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Original contribution

Clear cell carcinoma of the endometrium: an immunohistochemical and molecular analysis of 45 cases☆

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Summary The aim of the present paper is to study a cohort of pure selected endometrial clear cell carcinomas (ECCCs) from an immunohistochemical and molecular perspective to provide new data about the molecular profile of this disease. In detail, 45 consecutive patients with a proven diagnosis of pure ECCC, according to World Health Organization criteria, were included into the study. We determined the incidence of *KRAS*, *BRAF*, and *PIK3CA* mutations as well as the immunohistochemical expression of mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, PMS2), estrogen, progesterone receptors, p16, and p53. Immunohistochemical analyses for α-methylacyl-coenzyme-A racemase and Napsin A were performed to support the diagnosis of ECC. All cases were positive for at least 1 of the 2 markers. In detail, 34 of 45 (75.5%) cases were positive for α-methylacyl-coenzyme-A racemase, and 40 of 45 (88.8%) cases showed positive staining for Napsin A. All selected cases exhibited negative immunostain for estrogen receptor and progesterone receptor, a "patchy" immunostain for p16, and a "wild-type" staining pattern for p53. Fifteen patients (15/45; 33.3%) showed loss of 1 or more MMR proteins by immunohistochemistry. Seven patients showed dual loss of MSH2 and MSH6, 4 patients (8.8%) showed isolated loss of MSH6, and the remaining 4 patients showed isolated loss of PMS2, respectively. Pyrosequencing analysis revealed the presence of 5 of 45 mutations (11%) at codon 12 in exon 2 of *KRAS* (3/5 p.G12D, 60%; 2/5 p. G12V, 40%) and 5 of 45 (11%) mutations in *PIK3CA* gene, of which 3 of 5 (60%) were in exon 9 of *PIK3CA* (2 p.E542K and 1 p.Q546K) and 2 of 5 (40%) were in exon 20 (p.H1047R). Two synchronous mutations affecting exon 9 of *PIK3CA* (p.Q546K) and exon

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2, codon 12 of *KRAS* (p.G12D) were found. No mutations were detected in the hot spot region of *BRAF*. In conclusion, we provided detailed immunohistochemical and molecular data in a series of ECC, demonstrating a high incidence (33%) of MMR deficiencies detected by immunohistochemistry as well as a synchronous mutation affecting *PIK3CA* and *KRAS* genes. A more extensive interrogation of the genomic features of a much larger series of clear cell carcinomas will be required to define the genomic landscape of this subtype and to determine whether there are molecular alterations that are unique to, or significantly enriched in, clear cell tumors compared to other subtypes.

1. Introduction

Endometrial clear cell carcinoma (ECCC) is a rare subtype of endometrial carcinoma accounting for only 2% to 5.5% of all endometrial malignancies [1]. It presents mainly in postmenopausal women and shows poor response to platinum-based chemotherapy and radiation therapy [2].

Typically, ECCC is considered a prototypical type II endometrial carcinoma; nevertheless, its biological behavior, natural history, and morphoimmunophenotypical characteristics are slightly different from the other type II endometrial carcinomas; therefore, it could be considered a distinct tumoral entity [3]. Moreover, ECCC presents many overlapping morphological aspects with ovarian clear cell carcinoma (OCCC), with the first being classified as type II endometrial carcinoma and the latter being considered a type I ovarian carcinoma [3,4].

Taking into consideration the rarity of ECCC, it remains a pathological diagnostic challenge, with significant interobserver variability and limited information on its genomic background.

In this scenario, to define new prognostic markers and to set up more innovative and efficacious targeted therapies, it is of fundamental importance to get an adequate molecular characterization of ECCC.

The aim of the present paper is to study a cohort of pure selected ECCCs from an immunohistochemical and genetic perspective to provide new data about the molecular profile of this disease. In detail, we determined the incidence of mutations in the hot spot regions of *KRAS*, *BRAF*, and *PIK3CA* genes as well as the immunohistochemical expression of mismatch repair (MMR) (MLH1, MSH2, MSH6, PMS2), estrogen, progesterone receptors, Napsin A, α-methylacyl-coenzyme-A racemase (p504S), p16, and p53 proteins.

2. Materials and methods

2.1. Ethic statement and data collection

The present research complied with the Helsinki Declaration and was approved by the Human Ethics Committee and the Research Ethics Committee of the Catholic University of the Sacred Heart, Rome. All patients provided written informed consent according to institutional guidelines. Patients were informed that the resected specimens were stored by the sections of Anatomic Pathology of the Catholic University of the Sacred Heart of Rome and might potentially be used for scientific research and that their privacy would be maintained.

