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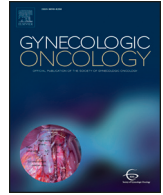
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Converging endometrial and ovarian tumorigenesis in Lynch syndrome: Shared origin of synchronous carcinomas

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HIGHLIGHTS

- Synchronous gynecological carcinomas from Lynch syndrome are molecularly concordant, suggesting shared origins.
- Complex hyperplasias without or with atypia molecularly resemble endometrial and ovarian carcinomas from the same patients.
- Joint involvement of endometrium and ovaries needs to be taken into account in clinical management of Lynch syndrome.

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ABSTRACT

Objective. The diagnosis of carcinoma in both the uterus and the ovary simultaneously is not uncommon and raises the question of synchronous primaries vs. metastatic disease. Targeted sequencing of sporadic synchronous endometrial and ovarian carcinomas has shown that such tumors are clonally related and thus represent metastatic disease from one site to the other. Our purpose was to investigate whether or not the same applies to Lynch syndrome (LS), in which synchronous cancers of the gynecological tract are twice as frequent as in sporadic cases, reflecting inherited defects in DNA mismatch repair (MMR).

Methods. MMR gene mutation carriers with endometrial or ovarian carcinoma or endometrial hyperplasia were identified from a nationwide registry. Endometrial ($n = 35$) and ovarian carcinomas ($n = 23$), including 13 synchronous carcinoma pairs, were collected as well as endometrial hyperplasias ($n = 56$) and normal endometria ($n = 99$) from a surveillance program over two decades. All samples were studied for MMR status, ARID1A and L1CAM protein expression and tumor suppressor gene promoter methylation, and synchronous carcinomas additionally for somatic mutation profiles of 578 cancer-relevant genes.

Results. Synchronous carcinomas were molecularly concordant in all cases. Prior or concurrent complex (but not simple) endometrial hyperplasias showed a high degree of concordance with endometrial or ovarian carcinoma as the endpoint lesion.

Conclusions. Our investigation suggests shared origins for synchronous endometrial and ovarian carcinomas in LS, in analogy to sporadic cases. The similar degrees of concordance between complex hyperplasias and endometrial vs. ovarian carcinoma highlight converging pathways for endometrial and ovarian tumorigenesis overall.

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1. Introduction

Endometrial and ovarian carcinomas are among the most common female cancers in the Western world. In the United States, >60,000 and 20,000 new cases, respectively, are expected to be diagnosed in 2018 [1]. Among gynecologic cancers, endometrial cancer is the most

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prevalent, whereas ovarian cancer is the leading cause of death. Endometrial and ovarian cancer may occur as part of Lynch syndrome (LS), in which inherited defects in DNA mismatch repair (MMR) underlie autosomal dominantly inherited predisposition to cancers of multiple organs [2]. While colorectal cancer is the most common cancer in LS overall, the incidence of endometrial cancer equals to or even exceeds that of colorectal cancer in female carriers of MMR gene mutations [3,4]. Up to 54% and 24% of female mutation carriers develop endometrial and ovarian cancer, respectively, at some point of their lives [3,4]. On the population level, 9% of endometrial cancer cases under 50 years of age [5] and 2% of ovarian cancer cases unselected for age [6] have been estimated to be due to germline mutations in MMR genes. Endometrial cancer in LS is of endometrioid histology in ~90% of cases and associated with earlier age at diagnosis (mean 50 vs. 68 years) and a higher prevalence of lower uterine segment involvement compared to sporadic cases [7,8]. Ovarian cancer in LS is likewise diagnosed at a younger age (mean 45 years, which is 15–20 years earlier than in sporadic cases), and 77% of epithelial ovarian carcinomas in LS are non-serous [9] in a marked contrast with the average population where the high-grade serous type predominates [10].

