



Original contribution

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



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Summary We focused on specific molecular events during the development of endometriosis-associated ovarian endometrioid carcinoma (OEmCa) and ovarian clear cell carcinoma (OCCC). Alterations in β -catenin (encoded by *CTNNB1*) and microsatellite instability (MSI), as well as changes in the expression levels of HNF-1 β 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 7 (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. Nonidentical *CTNNB1* status between 2 different epithelial lesions within endometriosis was observed in 8 of 10 informative endometriosis cases that had adjacent OEmCa. Similar findings for MSI features were also found in 2 of 3 informative cases, suggesting that endometriotic lesions may predominantly consist of polyclonal cells. In contrast, high HNF-1 β expression was significantly associated with SLC3A1 expression, which plays a major role in HNF-1 β -triggered induction of reactive oxygen species in OCCCAs, independent of abnormalities in both β -catenin and MSI/MMR status. Finally, 4 inflammatory parameters associated with repeated hemorrhaging in endometriosis were significantly higher in endometriosis with MSI-high when compared with that with MSS, independent of both β -catenin and HNF-1 β status. In conclusion, different molecular pathways including alterations in β -catenin, MSI, and HNF-1 β levels may contribute to tumorigenesis in endometriosis-associated carcinoma. © 2019 Elsevier Inc. All rights reserved.

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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% to 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% to 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, hepatocyte nuclear factor 1 homeobox B (HNF-1 β), which is highly expressed in OCCCa, acts as a prosurvival factor in endometriosis and thereby contributes to tumorigenesis. This is due to its ability to exert antioxidant 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 MMR 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-1 β , 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 and endometriotic epithelium, as described by Yamamoto *et al* [20], with minor modification. 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 carcinoma 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 carcinomas were subdivided into 2 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 [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/ β -Catenin; 1:1000 dilution) and anti-HNF-1 β (25/HNF-1 β ; 1:1000 dilution) were purchased from BD Biosciences (San Jose, CA). 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, United Kingdom). Anti-SLC3A1 antibody (ab196552; \times 1:100 dilution) was obtained from Abcam (Cambridge, United Kingdom).

2.3. Immunohistochemistry

Immunohistochemistry (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 minutes and incubated overnight at 4 °C with optimized dilution of primary antibodies.

Scoring of nuclear β -catenin and HNF-1 β immunoreactivity and cytoplasmic SLC3A1 immunostaining, respectively, was performed to evaluate the IHC findings. Briefly, the percentages of immunopositive cells were subdivided into 5 categories as follows: 0, all negative; 1, <10% positive cells; 2, 10% to 30%; 3, 30% to 50%; and 4, >50%. The immunointensity was also subclassified into 4 groups as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. IHC scores were generated by multiplying the values of the 2 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

Table 1 Summary of CTNNB1, MSI, and MMR protein status in endometriosis and the associated carcinomas

