Alterations in β-catenin, microsatellite instability, and HNF-1β levels are independently associated with ovarian endometriosis-associated tumorigenesis

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Revised

Alterations in β-catenin, microsatellite instability, and HNF-1β levels are independently associated with ovarian endometriosis-associated tumorigenesis

Running title: ovarian endometriosis-carcinoma sequence

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Conflict of interest

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Abstract

We focused on specific molecular events during the development of endometriosis-associated ovarian endometrioid carcinoma (OEmCa) and ovarian clear cell carcinoma (OCCCa). Alterations in β-catenin (encoded by *CTNNB1*) and microsatellite instability (MSI), as well as changes in the expression levels of HNF-1β and DNA mismatch repair (MMR) proteins were investigated in 50 OEmCas and 21 OCCCas with endometriotic lesions. Mutations of *CTNNB1* were identified in 28 (56%) of the 50 OEmCa cases and 26 (41.9%) of the 62 coexisting endometriosis lesions. MSI-high (H) was observed in seven (14.6%) of the 48 OEmCa and 14 (23.3%) of the 60 coexisting endometriosis, and was significantly associated with loss of MMR protein expression, but not *CTNNB1* mutations. Non-identical *CTNNB1* status between two different epithelial lesions within endometriosis was observed in eight out of 10 informative endometriosis cases that had adjacent OEmCa. Similar findings for MSI features were also found in two out of three informative cases, suggesting that endometriotic lesions may predominantly consist of polyclonal cells. In contrast, high HNF-1β expression was significantly associated with SLC3A1 expression, which plays a major role in HNF-1β-triggered induction of reactive oxygen species (ROS) in OCCCas, independent of abnormalities in both β-catenin and MSI/MMR status. Finally, four inflammatory parameters associated with repeated hemorrhaging in endometriosis were significantly higher in endometriosis with MSI-H when compared to that with MSS, independent of both β-catenin and HNF-1β status. In conclusion, different molecular pathways including alterations in β-catenin, MSI, and HNF-1β levels may contribute to tumorigenesis in endometriosis-associated carcinoma.

Key words: β-catenin, HNF-1β, microsatellite instability, endometriosis, ovarian carcinoma

1. Introduction

Endometriosis is a common gynecological disorder, classically defined as the presence of endometrial-like glands and stroma outside the uterus, and is thought to affect approximately 10-15% of all premenopausal women [1,2]. Ovarian endometriosis is a benign lesion, but its potential for malignancy and specific association with ovarian carcinoma (endometriosis-associated ovarian carcinoma: EAOCa) has been proposed based on the following clinicopathological evidence. Approximately 60% of EAOCas are adjacent to, or arise directly from, endometriosis lesions [3,4]; 5-10% of women with endometriosis have ovarian carcinomas with the predominant types being ovarian clear cell carcinoma (OCCCa, 40-50%) and endometrioid carcinoma (OEmCa, 20-40%), and endocervical-like type mucinous borderline tumors (<10%) [4-6].

Endometriosis lesions contain fluid comprising an excess of free iron due to repeated hemorrhaging [7]. High concentrations of free iron leading to increased levels of reactive oxygen species (ROS) in endometriosis can trigger oxidative damage, along with the activation of macrophages and release of cytokines into the stroma [8-10]. Circumstantial evidence that iron overload in experimental animals enhances epithelial cell proliferation and causes malignant tumors suggests that a similar tumorigenic mechanism occurs in endometriosis [11,12]. In contrast, HNF-1β (hepatocyte nuclear factor 1 homeobox B), which is highly expressed in OCCCa, acts as a pro-survival factor in endometriosis, and thereby contributes to tumorigenesis. This is due to its ability to exert ant-oxidant effects by altering glucose metabolism [13,14].

Instability of short tandem repeats or microsatellite instability (MSI) is a molecular phenotype that occurs due to a defective DNA mismatch repair (MMR)

system [15]. The MMR proteins recognize and fix DNA single base mismatches and small insertion/deletion loops that arise during DNA replication, homologous recombination, or other forms of DNA damage, such as oxidative stress [16,17]. Such mismatch repair abnormalities can be induced in endometriosis due to the prolonged inflammation in the microenvironment: ultimately this may result in ovarian tumorigenesis [18].

We have previously demonstrated that oncogenic mutations affecting the phosphorylation site of β-catenin (encoded by *CTNNB1*) occur during the relatively early stages of EAOCa development, particularly the OEmCa phenotype [19]. In the present study, we further investigated the molecular associations among β-catenin, HNF-1β, and MSI/MMR abnormalities during endometriosis-carcinoma (OEmCa and OCCCa) sequences.

2. Materials and methods

2.1. Clinical cases

The EAOCa was defined as endometriosis with OEmCa or OCCCa regardless of the presence of histological continuity between carcinoma (Ca) and endometriotic epithelium, as described by Yamamoto *et al.*, with minor modification [20]. Based on this definition and according to the criteria of the 2014 World Health Organization classification [21], we selected a total of 71 cases of EAOCa including 50 OEmCa and 21 OCCCa subtypes along with 20 cases of endometriosis without Ca surgically resected at Kitasato University Hospital from 2000 to 2018. Twenty OEmCa, but not OCCCa, cases overlapped with those used in our previous study [19]. Endometriosis with Cas were subdivided into two groups, endometriosis-1 and endometriosis-2 (E1 and E2)

lesions, depending on whether the endometriosis lesions were located adjacent to or distant from the cancerous lesion, respectively. The mean age of the patients was 53.1 years (range 22-80 years). Forty-nine cases were subcategorized as stage I and 22 as stage II to IV (Table 1), according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) [22]. None of the patients had undergone chemotherapy or radiation therapy before surgery. The tissues were routinely fixed in 10% formalin and processed for embedding in paraffin. Approval for this study was given by the Ethics Committee of the Kitasato University School of Medicine (B18-055).

2.2. Antibodies

Anti-β-catenin (clone 14/Beta-Catenin; 1/1000 dilution) and anti-HNF-1β (25/HNF-1β; 1/1000 dilution) were purchased from BD Biosciences (San Jose, CA, USA). Anti-MLH1 (ES05; 1/100 dilution), anti-MSH2 (25D12; 1/50 dilution), anti-MSH6 (PU29; 1/100 dilution), and anti-PMS2 (MoR4G; 1/100 dilution) antibodies were obtained from Novocastra (Leica Biosystems, Milton Keynes, UK). Anti-SLC3A1 antibody (ab196552; x1/100 dilution) was obtained from Abcam (Cambridge, UK).

2.3. Immunohistochemistry (IHC)

IHC was performed using a combination of the microwave oven heating and Histofine Simple Stain MAX-PO (MULTI: Nichirei Biosciences, Tokyo, Japan) methods. Briefly, after deparaffinization, the slides were treated by heating in a microwave oven in 10 mM citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) for 15 min, and incubated overnight at 4℃ with optimized dilution of primary

antibodies.

Scoring of nuclear β-catenin and HNF-1β immunoreactivity and cytoplasmic SLC3A1 immunostaining, respectively, was performed to evaluate the IHC findings. Briefly, the percentages of immunopositive cells were subdivided into five categories as follows: 0, all negative; 1, <10% positive cells; 2, 10-30%; 3, 30-50%; and 4, >50%. The immunointensity was also sub-classified into four groups as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. IHC scores were generated by multiplying the values of the two parameters together.

Loss of MMR expression was also defined as the absence of immunoreactivity in >90% of tumor nuclei, with intact nuclear expression in non-tumor cells (internal positive control). Cases with subclonal loss or heterogeneity of MMR expression were noted, but were classified as intact staining, as described by Xiao *et al*., with minor modifications [23].

2.4. Endometriosis-inflammatory (E-I) score

To evaluate the morphological alterations that occurred during repeated hemorrhaging in endometriosis, the lesions were examined and evaluated according to the following four parameters: 1) hemorrhagic area, 2) accumulation of macrophages, 3) degree of erosive change, and 4) degree of inflammatory cell infiltration. Each parameter was scored based on the extent of lesion in each section as follows: 0 (<10% of a section), 1 (10-30%), 2 (30-50%), and 3 (>50%). The E-I score was graded by counting the scores of each parameter.

