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Molecular association of functioning stroma with carcinoma cells in the ovary: A preliminary study

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Abstract. The cancer stroma serves an important role in tumour behaviours, including invasion, metastasis, and response to chemotherapy. The stroma of ovarian carcinoma is sometimes specialized, with luteinisation and/or hyperthecosis, and is designated as the 'functioning stroma' because it exerts endocrine function and produces sex steroid hormones. In the present study, 14 ovarian cancers with functioning stroma, comprising 7 endometrioid carcinomas and 7 clear cell carcinomas, were analysed to evaluate the molecular association of the functioning stroma with carcinoma cells. The median age of the patients was 67 years (range, 52-85 years); 13 patients were postmenopausal, and one was in perimenopause. Serum oestrogen values ranged from 10 to 129 ng/ml, with a median of 51 ng/ml. Sequence abnormalities in *AT-rich interaction domain 1A* (*ARID1A*), *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α* (*PIK3CA*), *Kirsten rat sarcoma viral proto-oncogene* (*KRAS*) and *phosphatase and tensin homolog (PTEN)* were examined in whole tumours. For cancers positive for sequence abnormalities, their localization in carcinoma cells and/or stromal cells was examined. A total of 8 mutations - *ARID1A* (L2155L), *PIK3CA* (H1047R), *KRAS* (Q12V, E31K, Q61L), and *PTEN* (C105fs*8) - were identified in the whole tumours of 5 patients. Seven of these eight mutations were detected only in carcinoma cells. However, one case of endometrioid carcinoma had a *KRAS* (E31K) mutation in both carcinoma and stromal cells. In conclusion, although functioning stromal cells of ovarian cancer are usually thought to be non-neoplastic, some may share an origin with carcinoma cells.

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Abbreviations: EOC, epithelial ovarian cancer; EMC, endometrioid carcinoma; CCC, clear cell carcinoma; FIGO, The International Federation of Obstetrics and Gynecology; FSH, follicle-stimulating hormone

Key words: ovarian cancer, functioning stroma, gene mutations, *Kirsten rat sarcoma viral proto-oncogene, AT-rich interaction domain 1A, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α, phosphatase and tensin homolog*

Introduction

The cancer stroma, which is generally thought to be derived from non-neoplastic cells represented by cancer-associated fibroblasts, plays an important role in various tumour behaviours, such as invasion, metastasis, and chemotherapeutic response (1-3). Carcinoma-associated fibroblasts promote cancer progression (4), angiogenesis (5), and metastasis (6). In several cancers, interactions between cancer cells and the associated stroma lead to drug resistance (7). Recent studies have suggested that some carcinoma cells and stromal cells may share an origin, based on gene landscapes shared by both cell types in certain cancers, including breast (8), colon (9), bladder (10), and ovarian cancers (11,12). However, the origin and role of cancer stroma have been controversial yet.

Epithelial ovarian cancer (EOC) is the leading cause of death arising from gynecological malignancies (13). EOCs, including serous carcinoma, endometrioid carcinoma (EMC), clear cell carcinoma (CCC), and mucinous carcinoma, have specific clinical and genetic features. EMC and CCC are histogenetically associated with endometriosis and are often characterized by *ARID1A* (30 and 40-60%, respectively), *PIK3CA* (40 and 51%, respectively), *KRAS* (33 and 20%, respectively), and *PTEN* (17 and 13%, respectively) (14-17). But the frequency of *TP53* mutation in EMC and CCC are considerably lower (7 and 13%, respectively), compared to the four genes (14-17). The stroma of EOCs sometimes consists of a specialized ovarian stroma with luteinisation and/or hyperthecosis with endocrine function, called a 'functioning stroma' (18). The relationship between functioning stroma and the response to chemotherapy or prognosis remains to be clarified. In addition, the histogenetic mechanism of functioning stroma is poorly defined. Functioning stroma is observed not only in mucinous carcinoma but also in

EMC and CCC (19). However, serous carcinoma characterized by *TP53* mutation rarely has a functioning stroma (19). Therefore, this study aimed to evaluate the localization of gene abnormalities commonly detected in EMC and CCC in carcinoma cells and functioning stromal cells separately. We believe that some of functioning stroma may share an origin with carcinoma cells.