Case no.	Age (y)	FIGO stage	β-Catenin mutation			MSI status			MMR IHC abnormality		
			Ca	E1	E2	Ca	E1	E2	Ca	E1	E2
OEmCa											
E1	69	Ia	GAC32GTC	GAC32GTC	ATC35ATT, TCT37TGT	MSS	MSS MSI-	MSS	Intact	Intact	Intact
E2	48	Ic	GAC32TAC	GAC32TAC	NE	MSS	L	NE	Intact	Loss	NE
E3	22	Ia	GGA34GTA	GGA34GAA	NE	NE	NE	NE	Intact	Intact	NE
E4	51	Ic	TCT45CCT	Wild	NE	MSS	H	NE	Intact	Intact	NE
E5	52	Ic	GGA34GTA	GGA34GTA	NE	NE	NE	NE	Intact	Intact	NE
E6	53	Ic	TCT37CCT	TCT37CTT	NE	MSS	H MSI-	NE	Intact	Intact	NE
E7	38	Ia	TCT37TGT	Wild	NE	MSS	H	NE	Loss	Loss	NE
E8	53	Ia	ACT40ATT	Wild	NE	MSS	MSS	NE	Intact	Loss	NE
E9	51	Ic	Wild	Wild	Wild	MSI- H	MSI- H	MSI- H	Loss	Loss	Loss
E10	39	Ic	TCT33TGT	Wild	NE	H	H	NE	Loss	Loss	NE
E11	56	Ic	Wild	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
E12	45	Ic	TCT37TTT	TCT37TGT	NE	H	H	NE	Intact	Intact	NE
E13	47	Ic	Wild	NE	NE	MSS	NE	NE	Loss	NE	NE
E14	63	IIc	Wild	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
E15	56	Ia	TCT33TGT	TCT33TGT	TCT37TGT, ACC41ACT	MSS	MSS MSI-	MSS	Intact	Intact	Intact
E16	41	Ic	Wild	Wild	NE	H	H	NE	Loss	Loss	NE
E17	53	Ic	TCT37TGT	AGT47GGT	Wild	MSS	MSS	MSS	Intact	Intact	Intact
E18	48	Ic	Wild	Wild	Wild	MSS	L MSI-	MSS	Intact	Intact	Intact
E19	67	IIc	Wild	Wild	Wild	MSS	L	MSS	Intact	Intact	Intact
E20	72	Ic	TCT37TTT	TCT37TTT	NE	MSS	MSS	NE	Intact	Intact	NE
E21	51	Ic	Wild	Wild	NE	H	MSS	NE	Loss	Loss	NE
E22	51	Ia	TCT33CCT	TCT33CCT	NE	MSS	MSS	NE	Loss	Intact	NE
E23	59	IIc	Wild	NE	NE	MSS	NE	NE	Intact	NE	NE
E24	88	Ic	Wild	NE	NE	MSI- L	NE	NE	Intact	NE	NE
E25	55	IIc	Wild	Wild	NE	H MSI-	H	NE	Loss	Loss	NE
E26	53	IIc	Wild	Wild	NE	L MSI-	NE	NE	Loss	Loss	NE
E27	71	Ic	TCT33TTT	TCT33TTT	NE	MSS	H	NE	Intact	Intact	NE
E28	68	IIc	Wild	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
E29	41	Ic	TCT37TTT	TCT33TTT, TCT45CCT	TCT37TTT	MSS	MSS MSI-	MSS	Intact	Intact	Intact
E30	57	IV	TCT37TTT	TCT37TTT	TCT37TTT	MSS	L	L	Intact	Intact	Intact
E31	48	Ia	GAC32TAC	NE	NE	MSS	NE	NE	Intact	NE	NE
E32	41	Ia	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
E33	40	IIc	GAC32GGC	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
E34	70	Ia	TCT45TTT	Wild	NE			NE	Intact	Loss	NE

Table 1 (continued)

Case no.	Age (y)	FIGO stage	β-Catenin mutation			MSI status			MMR IHC abnormality		
			Ca	E1	E2	Ca	E1	E2	Ca	E1	E2
E35	62	Ic	TCT45TTT	Wild	NE	MSS	H	NE	Intact	Loss	NE
E36	39	IIc	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Loss	Loss
E37	55	Ic	Wild	Wild	NE	MSS	MSS	NE	Loss	Loss	NE
E38	79	Ia	GAC32AAC	TCT29TTT, TCT33TTT, TCT45CCT	NE	MSS	L	NE	Intact	Intact	NE
E39	57	Ic	Wild	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
E40	47	IIIa	Wild	Wild	NE	MSS	H	NE	Intact	Intact	NE
E41	59	Ic	Wild	Wild	TCT33TTT	MSS	MSS	MSS	Intact	Loss	Intact
E42	46	Ic	TCT33TTT TCT33TTT, GGA34GTA,	TCT29TTT, TCT45CCT	NE	MSS	L	NE	Intact	NE	NE
E43	52	IIb	TCT45CCT	TCT33TTT	TCT33TTT, TCT45CCT	MSI- H	MSI- H	MSI- L	Loss	Intact	Loss
E44	73	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
E45	42	Ic	TCT45TTT	TCT33TTT	Wild	MSI- L	MSI- L	MSI- L	Intact	Intact	Intact
E46	69	Ic	TCT33TGT	TCT33TGT	TCT33TTT, TCT45CTT TCT33TTT,	MSS	MSS	MSS	Intact	Intact	Intact
E47	44	Ia	TCT37TGT	TCT37TGT	TCT45TCC	MSS	MSS	L	Intact	Intact	Intact
E48	75	Ic	Wild	Wild	Wild	MSS	MSS	H	Loss	Loss	Intact
E49	54	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
E50	43	Ia	GAC32AAC	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
OCCC _a											
C1	54	Ic	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	L	MSS	Intact	Intact	Intact
C5	64	IIIc	Wild	Wild	Wild	MSS	L	MSS	Loss	Intact	Intact
C6	53	IIIb	Wild	Wild	Wild	MSS	MSS	MSS	Loss	Intact	Intact
C7	66	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C8	62	IIc	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Loss
C9	35	IIIc	Wild	Wild	Wild	MSI- L	MSS	MSS	Intact	Intact	Intact
C10	49	Ic	Wild	TCT33TTT	TCT33TTT, TCT45CCT	MSS	MSS	NE	Intact	Intact	Intact
C11	72	IIIc	Wild	TCT45CCT	Wild	L	NE	NE	Intact	Intact	Intact
C12	52	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C13	69	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C14	60	Ic	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C15	45	Ia	Wild	TCT33TTT	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C16	72	IIIc	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C17	62	Ia	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact
C18	49	Ic	TCT37TGT	Wild	NE	MSS	MSS	NE	Intact	Intact	NE
C19	76	IIb	Wild	Wild	Wild	MSS	MSS	MSS	Intact	Intact	Intact