2.5. Mutation analysis for *CTNNB1*

A 10-μm paraffin-wax section was reviewed and carcinoma cell-rich areas were manually dissected under microscopic guidance, avoiding contamination by stromal components. Their matched endometriotic lesions were also microdissected using an Arcturus XT (Applied Biosystems, Foster City, CA, USA). Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). Exon 3 of the *CTNNB1* was amplified by PCR, as described previously [19]. Subsequently, the PCR products were subjected to direct sequencing PCR with BigDye terminator v 3.1/1.1 cycle sequencing reagents (Applied Biosystems). The samples were finally analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

2.6. MSI testing

MSI was tested using the Bethesda Consensus Conference reference panel containing five markers (*BAT-25, BAT-26, D2S123, D5S326*, and *D17S250*) as described previously [24]. The amplification products were analyzed by capillary electrophoresis using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Lesions were classified as MSI-high (MSI-H) if two or more loci displayed MSI, or MSI-low (MSI-L) if only one locus displayed MSI. Lesions in which MSI was not exhibited in any of the loci were termed as microsatellite stable (MSS) [25].

2.7. Statistical analysis

Comparative data were analyzed using the Mann-Whitney *U*-test, Chi-square test, and the Spearman's correlation coefficient, where appropriate. The cutoff for statistical significance was set as $p < 0.05$.

3. Results

3.1. Nuclear β-catenin and HNF-1β expression in endometriosis and EAOCa

Distinct nuclear immunostaining for both β-catenin and HNF-1β was observed in the epithelial cells in Ca and their coexisting endometriotic lesions. Nuclear staining for β-catenin, but not HNF-1β, was also found in a few stromal cells in the endometriotic lesions (Figure 1A)

The average nuclear β-catenin score showed a significantly stepwise increase from endometriosis without Ca, through endometriosis with OEmCa, to OEmCa: this was in contrast to the complete lack of staining in OCCCa and the coexisting endometriosis (Figure 1B). The HNF-1β score showed a significantly stepwise decrease from endometriosis without Ca, through endometriosis with OEmCa, to OEmCa. In contrast, the score was significantly higher in OCCCa and the coexisting endometriosis when compared to samples of endometriosis without Ca and endometriosis with OEmCa (Figure 1C).

3.2. Mutations of the *CTNNB1* and HNF-1β status in endometriosis and EAOCas

Representative IHC findings for β-catenin and HNF-1β, sequence analysis of the *CTNNB1*, and MSI status in the OEmCas and coexisting endometriotic lesions (cases E27) are shown in Figure 2A.

Mutations of exon 3 in *CTNNB1* were significantly higher in OEmCas and the coexisting endometriosis when compared to OCCCa with coexisting endometriosis, as well as endometriosis without Ca lesions (Table 2 and

Supplementary Table S1). Eighteen out of 37 informative endometriosis with OEmCa lesions harboring *CTNNB1* mutations demonstrated identical single nucleotide substitutions between carcinoma and coexisting endometriosis (E1 or E2) lesions, whereas the mutation loci detected in eight lesions (E1, E3, E15, E17, E38, E42, E45, and E47) were different. Five (E1, E15, E29, E46, and E47) out of 10 informative endometriotic lesions with OEmCa also harbored different mutations between the E1 and E2 lesions. In contrast, *CTNNB1* mutations were detected in four endometriotic lesions adjacent to OCCCas (C10, C11, C10), but not within the OCCCas. In addition, double and triple point mutations were identified in two OEmCas (E4 and E43) and nine endometriotic lesions with OEmCas (E1, E3, E15, E29, E38, E43, E46, E47) or OCCCa (C10) (Table 1 and Supplementary Table S2).

The nuclear β-catenin score was significantly higher in endometriosis adjacent to OEmCa and OEmCas, in line with the presence of the *CTNNB1* mutations, whereas HNF-1β scores were significantly lower in OEmCas with *CTNNB1* mutations when compared with the wild-type lesions. Such associations were not observed in samples of endometriosis with OCCCa (Figure 2B).

3.3. MSI and MMR protein status in endometriosis and EAOCas

A comparison of the frequency of instability seen in endometriosis and carcinoma lesions showed that alterations in the dinucleotide markers D5S346 and D2S123 were relatively frequent when compared with the mononucleotide markers BAT25 and BAT26, particularity in endometriosis lesions (Supplementary Table S3). In contrast, MSI-H was significantly associated with instability at mononucleotide repeats with or without a dinucleotide locus when

compared with the dinucleotide locus only (Supplementary Table S4). This is in line with previous reports that show mononucleotide repeats are more sensitive than dinucleotide loci when used for detecting MSI [26,27].

As shown in Table 2, MSI-H status was significantly higher in endometriotic lesions with OEmCa as compared to that of endometriosis with OCCCa or without Ca lesions, while such difference was not evident between OEmCa and OCCCa (Table 2). No differences in MSI status were also found between endometriosis and Ca lesions (Supplementary Table S1). Of the 16 informative lesions, seven demonstrated identical MSI-H status between OEmCas and the coexisting endometriosis, while nine were not identical. In addition, in two cases (E43 and E48), the MSI-H status was not identical between the E1 and E2 lesions adjacent to OEmCas (Table 1 and Supplementary Table S2). There was no association between MSI status and either nuclear β-catenin or HNF-1β scores in both OEmCas and OCCCas with endometriosis (Figure 2C).

Representative IHC findings for MMR proteins including MLH1, MSH2, MSH6, and PMS2 in endometriosis, OEmCa, and OCCCa are shown in Figure 3A. As shown in Table 2, abnormalities in MMR protein expression, including a complete loss of expression or reduced expression (<10% tumor nuclei staining) of at least one MMR protein, were significantly different among endometriosis with OEmCa or OCCCa and without Ca lesions. In contrast, no differences in MMR protein status were observed between OEmCa and OCCCa, and were also evident between endometriosis and Ca lesions (Supplementary Table S1).

Among the MMR protein-negative lesions, 12 endometriosis, six OEmCa, and two OCCCa lesions lost both MSH2 and MSH6; these proteins form the MutSα complex, which recognizes as single base mismatches and short insertion-deletion loops in DNA. Moreover, nine endometriosis and five OEmCa lesions had abnormalities in both the proteins that form the MutLa complex

(MLH1 and PMS2), which mediate the progression from mismatch recognition to activation of downstream activities during the mismatch repair process. Isolated protein loss was observed in lesions of eight endometriosis cases, two OEmCa, and one OCCCa (for loss of MSHX expression, see Supplementary Table S5).

The MMR protein abnormalities were significantly associated with MSI status in both endometriosis and Ca lesions (Supplementary Table S6). The nuclear β-catenin score was significantly lower in endometriosis with OEmCa and OEmCa with MMR abnormality when compared to those with intact MMR protein expression. Similar findings regarding HNF-1β score were also observed in endometriosis with OCCCa (Figure 3B). In contrast, the *CTNNB1* mutations were not associated with MMR/MSI abnormalities in both endometriosis and Ca lesions (Supplementary Table S7).

Finally, neither MSI nor MMR protein status was associated with clinicopathological factors, including patient age and clinical stage in endometriosis and Cas, whereas the *CTNNB1* mutations were significantly associated with young age and early clinical stage in the Ca categories (Supplementary Table S8).

3.4. SLC3A1 expression in endometriosis and EAOCa

Since SLC3A1 (rBAT) plays a major role in HNF-1β-triggered ROS resistance [13,14], we examined whether SLC3A expression was associated with nuclear β-catenin and HNF-β statuses in Cas and their coexisting endometriotic lesions. A distinct cytoplasmic immunoreactivity was mainly observed in epithelial components, particularity in endometriosis (Figure 4A). SLC3A1 scores were significantly higher in endometriosis adjacent to the OEmCa or OCCCa when compared with the endometriosis without Ca lesions

and OEmCas, but not when compared to the OCCCas (Figure 4B). The SLC3A1 score was positively associated with nuclear β-catenin in endometriosis and HNF-1β score in OCCCa (Supplementary Table S9). In contrast, there was no association between *CTNNB1* mutations and MSI/MMR status (data not shown).