Materials and methods

Patients and samples. Subjects eligible for this study had histologically confirmed ovarian EMC or CCC with functioning stroma (Fig. 1). Patient and clinicopathological data, including age, menopause, International Federation of Obstetrics and Gynecology (FIGO) stage, histological subtype, histological grade, surgery (optimal, residual tumour <1 cm; suboptimal, residual tumour ≥1 cm), serum oestrogen level, serum follicle-stimulating hormone (FSH) level, recurrence, and death, were reviewed. Serum levels of oestrogen (Eclusys E2 IV; Roche Diagnostics, Tokyo, Japan) and FSH (FSH II; Roche Diagnostics) were analysed by enzyme immunoassays. All patients had a follow-up period of at least three years. The study was approved by the Institutional Review Board of the Saitama Medical University International Medical Center (Saitama, Japan), and written informed consent was obtained from all patients.

Laser microdissection and DNA extraction. Formalin-fixed, paraffin-embedded sections (10 μm) prepared from tumour tissue specimens were affixed to 2-μm-thick LCM Film glass slides (Membrane Slides PEN Membrane 2; Leica, Wetzlar, Germany) and stained with 0.05% toluidine blue solution (pH 2.5; Wako, Osaka, Japan). Stained sections were microdissected using a Leica LMD7000 laser microdissection microscope. Carcinoma cells and adjacent functional stromal cells were visualized under the microscope and were selectively detached by activation of the laser (Fig. 2). DNA was extracted using the Maxwell RSC DNA FFPE kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Amplification and sequence analysis of ARID1A, PIK3CA, KRAS, and PTEN. We analysed *ARID1A* (exons 18 and 20), *PIK3CA* (exons 9 and 20), *KRAS* (exons 2 and 3), and *PTEN* (exons 5-8) sequences in DNA extracted from the whole tumours of 14 patients. For cases with mutations, carcinoma cells and functioning stromal cells were analysed separately to clarify the histological localization of the mutations. Target sequences were PCR-amplified using Accuprime Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) on a 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Primer sequences are shown in Table I. The thermal cycles were as follows: 95˚C for 10 min, followed by 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 68˚C for 60 sec. Products were electrophoresed on a 2% agarose gel. Purified products were subjected to direct sequencing on an ABI PRISM 3100 (Applied Biosystems) using the ABI PRISM Big Dye Terminator Ver3.1 Cycle Sequencing kit according to the manufacturer's instructions. Sequencing was conducted twice to confirm reproducibility of the results.

Results

Patient characteristics. The median age of the 14 patients was 67 years (range, 52 to 85 years); 13 patients were postmenopausal, and one was in perimenopause. Serum oestrogen levels ranged from 10 to 129 ng/ml, with a median of 51 ng/ml, before surgery, but were reduced to less than 10 ng/ml in all available postoperative patients. Serum FSH levels ranged from 6 to 89 mIU/ml, with a median of 32 mIU/ml, preoperatively, but increased after surgery, ranging from 62 to 96 mIU/ml and with a median of 82 mIU/ml. Ten patients had FIGO stage I cancer, two patients had stage II, and two patients had stage IV. Five patients with EMC had grade 1 cancer, and two patients with EMC had grade 2 cancer. Two patients with FIGO stage IV had suboptimal surgeries, and the others had optimal surgeries. Among CCC patients, four patients experienced recurrence, and three of them died of the disease. In EMC, there was no recurrence or death.

Localization of gene mutations. As shown in Table II, one patient had an *ARID1A* mutation (7%), two patients had *PIK3CA* mutations (14%), three patients had *KRAS* mutations (21%), and one patient had a *PTEN* mutation (7%). In EMC, three patients had *KRAS* mutations (43%), one patient had a *PIK3CA* mutation (14%), and none of the patients had *ARID1A* or *PTEN* mutations (0%). In CCC, one patient had both *PIK3CA* and *PTEN* mutations (14%), one patient had an *ARID1A* mutation (14%), and none of the patients had *KRAS* mutations (0%).

In total, eight mutations were detected in whole tumours of five patients: *ARID1A* (L2155L), *PIK3CA* (H1047R), *KRAS* (Q12V, E31K, Q61L), and *PTEN* (C105fs*8) (Table III). Seven of the eight mutations were detected in only carcinoma cells; thus, only one patient (case 4) had a *KRAS* (E31K) mutation in both carcinoma and functioning stromal cells. The *KRAS* mutation in this case (Fig. 3) was repeatedly confirmed. In non-functioning stroma, composed of non-specific fibroblasts, no *KRAS* mutation was detected in this patient. Moreover, germline analysis of this patient revealed no *KRAS* mutation.