(continued on next page)

Table 1 (continued)

Case no.	Age (y)	FIGO stage	β-Catenin mutation			MSI status			MMR IHC abnormality		
			Ca	E1	E2	Ca	E1	E2	Ca	E1	E2
C20	57	Ic	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	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

Abbreviations: Ca, carcinoma lesion; case number; ;FIGO, International Federation of Gynecology and Obstetrics; NE, not examined; w/o Ca, without carcinoma Ca, carcinoma lesion; E1, endometriotic lesion-1; E2, endometriotic lesion-2;.

expression were noted but were classified as intact staining, as described by Xiao et al [23], with minor modifications.

2.4. Endometriosis-inflammatory score

To evaluate the morphologic alterations that occurred during repeated hemorrhaging in endometriosis, the lesions were examined and evaluated according to the following 4 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 endometriosis-inflammatory (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). 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 5 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 2 or more loci displayed MSI, or MSI-low (MSI-L) if only 1 locus displayed MSI. Lesions in which MSI was

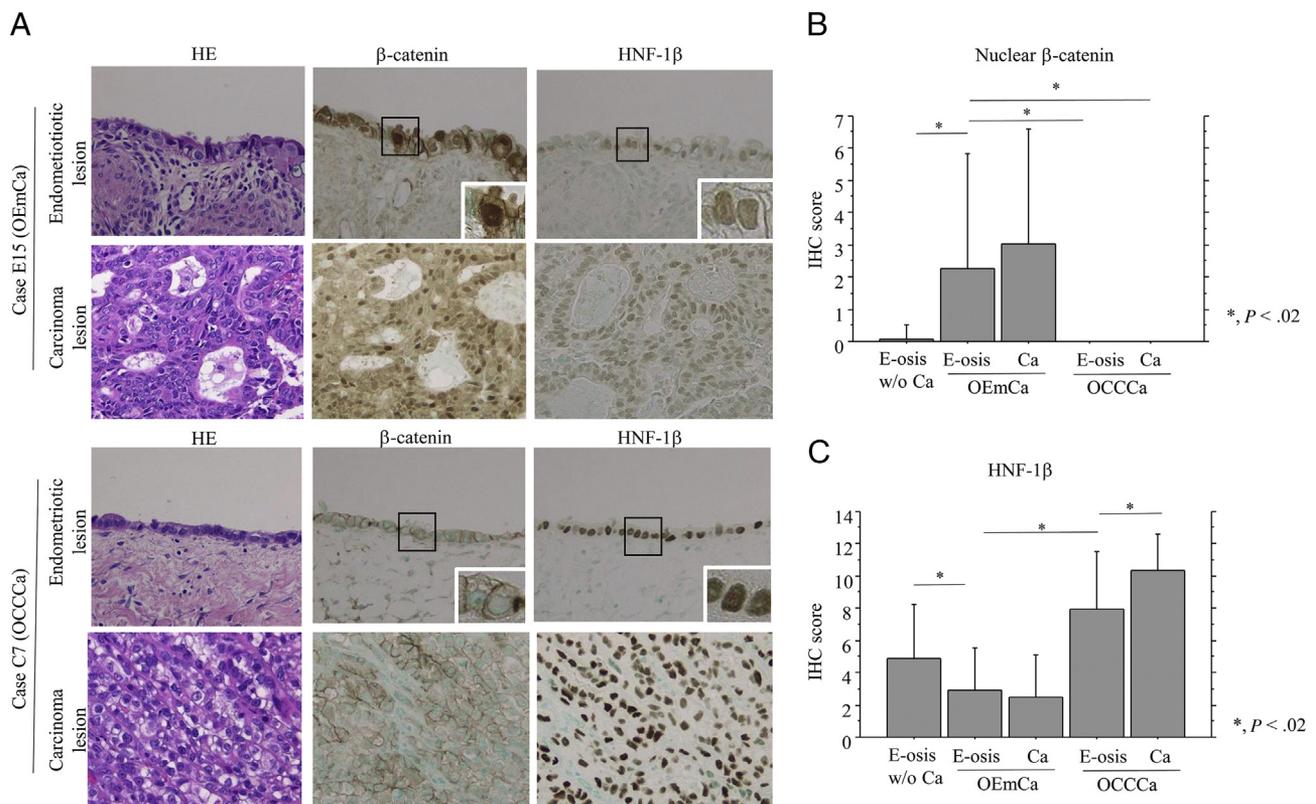


Fig. 1 IHC findings of β -catenin and HNF-1 β in endometriosis and the associated carcinomas. A, Micrographs showing hematoxylin and eosin and IHC staining for β -catenin and HNF-1 β in endometriotic and carcinoma lesions in OEmCa (upper) and OCCCa (lower). Note the nuclear β -catenin accumulation in both OEmCa and the coexisting endometriosis, in contrast to the strong HNF-1 β staining in both OCCCa and coexisting endometriosis. Closed boxes are magnified in the insets. Original magnifications $\times 100$ and $\times 400$ (inset). B, IHC scores for nuclear β -catenin (upper) and C, HNF-1 β (lower) in endometriosis (E-osis) without (w/o) carcinoma, endometriosis adjacent to carcinoma, and carcinoma lesions. Data shown as mean \pm SD.

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, χ^2 test, and the Spearman correlation coefficient, where appropriate. The cutoff for statistical significance was set as $P < .05$.

3. Results

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

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

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

