Synchronous endometrioid endometrial and ovarian carcinomas are biologically related: A clinico-pathological and molecular (next generation sequencing) study of 22 cases

NIKOLA HÁJKOVÁ[1], IVANA TICHÁ[1], JAN HOJNÝ[1], KRISTÝNA NĚMEJCOVÁ[1],
MICHAELA BÁRTŮ[1], ROMANA MICHÁLKOVÁ[1], MICHAL ZIKÁN[2], DAVID CIBULA[3],
JAN LACO[4], TOMÁŠ GERYK[4], GÁBOR MÉHES[5] and PAVEL DUNDR[1]

[1]Institute of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, 12800 Prague;
[2]Department of Obstetrics and Gynecology, Bulovka Hospital, First Faculty of Medicine, Charles University, 18081 Prague;
[3]Department of Obstetrics and Gynecology, Oncogynecological Centre, First Faculty of Medicine, Charles University and
General University Hospital in Prague, 12000 Prague; [4]The Fingerland Department of Pathology,
Charles University Faculty of Medicine and University Hospital Hradec Kralove, 50005 Hradec Kralove,
Czech Republic; [5]Department of Pathology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Abstract. The criteria for distinction between independent primary tumors and metastasis from one site to the other in synchronous endometrioid endometrial and ovarian carcinoma (SEO) has been a matter of dispute for a long time. In our study we performed a comprehensive clinico-pathological and molecular analysis of 22 cases of SEO. Based on conventional clinico-pathological criteria the cases were classified as independent primary tumors (10 cases) and metastasis from one location to the other (12 cases). All tumors were analyzed by NGS with a panel of 73 genes (219 kbp). Clonal origin was confirmed in all cases by at least one shared mutation in *PTEN*, *AKT1*, *PIK3CA*, *KRAS*, *TP53* and *ARID1A*. Two patients carried germline pathogenic mutation in cancer-predisposing genes *BRCA1* or *BARD1*. Microsatellite instable phenotype was detected in 5/22 (22.7%) SEO, but in one case only in the endometrial tumor. In conclusion, our results showed that all 22 SEOs were clonally related, irrespectively of their clinico-pathological features. Even low grade and low stage tumors classified as independent primaries, according to the conventional morphological criteria, have a clonal origin. From the practical point of view, only the conventional morphological criteria should be used for the classification (staging) of these tumors. However, molecular profiling of these tumors may have prognostic and predictive meaning.

Correspondence to: Dr Ivana Tichá or Professor Pavel Dundr, Institute of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Studnickova 2, 12800 Prague, Czech Republic
E-mail: ivana.ticha@vfn.cz
E-mail: pavel.dundr@vfn.cz

Introduction

Synchronous endometrial and ovarian carcinomas occur in approximately 5% of endometrial carcinomas and 10-20% of ovarian carcinomas, respectively (1,2). The distinction between independent primary tumors and metastasis from one site to the other (endometrium to the ovary or ovary to the endometrium) can be complicated but it is clinically significant. Historically, the criteria for this distinction evolved and were based mostly on morphological features (3,4). However, a great benefit has been expected from ancillary methods (especially molecular analyses) which are now becoming, together with methodological development, more complex and also more available. Surprisingly, the results of recent molecular studies have shown that most SEOs share clonal origin irrespectively of their clinico-pathological features (5-7). The benefit of molecular analysis regarding differential diagnosis between independent primary tumors and metastatic disease is, from this point of view, disputable. Nevertheless, in one recent study the authors suggested that molecular profiling might be beneficial in this setting (8). However, this suggestion was based on molecular profiling of one SEO only. In our study we focused on a comprehensive clinico-pathological and molecular study of 22 cases of SEO.

