant phenotypes, respectively, in both, primary and metastatic lesions. Among the 21 cases of wild-type in the primary lesion, one showed aberrant p53 expression in the metastatic lesion, and among the 12 cases of aberrant p53 expression in the primary lesion, one case showed wild-type p53 expression in the metastatic lesions. Therefore, the concordance rate between primary and metastatic lesions was 93.9% (31/33). Inter-lesion heterogeneity was observed in eight cases (24.2%). Representative images of p53 are shown in Figure 2.

Profiles of MMR proteins. As confirmed by the IHC analysis, 16 cases (48.5%) were classified as dMMR and 17 (51.5%) as pMMR. No cases of discordant MMR status between primary and metastatic lesions were observed. Among the 16 dMMR cases, 14 cases (87.5%) showed diffuse loss of both MLH1 and PMS2, one (6.3%) showed subclonal loss of MSH6 alone, and the last case showed

subclonal loss of all four MMR proteins. Of the 14 dMMR cases with diffuse loss of MLH1 and PMS2, eight (57.1%) presented heterogeneous staining of MSH2 and/or MSH6. Finally, inter-lesion heterogeneity was observed in eight cases (24.2%). Representative examples of IHC staining for MMR proteins are shown in Figure 3.

According to the heterodimer formation pattern of the MMR proteins, dMMR was divided into two main classifications: MLH1-PMS2 deficiency and MSH2-MSH6 deficiency. The dMMR cases were further divided into two groups: the first (1) consisted of cases with MLH1-PMS2 or MSH2-MSH6 deficiency and the second (2) consisted of cases with MLH1-PMS2 and MSH2-MSH6 deficiency. Among the 16 dMMR cases, seven cases fell into the first classification and nine cases fell into the second classification.

Survival analysis with respect to molecular stratification. Survival analysis revealed that patients with aberrant p53 expression in primary lesions and metastatic LNs had shorter PFS than those with wild-type p53 expression (p=0.068 and p=0.008, respectively) (Figure 4A and 4B). MMR expression was not significantly associated with PFS (p=0.144) (Figure 4C). Of the dMMR cases, one of the seven cases (5.9%) falling into classification (1) and four of the nine cases (44.4%) falling into classification (2) experienced recurrence; however, these findings were not statistically significant (p=0.273). Wild-type p53 in primary lesion with inter-lesion heterogeneity in either p53 or MMR proteins had significant worse PFS than those without inter-lesion heterogeneity (p=0.049) (Figure 4D).

Receiver operating characteristic (ROC) curve of LNR. Although LNR is not considered as a risk factor for recurrence in the current JSGO guideline, several studies have proposed the association of LNR and prognosis (17-19). Since we focused on EC with LN metastases, we considered LNR as one of the clinical characteristics in this study. The ROC curve analysis was used to determine the

optimal cutoff values of LNR for predicting recurrence. The analysis identified an LNR ≥0.11 (area under the curve =0.691, sensitivity: 71.4%, specificity: 63.2%) as the most accurate cutoff value for predicting recurrence in this cohort (Figure 5).

Correlation between aberrant p53, LNR, and patient characteristics. Aberrant p53 expression in metastatic lesion was significantly associated with older age, high-grade histology and recurrence (Table II). An LNR ≥0.11 significantly correlated with older age and recurrence (Table III).

Cox univariate analysis of the association between patient characteristics and PFS. Aberrant p53 status in metastatic lesions were significantly associated with PFS (p=0.013) (Table IV).

Discussion

Recently, due to next-generation sequencing technology, the molecular characteristics and mutational profiles of EC have been identified, especially in the TCGA study (7).

During initial management of EC, surgery is often the standard of care, and identifying the risk of relapse based on the pathological findings has a strong influence on postoperative treatment (5, 15). The pathological findings include histology, degree of muscle invasion, LVSI, and the presence or absence of extrauterine lesions (15). However, these factors do not take into account the importance of the molecular and mutational signatures of EC in prognosis.

Intra-tumor heterogeneity could be present in the earliest days of carcinogenesis (12, 13), and metastatic lesions could possess heterogeneous characteristics even at the start of treatment. Therefore, analysis of only primary lesions could result in a misinterpretation of the metastatic tumors.

TP53 mutation occurs early in tumorigenesis and is one of the most important molecular factors associated with unfavorable prognosis (2, 13, 20). According to TCGA, analysis of dMMR is also

crucial for prognosis and MMR deficiency arises in the early stages of tumorigenesis (5, 7, 16, 21, 22). Next-generation DNA sequencing is expensive and impractical to apply in routine clinical settings. Moreover, metastatic lesions like LNs often contain relatively small amount of tumor. In this situation, DNA sequencing could not be applied compared to IHC, which could be easily performed. IHC can reveal the status of *TP53* mutation and MMR deficiency (20, 22). To the best of our knowledge, this is the first study to analyze the association of the clinical outcomes with p53 and MMR protein status of primary lesions and all the metastatic LNs.