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

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

Mutations of exon 3 in *CTNNB1* were significantly higher in OEmCas and the coexisting endometriosis when compared

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

	n	<i>CTNNB1</i> mutation		<i>P</i>	n	MSI status			<i>P</i>	n	MMR status		<i>P</i>
		Positive, n (%)	Negative, n (%)			MSS, n (%)	MSI-L, n (%)	MSI-H, n (%)			Intact	Loss	
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)	
OCCCca	40	4 (10)	36 (90)	<.0001	37	35 (94.6)	2 (5.4)	0	.002	40	37 (92.5)	3 (7.5)	.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)	<.0001	48	37 (77.1)	4 (8.3)	7 (14.6)	.18	50	38 (76)	12 (24)	.36
OCCCca	21	1 (4.8)	20 (95.2)		21	19 (90.5)	2 (9.5)	0		21	18 (85.7)	3 (14.3)	

Abbreviation: w/o Ca, without carcinoma; .

with OCCCa with coexisting endometriosis, as well as endometriosis without carcinoma lesions (Table 2 and Supplementary Table S1). Eighteen 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 8 lesions (E1, E3, E15, E17, E38, E42, E45, and E47) were different. Five (E1, E15, E29, E46, and E47) of 10 informative endometriotic lesions with OEmCa also harbored different mutations between the E1 and E2 lesions. In contrast, *CTNNB1* mutations were detected in 4 endometriotic lesions adjacent to OCCCas (C10, C11, C10), but not within the OCCCas. In addition, double- and triple-point mutations were identified in 2 OEmCas (E4 and E43) and 9 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-1 β 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 (Fig. 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, particularly 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 showing that 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 with that of endometriosis with OCCCa or without carcinoma lesions, whereas such difference was not evident between OEmCa and OCCCa (Table 2). No differences in MSI status were also found between endometriosis and carcinoma lesions (Supplementary Table S1). Of the 16 informative lesions, 7 demonstrated identical MSI-H status between OEmCas and the coexisting endometriosis, whereas 9 were not identical. In addition, in 2 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-1 β scores in both OEmCas and OCCCas with endometriosis (Fig. 2C).