Materials and methods

Patients and materials. Archive files of the: i) Institute of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic; ii) The Fingerland Department of Pathology, Charles University Faculty of Medicine and University Hospital Hradec Králové, Czech Republic; and iii) Department of Pathology, University of Debrecen, Hungary, were searched for synchronous cases of endometrioid endometrial and ovarian carcinomas. Twenty-two SEOs were found with corresponding formalin-fixed paraffin-embedded tissue (FFPE) blocks from both endometrial and ovarian tumors. One patient with SEO also had synchronous CCCO arising in the second ovary. Another patient had endometrioid carcinoma of the left ovary and after 49 months she developed SEO (of the endometrium and right ovary). Histological type, staging and grading of EEC and EOC were assessed for each tumor separately according to the standard criteria (9). A review of the hematoxylin and eosin stained slides was performed in all cases, and the area of tumor tissue for macrodissection was marked (percentage of tumor cells in selected area ranges between 30 and 90%).

DNA from FFPE blocks was isolated using standard procedures implementing QIAamp DNA Tissue kit (Qiagen, Hilden, Germany) or cobas® DNA Sample Preparation kit (Roche, Basel, Switzerland), respectively.

Sequencing analysis. The whole project and all auxiliary files are designed for genome build GRCh37 (hg 19) coordinates. Samples for sequence capture NGS (massive parallel sequencing) were prepared using the KAPA HyperPlus kit. Target sequences were enriched using commercial hybridization probes (Nimblegen, Roche) designed for human DNA regions of our interest (73 genes or gene parts; 219 kbp). The panel included the following genes: *AKT1, AKT3, ARID1A, ARID2, ATM, BAP1, BARD1, BIRC5* (promoter), *BRAF* (exon 11,15), *BRCA1, BRCA2, BRIP1, CCND2, CCND3, CDH1, CDK4, CDKN2A, CYP19A1, ERBB2, ERCC3, ESR1, ESR2, F11R, FOXL2, GNA11, GNAQ, HNF1B, HRAS, IDH1, JAM2, JAM3, KDR, KIT, KRAS, MAP2K1, MAP2K2, MAPK1, MAPK3, MDM2, MET, MITF, MLH1, MLH3, MSH2, MSH6, MYC, NBN, NRAS, PALB2, PARD3, PDGFRA* (exon 12,14,18), *PIK3CA, POLE, POT1, PPM1D, PPP6C, PTEN, RAD51C, RAD51D, RB1, SF3B1, SMARCA4, SMARCB1, SNAI1, SNAI2, SNAI3, TERT* (promoter), *TJP1, TP53, TWIST1, TWIST2, ZEB1, ZEB2*. The library was pair-end sequenced by MiSeq instrument (Illumina, Inc., San Diego, CA, USA).

Selected germline variants and selected variants with a frequency higher than 10% were confirmed by direct Sanger sequencing using BigDye v3.1 and ABI3500 analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The processing of raw sequencing data was performed to analyze the spectrum of genetic variants, such as single nucleotide variants and short insertions or deletions using NextGENe software (SoftGenetics LLC, State College, PA, USA) according to the standardized biostatistical methods for NGS data. Primary raw data were trimmed and demultiplexed by MiSeq system during post-sequencing process. Output in .fastq format was complexly analyzed by NextGENe software (SoftGenetics LLC). The PCR duplicate reads were removed by Sequence Operation Tool (default settings), then .fastq files were converted by using Format Conversion Tool to .fasta format. During conversion, reads with low quality were removed (Settings: Median score threshold ≥25; Max # of uncalled bases ≤2; Called base number of each read ≥40; Trim or reject read when ≥3 base(s) with score ≤2). After format conversion, reads were mapped on genome by Project Wizzard Tool (Settings: Instrument type-Illumina; Application type-SNP/Indel discovery; Steps-Sequence Alignment; Reference file-Human_GRCh_v37p10_dbsnp135; Allowable mismatched bases-0; Allowable ambiguous alignments-10; Seeds: 21 bases, move step-1 base; Allowable alignments-80; Overall matching base percentage ≥95%). Results of NextGENe software analysis (mutation report, expression report, coverage report, CNV analysis report) were filtered for the region of interest and mutation report additionally for the frequency of mutation allele >5%.

A variant comparison tool (NextGENe software; SoftGenetics, LLC) was used for the evaluation of shared/different mutations between the tumors of respective patients. Nonsynonymous variants in exons and adjacent intronic regions with frequency ≥10% in at least one tumor were evaluated and manually controlled using an IGV viewer (Broad Institute). Mutations detected with coverage under 100x, which is caused by a limitation of the DNA quality from FFPE tissue, were manually controlled using an IGV viewer in both endometrial and ovarian tumors. These mutations are considered as true variants (not artefacts) if: i) The mutation was detected in both tumors of respective patient (in case of shared mutation); ii) reads with mutation excluded duplicates; and iii) variant was detected in both strands with different orientation of paired-end reads.