TP53 mutation is the single most influential factor affecting prognosis (20) and aberrant p53 expression was observed in 39.4% of patients in our cohort. Previously reported frequencies of p53 overexpression vary widely for a range of reasons, including the study cohort. For example, Haraga *et al.* reported a p53 overexpression rate of 14.7% in a cohort of patients with all stages of EC, and Saijo *et al*. reported aberrant p53 status in 63% of patients with endometrial carcinosarcoma (16, 23).

William *et al*. reported a variation in the number of mutations in primary lesions and their paired metastases; even among common driver mutations, the concordance rate of primary and metastatic lesions was 83% (2). In the current study, the concordance rate of p53 status between primary and metastatic lesions was 93.9%; and eight cases exhibited inter-lesion heterogeneity including six cases defined as the same classification both primary and metastatic lesions. Four out of eight (50%) cases contained low-grade endometrioid histology, and two out of the four cases exhibited dMMR. Köbel *et al*. reported the possibility of later acquisition of *TP53* mutation in endometrioid carcinoma, especially in mutator phenotype like dMMR [20]. The heterogeneous expression revealed in our study could be attributable to the subsequent occurrence of *TP53* mutations, especially in low-grade endometrioid and dMMR cases.

In previous studies, approximately 20-40% of patients with EC demonstrate dMMR, largely resulting from promoter hypermethylation and silencing of MLH1 (3, 22, 24, 25). Ida *et al*. reported

a dMMR rate of 60% in mixed endometrial carcinomas, while Saijo *et al*. reported a dMMR rate of only 10.5% in only endometrial carcinosarcoma (3, 16, 23-25). Our cohort displayed a relatively high dMMR rate of 48.5%, possibly due to inclusion of different cohorts. The tumor volume might be another possible cause for this variation. For example, Casey *et al*. reported that epigenetic silencing of MLH1 is significantly associated with large tumor volume (26). Since our study evaluated patients with EC at an advanced stage, we observed considerably large tumor volumes with a median tumor size of 42.0 cm^3. Tumor size is a significant prognostic factor, and the cutoff value of a diameter of 2 cm exists between large and small tumors (27). Our study observed diameters much longer than the cutoff value, and in all cases, the tumor size was ≥2 cm.

We speculate that the large tumor volume is also related to the heterogeneous staining pattern of MMR proteins. Of the 16 cases of dMMR in this study, eight presented homogenous loss of MLH1/PMS2 and heterogeneous expression of MSH2/MSH6. A similar expression pattern is often observed in colorectal cancer, and the possibility of secondary MSH2/MSH6 inactivation has been proposed (28). Since stage IIIC EC is an advanced disease, the primary lesion volume tends to be large owing to repeated cell division. Additionally, because patients with EC with dMMR have a faulty DNA MMR system, more cell division occurs, resulting in increased DNA replication errors and mutations, ultimately resulting in heterogeneous MMR protein expression.

Considering the prognostic value of p53, Kaplan–Meier and Cox univariate analyses showed that p53 expression in metastatic lesions was significantly associated with PFS in our study. Aberrant p53 status in metastatic lesions could be a superior prognostic predictor to that in primary lesions. The association between MMR deficiency and survival outcomes remains controversial (3). Since EC with dMMR is characteristically hypermutated according to a TCGA study (7), it displays large amounts of neoantigens and potentially high immunogenicity. These signatures could result in prognostic differences between patients with MMR proficient and deficient EC. Among the dMMR

cases, although we failed to demonstrate significant differences between the observed classifications ([1] MLH1-PMS2 or MSH2-MSH6, [2] MLH1-PMS2 and MSH2-MSH6) (p=0.273), the recurrence rate in cases from classification two (44.4%) was higher than in cases from classification one (5.9%). We believe that the observed classifications could explain the prognostic differences. Moreover, intra- and inter-lesion heterogeneity attributed to genetic instability is considered to possess two aspects: one is favorable; increased immunogenicity resulting in better prognosis, and the other is unfavorable; unfavorable additional mutation might occur resulting in worse prognosis. In this study, even the cases with wild-type p53 expression in primary lesion had worse PFS when complicated with inter-lesion heterogeneity, possibly because the unfavorable aspect might be highlighted.