Representative IHC findings for MMR proteins including MLH1, MSH2, MSH6, and PMS2 in endometriosis, OEmCa, and OCCCa are shown in Fig. 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 1 MMR protein, were significantly different among endometriosis with OEmCa or OCCCa and without carcinoma lesions. In contrast, no differences in MMR protein status were observed between OEmCa and OCCCa, and were also evident between endometriosis and carcinoma lesions (Supplementary Table S1).

Among the MMR protein-negative lesions, 12 endometriosis, 6 OEmCa, and 2 OCCCa lesions lost both MSH2 and MSH6; these proteins form the MutS α complex, which recognizes as single-base mismatches and short insertion-deletion loops in DNA. Moreover, 9 endometriosis and 5 OEmCa lesions had abnormalities in both the proteins that form the MutL α complex (MLH1 and PMS2), which mediate the progression from mismatch recognition to activation of downstream activities during the MMR process. Isolated protein loss was observed in lesions of 8 endometriosis cases, 2 OEmCa, and 1 OCCCa (for loss of MSHX expression, see Supplementary Table S5).

The MMR protein abnormalities were significantly associated with MSI status in both endometriosis and carcinoma

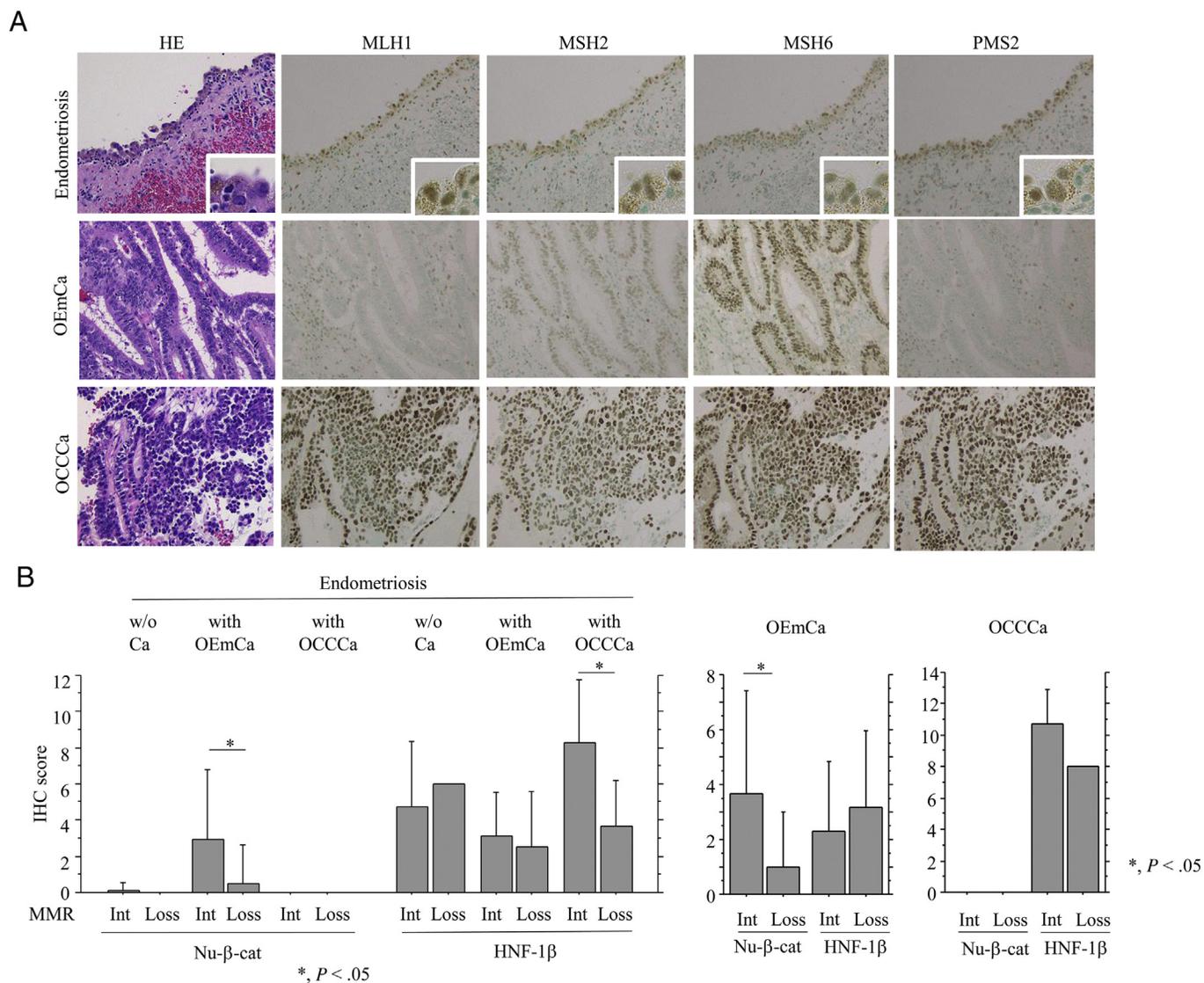


Fig. 3 IHC of MMR protein expression in endometriosis and the associated carcinomas. A, Hematoxylin and eosin and IHC staining for 4 MMR markers including MLH1, MSH2, MSH6, and PMS2 in endometriosis (upper panels), OEmCa (middle panel), and OCCa (lower panel). Note the lack of nuclear immunostaining for MLH1, MSH2, and PMS2 in OEmCa, in contrast to the distinct nuclear staining in endometriosis