False calls of detected mutation in *PIK3CA* evaluated by NextGENe: p.(R524K), p.(Y644H), p.(E707K) were filtered out. The presence of a *PIK3CA* pseudogene on chromosome 22, with >95% sequence homology interferes with the detection of these variants (10).

The single nucleotide polymorphisms (SNPs; mutation allele frequency-MAF-above 0.01, i.e., above 1% in population) were filtered out according to the data from the SNP databases (ExAC, 1,000 g, ESP6500) which are part of the mutation report generated by the NextGENe® Software. In order to assess the impact of the detected missense variants, several widely used in silico prediction programs or databases imported in NexteGENe Software were employed, comprising: ClinVar database, COSMIC database, and dbNSFP database (MetaSVM, MetaLR, RS_DBSNP141, SIFT, Polyphen2_HDIV, Polyphen2_HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN GERP++, phyloP46, SiPhy). Clinical significance and ensemble prediction scores were included in the NextGENe mutation reports.

Germline variants possibly associated with cancer predisposing syndromes were selected according to the following rules: i) VAF >40% in both tumors; ii) variants are not described as SNPs; and iii) clinical significance of the variant was assessed to be pathogenic and/or of uncertain significance according to the ClinVar database. These variants were confirmed as germline mutations by Sanger sequencing of DNA isolated from non-tumor tissue.