3.5. Association between inflammatory reactions, β-catenin, and MSI status in endometriosis

Representative images for four inflammatory parameters including hemorrhage, macrophage accumulation, erosion, and inflammatory cell infiltration in response to repeated hemorrhaging within endometriosis are shown in Figure 5A. These parameters and E-I scores showed significant stepwise decreases from endometriosis without Ca, through endometriosis with OEmCa, to endometriosis with OCCCa lesions (Figure 5B). The hemorrhage and inflammatory cell infiltration parameters, as well as the E-I scores, were significantly higher in endometriosis with MSI-H when compared to MSS (Figure 5C). The parameters of inflammatory cell infiltration were also significantly higher in endometriosis with instability at the mononucleotide locus when compared with the dinucleotide repeats (Figure 5D). In contrast, no associations between *CTNNB1* status and any of the endometriosis-related factors were noted (data not shown).

4. Discussion

The present study provides evidence that *CTNNB1* mutations and nuclear accumulation are frequently observed in OEmCa and the coexisting endometriosis, as reported previously [19]. Interestingly, the E-I scores, including

the four endometriosis-related inflammatory parameters, were significantly higher in endometriosis adjacent to OEmCas when compared to the OCCCas. Owing to the relatively minor events that occur due to *CTNNB1* mutations in OCCCas with endometriosis, we presume that the genetic abnormality may be attributed to local inflammatory reactions involving oxidative stress, which can induce genomic mutations [11,12]. However, the gene mutation status was not associated with any of the endometriosis-related inflammatory factors, indicating that other factors may contribute to the *CTNNB1* mutation profiles during endometriosis-OEmCa progression. Interestingly, it has been reported that abnormal β-catenin expression correlates with cyclin D1 overexpression but inversely with *KRAS* mutation in OEmCa associated with endometriosis, indicating a functional cross-talk or shared down-stream effector of the MAPK (*KRAS*) and Wnt (*CTNNB1*) pathways [28].

The findings of this study also demonstrate that MSI-H is predominantly detected in OEmCa, but not OCCCa, and is significantly associated with alterations in MMR expression levels. Moreover, MSI-H in endometriosis was significantly associated with higher E-I scores, including the hemorrhage and inflammatory cell infiltration parameters. This finding is in line a reported association between MSI-H and elevated levels of C-reactive protein in the serum and high white blood cell counts [29]. Given the notion that the accumulation of target gene mutations due to MSI plays a major role in malignant transformation [30], we suggest that MSI/MMR abnormalities due to inflammatory reactions may, in part, be involved in the malignant transformation of endometriosis into the OEmCa phenotype.

Mutations of *CTNNB1* occur frequently in MSI-H colorectal carcinomas when compared with MSS/MSI-L-colorectal carcinoma, suggesting that these mutations are relatively specific to genomically unstable MMR-deficient tumors

[31]. However, we failed to demonstrate such associations in the current study. Moreover, MMR abnormality was significantly associated with lower nuclear β-catenin scores in OEmCas and the coexisting endometriosis. In agreement with other reports [31,32], we conclude that alterations in *CTNNB1* and the MSI/MMR system may be independent during progression from endometriosis to OEmCa.

In contrast, the HNF-1β scores were significantly higher in both OCCCa and the coexisting endometriosis when compared to the OEmCas with endometriosis. Moreover, the scores were inversely associated with *CTNNB1* mutations in OEmCas and abnormal MMR status in endometriosis adjacent to OCCCa. Given our results showing the absence of any association between HNF-1β expression and MSI status in both OEmCas and OCCCas with endometriosis, we suggest that HNF-1β is the main contributor to the development of OCCCa arising from the coexisting endometriosis, independent of β-catenin and MSI/MMR status. Interestingly, the identification of a single nucleotide polymorphism (rs11651755) in *HNF-1β* has implicated HNF-1β in the early stages of tumorigenesis in OCCCa, even before the development of endometriosis [33].

HNF-1β also acts as an inhibitor of cellular oxidative stress under stressful environments by regulating the expression of SLC3A1 (rBAT) [13,14]. In the present study, SLC3A1 scores were positively correlated with HNF-1β scores in OCCCas, but not OEmCas. In addition, E-I scores, including some inflammatory parameters, were significantly lower in endometriosis adjacent to OCCCas with high HNF-1β expression when compared to OEmCas with endometriosis and low HNF-1β expression. These findings implicate ROS resistance driven by the HNF-1β/SLC3A1 axis as an essential step in the endometriosis to OCCCa progression. However, further studies are required in order to validate this

hypothesis.

Finally, endometriosis is assumed to have premalignant potential, a hypothesis recently underscored by studies reporting 100% monoclonality in endometriosis cysts [34,35]. In this study, non-identical patterns for both *CTNNB1* mutations and MSI status were frequently observed in the two different epithelial lesions within endometriosis adjacent to the OEmCas. This indicates that the bulk of endometriosis tissues might consist of polyclonal cells. In support of this, only 7% of the endometriosis samples exhibited a monoclonal phenotype based on the findings of a polymorphism in the *X-linked phosphoglycerate kinase 1* gene [36].

Taken together, the findings of the present study indicate the existence of different molecular pathways that are operate in the progression of endometriosis to OEmCa or OCCCa (Figure 6). High concentrations of free iron due to repeated hemorrhaging within endometriosis may directly lead to the production of oxidative stress, which may induce DNA damage. This damage, along with other unknown factors and MMR abnormalities, may independently cause alterations in *CTNNB1* or MSI, resulting in the development of OEmCa arising from the coexisting endometriosis. In contrast, the HNF-1β/SLC3A1 axis may contribute to the endometriosis-OCCCa sequence via inhibition of oxidative stress. Thus, different molecular pathways including alterations in *CTNNB1*, MSI, and HNF-1β levels may contribute to tumorigenesis in endometriosis-associated carcinoma. Recently, alterations in several candidate genes including *ARID2, PBRM1, CTNNA1, PPP2R1A, NOTCH1, NOTCH2*, and *ETS1*, as well as *ARID1A, PIK3CA, KRAS*, and *TP53,* have been reported as predisposing factors in the progression of endometriotic lesions to malignantly transformed OCCCa or OEmCa [37].

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Table 1 Summary of CTNNB1, MSI, and MMR protein status in endometriosis and the a+B3:P97ssociated carcinomas