and OCCa. Closed boxes are magnified in the insets. Original magnification $\times 100$ and $\times 400$ (inset). B, Association between IHC scores for nuclear (Nu)- β -catenin and HNF-1 β , and MMR protein status in endometriotic and the associated carcinoma lesions. Int, intact of MMR protein expression; ;Int, intact of MMR protein expression; Loss, loss of MMR protein expression; w/o carcinoma, without carcinoma. Data shown as mean \pm SD.

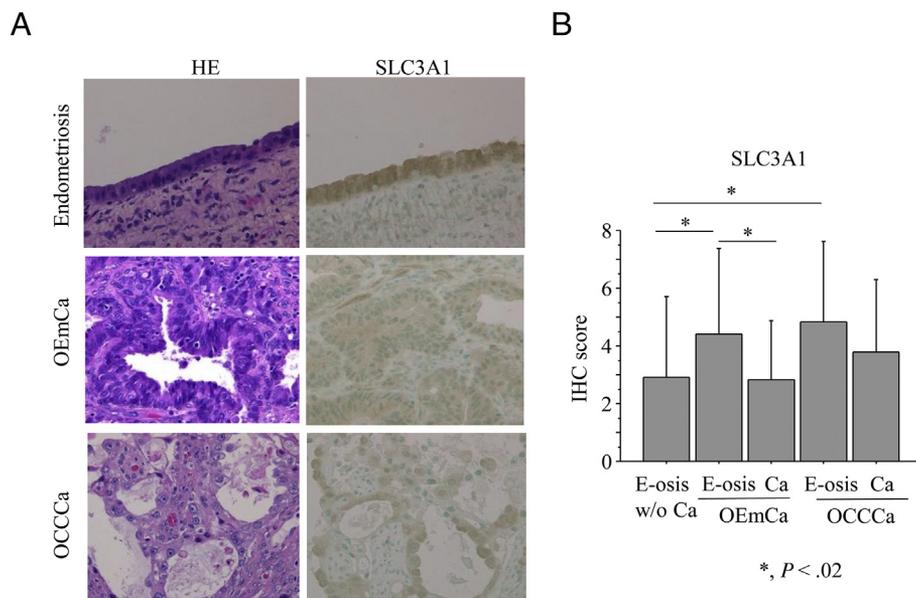


Fig. 4 IHC of SLC3A1 in endometriosis and the associated carcinomas. A, Micrographs showing hematoxylin and eosin and IHC staining for SLC3A1 in endometriosis (upper), OEmCa (middle), and OCCCa (lower). Note the distinct cytoplasmic SLC3A1 immunostaining in epithelial components of endometriosis, in contrast to the weak staining in OEmCa and OCCCa. Original magnification $\times 200$. B, IHC scores for SLC3A1 in endometriosis and the associated carcinomas. E-osis w/o carcinoma, endometriosis without carcinoma. Data shown as mean \pm SD.

lesions (Supplementary Table S6). The nuclear β -catenin score was significantly lower in endometriosis with OEmCa and OEmCa with MMR abnormality when compared with those with intact MMR protein expression. Similar findings regarding HNF-1 β score were also observed in endometriosis with OCCCa (Fig. 3B). In contrast, the *CTNNB1* mutations were not associated with MMR/MSI abnormalities in both endometriosis and carcinoma 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 carcinomas, whereas the *CTNNB1* mutations were significantly associated with young age and early clinical stage in the carcinoma categories (Supplementary Table S8).