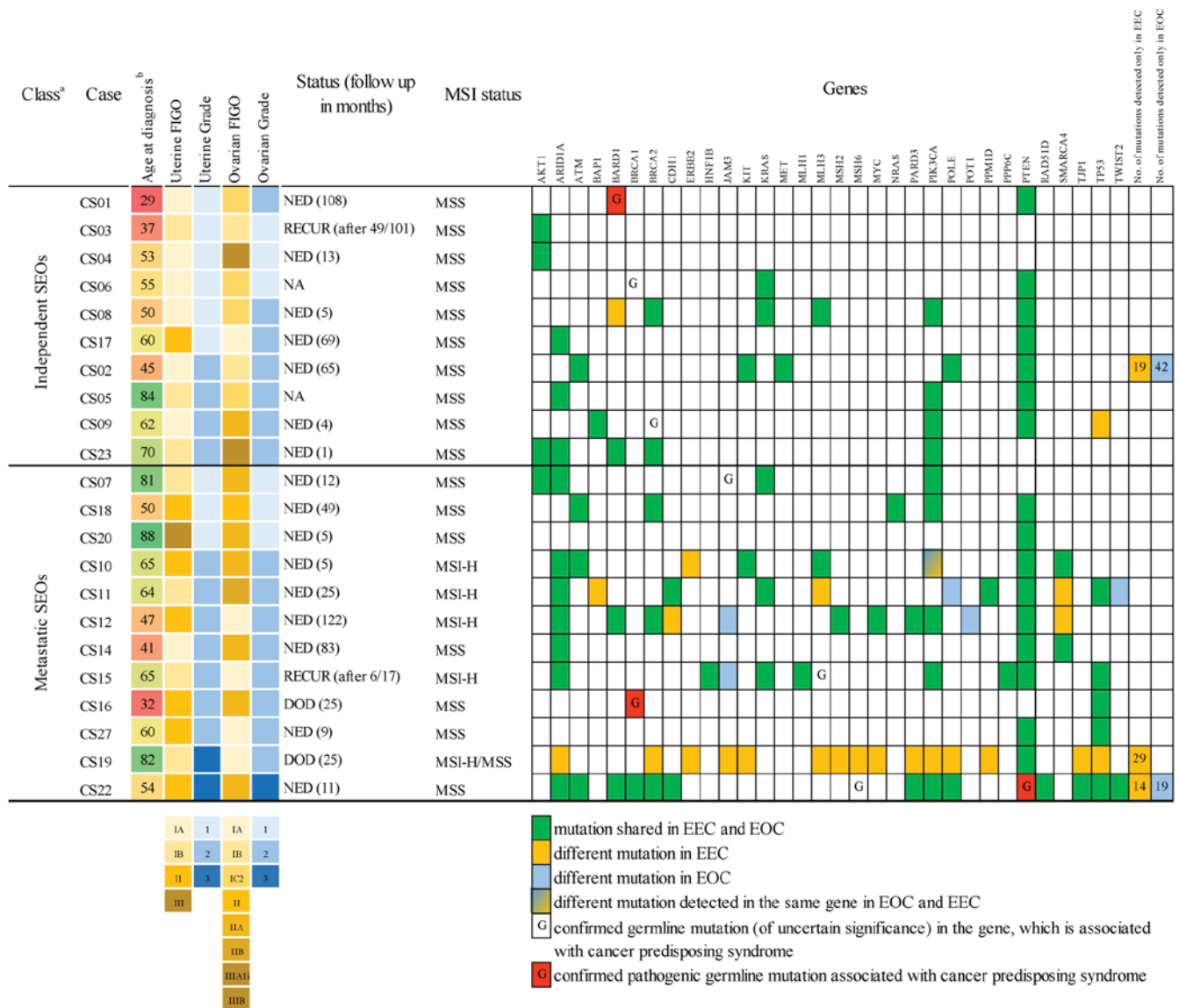
Figure 1. Spectrum of detected mutations. Tumor CS11 spreads to the peritoneum, CS18 spreads to the peritoneum, cervix and fallopian tube and CS22 spreads to the peritoneum and cervix. Patient CS12 was diagnosed with colorectal carcinoma 15 months after primary EEC/EOC diagnosis, and patient CS15 was diagnosed with EEC/EOC 41 months after a previous follicular lymphoma diagnosis. [a]Class according to the examination by pathologist using current criteria. Independent SEOs are tumors classified as independent synchronous endometrial and ovarian carcinoma. Metastatic SEOs are tumors classified as metastasis from one location to the other. [b]Colored scale from red to green indicates the age of patients at diagnosis. CS, case specific; NED, no evidence of disease; RECUR, the recurrent disease was found during follow-up; DOD, died of disease; NA, not available; MSS, microsatellite stable in EEC/EOC; MSI-H, microsatellite instable in EEC/EOC; MSI-H/MSS, case CS19 MSI-H in EEC and MSS in EOC; EEC, endometrioid endometrial carcinoma; EOC, endometrioid ovarian carcinoma; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; SEO, synchronous endometrial and ovarian carcinoma; MSI, microsatellite instability.