In 10% of sporadic cases [11] and 20% of LS cases [7,12], carcinomas are diagnosed in both the uterus and the ovary simultaneously, raising the question of tumor origins: do the two cancers arise independently or one as a metastasis of the other? In the sporadic setting, two recent studies addressed this question by targeted sequencing, and shared profiles of somatic mutations suggested that synchronous tumors represented metastatic disease from one site to the other [13,14]. However, synchronous endometrial and ovarian carcinomas from an additional LS case lacked somatic mutations in common, implying that LS might constitute an exception to the general rule [14]. Epidemiological observations suggest that the developmental pathways to endometrial and ovarian carcinoma may cross far prior to malignant transformation. Up to 42% of women in whom endometrial sampling reveals atypical endometrial hyperplasia are found to have simultaneous endometrial cancer in hysterectomy specimens [15] consistent with the idea that endometrioid endometrial carcinoma evolves via endometrial hyperplasia [16]. Interestingly, some 50% of patients with endometrioid ovarian carcinoma, too, display concurrent atypical endometrial hyperplasia [17], the significance of which remains to be clarified: does endometrial hyperplasia represent an early step of synchronous endometrial tumorigenesis or have relevance for ovarian cancer development as well, given that endometrial epithelial cells are considered to be the origins of endometrioid and clear cell carcinomas of the ovary [18]?

We took advantage of synchronous cancers arising in LS individuals and consecutive endometrial biopsy specimens from lifelong surveillance of MMR gene mutation carriers to examine the relationship between endometrial and ovarian tumorigenesis. Our results define the developmental routes of endometrial and ovarian cancer and are clinically relevant.

2. Materials and methods

2.1. Patients and samples

The nation-wide Hereditary Colorectal Cancer Registry of Finland was used as a source to identify LS individuals with endometrial or ovarian carcinoma or endometrial hyperplasia. Tumor and preceding surveillance specimens were available from 66 mutation carriers (*MLH1* 52, *MSH2* 10, and *MSH6* 4), including a total number of 213 samples (Supplementary Table S1). Endometrial hyperplasia specimens were classified into four categories (simple hyperplasia, SH; simple atypical hyperplasia, SAH; complex hyperplasia without atypia, CH; and complex hyperplasia with atypia, CAH) in accordance with the WHO1994/2003 classification, since it was the original schema used in sample diagnostics [19,20]. A category including SAH was omitted because only one SAH sample was identified.

A gynecological pathologist had originally determined the histology of specimens and the diagnosis was verified after sample collection by a gynecological pathologist (R.B.). Hematoxylin and eosin was used to stain formalin-fixed paraffin-embedded (FFPE) tissue sections for visual inspection and tumor sections containing >60% of tumor cells were chosen for DNA extraction performed by a customized protocol [21]. Manual microdissection was used to carefully separate normal, hyperplasia and tumor samples. The study was approved by the Institutional Review Boards of the Departments of Surgery (466/E6/01) and the Obstetrics and Gynecology (040/95) of the Helsinki University Central Hospital (Helsinki, Finland) and Jyväskylä Central Hospital (Jyväskylä, Finland) (Dnro 5/2007). The National Supervisory Authority for Welfare and Health (Valvira/Dnro 10741/06.01.03.01/2015) approved the collection of archival samples.

2.2. Immunohistochemistry (IHC) for L1CAM and ARID1A

PT-Module (Lab Vision, CA, USA) was obtained to perform antigen retrieval on 4 μ m deparaffinized tissue slides at 98°C/20 min in Envision TM Flex Target Retrieval solution pH 9 for L1CAM and pH 6.1 for ARID1A (Agilent Technologies, USA). The antibodies used were Covance SIG-39110-200 produced in mouse for L1CAM (1:40/20 min, CD171, clone 1E11, Covance) and anti-ARID1A antibody produced in rabbit (1:200/20 min, HPA005456, polyclonal, Lot D104841, Sigma-Aldrich, USA). Slides were stained with Autostainer 480 automated immunostainer (Lab Vision, CA, USA) and hematoxylin (Mayers HTX, Histolab) was used to counterstain tissue sections. Protein expression was evaluated and scored from stained slides by two pathologists (R.B. and A.P.). Membranous L1CAM staining of cells was scored as positive/abnormal when >10% of tumor cells expressed L1CAM. ARID1A expression was scored as negative/abnormal when all tumor cell nuclei stained negative but positive expression was preserved in stromal cells.