Trends in prognosis, hormone levels, and gene mutations. Five patients with high serum oestrogen levels (≥50 ng/ml) survived, whereas three patients (38%) with low serum oestrogen levels (<50 ng/ml) died of the disease. In the four patients with recurrence, one patient (case 10) with a high serum oestrogen level (88 ng/ml) and low FSH level (6 mIU/ml) experienced disease-free survival after chemotherapy, but the other three patients died of the disease.

Discussion

In EOC, the cancer stroma is classified as either non-specific fibroblastic type or functioning stroma, the latter of which frequently occurs in postmenopausal EOCs. However, functioning stroma had not yet been thoroughly characterized in terms of histogenesis, response to chemotherapy, and prognosis. A close association between mucinous tumours and functioning stroma has been reported (19), and Katoh *et al* (20) found that functioning stroma is more common in EMCs resembling sex cord-stromal tumours. In the seven EMC and seven CCC

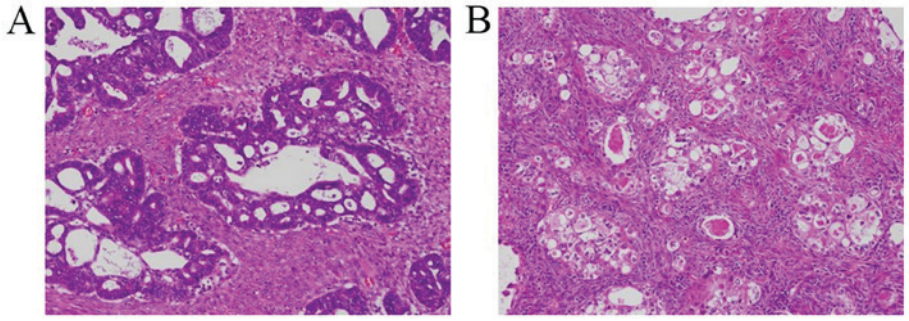

Figure 1. Histological findings. (A) Functioning stroma (case 4) in EMC and (B) CCC (case 10). Magnification, x20. EMC, endometrioid carcinoma; CCC, clear cell carcinoma.

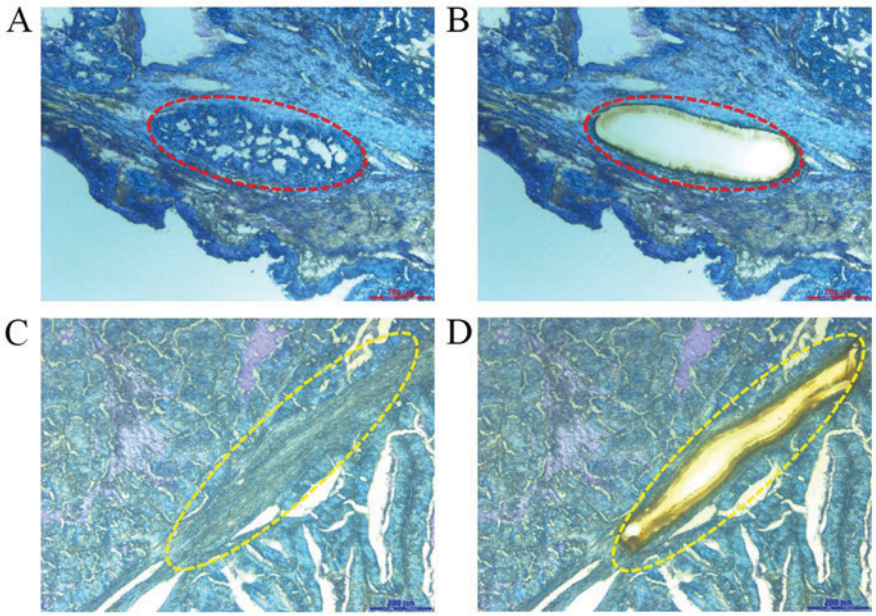

Figure 2. Laser microdissection from toluidine blue-stained sections (case 4). Carcinoma (A) prior to (red outlined area) and (B) following (red outlined area) dissection. Functioning stroma (C) prior to (yellow outlined area) and (D) following (yellow outlined area) dissection. Magnification, x20.