Analysis of microsatellite instability. Analysis of MSI was performed by fragmentation analysis on ABI 3500 (Thermo Fisher Scientific, Inc.) with the set of five quasimonomorphic mononucleotide microsatellite markers BAT-26, BAT-25, NR-21, NR-22 and NR-24. The phenotype MSI-high (MSI-H) was defined as the presence of at least two instable loci and MSI-low as the presence of one instable locus, respectively. Microsatellite stable (MSS) tumors showed no instability.

Results

Clinico-pathological findings. The characteristics of the patients and tumors are summarized in Fig. 1. From 22 cases, ten SEO cases were classified based on conventional

morphological criteria as independent tumors, and twelve cases were considered as single primary tumors with metastasis to the other location (ovary or endometrium). In three cases classified as metastasis from one location to the other peritoneal involvement was also found, and in one of these cases there was Fallopian tube involvement. A lymphadenectomy procedure was performed in 11 patients, and only in one patient there was a metastasis found in one iliac lymph node.

The average age of diagnosis was 58 years (range, 29-88 years). The average follow-up for all patients with available data (20 from 22 patients) was 37 months (range, 1-122 months). Only 1 of the 10 (10%) patients (CS03) classified as independent SEO developed recurrent disease. This patient had a well-differentiated endometrioid carcinoma
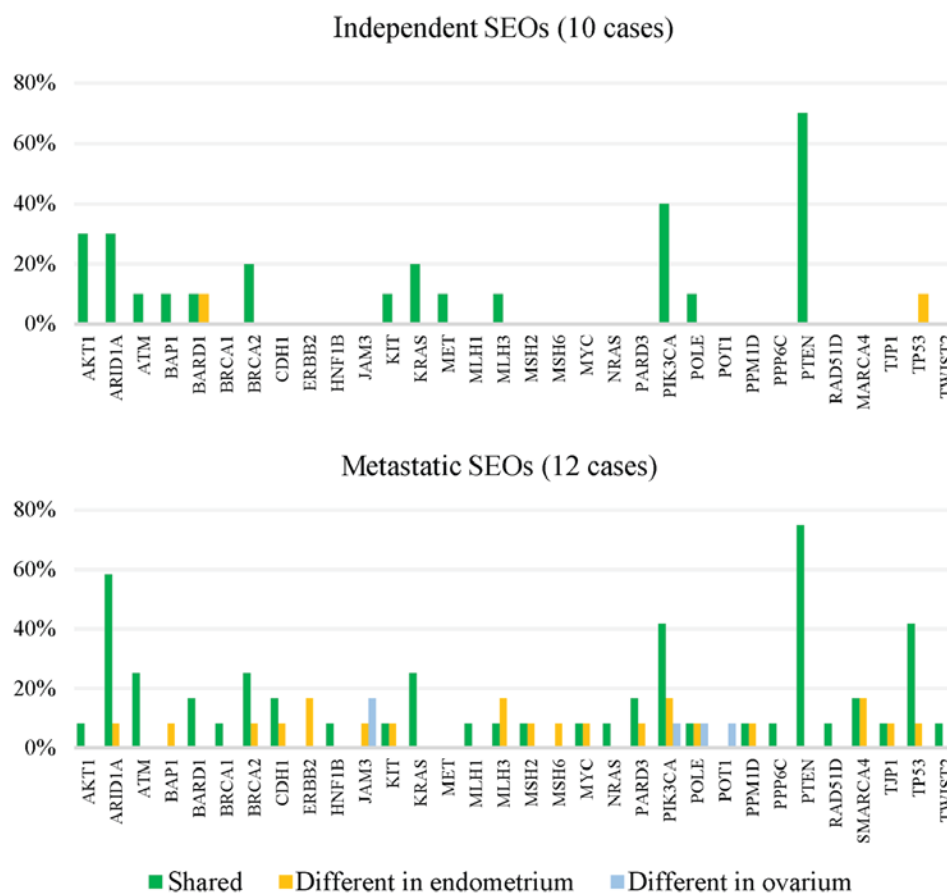
Figure 2. Frequency of targeted gene mutations in metastatic and independent SEOs classified according to the current pathological criteria. SEO, synchronous endometrial and ovarian carcinoma.

of the left ovary treated by adnexectomy with subsequent pelvic and para-aortic lymphadenectomy, omentectomy and appendectomy. The SEO was found after 49 months in the form of a well-differentiated endometrioid carcinoma in the contralateral (right) ovary and in the endometrium, and it was treated by a combined radical hysterectomy and right adnexectomy with subsequent chemotherapy [6 cycles of paclitaxel (PTX) and carboplatin (CBDCA)]. The patient is without any signs of disease 52 months after surgery. No patient from this group died of the disease. On the contrary, 2/12 patients with SEO classified as metastasis from one location to the other died of the disease. Another patient from this group developed metastatic disease 6 months after the diagnosis, the metastatic lesion was located in the abdominal wall and treated by a surgical excision. This patient was without any signs of the disease 11 months after the excision and then was lost to follow-up.

Molecular findings. The detected mutations are summarized in Fig. 1. Comparison of molecular profiles of independent and metastatic SEOs are shown in Fig. 2.

All of the SEOs (100%, 22/22) showed to be clonally related according to the presence of shared mutations (at least one). In 77.3% cases (17/22) a shared mutation in *PTEN* was detected and out of those, eight SEOs shared additional *ARID1A* mutation. Two SEOs (9%, 2/22) carried mutations in *ARID1A*, *AKT1* and *PIK3CA* while *PTEN* was a wild-type.