2.3. Mismatch repair (MMR) status

Sample DNA was investigated by polymerase chain reaction (PCR) using fluorescently labeled mononucleotide repeat markers BAT25 and BAT26. If both markers were stable, the interpretation was microsatellite stability (MSS), whereas one or two unstable markers indicated microsatellite-instability (MSI) [22]. Immunohistochemistry (IHC) was performed to investigate MMR protein expression as described [23]. MMR was regarded deficient by the presence of MSI, absence of MMR protein, or both.

2.4. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

Samples were investigated using methylation-specific (MS)-MLPA SALSA MLPA ME001-C2 test (MRC-Holland, Amsterdam, The Netherlands) as described [23] to analyze methylation patterns of 24 general tumor suppressor genes (TSGs) (listed at <http://www.mrc-holland.com>) often methylated in several cancers. In addition, custom designed MS-MLPA probe mix including 7 gene probes supplemented with Salsa MLPA kit P-300-B1 human DNA reference-2 reagents was used to analyze methylation alterations in genes often methylated specifically in endometrial and ovarian cancer as described [24]. The test produces a methylation dosage ratio (Dm), which varies between 0 and 1.0 and reflects the percentage of methylated DNA. The Dm value was calculated individually for each sample as previously described [25]. The Dm value of 0.15 or above was set as the technical threshold for indication of hypermethylation for all genes included in the 24 TSG MS-MLPA test [25], except for *CDKN2B*. The hypermethylation thresholds for each of the seven endometrial and ovarian carcinoma-related genes included in the custom MS-MLPA test and for *CDKN2B* included in the commercial test were determined using LS normal endometrial

samples and calculated as the mean Dm in normal endometria plus 1 standard deviation.

2.5. Targeted sequencing for somatic mutations

Tumors and corresponding normal DNA samples were sequenced at the Institute for Molecular Medicine Finland (FIMM; Helsinki, Finland). Sequencing was performed on Illumina HiSeq 2500 platform (San Diego, CA) using the Nimblegen Comprehensive Cancer Panel (Roche Diagnostics), a 4 Mb design with 578 cancer-related genes, as described [26]. In brief, libraries were prepared using ThruPLEX® DNA-seq Kit, and the exons captured according to the manufacturer's protocol (Rubicon Genomics). The mean target coverage for tumors was 106-fold (Supplementary Table S2). The variant calling pipeline is described in Sulonen et al. [27]. VarScan 2 mutation detection algorithm version 2.3.2 was used identification of the non-synonymous somatic mutations from the paired normal and tumor data [28] as described [26]. Variants with VarScan somatic *p*-value below 0.01 were selected for subsequent analyses.

VarSeq (GoldenHelix®) was used to conduct in silico evaluation of somatic single nucleotide variants. The algorithms used to predict the effect of amino acid substitution on protein function were SIFT, PolyPhen-2, MutationTaster, MutationAssessor, FATHMM, and FATHMM MKL Coding. Mutation ID in COSMIC was provided if the mutation was present in the Catalogue of somatic mutations in cancer (COSMIC v71, GRCh 37; <http://grch37-cancer.sanger.ac.uk/cosmic>).

2.6. Statistical analyses

SPSS software version 22.0 (IBM® SPSS® Statistics, Inc. Chicago, IL, USA) was used for statistical evaluations. Frequency data was analyzed by Fisher's exact test. Shapiro-Wilk test was performed to test normality of data. Comparisons between two groups including numbers of methylated genes or Dm values were evaluated by the Student's *t*-test (for normally distributed samples) or nonparametric Mann-Whitney *U* test (for not normally distributed samples). *p* values < 0.05 (2-tailed) were considered significant.