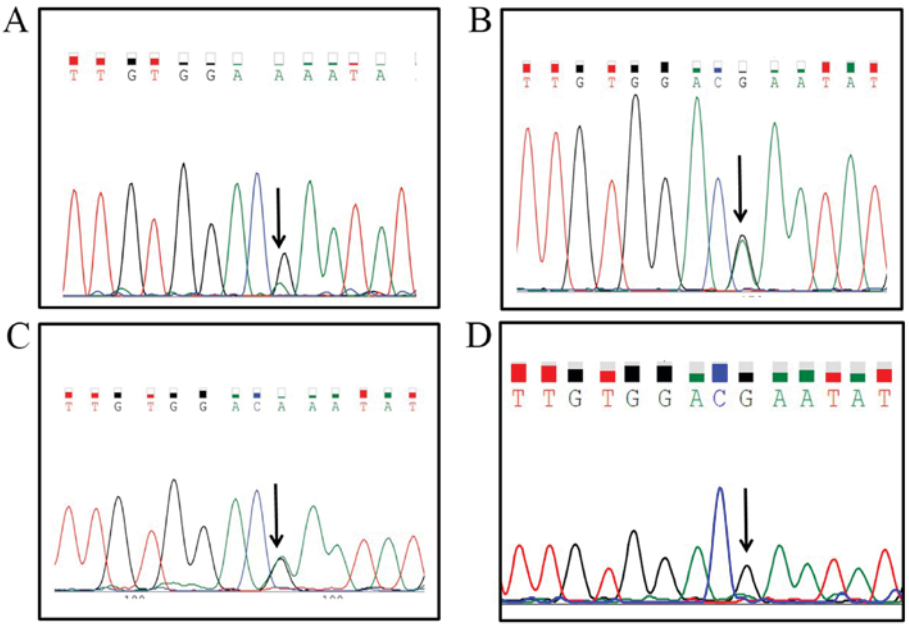

Figure 3. In case 4, the *KRAS* mutation was detected in (A) whole tumour, (B) carcinoma, and (C) functioning stroma, but not in (D) non-tumour tissues of the uterus. *KRAS, Kirsten rat sarcoma viral proto-oncogene*.

Table I. Sequence information for primers used to amplify *ARID1A*, *PIK3CA*, *KRAS* and *PTEN*.

A, *ARID1A*

Gene	Primer (5'-3')
Exon 18	
S	TGGCATCTGTGGGCTTTATGT
AS	CCATACTGGTTGTATACATCTTGCT
S	GGAGATGTACAGCGTGCCATA
AS	TTGTGGTGGCATGTTTTGCTG
S	CAGAACCAATTTCCATTCCAGT
AS	GTGTGCAGCATTTCATCTGTTC
S	GGGGCGTAATGACATGACCTAT
AS	AATGTGATTCTGCATGCTTGGTG
S	CAAGGCCCCCTCCATCTAAC
AS	TGCTAGGAGAGGTGCGGTTC
S	ATGCAGAATCACATTCCTCAGGTAT
AS	GGCAGATTAGGCAACCGAATG
Exon 20	
S	GGGGAGGTCTCTCAAGTCAAT
AS	ATGGGAGCTGGACTAGACAC
S	GGACAGAGAACGCTACTGGAT
AS	AATGGATCATTCTTCTGTACGATCT
S	GAGGAGAAGCTGATCAGTAAGTTTG
AS	CTGCTGTTGTCACATGCTTCC
S	CTGAGCATATCCAGACCCACTTC
AS	GCCTCTGAACTCTTAGCTCCATC
S	CAGCCACTATGGATGACATGTT
AS	GGTGTTTGGACATCTCAAAGTCA
S	GCGTCTGTGTGTCCAATACCA
AS	CACTCCACTTTGTTGCAGCTC
S	CAGGCACCACTAACTTATGAAAAGG
AS	GAGTTTGCTGAGGGTTTCCAAGA
S	TCCTTTCCCCGCAGAGACT
AS	CCAGGAGGTTGCCGATACTG
S	GTGCCATTGCAGTGCAGAA
AS	GAGATGTCCAACAGCCGTGAT
S	TGGACGAGAACCACTCAGAGTTTAC
AS	AGGGCAACAGTCAGTTTCTAAGTTC

B, *PIK3CA*

Gene	Primer (5'-3')
Exon 9	
S	TGTAAAACGACGGCCAGTGGGAAA AATATGACAAAGAAAGC
AS	CAGGAAACAGCTATGACCTGAGAT CAGCCAAATTCAGTT
Exon 20	
S	TGTAAAACGACGGCCAGTCTCAATG ATGCTTGGCTCTG
AS	CAGGAAACAGCTATGACTGGAATCC AGAGTGAGCTTTC

Table I. Continued.