	Age	FIGO	β-catenin mutation			MSI status			MMR IHC abnormality		
No.	(years)	stage	Ca	E1	E2	Ca	E1	E2	Ca	E1	E2
OEmCa											
E1	69	I a	GAC32GTC	GAC32GTC	ATC35ATT,TCT37TGT	MSS	MSS	MSS	Intact	Intact	Intact
E2	48	I c	GAC32TAC	GAC32TAC	NE	MSS	MSI-L	NE	Intact	Loss	NE
E3	22	I a	GGA34GTA	TCT33TTT, GGA34GAA	NE	NE	NE	NE	Intact	Intact	NE
E4	51	I c	TCT33TTT, TCT33TGT, TCT45CCT	wild	NE	MSS	MSI-H	NE	Intact	Intact	NE
E5	52	I c	GGA34GTA	GGA34GTA	NE	NE	NE	NE	Intact	Intact	NE
E6	53	I c	TCT37CCT	TCT37CTT	NE	MSS	MSI-H	NE	Intact	Intact	NE
E7	38	I a	TCT37TGT	wild	NE	MSS	MSI-H	NE	Loss	Loss	NE
E8	53	I a	ACT40ATT	wild	NE	MSS	MSS	NE	Intact	Loss	NE
E9	51	I c	wild	wild	wild	MSI-H	MSI-H	MSI-H	Loss	Loss	Loss
E10	39	I c	TCT33TGT	wild	NE	MSI-H	MSI-H	NE	Loss	Loss	NE
E11	56	I c	wild	wild	NE	MSS	MSS	NE	Intact	Intact	NE
E12	45	I c	TCT37TTT	TCT37TGT	NE	MSI-H	MSI-H	NE	Intact	Intact	NE
E13	47	I c	wild	NE	NE	MSS	NE	NE	Loss	NE	NE
E14	63	II c	wild	wild	NE	MSS	MSS	NE	Intact	Intact	NE
E15	56	I a	TCT33TGT	TCT33TGT	TCT37TGT,ACC41ACT	MSS	MSS	MSS	Intact	Intact	Intact
E16	41	I c	wild	wild	NE	MSI-H	MSI-H	NE	Loss	Loss	NE
E17	53	I c	TCT37TGT	AGT47GGT	wild	MSS	MSS	MSS	Intact	Intact	Intact
E18	48	I c	wild	wild	wild	MSS	MSI-L	MSS	Intact	Intact	Intact
E19	67	II c	wild	wild	wild	MSS	MSI-L	MSS	Intact	Intact	Intact
E20	72	I c	TCT37TTT	TCT37TTT	NE	MSS	MSS	NE	Intact	Intact	NE
E21	51	I c	wild	wild	NE	MSI-H	MSS	NE	Loss	Loss	NE
E22	51	I a	TCT33CCT	TCT33CCT	NE	MSS	MSS	NE	Loss	Intact	NE
E23	59	II c	wild	NE	NE	MSS	NE	NE	Intact	NE	NE
E24	88	I c	wild	NE	NE	MSI-L	NE	NE	Intact	NE	NE
E25	55	II c	wild	wild	NE	MSI-H	MSI-H	NE	Loss	Loss	NE
E26	53	II c	wild	wild	NE	MSI-L	NE	NE	Loss	Loss	NE
E27	71	I c	TCT33TTT	TCT33TTT	NE	MSS	MSI-H	NE	Intact	Intact	NE
E28	68	II c	wild	wild	NE	MSS	MSS	NE	Intact	Intact	NE
E29	41	I c	TCT37TTT	GAC32GAA, TCT33TTT, TCT45CCT	TCT37TTT	MSS	MSS	MSS	Intact	Intact	Intact
E30	57	IV	TCT37TTT	TCT37TTT	TCT37TTT	MSS	MSI-L	MSI-L	Intact	Intact	Intact
E31	48	I a	GAC32TAC	NE	NE	MSS	NE	NE	Intact	NE	NE
E32	41	I a	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
E33	40	II c	GAC32GGC	wild	NE	MSS	MSS	NE	Intact	Intact	NE
E34	70	I a	TCT45TTT	wild	NE	MSI-L	MSI-L	NE	Intact	Loss	NE
E35	62	I c	TCT45TTT	wild	NE	MSS	MSI-H	NE	Intact	Loss	NE
E36	39	II c	wild	wild	wild	MSS	MSS	MSS	Intact	Loss	Loss
E37	55	I c	wild	wild	NE	MSS	MSS	NE	Loss	Loss	NE
E38	79	I a	GAC32AAC	TCT29TTT, TCT33TTT, TCT45CCT	NE	MSS	MSI-L	NE	Intact	Intact	NE
E39	57	I c	wild	wild	NE	MSS	MSS	NE	Intact	Intact	NE
E40	47	IIIa	wild	wild	NE	MSS	MSI-H	NE	Intact	Intact	NE
E41	59	I c	wild	wild	TCT33TTT	MSS	MSS	MSS	Intact	Loss	Intact
E42	46	I c	TCT33TTT	TCT29TTT, TCT45CCT	NE	MSS	MSI-L	NE	Intact	NE	NE
E43	52	II b	TCT33TTT, GGA34GTA, TCT45CCT	TCT33TTT	TCT33TTT, TCT45CCT	MSI-H	MSI-H	MSI-L	Loss	Intact	Loss
E44	73	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
E45	42	I c	TCT45TTT	TCT33TTT	wild	MSI-L	MSI-L	MSI-L	Intact	Intact	Intact
E46	69	I c	TCT33TGT	TCT33TGT	TCT33TTT, TCT45CTT	MSS	MSS	MSS	Intact	Intact	Intact
E47	44	I a	TCT37TGT	TCT37TGT	TCT33TTT, TCT45TCC	MSS	MSS	MSI-L	Intact	Intact	Intact
E48	75	I c	wild	wild	wild	MSS	MSS	MSI-H	Loss	Loss	Intact
E49	54	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
E50	43	I a	GAC32AAC	wild	NE	MSS	MSS	NE	Intact	Intact	NE
OCCCa											
C1	54	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C2	61	IV	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C3	76	IIIc	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C4	56	IIIb	wild	wild	wild	MSS	MSI-L	MSS	Intact	Intact	Intact
C5	64	IIIc	wild	wild	wild	MSS	MSI-L	MSS	Loss	Intact	Intact
C6	53	IIIb	wild	wild	wild	MSS	MSS	MSS	Loss	Intact	Intact
C7	66	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C8	62	II c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Loss
C9	35	IIIc	wild	wild	wild	MSI-L	MSS	MSS	Intact	Intact	Intact
C10	49	I c	wild	TCT33TTT	TCT33TTT, TCT45CCT	MSS	MSS	NE	Intact	Intact	Intact
C11	72	IIIc	wild	TCT45CCT	wild	MSI-L	NE	NE	Intact	Intact	Intact
C12	52	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C13	69	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C14	60	I c	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C15	45	I a	wild	TCT33TTT	wild	MSS	MSS	MSS	Intact	Intact	Intact
C16	72	IIIc	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C17	62	I a	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C18	49	I c	TCT37TGT	wild	NE	MSS	MSS	NE	Intact	Intact	NE
C19	76	II b	wild	wild	wild	MSS	MSS	MSS	Intact	Intact	Intact
C20	57	I c	wild	wild	NE	MSS	MSS	NE	Intact	Intact	NE
C21	64	IIIc	wild	wild	wild	MSS	MSS	MSS	Loss	Loss	Loss
Endometriosis w/o Ca											
w/o1	45		wild	NE	NE	NE	MSI-L	NE	Intact	NE	NE
w/o2	55		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o3	46		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o4	40		wild	NE	NE	NE	MSI-H	NE	Intact	NE	NE
w/o5	50		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o6	43		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o7	36		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o8	42		wild	NE	NE	NE	MSI-H	NE	Intact	NE	NE
w/o9	43		wild	wild	NE	NE	MSS	MSI-L	Intact	Intact	NE
w/o10	40		AAT51AAC	NE	NE	NE	MSS	NE	Loss	NE	NE
w/o11	45		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o12	50		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o13	42		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o14	44		wild	NE	NE	NE	MSS	MSS	Intact	Intact	NE
w/o15	42		wild	NE	NE	NE	MSI-L	MSS	Intact	Intact	NE
w/o16	42		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o17	40		wild	NE	NE	NE	MSS	NE	Loss	NE	NE
w/o18	47		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o19	42		wild	NE	NE	NE	MSS	NE	Intact	NE	NE
w/o20	35		wild	NE	NE	NE	MSS	NE	Intact	NE	NE

Case No; case number; NE, not examined; w/o Ca, without carcinoma; Ca, carcinoma lesion; E1, endometriotic lesion-1; E2, endometriotic lesion-2

Table 2 *CTNNB1* mutation, MSI, and MMR protein statues in endometriosis and the associated carcinomas

			CTNNB1 mutation				MSI status					MMR status		
			Positive	Negative			MSS	MSI-L	MSI-H					
		n	n (%)	n (%)	*p*-value	n	n (%)	n (%)	n (%)	*p*-value	n	Intact	Loss	*p*-value
Endometriosis lesion along with	OEmCa	62	26 (41.9)	36 (58.1)		60	35 (39.8)	11 (18.3)	14 (23.3)		63	45 (71.4)	18 (28.6)	
	OCCCa	40	4 (10)	36 (90)	<0.0001	37	35 (94.6)	2 (5.4)	0	0.002	40	37 (92.5)	3 (7.5)	0.01
	w/o Ca	21	1 (4.8)	20 (21.8)		23	18 (78.3)	3 (13)	2 (8.7)		23	21 (20.4)	2 (8.6)	
Carcinoma lesion	OEmCa	50	28 (56)	22 (44)		48	37 (77.1)	4 (8.3)	7 (14.6)		50	38 (76)	12 (24)	
	OCCCa	21	1 (4.8)	20 (95.2)	<0.0001	21	19 (90.5)	2 (9.5)	0	0.18	21	18 (85.7)	3 (14.3)	0.36

n, number of cases; w/o Ca, without carcinoma; n, number of lesions

Figure legends

Figure 1. Immunohistochemistry (IHC) findings of β-catenin and HNF-1β in endometriosis and the associated carcinomas. (A) Micrographs showing hematoxylin and eosin (HE) and IHC staining for β-catenin and HNF-1β in endometriotic and Ca lesions in OEmCa (upper) and OCCCa (lower). Note the nuclear β-catenin accumulation in both OEmCa and the coexisting endometriosis, in contrast to the strong HNF-1β staining in both OCCCa and coexisting endometriosis. Closed boxes are magnified in the insets. Original magnifications: x100 and x400 (inset). (B) IHC scores for nuclear β-catenin (upper) and HNF-1β (lower) in endometriosis (E-osis) without (w/o) Ca, endometriosis adjacent to Ca, and Ca lesions. Data shown as mean ± SD.