Forty-five cases of ECCC diagnosed between 2001 and 2014 were collected for this study from our institution (Gynaecologic Oncology Unit of the Catholic University of the Sacred Heart). In all cases, an experienced gynecological pathologist team (G. F. Z., G. A., A. S.) established histological diagnosis at the Department of Pathology of our institution after an extensive and careful evaluation of tumor specimens. In all cases, a final diagnosis of primary clear cell carcinoma of the endometrium was made according to World Health Organization morphological criteria: presence of polygonal or hobnail or cells with clear or eosinophilic/oxyphilic cytoplasm and nuclear atypia, with different pattern of growth, such as papillary, tubulocystic, or solid. Papillae with hyalinized stroma and hyaline bodies were also important diagnostic clues [5]. Other histopathological parameters have been also evaluated: the presence of lymphocytic infiltration, the mitotic index (number of mitoses per 10 high-power fields), and the nuclear grade with a 3-tiered scale based on nuclear pleomorphism [6].

For inclusion in the study, tumors were considered as pure ECCC when showing the mentioned histopathological features according to World Health Organization criteria [5], in addition to the following immunohistochemical profile: estrogen receptor (ER)/progesterone receptor (PR) absent or focal, and Napsin A and/or p504S positive.

Other cases lacking diagnostic consensus between pathologists, and/or mixed epithelial carcinoma with a clear cell component, and/or cases with focal cytoplasmic clearing without consistent morphological characteristics for pure clear cell phenotype, together with a discordant immunohistochemical profile (eg, ER/PR diffusely positive, and Napsin A and p504S negative), were initially excluded from our selection.

All women received complete surgical staging, including the following: peritoneal washing, total hysterectomy, bilateral salpingo-oophorectomy, total omentectomy, peritoneal biopsies, and pelvic/para-aortic lymphadenectomy up to the renal vessels.

2.2. Immunohistochemical analysis

Immunohistochemistry was performed with the labeled streptavidin-biotin peroxidase detection system using the Ventana automated immunostainer (Ventana Medical Systems, Tucson, AZ).

After being independently morphologically reviewed by 3 expert pathologists (G. F. Z., G. A., A. S.), all cases were immunohistochemically tested for p504s and Napsin A to buttress the diagnosis of ECC.

All cases were also tested for mismatch repair proteins, p53, p16 protein, ER, and PR.

Mismatch repair proteins status was determined with the following antibodies: MSH2 (clone G219-1129; Ventana), MSH6 (clone SP93; Ventana), MLH1 (clone M1; Ventana), and PMS2 (clone A16-4; Ventana) in the setting of intact control stromal/lymphocyte staining. Cases were considered as showing stable immunophenotype (MSI+) if any tumor cell nuclei showed positive staining and unstable immunophenotype (MSI–) if all tumor cell nuclei were negative in the presence of internal positive control immunoreactivity [7]. Stromal/lymphocyte staining as well as non-neoplastic endometrial glands served as positive internal controls.

p53 immunostaining was assessed according to previously published works regarding staining patterns that correlate with an underlying *TP53* mutation [8]. Cases were classified as "p53 wild type" (p53-wt: focal and/or heterogeneous staining pattern) or "p53 positive" (diffuse expression in at least 75% of tumor cell nuclei), and cases showing complete absence of staining in tumoral nuclei were considered as "null phenotype." Immunoexpression of ER and PR was evaluated using a semiquantitative score (0 = 0%, 1 = 1%-10%, 2 = 11%-50%, and 3 = 51%-100% positive). Immunostaining for p16 was considered as diffuse if 90% or greater of tumor cells were stained and patchy (nondiffuse) if staining was observed in less than 90% of tumor cells.

2.3. Pyrosequencing analysis

The PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of hot spot regions in all 3 genes investigated (*KRAS* NM_004985.4, *PIK3CA* NM_006218.2, *BRAF* NM_004333.4), starting from 2.5-μm sections of formalin-fixed paraffin-embedded tissue of corresponding primary ECCC. A pathologist evaluated the percentage of tumor cells (at least >40% tumor cells) before performing the manual dissection of tumor area, thus avoiding the samples containing tumor necrosis or few neoplastic cells. Then, all tumors underwent genomic DNA extraction using QIAamp DNA FFPE tissue (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. A custom Pyro assay was designed for sequencing of exon 9 and 20 of *PIK3CA* gene biotinylated 5′-GGGAAAATGACAAAGAACAGCT-3′ and unlabeled reverse 5′-ACCTGTGACTCCATAGAAAATCTT-3′ for exon 9 and biotinylated forward 5′-ATTCGAAAGACCCTAGCCTTAGA-3′ and unlabeled reverse 5′TGCTGTTTAATTGTGTGGAAGATC-3′ for exon 20, respectively. The sequencing analysis was carried out using the following primers: 5′-CCATAGAAAATCTTTCTCCT-3′ targeting codons 542, 545, and 546 in exon 9 and 5′-GTTGTCCAGCCACC-3′ targeting codons 1043-1047 in exon 20. For analysis of hot spot regions of *KRAS* (codons 12 and 13) and *BRAF* (codon 600), Therascreen *KRAS* and *BRAF* Pyro Kits CE (Qiagen GmbH, Hilden, Germany) were used. Data analysis was performed using the PyroMark Q24 software (Qiagen GmbH, Hilden, Germany).