C, *KRAS*

Gene	Primer (5'-3')
Exon 2	
S	TAACCTTATGTGTGACATGTTC
AS	ATGCATATTAAAACAAGATTTACC
Exon 3	
S	CTCCCTTCTCAGGATTCCTA
AS	AGTCCTCATGTACTGGTCCC

D, *PTEN*

Gene	Primer (5'-3')
Exon 5	
S	ACCTGTTAAGTTTGTATGCAAC
AS	TCCAGGAAGAGGAAAGGAAA
Exon 6	
S	CATAGCAATTTAGTGAAATAACT
AS	GATATGGTTAAGAAAACTGTTC
Exon 7	
S	TGACAGTTTGACAGTTAAAGG
AS	GGATATTTCTCCCAATGAAAG
Exon 8	
S	ACACATCACATACATACAAGTC
AS	GTGCAGATAATGACAAGGAATA

S, sense; AS, antisense; *ARID1A, AT-rich interaction domain 1A; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; KRAS, Kirsten rat sarcoma viral proto-oncogene; PTEN, phosphatase and tensin homolog*.

patients recruited in the present study, functioning stroma was identified histologically and endocrinologically. Five EOCs in four patients carried mutations in *ARID1A*, *PIK3CA*, *KRAS*, and *PTEN* in carcinoma cells, but not in functioning stromal cells. However, one case of ovarian EMC (case 4) harboured an identical *KRAS* mutation (E31K) in both carcinoma and functioning stromal cells, whereas no mutation was detected in the tissue surrounding the tumour. Akahane *et al* (11) reported a case of ovarian EMC with carcinoma and stromal cells harbouring the same mutation in *TP53* (R2489) as indicated by direct sequence analysis. Tuhkanen *et al* (12) reported 39 similar genetic alterations in carcinoma and stromal cells in 11 EOC tumours based on multiplex ligation-dependent probe amplification. These findings suggest that the cancer stroma may contain cells of epithelial origin that are generated by epithelial-mesenchymal transition and that this transformed tumour stroma may have an effect on epithelial-stromal cell interactions and tumorigenesis (12). Therefore, it may be possible to elucidate the mechanisms of tumour initiation by evaluating the association between carcinoma cells and functioning stromal cells.

Table II. Demographic and clinicopathological data for 14 patients, and gene mutations in whole tumours in the 14 cases.

Case	Age, years	Age at menopause, years	Histology	FIGO stage	FIGO grade	Surgery	Recurrence	Death	Oestrogen, ng/ml		FSH, mIU/ml		*ARID1A*	*PIK3CA*	*KRAS*	*PTEN*
									Pre	Post	Pre	Post				
1	79	53	EMC	1C	1	Opt	No	No	50	<10	23	62	WT	WT	Q12V	WT
2	80	50	EMC	2B	2	Opt	No	No	50	<10	28	84	WT	WT	WT	WT
3	63	48	EMC	1C	1	Opt	No	No	103	<10	16	85	WT	WT	E31K, Q61L	WT
4	74	50	EMC	4A	1	Sub	No	No	36	NA	35	NA	WT	H1047R	E31K	WT
5	70	50	EMC	1C	1	Opt	No	No	36	<10	39	89	WT	WT	WT	WT
6	65	52	EMC	1A	2	Opt	No	No	48	NA	NA	NA	WT	WT	WT	WT
7	85	47	EMC	1C	1	Opt	No	No	129	NA	NA	NA	WT	WT	WT	WT
8	68	50	CCC	1A	NA	Opt	No	No	31	NA	29	96	WT	WT	WT	WT
9	64	52	CCC	2A	NA	Opt	Yes	Yes	29	NA	NA	NA	WT	H1047R	WT	C105fs*8
10	62	51	CCC	1C	NA	Opt	Yes	No	88	<10	6	76	WT	WT	WT	WT
11	63	53	CCC	1C	NA	Opt	Yes	Yes	21	NA	55	NA	L2155L	WT	WT	WT
12	62	52	CCC	1A	NA	Opt	No	No	10	NA	79	NA	WT	WT	WT	WT
13	52	51	CCC	1C	NA	Opt	No	No	NA	NA	NA	NA	WT	WT	WT	WT
14	52	Peri	CCC	4B	NA	Sub	Yes	Yes	34	NA	12	NA	WT	WT	WT	WT

FIGO, International Federation of Obstetrics and Gynecology; FSH, follicle-stimulating hormone; Pre, preoperative; Post, postoperative; Peri, perimenopause; EMC, endometrioid carcinoma; CCC, clear cell carcinoma; Opt, optimal surgery (residual tumour <1 cm); Sub, suboptimal surgery (residual tumour ≥1 cm); NA, not available; WT, wild-type; *ARID1A, AT-rich interaction domain 1A; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; KRAS, Kirsten rat sarcoma viral proto-oncogene; PTEN, phosphatase and tensin homolog*.