Figure 2. IHC, sequence analysis of *β-catenin*, and MSI status in EAOCa and the coexisting endometriosis. (A) OEmCa (case E37). Upper left: micrographs showing hematoxylin and eosin (HE) as well as IHC staining for β-catenin and HNF-1β. Note the strong nuclear β-catenin staining (indicated by arrows) in both endometriotic and the associated carcinoma lesions, in contrast to the weak or negative HNF-1β staining. Original magnification: x200. Upper right: MSI analyses using five markers including BAT-25, BAT-26, D2S123, D5S326, and D17S250 (indicated by arrows and bracket in upper panel). Note the MSI in BAT-26, D5S346, and D2S123 markers (indicated by thick arrows) and loss of heterozygosity in D2S123 (indicated by arrow head) in endometriotic (middle), but not in the associated Ca (lower), lesions when compared with the normal tissue (upper). Lower: sequence analysis of exon 3 of the *CTNNB1*. Heterozygous substitution mutations (indicated by the arrows) seen in endometriotic and the associated Ca lesions. (B) Association between *CTNNB1*

mutations and IHC scores for nuclear (Nu) β-catenin and HNF-1β in endometriotic and the associated Ca lesions. Data shown as mean ± SD. (C) Association between MSI status and IHC scores for Nu β-catenin and HNF-1β in endometriotic and the associated carcinoma lesions. P, positive; N, negative; S/L, MSS/MSI-L; H, MSI-H, w/o Ca, without carcinoma; Ca, carcinoma. Data shown as mean ± SD.

Figure 3. IHC of MMR protein expression in endometriosis and the associated carcinomas. (A) HE and IHC staining for four MMR markers including MLH1, MSH2, MSH6, and PMS2 in endometriosis (upper panels), OEmCa (middle panel), and OCCCa (lower panel). Note the lack of nuclear immunostaining for MLH1, MSH2, and PMS2 in OEmCa, in contrast to the distinct nuclear staining in endometriosis and OCCCa. Closed boxes are magnified in the insets. Original magnification x100 and x400 (inset). (B) Association between IHC scores for nuclear (Nu)-β-catenin and HNF-1β, and MMR protein status in endometriotic and the associated Ca lesions. w/o Ca, without carcinoma; Int, intact of MMR protein expression; Loss, loss of MMR protein expression. Data shown as mean ± SD.

Figure 4. IHC of SLC3A1 in endometriosis and the associated carcinomas. (A) Micrographs showing HE and IHC staining for SLC3A1 in endometriosis (upper), OEmCa (middle), and OCCCa (lower). Note the distinct cytoplasmic SL3CA1 immunostaining in epithelial components of endometriosis, in contrast to the weak staining in OEmCa and OCCCa. Original magnification: x200. (B) IHC scores for SL3CA1 in endometriosis and the associated carcinomas. E-osis w/o Ca, endometriosis without carcinoma. Data shown as mean ± SD.

Figure 5. Association between inflammatory features and MSI status in endometriosis. (A) Micrographs showing HE staining in endometriosis. Upper left: hemorrhagic parameter: note the hemorrhagic area (indicated by arrow) within the cystic wall. Upper right: accumulation of macrophages: note the accumulation of macrophages with massive cytoplasmic hemosiderin levels. Lower left: inflammatory cell infiltration parameter: note the lymphoid cell infiltration within the cystic wall in endometriosis. Lower right: erosion parameter: note the erosive changes (indicated by arrows), along with macrophage accumulation within the cystic wall. Closed boxes are magnified in the insets. Original magnifications: x100 and x400 (inset). (B) Scores of the four inflammatory parameters and endometriosis-inflammatory (E-I) scores in endometriosis w/o Ca, endometriosis without carcinoma; E, endometriosis adjacent to OEmCa; C, endometriosis adjacent to OCCCa. Data shown as mean ± SD. (C) Associations between the four inflammatory parameters as well as E-I scores and the MSI status. S, MSS; L, MSI-L; H, MSI-H. Data shown as mean ± SD. (D) Associations between the four inflammatory parameters as well as E-I scores and alterations in repeat sequence patterns. S, MSS; Mo, mononucleotide repeat; Di, dinucleotide repeat. Data shown as mean ± SD.

Figure 6. Schematic representation of associations between β-catenin, HNF-1β, and MSI status in endometriosis-associated OEmCa and OCCCa.

Highlight

・β-catenin and MSI serve as key roles for endometriosis-associated endometrioid carcinoma sequence.

・HNF-1β/SLC3A1 axis acts as crucial role for endometriosis-associated clear cell carcinoma sequence.

・These factors may contribute independently to the tumorigenesis of endometriotic lesions.