3.4. SLC3A1 expression in endometriosis and EAOCa

Because SLC3A1 (rBAT) plays a major role in HNF-1 β -triggered ROS resistance [13,14], we examined whether SLC3A expression was associated with nuclear β -catenin and HNF- β statuses in carcinomas and their coexisting endometriotic lesions. A distinct cytoplasmic immunoreactivity was mainly observed in epithelial components, particularly in endometriosis (Fig. 4A). SLC3A1 scores were significantly higher in endometriosis adjacent to the OEmCa or OCCCa when compared with the endometriosis without carcinoma lesions and OEmCas, but not when compared with the OCCCas (Fig. 4B). The SLC3A1 score was positively associated with nuclear β -catenin in endometriosis and HNF-1 β 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 4 inflammatory parameters including hemorrhage, macrophage accumulation, erosion, and inflammatory cell infiltration in response to repeated hemorrhaging within endometriosis are shown in Fig. 5A. These parameters and E-I scores showed significant stepwise decreases from endometriosis without carcinoma, through endometriosis with OEmCa, to endometriosis with OCCCa lesions (Fig. 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 with MSS (Fig. 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 (Fig. 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 4

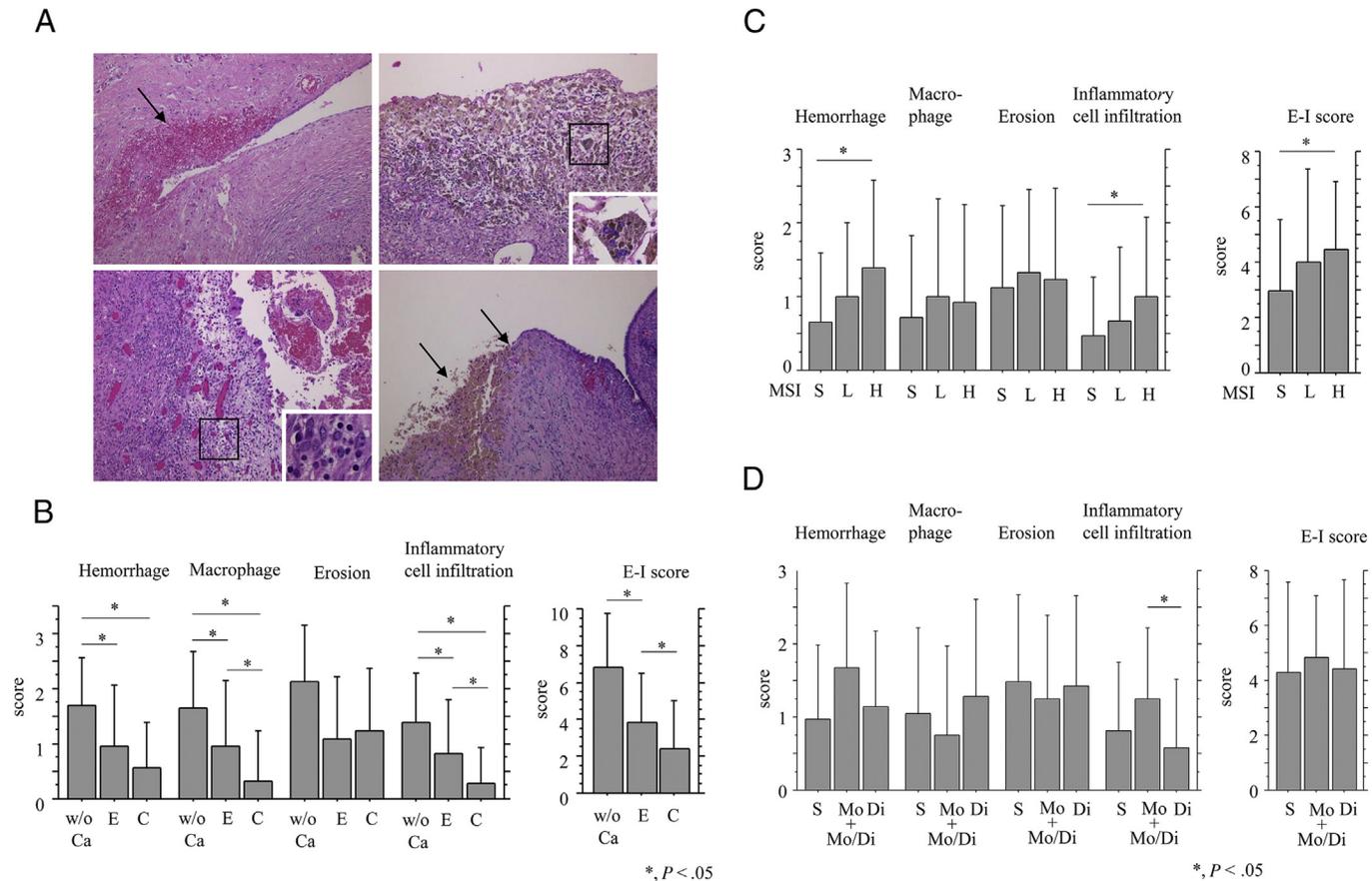


Fig. 5 Association between inflammatory features and MSI status in endometriosis. A, Micrographs showing hematoxylin and eosin staining in endometriosis. Upper left: hemorrhagic parameter: note the hemorrhagic area (indicated by arrow) within the cystic wall. Upper right: accumulation of macrophages: 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 $\times 100$ and $\times 400$ (inset). B, Scores of the 4 inflammatory parameters and E-I scores in endometriosis w/o Ca (endometriosis without carcinoma); C, endometriosis adjacent to OCCa; E, endometriosis adjacent to OEmCa. Data shown as mean \pm SD. C, Associations between the 4 inflammatory parameters as well as E-I scores and the MSI status. L, MSI-L; ;L, MSI-L; H, MSI-H; S, MSS. Data shown as mean \pm SD. D, Associations between the 4 inflammatory parameters as well as E-I scores and alterations in repeat sequence patterns. Mo, mononucleotide repeat; ;Di, dinucleotide repeat; Mo, mononucleotide repeat; S, MSS. Data shown as mean \pm SD.