3. Results

3.1. Study rationale and frequencies of molecular alterations

Prompted by the discordant preliminary observations between sporadic and LS-associated synchronous carcinomas [13,14], we undertook this study to explore the relationship between endometrial and ovarian tumorigenesis in LS. Our investigation is based on 213 specimens from 66 carriers of MMR gene mutations and includes carcinomas of the endometrium (endometrioid) and ovary (endometrioid and clear cell), as well as consecutive specimens of non- and premalignant endometrial tissues from a surveillance program operative since 1996 [29] (Supplementary Table S1). We recently used this series to investigate the frequencies of MMR, ARID1A, and TSG methylation alterations against

the progressive histological abnormality of endometrial specimens from LS and sporadic cases [30]. We now focus on synchronous carcinomas (13 pairs from equally many individuals) and endometrial hyperplasia – endometrial/ovarian carcinoma combinations (35 pairs from 22 mutation carriers) to explore their clonal relatedness. LS synchronous carcinomas are additionally compared by targeted sequencing. All LS specimens are investigated for the protein expression of L1CAM, an adhesion molecule connected to invasion and metastatic potential [31–33], to supplement our previous set of markers [30] monitoring early alterations.

Table 1 shows the frequencies of molecular changes detected in the LS sample series (this study and [30]). Compared to the very high frequencies (up to 100%) for MMR defects and ARID1A expression loss, L1CAM aberrations were less prominent. The highest frequencies were seen in ovarian clear cell carcinomas (OvCC), of which 43% (3/7) displayed L1CAM overexpression. Representative examples of immunohistochemical staining results of L1CAM are given in Supplementary Fig. S1.

3.2. Pairwise evaluation of synchronous gynecological carcinomas for concordance

Our LS series included 13 pairs of synchronous carcinomas (9 endometrial plus ovarian carcinoma pairs, 3 cases with bilateral ovarian carcinomas, and one pair with endometrial and endocervical carcinoma). Fig. 1 depicts case by case the molecular alterations discovered in the tumors. To systematically compare the paired tumors for concordance, evaluation criteria were developed taking 5 parameters (MMR status, ARID1A protein expression, L1CAM protein expression, hypermethylation status of 7 endometrial and ovarian cancer-related TSGs and hypermethylation status of 24 general TSGs) into account (please see footnote of Fig. 1). A pair was regarded concordant if at least 3 of 5 (or at least 50%) of parameters were concordant. By these criteria, all synchronous cases were deemed concordant. Synchronous tumors invariably shared the same MMR and ARID1A expression status, and TSG hypermethylation patterns also exhibited high intra-pair concordance (Fig. 1). Our findings thus suggested that the synchronous LS cancers had shared origins (i.e., in each pair, one tumor was likely to be a metastasis of the other).

To further explore the relationship between those synchronous carcinomas that affected different organs (the ovary and the endometrium), the 9 synchronous ovarian and endometrial carcinomas were statistically compared as groups relative to each other and to non-synchronous ovarian and endometrial carcinomas (Table 2). As molecular parameters, the frequencies of MMR, ARID1A, and L1CAM alterations and the numbers of hypermethylated TSGs (representing two gene panels), and the methylation dosage ratios (Dm values) for 6 genes most commonly methylated in our series (*RSK4*, *SPARC*, *HOXA9*, *HOXA10*, *RASSF1* and *CDH13*) were considered. No significant differences between the synchronous tumors were found (Fisher's exact test was used for frequencies and paired *t*-test for Dm values). Some significant differences were observed in synchronous vs. non-synchronous

Table 1
Molecular alterations in endometrial and ovarian carcinomas and in non- and pre-malignant endometrial specimens.