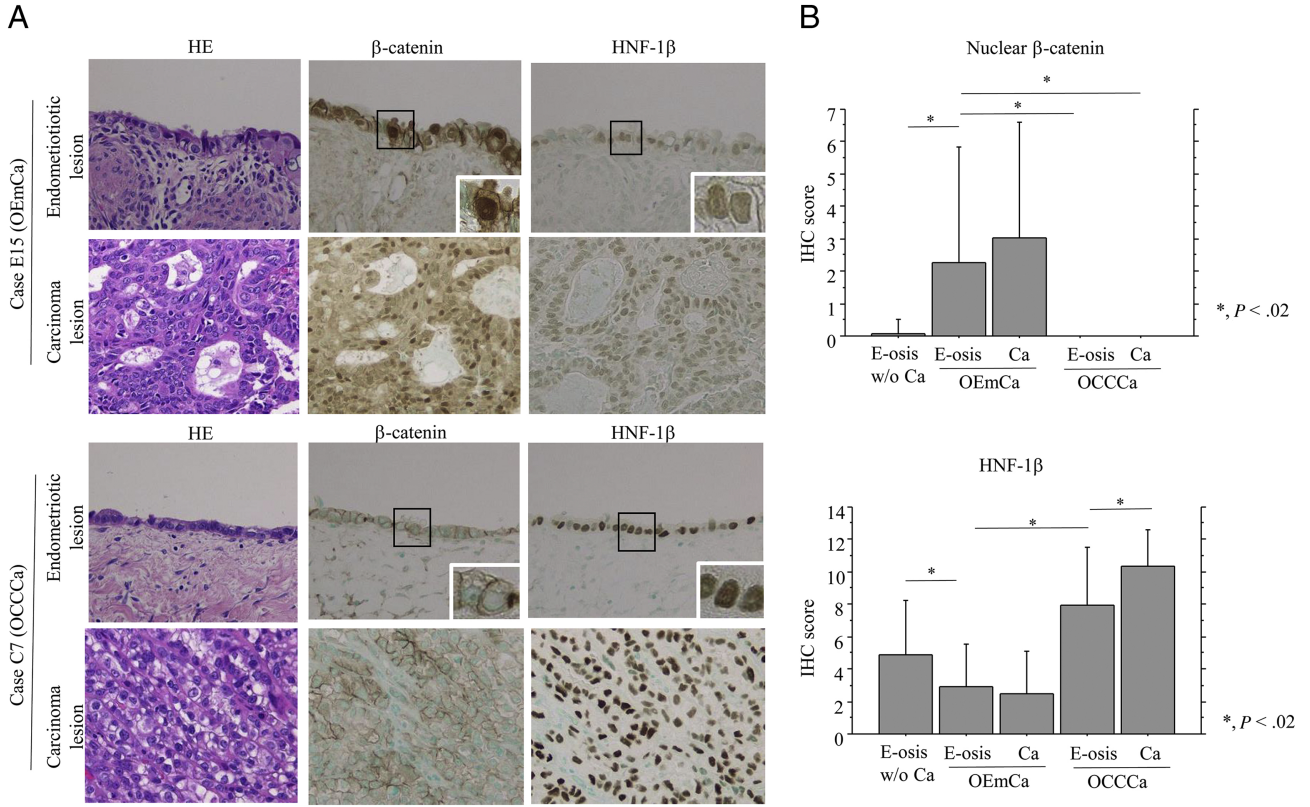

A
HE
β-catenin
HNF-1β
Case E15 (OEmCa)
Endometiotiotic lesion
Carcinoma lesion
HE
β-catenin
HNF-1β
Case C7 (OCCCa)
Endometriotic lesion
Carcinoma lesion
B
Nuclear β-catenin
IHC score
0
1
2
3
4
5
6
7
*
*
*
*, P < .02
E-osis w/o Ca
E-osis
Ca
OEmCa
E-osis
Ca
OCCCa
HNF-1β
IHC score
0
2
4
6
8
10
12
14
*
*
*
*, P < .02
E-osis w/o Ca
E-osis
Ca
OEmCa
E-osis
Ca
OCCCa

Figure 1

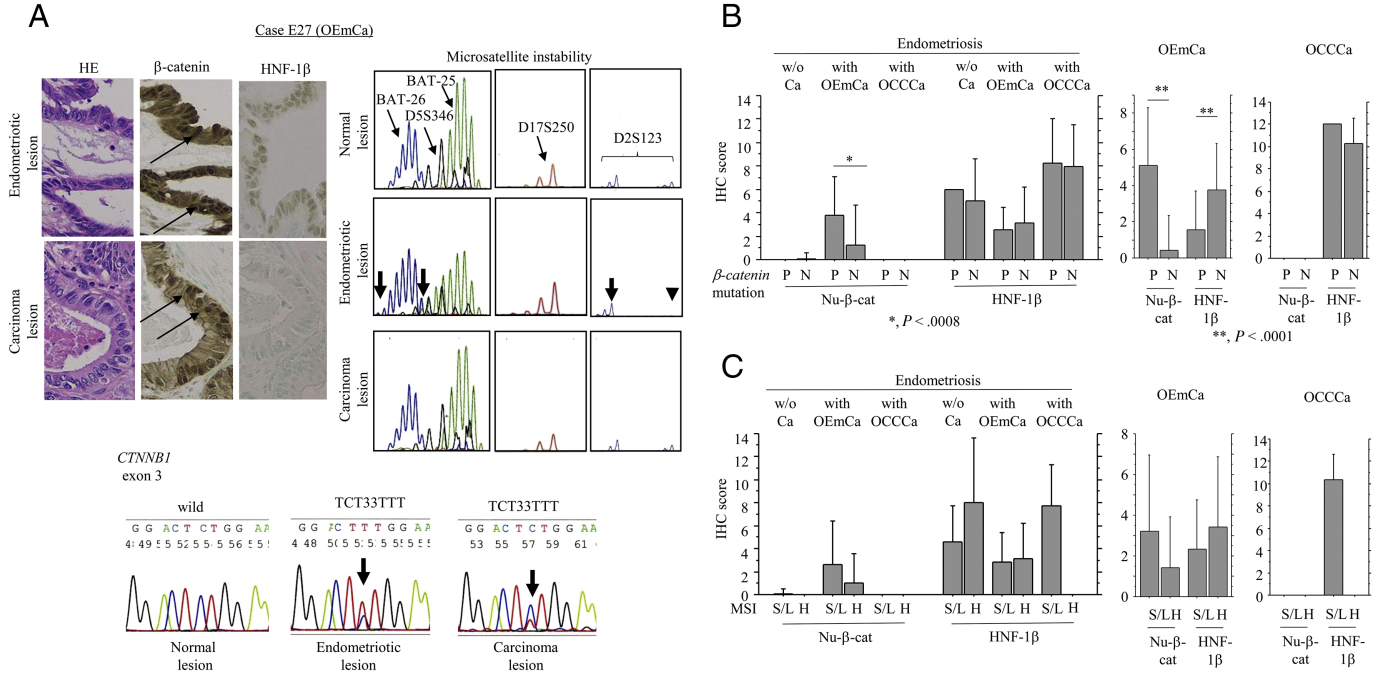

A
Case E27 (OEmCa)
HE
β-catenin
HNF-1β
Endometriotic lesion
Carcinoma lesion
Microsatellite instability
BAT-25
BAT-26
D5S346
D17S250
D2S123
Normal lesion
Endometriotic lesion
Carcinoma lesion
CTNNB1
exon 3
wild
TCT33TTT
TCT33TTT
Normal lesion
Endometriotic lesion
Carcinoma lesion
B
Endometriosis
w/o Ca
with OEmCa
with OCCCa
w/o Ca
with OEmCa
with OCCCa
OEmCa
OCCCa
IHC score
β-catenin mutation
P N
Nu-β-cat
HNF-1β
*, P < .0008
**, P < .0001
C
MSI
S/L H
Nu-β-cat
HNF-1β