Another two cases carried only *AKT1* mutation, which is a known pathogenic hot-spot p.(E17K) variant. Interestingly, one microsatellite instable SEO (CS15, both tumors of grade 2) shared pathogenic somatic mutation in *HNF1B* NM_000458.2: c.1561_1562insC, p.(Q521Pfs*30), both tumors carried mutations in *MLH3*, *MLH1* and also further mutations in *PTEN* NM_000314.4: c.955_958del, p.(T319X), *ARID1A* NM_006015.4: c.5981delT, p.(V1994Gfs*21) and c.3667C>T, p.(R1223C), *KRAS* NM_004985.3: c.35G>A, p.(G12D) and *TP53* NM_001126112.2: c.960_961del, p.(K321Tfs*15).

Two SEOs (CS02 and CS22) shared *POLE* mutation causing hypermutator phenotype (24 in EEC and 42 in EOC, 27 in EEC and 32 in EOC nonsynonymous mutations, respectively). As the evidence suggests, POLE hypermutator phenotype defines a unique subclass of endometrioid tumors. Moreover, in one patient (54-year-old patient-CS22) a germline *PTEN* mutation NM_000314.4: c.389G>A, rs121909229, p.(R130Q) was detected. Germline pathogenic *PTEN* mutations are usually associated with Cowden syndrome (PTEN hamartoma tumor syndrome). The association of the detected germline *PTEN* mutation with carcinogenesis in this particular patient is however questionable, due to the relatively high patient's age at the time of diagnosis and *POLE* hypermutator phenotype. In patient CS19, *POLE* mutation (29 nonsynonymous mutations detected in endometrial tumor), and microsatellite instability were detected in the endometrial tumor but not in the paired ovarian tumor, while both tumors shared the *PTEN* mutation.

Fragment analysis showed microsatellite instable phenotype in 22.7% (5/22) of cases, including the above-mentioned case which was microsatellite instable only in the endometrial tumor.

Several germline mutations (germline status was confirmed by Sanger sequencing of DNA isolated from non-tumor tissue) in the genes which have been previously associated with cancer predisposing syndromes in the literature were detected. Two patients carried the pathogenic germline variant in *BARD1* or *BRCA1*. The tumors of a 29-year-old patient (CS01) shared pathogenic nonsense germline mutation in *BARD1* NM_000465.2: c.1690C>T, rs587780021, p.(Q564X) together with shared somatic *PTEN* mutation. Another (32-year old patient-CS16) shared pathogenic frameshift germline mutation in *BRCA1* NM_007294.3: c.5266_5267insC, rs80357906, p.(Q1756Pfs*73) together with somatic *TP53* mutation. Other germline mutations of uncertain clinical significance were detected in the genes *BRCA1*, *BRCA2*, *MLH3* or *MSH6*.

In the case of CS06, a patient diagnosed with clear cell carcinoma of the ovary (CCCO) together with SEO, a different mutation profile was found in the respective tumors. The SEO shared common mutation in *PTEN* and *KRAS*, which was absent in CCCO, while the MSH6 mutation NM_000179.2: c.2873_2874del, p.(Q958Pfs*7) was detected only in CCCO.

Discussion

Synchronous endometrioid endometrial and ovarian carcinomas have been a matter of dispute especially because of the difficulties in differential diagnostics between two independent primary tumors and metastasis from one site to the other. The morphological criteria involved in their diagnostics comprise mainly the grade and stage of the diseases (3,4,8). In endometrial tumors the criteria include: Size of the tumor and depth of invasion, direct extension to the adnexa, lymphovascular space invasion, presence of atypical hyperplasia in the surrounding endometrium, and grading. In ovarian tumors the criteria include: The presence of endometriosis, size and laterality of the tumor, surface implants, hilar location, lymphovascular space invasion, and multinodularity. In general, several studies have confirmed that low stage (organ confined) and low-grade SEOs behave clinically as independent primary tumors. Moreover, it has been shown that women with stage I endometrioid endometrial cancer with synchronous stage I endometrioid ovarian cancer have a survival outcome similar to those patients with stage I endometrioid endometrial cancer without synchronous ovarian cancer (1,11-21). This is in concordance with the results of our study, as only 1/10 patients classified as independent SEO developed recurrent disease and none of these patients died of the disease.