	Endometrial endometrioid carcinoma (EnCa)	Ovarian clear cell carcinoma (OvCC)	Ovarian endometrioid carcinoma (OvE)	Normal endometrium	Simple hyperplasia (SH)	Complex hyperplasia without atypia (CH)	Complex atypical hyperplasia (CAH)
MMR-deficient	30/31 (97%)	9/9 (100%)	14/14 (100%)	12/99 (12%)	5/12 (42%)	5/6 (83%)	33/38 (87%)
Loss of ARID1A expression	14/23 (61%)	9/9 (100%)	12/14 (86%)	0/22 (0%)	0/6 (0%)	1/4 (25%)	6/30 (20%)
Overexpression of L1CAM	3/22 (14%)	3/7 (43%)	2/13 (15%)	N/A	0/4 (0%)	1/4 (25%)	1/28 (4%)
Average number of methylated endometrial and ovarian cancer related genes out of total 7	2.3	3.7	3.2	0.70	1.2	2.3	2.0
Average number of methylated tumor suppressor genes out of total 24	3.7	3.8	3.4	2.4	2.3	3.0	3.8

Abbreviations: N/A = not applicable. Proportions are based on cases that could be successfully analyzed.

Table 3
Summary of somatic mutations shared by synchronous Lynch carcinomas, based on deep sequencing of 578 cancer-relevant genes.

Case ID	Tumor 1	Tumor 2	Shared	Shared (relaxed criteria) ^a	Examples of shared somatic mutations predicted damaging ^b
LOC3	2915 (OvCC)	72 (EnCa)	2	N/A	TGFB2 fs, JAK1 fs
LOC16	23 (OvE)	132 (EnCa)	0	4	ARID2 R1679Q, MAP2K2 E328K, SMARCB1 R350W
LOC18	38 (OvE)	1544 (EnCa)	10	N/A	PTEN K332*, PTEN fs
LOC13	65 (OvE)	78 (OvE)	41	N/A	PIK3CA R88Q, PTEN fs, ARID1A fs (two different)
LOC6	37 (OvCC)	41 (OvEB)	0	1	CTNNB1 R565H

Abbreviations: fs, frameshift; N/A, not applicable.

^a Somatic VarScan $p < 0.05$ in both tumors (indicated for cases without any shared mutations with $p < 0.01$).

^b A complete list of mutations is given in Supplementary Table S3.

the degree of molecular sharing between endometrial and ovarian tumorigenesis may be higher than appreciated before.

4. Discussion

The epidemiology of endometrial and ovarian cancer is intertwined, and several possible mechanisms, including hormonal and inflammation and immune system-related, may underlie this phenomenon [34]. LS provides an efficient tool to investigate the pathogenesis of endometrial and ovarian carcinoma for the following reasons: (i) the lifetime risks of these cancers are significantly elevated in LS compared to the average population [3,4], (ii) multiple lesions (both malignant and benign) in the same individuals are common [7,12], and (iii) lifelong surveillance against gynecological cancer results in consecutive specimens that are invaluable for research [35].

In LS, the clinically important issue of independent primary tumors vs. metastatic disease in the case of synchronous endometrial and ovarian cancers is unsettled, so far. We evaluated 13 synchronous carcinomas (9 endometrial plus ovarian carcinoma pairs, 3 cases with

bilateral ovarian carcinomas, and one pair with endometrial and endocervical carcinoma) (Fig. 1) and our results indicate shared origins. Frequent L1CAM overexpression among the synchronous tumors was in agreement with the interpretation of metastatic disease. The available data are thus consistent with metastatic disease in synchronous cases from sporadic [14] and LS cases (this study); however, the direction of metastasis is unknown. Kelemen et al. [11] explored the patterns of molecular alterations (PTEN and MMR protein expression) in three groups of sporadic tumors: ovarian carcinomas synchronous with endometrial carcinoma, non-synchronous endometrial carcinomas, and non-synchronous ovarian carcinomas. They found that ovarian carcinomas synchronous with endometrial carcinoma showed a greater similarity relative to non-synchronous endometrial carcinoma than non-synchronous endometrioid ovarian carcinoma, possibly suggesting a metastatic spread from the endometrium to the ovary. In our LS series, synchronous ovarian carcinoma (or endometrial carcinoma) showed the closest molecular similarity to its synchronous pair, and a closer similarity to non-synchronous cancer of the same rather than the different organ (Table 2). Molecular similarity between tumors within each