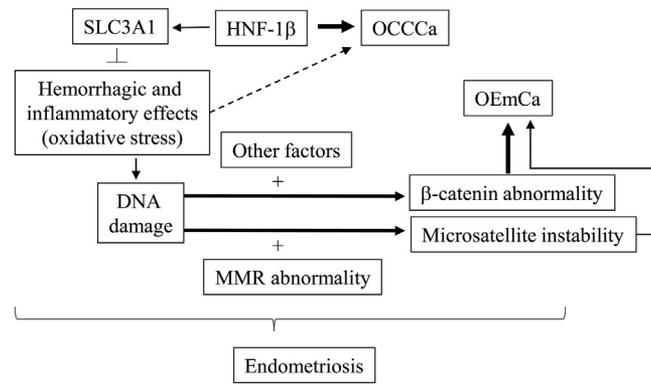


Fig. 6 Schematic representation of associations between β -catenin, HNF-1 β , and MSI status in endometriosis-associated OEmCa and OCCCa.

endometriosis-related inflammatory parameters, were significantly higher in endometriosis adjacent to OEmCas when compared with 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 downstream 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 with 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-1 β scores were significantly higher in both OCCCa and the coexisting endometriosis when compared with 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-1 β expression and MSI status in both OEmCas and OCCCas with endometriosis, we suggest that HNF-1 β 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-1 β in the early stages of tumorigenesis in OCCCa, even before the development of endometriosis [33].

HNF-1 β 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-1 β 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-1 β expression when compared with OEmCas with endometriosis and low HNF-1 β expression. These findings implicate ROS resistance driven by the HNF-1 β /SLC3A1 axis as an essential step in the endometriosis to OCCCa progression. However, further studies are required 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, nonidentical patterns for both *CTNNB1* mutations and MSI status were frequently observed in the 2 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 operate in the progression of endometriosis to OEmCa or OCCCa

(Fig. 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-1 β /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-1 β 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 malignant transformed OCCCa or OEmCa [37].

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.humpath.2019.04.001>.

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