Figure 2

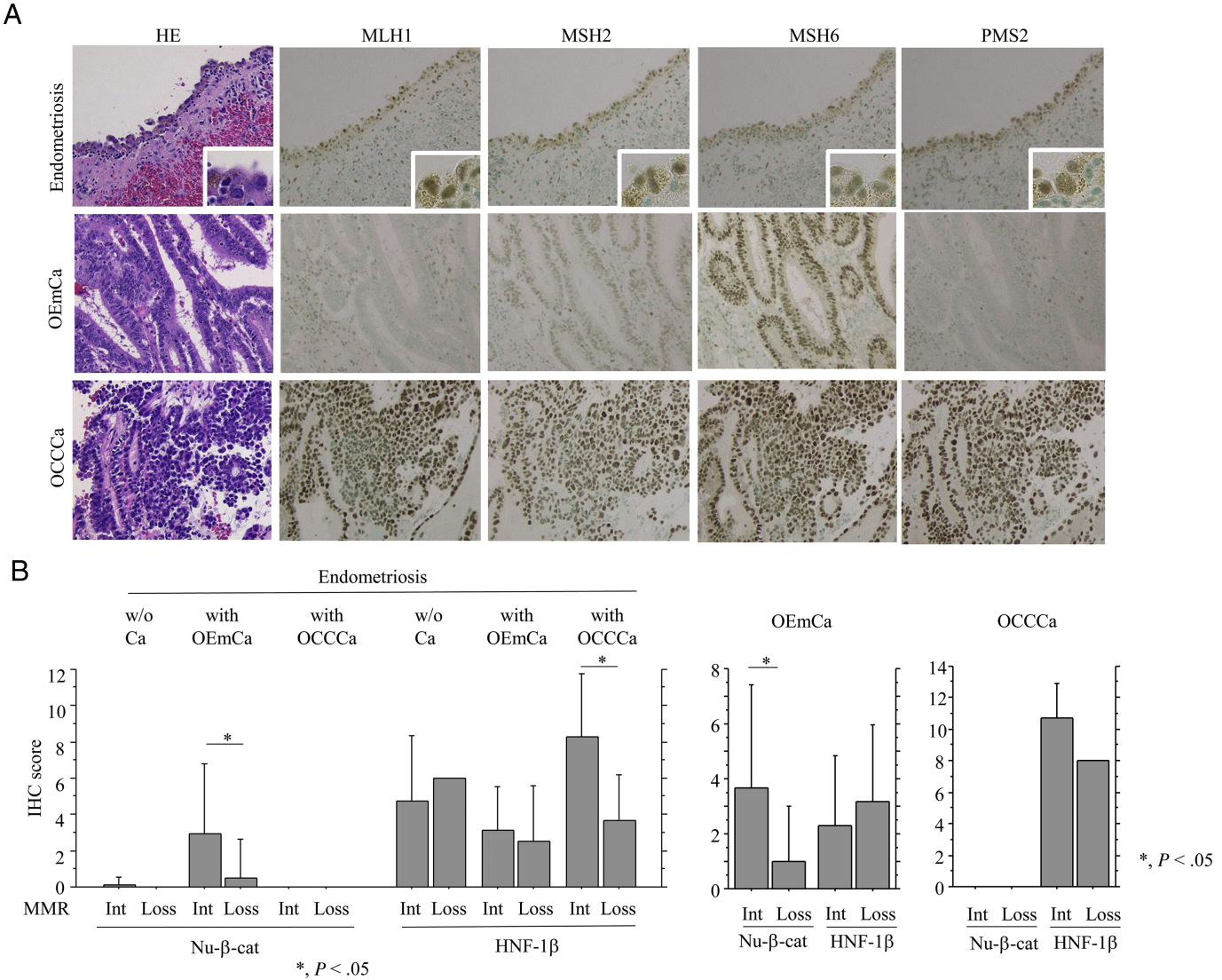
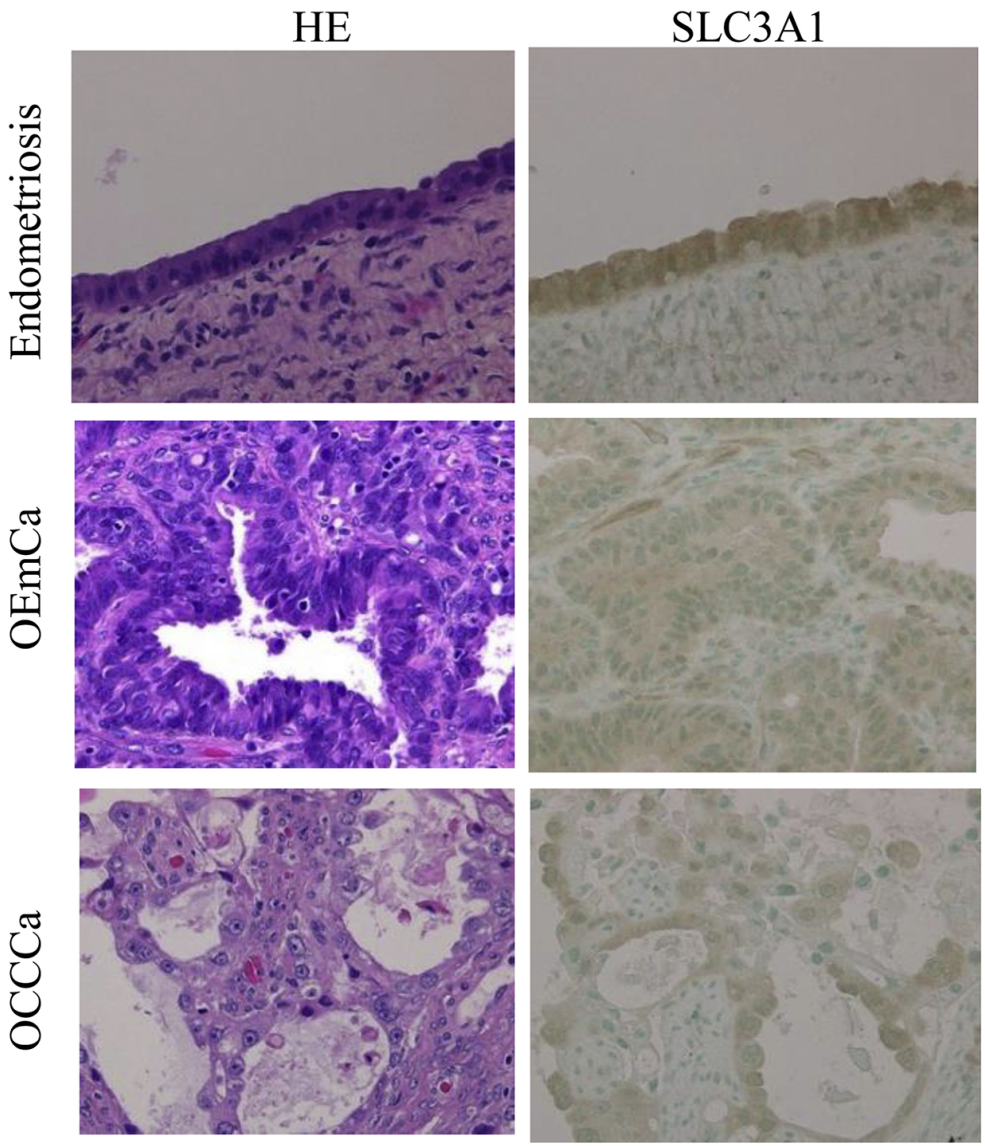

A
HE
MLH1
MSH2
MSH6
PMS2
Endometriosis
OEmCa
OCCCa
B
Endometriosis
w/o Ca
with OEmCa
with OCCCa
w/o Ca
with OEmCa
with OCCCa
IHC score
MMR
Int Loss Int Loss Int Loss Int Loss Int Loss Int Loss
Nu-β-cat
HNF-1β
*, P < .05
OEmCa
OCCCa
Int Loss Int Loss
Nu-β-cat
HNF-1β
*, P < .05

Figure 3

A


HE
SLC3A1
Endometriosis
OEmCa
OCCCa

B

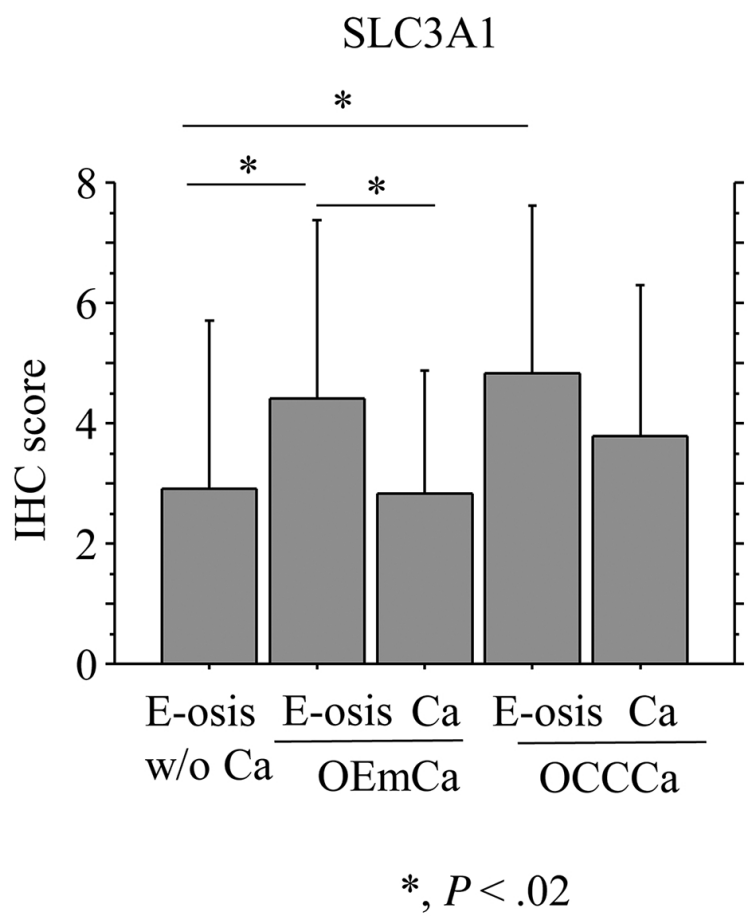

SLC3A1
IHC score
0
2
4
6
8
*
*
*
E-osis w/o Ca
E-osis
Ca
OEmCa
E-osis
Ca
OCCCa
*, P < .02