Few studies have focused on which ancillary methods may be potentially helpful in the decision process, including immunohistochemical and molecular studies. The use of immunohistochemistry seems to be very limited and only few studies focused on assessing the expression of selected markers. One such study assessed the expression of cytokeratins, vimentin, CEA, Ca125 and Ca19.9 (22). The results between endometrial and ovarian tumors showed overlapping features and the authors concluded that in this setting, immunohistochemistry is not beneficial. In another study, the authors used different antibodies including ER, PR, HER2, p53, and Ki-67. They found out that some antibodies (ER, PR, bcl2) showed different immunostaining patterns between two primaries and can be used as a surrogate marker in the distinction of these tumors (23). Other study focused on vimentin expression in endometrial and ovarian endometrioid carcinoma (24). In this study the authors found that vimentin was negative in 97% of primary ovarian carcinomas and positive in 82% of primary endometrial carcinomas. In SEOs the expression of vimentin was discordant in 53% of cases. They concluded that the expression of vimentin between endometrial and ovarian endometrioid carcinoma is different. However, the practical meaning of immunohistochemistry in this setting is disputable in light of the current knowledge that almost all EOC are clonal lesions and also, with respect to immunophenotypic tumor heterogeneity, can also be influenced by tumor microenvironment.

Molecular studies used variable approaches, including the assessing of microsatellite instability (MSI), the pattern of X chromosome inactivation, loss of heterozygosity (LOH), and mutation of single or small group of genes, especially *PTEN* and *CTNNB1* (22,25-35). Most of the studies failed to identify the clonal origin in most of the cases analyzed. The results of these studies are disputable and limited, particularly with regards to the used methodology. Analysis of a limited number of markers did not allow a comprehensive evaluation and comparison of possible clonal origin of all tumors. In view of current knowledge, it is clear that the intratumoral and intermetastatic heterogeneity is relatively common and can be influenced by several factors such as tumor type, presence of driver mutations, mutation load, and also by immune host reaction with clonal selection. Only three recent studies focused on the comparison between endometrial and ovarian tumors by NGS approach (5-7). One of these studies analyzed 18 SEO cases (all but two were endometrioid in type; the two exceptions were ovarian clear cell carcinoma and endometrial endometrioid carcinoma), 11 of them were classified based on conventional criteria as independent primary tumors. The authors used targeted sequencing of 35 genes commonly altered in endometrial and ovarian tumors. Their results showed that 17/18 cases were of clonal origin, including 10/11 cases based on conventional criteria classified as independent tumors (6). The second study analyzed 23 SEOs, with 15 of being classified as independent primary tumors. Five of these cases were analyzed by whole-exome sequencing, the remaining 18 cases were analyzed by NGS targeting of 341 (n=4) or 410 (n=14) genes, respectively. Their results showed that all sporadic SEOs were clonally related. The only exception was a SEO in patient with Lynch syndrome with germline *MSH6* mutation in which the somatic mutations were different. The last study analyzed 14 cases by NGS targeting of 409 genes and by genome-wide copy number analysis by MIP microarrays (7). Thirteen of their cases showed evidence of clonality. In our present study we found that all 22 synchronous EECs and EOCs shared nonsynonymous mutations in at least one gene: *PTEN*, *AKT1*, *PIK3CA*, *KRAS*, *TP53* and *ARID1A*. These findings suggest that the relatively small panel of genes included in the NGS analysis can be a very useful approach to confirming clonal relation of SEOs.

The results of all these 3 molecular studies were very similar and are in concordance with the results of our study, the most important findings being: i) almost all SEOs are of clonal origin and even low stage and low-grade tumors seem to represent dissemination from one site to the other (however, without a possibility to conclusively assess the directionality); ii) all sporadic SEOs shared nonsynonymous mutations in at least one cancer driver gene of EEC and/or EOC. The authors suggest that the low grade and low stage SEOs, despite being clonal in origin, probably represent phenomenon of restricted dissemination colonizing only certain types of microenvironments and represent clinically indolent spread and not a sign of a 'fully' metastatic disease. A possible explanation is that SEOs classified as independent tumors, using conventional criteria, represent mostly primary indolent endometrial tumors with spread through the Fallopian tube and seeding of the implant into the ovary. This explanation is supported by the finding that SEO tumors showed a lower frequency of ovarian endometriosis than sporadic ovarian endometrioid carcinomas (36).