For clinical data, LNR has been proposed as a meaningful prognostic factor in patients with stage IIIC EC (17-19). Under our cutoff value by ROC curve analysis, LNR ≥0.11 showed a significant association with recurrence and marginal correlation with PFS in Cox univariate analyses (p=0.049 and p=0.068, respectively). Our finding of LNR corresponded to previous findings (17-19).

Although our study cohort was relatively small and confined to a single Institution, this study provides a thorough evaluation and interpretation of p53 and MMR proteins in primary and all involved LNs in patients with stage IIIC EC. We could validate the polyphyletic evolution of different mutational signatures from primary lesions occurring even in fundamental molecules, such as p53 and MMR proteins. When diagnosing stage IIIC EC, an evaluation of p53 and MMR proteins in metastatic lesions in addition to primary lesion should be considered. When heterogeneity is identified in metastatic lesions, it is advisable to consider the possibility of an unfavorable prognosis and increase the level of surveillance after initial treatment.

In conclusion, polyclonal development from the primary lesion to individual LNs was validated. This heterogeneity could impact the prognosis. Aberrant p53 expression in the metastatic lesion had worse PFS than those with wild-type expression. Even the case with wild-type p53 status in primary

lesion had worse PFS when complicated with inter-lesion heterogeneity of either p53 or MMR proteins. Therefore, evaluation of p53 and MMR proteins in metastatic lesions in addition to primary lesion could provide superior beneficial information than examination of only primary lesion in patients with stage IIIC EC.

Conflicts of Interest

The Authors declare no conflict of interest.

Authors' Contributions

K.O. and K.N. conceived this study. K.O. conducted the experiments. J.H. assisted in the experiments. K.N. double-checked experiment results. K.O. and K.N. analyzed the results. K.O. and K.N. wrote the main manuscript and prepared the Tables and Figures. H.M. supervised the entire study. All the authors reviewed and approved the manuscript.

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Figure Legends

Figure 1. ~~Patient clinicopathological and molecular characteristics, as well as prognostic outcomes.~~ Clinicopathological and molecular landscape of 33 patients with EC in this study cohort. The first to

the third line from the top shows expression status of p53 in primary and metastatic lesion, and the existence of inter-lesion heterogeneity. The fourth to the 12th lines indicate expression status of MMR proteins in primary and metastatic lesion, and the existence of inter-lesion heterogeneity. Comprehensive evaluation of p53 and MMR proteins in primary and metastatic lesion, and their heterogeneity are shown in the 13th to 15th lines. Then clinical data (FIGO stage, Grade, Histology and Lymph Node Ratio) and prognostic data (Recurrence and Death) are shown.

Figure 2. Representative images of p53 expression status by immunohistochemistry (IHC). (A–B): wild type expression, (C–F): aberrant type expression. (A): positive nuclear staining <10%, (B): positive staining 10–80%, (C): positive staining >80%, (D–E): focal positive staining >80%, (F): complete negative staining (null pattern).

Figure 3. Representative examples of immunohistochemical staining of mismatch repair (MMR) proteins. The surrounding stromal cells adjacent to the tumor cells were used as a positive control. (A): Diffuse positive nuclear staining of the tumor cells considered as pMMR, (B): Diffuse negative staining of the tumor cells (lower side) considered as dMMR, surrounding stromal cells and lymph follicle (upper side) are positive control, (C): focal loss of staining regarded as dMMR. (D–F): case12, MSH6, (D): primary lesion; focal loss, (E): one of the right obturator LNs; diffuse loss, (F): one of the right obturator LNs: diffuse positive.

Figure 4. Kaplan–Meier curves of progression-free survival (PFS). (A): p53 status in primary lesion, (B): p53 status in metastatic lesions, (C): mismatch repair (MMR) status, (D): with or without inter-lesion heterogeneity in p53 and MMR proteins.

Figure 5. Receiver-operating characteristic curve analysis to determine the cutoff value of lymph node ratio (LNR) for the prediction of recurrence.

Table Legends

Table I. Patient clinicopathological characteristics.

Abbreviations used in the Table I. Values: median (range) or number (%). G: Grade; LVSI: lymphovascular space invasion; LN: lymph node; LNR: lymph node ratio.

Table II. p53 status in metastatic lesion and patients' characteristics.
Abbreviations used in the Table II. LNR: lymph node ratio. *p<0.05; **p<0.01.

Table III. LNR and patients' characteristics.
Abbreviations used in the Table III. LNR: lymph node ratio; LN: lymph node; SD: standard deviation; MMR: mismatch repair. *p<0.05.

Table IV. Prognostic factors for progression-free survival by Cox univariate analysis.
Abbreviations used in the Table IV. PAN: para-aorta lymph node; LNR: lymph node ratio; MMR; mismatch repair. *p<0.05.