Table III. Localization of *ARID1A*, *PIK3CA*, *KRAS* and *PTEN* mutations.

		ARID1A			*PIK3CA*			*KRAS*			*PTEN*		
Case	Histology	Whole	CA	FS	Whole	CA	FS	Whole	CA	FS	Whole	CA	FS
1	EMC	WT	WT	WT	WT	WT	WT	Q12V	Q12V	WT	WT	WT	WT
3	EMC	WT	WT	WT	WT	WT	WT	E31K, Q61L	E31K, Q61L	WT	WT	WT	WT
4	EMC	WT	WT	WT	H1047R	H1047R	WT	E31K	E31K	E31K	WT	WT	WT
9	CCC	WT	WT	WT	H1047R	H1047R	WT	WT	WT	WT	C105fs*8	C105fs*8	WT
11	CCC	L2155L	L2155L	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

Whole, whole tumour; CA, carcinoma; FS, functioning stroma; EMC, endometrioid carcinoma; CCC, clear cell carcinoma; WT, wild-type; *ARID1A*, *AT-rich interaction domain 1A*; *PIK3CA*, *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α*; *KRAS*, *Kirsten rat sarcoma viral proto-oncogene*; *PTEN*, *phosphatase and tensin homolog*.

KRAS mutations are typically detected in 20-30% of EMC cases (14,21); however, in the present study, EMC with functioning stroma had a higher frequency (43%) of *KRAS* mutations. *KRAS* mutation is more common in endometriosis-associated EMC than in non-endometriosis-associated EMC (21). These findings suggest that EMC with functioning stroma may be associated with endometriosis. Mutations in *ARID1A*, which are distributed evenly across the gene, are detected in 46% of ovarian CCC and 30% of EMC cases (16). In the present study, there was only one case of CCC (7%) with *ARID1A* mutation. However, we immunohistochemically confirmed the deletion of *ARID1A* in eight of the 14 patients (57%) (data not shown). This discrepancy may be explained by the fact that only exons 18 and 20 were sequenced in the present study. EOC patients with functioning stroma exhibited elevated serum oestrogen levels and reduced serum FSH levels. Following surgery, the levels of both hormones were restored to normal postmenopausal levels. Patients with high oestrogen levels (≥50 ng/ml) experienced no death from the disease, as demonstrated by one patient with EMC (case 10) who had a long period of disease-free survival after chemotherapy for recurrence; however, patients with low oestrogen levels (<50 ng/ml) exhibited poor outcomes. Thus, the functioning stroma, characterized by morphological and endocrinological differentiation, may be related to the biological behaviours of cancer.

Our study had several limitations. The sample size was small and the range of genetic mutations analysed was narrow. In addition, the detection of identical mutations in both carcinoma and functioning stromal cells was not replicated, and differences between EMC with and without functioning stroma were not assessed. Therefore, this research should be considered a preliminary study. Confirmation of our findings in a larger population, including patients with and without functioning stroma, is warranted.

In conclusion, we are the first to report a case of EMC in which the same *KRAS* mutation was observed in both carcinoma cells and functioning stromal cells. This suggests that some regions of the tumour and stroma may have a common origin. Further studies are needed to clarify the molecular association of functioning stroma with carcinoma cells in a larger population.

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Availability of data and materials

All datasets generated in this study are available from the corresponding author upon reasonable request.

Authors' contributions

MN took part in the study conception and design, as well as the acquisition, analysis and interpretation of data. MiY took part in the interpretation of data and drafting of the manuscript. KK and TK took part in the acquisition and analysis of data. MS and KI took part in the conception and design of the study, and revised the manuscript for important intellectual content. HK participated in the analysis of data and critically revised the manuscript for important intellectual content. MaY took part in the conception and design of the study, critically revised the manuscript for important intellectual content, and supervised the study.

Ethics approval and consent to participate

The study protocol was approved by the institutional review board of Saitama Medical University International Medical Center (Saitama, Japan), and written informed consent was obtained from all patients.

Patient consent for publication

The present study obtained written informed consent for publication from the all patients.

Competing interests

The authors declare that they have no competing interests.

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