Based on the results of our study and other three recent studies focusing on this topic the benefit of molecular analysis used for differential diagnosis between independent primary tumors and metastatic disease is disputable (5-7). The finding of clonal origin of almost all SEOs is very important and contrary to the belief that molecular testing could be used as a tool for differential diagnosis. Still, this issue has not been solved and in one recent study the authors suggest that molecular profiling may be beneficial in this setting (8). However, this suggestion was based on whole exome sequencing of only one case of SEO. In their case the authors detected 253 shared mutations (including *ARID1A*, *PIK3CA*, *MSH6* and intronic mutation in *PTEN*), but more mutations were found only in EEC or EOC. Despite the findings of shared mutations, they proposed that these tumors were clonally unrelated and classified them as synchronous independent tumors.

Regardless of the significance in assessing the clonality of an SEO, our results showed some interesting findings with respect to the spectrum of molecular changes occurring in the analyzed tumors. Pathogenic germline mutations in *BARD1* or *BRCA1*, which are usually associated with cancer predisposing syndromes, were detected in two patients with SEOs. Germline *BARD1* mutation p.(Q564X) has been previously described in families with members affected by breast, colon and uterine cancer (37). Germline *BRCA1* mutation p.(Q1756Pfs*73) is a known founder mutation in the Ashkenazi Jewish population and in several European countries including the Czech Republic (38,39). This finding is important for proper genetic counseling besides Lynch syndrome, the common predisposing syndrome associated with uterine tumors.

Pathogenic somatic *HNF1B* mutation NM_000458.2: c.1561_1562insC, p.(Q521Pfs*30) shared in the SEO of one patient (CS15) represents the insertion of one nucleotide (cysteine affecting codon 521 located in exon 8 polyC (C)7 sequence) resulting in frameshift and protein truncation. This mutation in the polyC segment might possibly be the result of the microsatellite instability of these tumors (mutated *MLH3* and *MLH1*). The tumors carried further pathogenic or likely pathogenic somatic mutations in *PTEN*, *ARID1A*, *KRAS*, and *TP53*. Nevertheless, the *HNF1B* p.(Q521Pfs*30) mutation has been previously described in two stomach adenocarcinomas (40) and in four renal clear cell carcinomas according to the TCGA atlas (study Kidney Renal Clear Cell Carcinoma) (41). All those tumors carried also at least one of the mutations in *ARID1A*, *TP53* (frameshift variant), *PTEN* and/or *KRAS*, which suggests a similar mutation profile in different types of tumors. The incidence and significance of *HNF1B* mutations in EEC is unknown. However, in our previous study we detected another pathogenic truncation mutation of this gene c.454C>T, p.(Q152X) in 1/30 EEC (42).

In conclusion, based on our results and the results of three previously published comprehensive molecular studies of SEOs, these tumors are clonally related in almost all cases irrespectively of their clinico-pathological features. Even low grade and low stage tumors classified as independent primaries, according to the conventional morphological criteria, have a clonal origin. From the practical point of view, only the conventional morphological criteria should be used for the classification of these tumors, while molecular profiling does not seem, in this context, helpful. However, analysis of more cases is needed to draw a definite conclusion. Despite this fact, the molecular studies of SEOs can help us to better understand the pathogenesis of SEOs and can be beneficial in clinical practice for example in cases of metachronous tumors affecting female genital organs and/or peritoneum, or in cases of metastatic tumors (43,44). Moreover, molecular profiling of SEOs can be also significant with respect to prognostic and also predictive meaning.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PD, NH and IT contributed to the conception and design of the study. DC, JL, TG, GM, KN, PD, MB and MZ acquired the data. PD, NH, KN, JH, IT and MB performed the experiments, and acquired and interpreted the data. NH, JH, RM and IT processed the bioinformatics data. PD, NH and IT drafted the manuscript. PD and IT proofread the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

In compliance with the Helsinki Declaration, the study has been approved by The Ethics Committee of General University Hospital in Prague (Czech Republic).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have co competing interests.

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