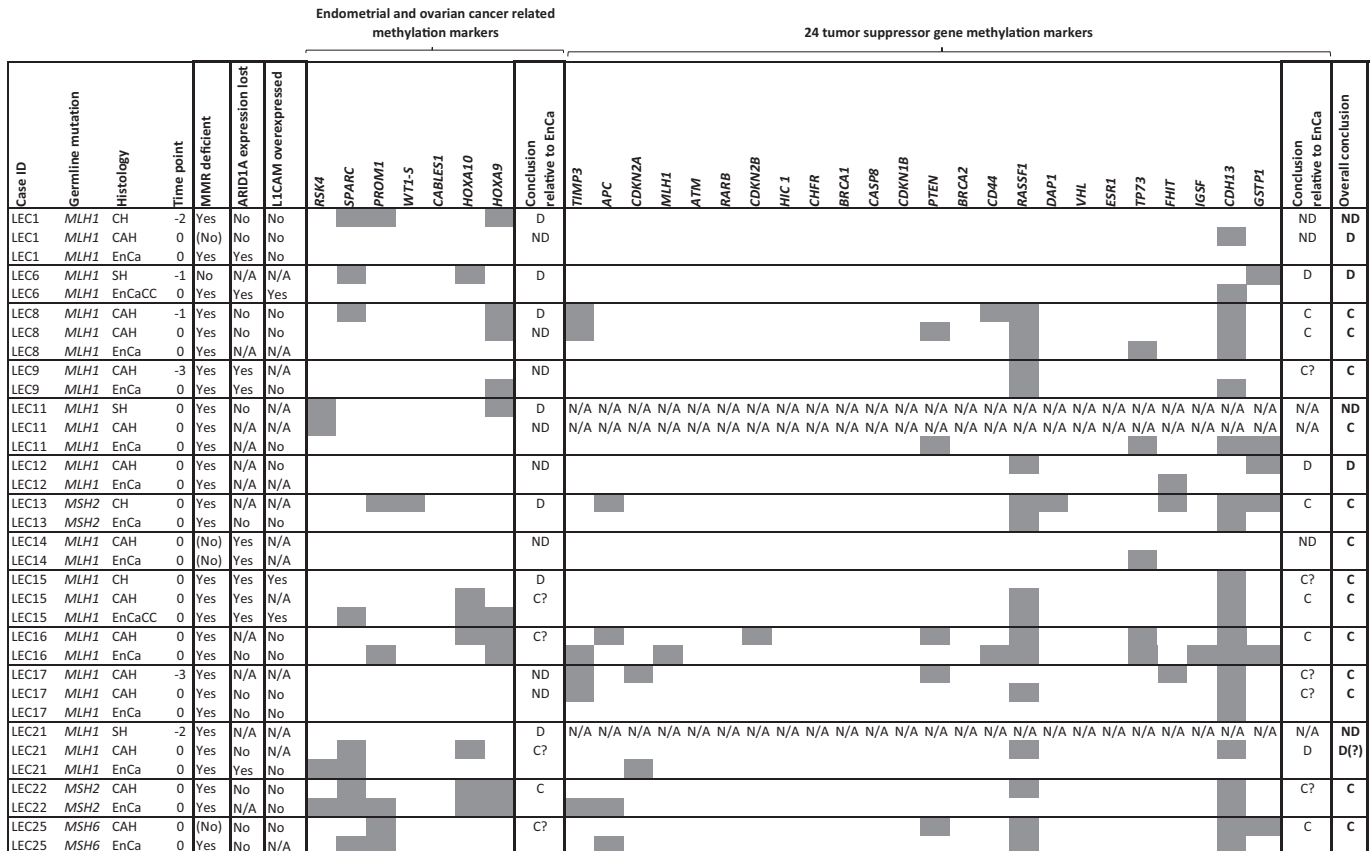


Fig. 2. Molecular characteristics of endometrial hyperplasias occurring prior to or concurrently with endometrial carcinoma from Lynch syndrome patients.

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