Figure 4

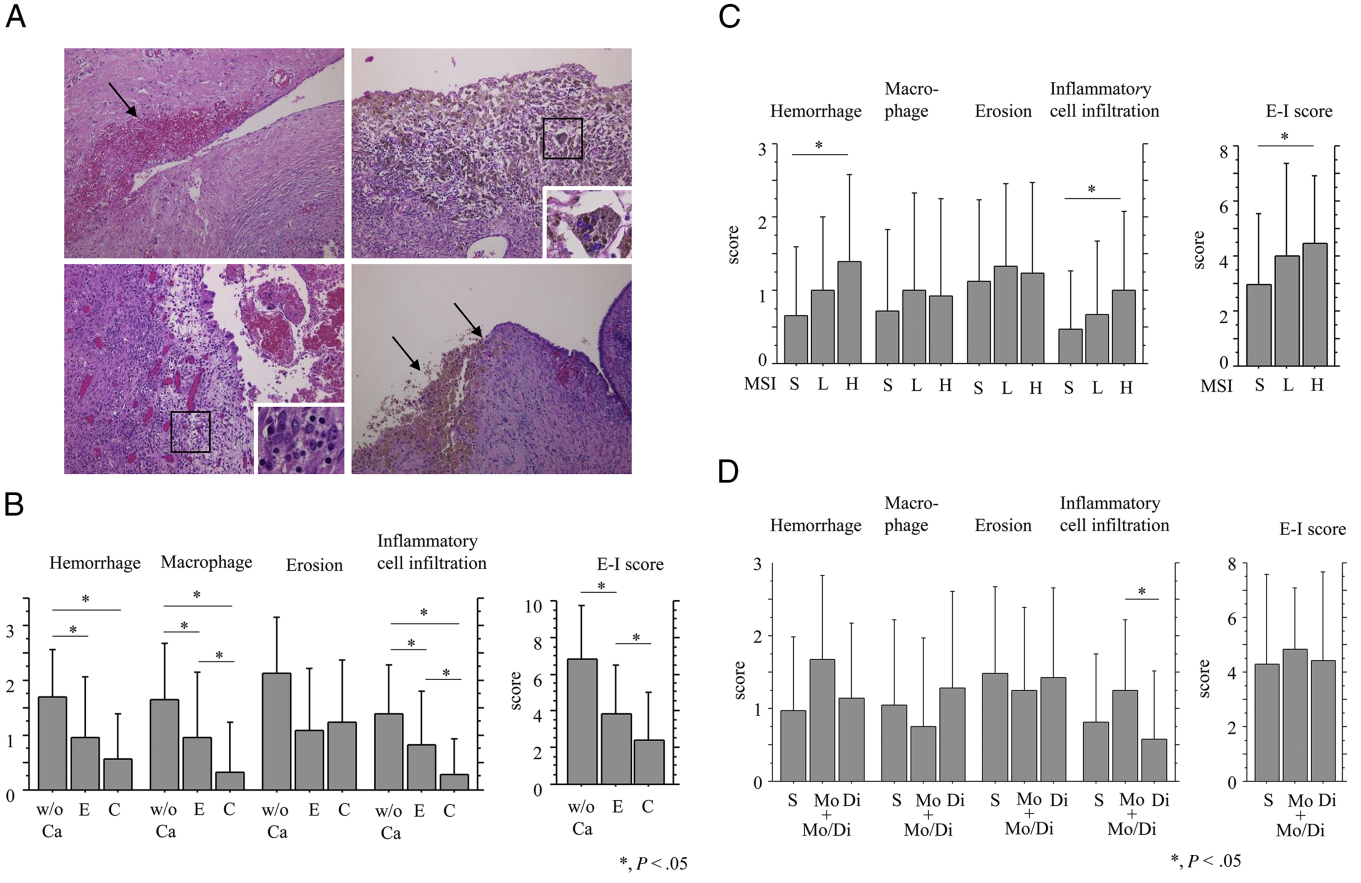

A
B
Hemorrhage
Macrophage
Erosion
Inflammatory cell infiltration
E-I score
score
w/o Ca
E
C
*, P < .05
C
Hemorrhage
Macro-phage
Erosion
Inflammatory cell infiltration
E-I score
score
MSI
S
L
H
D
Hemorrhage
Macro-phage
Erosion
Inflammatory cell infiltration
E-I score
score
S
Mo + Mo/Di
Di
*, P < .05

Figure 5

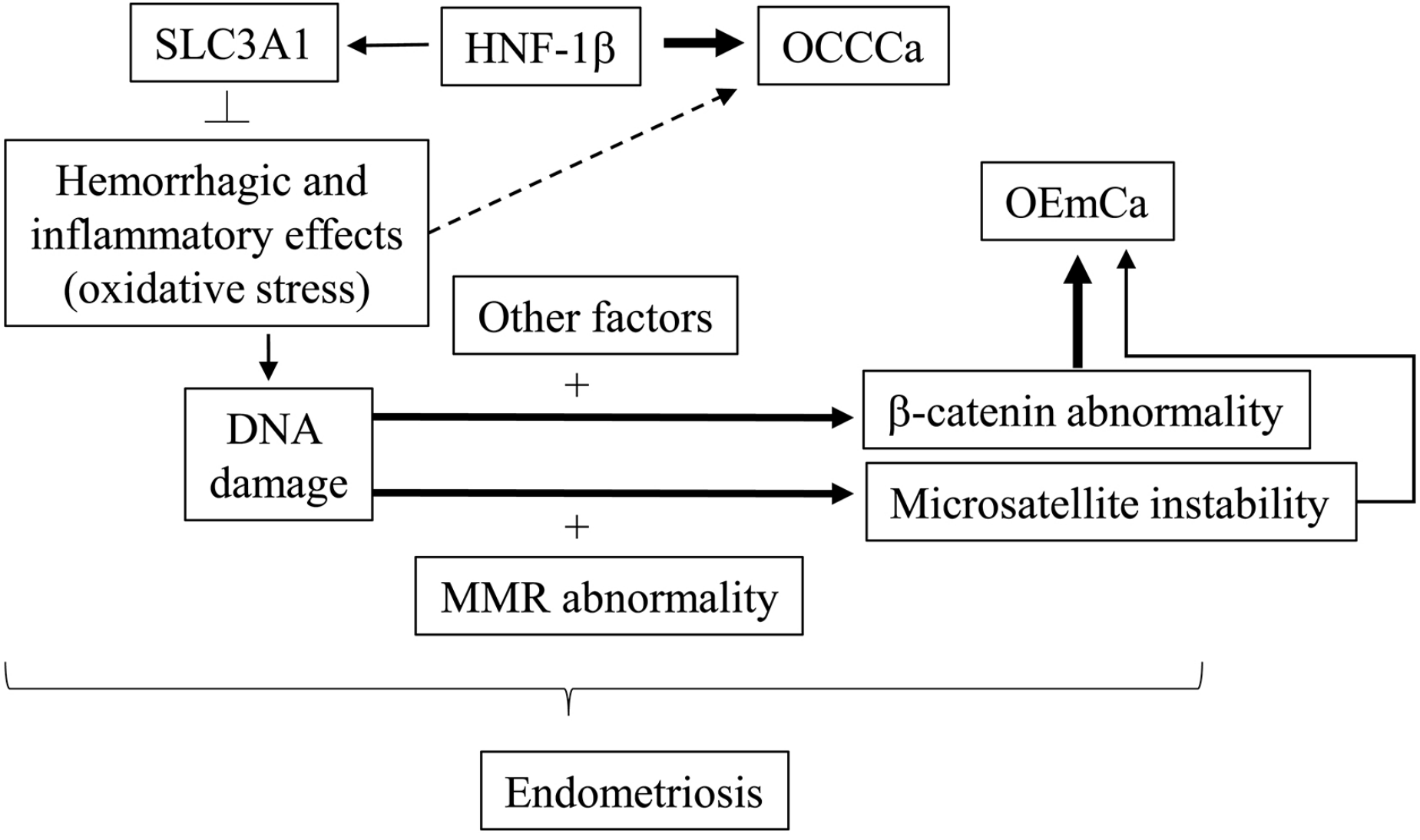

SLC3A1
HNF-1β
OCCCa
Hemorrhagic and inflammatory effects (oxidative stress)
OEmCa
Other factors
+
DNA damage
β-catenin abnormality
Microsatellite instability
+
MMR abnormality
Endometriosis

Figure 6