3. Results

3.1. Clinicopathological characteristics

A diagnosis and a careful pathological review of pure ECCC were rendered in 45 cases, whose characteristics are summarized in the Table. Our series included 57.8% ECCCs at an early stage (I-II) and 42.2% of patients showing late-stage (II-IV) disease at diagnosis. The ECCCs included in this study showed different uterine sites of origin, with the fundus and/or the body being the most prevalent (71%, 32/45). In 9 cases, the neoplasia was located in the isthmus; in 2 cases, it involved the entire cavity; and in the remaining 2 cases, the site was not specified. Median age of our patients was 64.8 years (range 27-89 yeas).

From a morphological point of view, we have observed a wide spectrum of cytoarchitectural features in our ECCC population. In particular, 38 cases (85%) showed a prevalent clear cell component admixed with focal oxyphilic neoplastic elements. The remaining 7 cases (15%) were completely constituted by neoplastic cells with eosinophilic granular cytoplasm and intense intratumoral lymphocytic infiltrate. Considering the architectural features, all cases consisted of an admixture of at least 2 patterns, the most frequently encountered being the papillary followed by the solid and the tubule-cystic. In detail, 34 cases (75%) showed a solid and papillary growth (papillary prevalent), and 11 (25%) were prevalently papillary with focal solid and tubule-cystic areas. All ECCCs predominantly showed grade 2 nuclei, and in 35% of the cases, focal areas with grade 3 nuclei were observed. The median mitotic index was 4 mitotic figures per 10 high-power fields (range 2-10).

3.2. Immunohistochemistry

Immunohistochemistry was carried out on all 45 selected ECCCs. All cases exhibited negative immunostaining for ER and PR, a "patchy" immunostaining for p16, and a "wild-type" staining pattern for p53 (Fig. 1B and C, and Fig. 2E and F), respectively.

Table Clinicopathological, immunohistochemical, and molecular features of the studied cohort

Characteristics	All cases n (%)	MLH1 loss	MLH2 loss	MSH6 loss	PMS2 loss	*PI3KCA* mutations, n (%)		*KRAS* mutations, n (%)	
						With	Without	With	Without
All cases	45	0	7	11	4	5 (11)	40 (89)	5 (11)	45 (89)	1 (100)
Age, median (range), y	64.8 (27-89)		61 (46-73)			61.8 (44-78)	65.2 (27-89)	71.8 (49-89)	63.9 (27-85)	62 (62)
FIGO stage										
I-II	26 (57.8)		6	8	2	4 (15.4)	22 (84.6)	2 (7.7)	24 (92.3)	1 (3.8)
III-IV	19 (42.2)		1	3	2	1 (5.3)	18 (94.7)	3 (15.8)	16 (84.2)	0
Localization										
Not specified	2 (4.4)					0	2 (4.4)	0	2 (4.4)	0
Fundus	3 (6.7)		1	2	1	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)	1 (100)
Body	19 (42.2)		1	2	1	1 (5.3)	18 (94.7)	3 (15.8)	16 (84.2)	0
Body/fundus	10 (22.2)					2 (20)	8 (80)	1 (10)	9 (90)	0
Isthmus	9 (20)		5	7	2	1 (11.1)	8 (88.9)	0	9 (100)	0
Entire cavity	2 (4.5)					0	2 (100)	0	2 (100)	0

Immunohistochemical analyses for p504S and Napsin A were performed to support the diagnosis of clear cell carcinoma. All cases were positive for at least 1 of the 2 markers. Regarding p504S, 34 of 45 (75.5%) cases were positive, with variable levels of staining intensity. Regarding Napsin A, we have observed 40 of 45 (88.8%) positive cases (Fig. 3).

Fifteen patients (15/45; 33.3%) showed loss of 1 or more MMR proteins by immunohistochemistry. In detail, 7 patients showed dual loss of MSH2 and MSH6 (Fig. 1E and F, and Fig. 2B and C), 4 patients (8.8%) showed isolated loss of MSH6, and the remaining 4 patients showed isolated loss of PMS2. The average age of patients with tumors harboring mismatch repair deficiencies was 61 years (range 46-73) compared to 74 years (range 51-85) for mismatch repair–intact tumors.

3.3. Molecular profile

All samples tested for the molecular analysis contained at least 40% tumor cells by histologic evaluation. Pyrosequencing analysis of *PIK3CA*, *KRAS*, and *BRAF* hot spot regions on 45 ECCC patients revealed interesting results. Briefly, our data confirmed a similar incidence of *PIK3CA* and *KRAS* hot spot mutations (5/45; 11%) among the cases, whereas no mutations in *BRAF* gene were detected.

The median age of *PIK3CA* mutated patients was 61.8 years compared to 65.2 years in *PIK3CA* wild-type ECCC patients. Women with *PIK3CA* mutated ECCC were preferentially in stage I-II according to the International Federation of Gynecology and Obstetrics classification. In detail, 2 mutations in exon 9 (c.1624G>A and p.E542K) and 2 mutations in exon 20 (c.3140A>G and p.H1047R) were, respectively, identified in different samples (Fig. 4B and C).

Regarding *KRAS* pyrosequencing hot spot regions analysis, the median age of mutated patients was 71.8 years compared to 63.9 years in *KRAS* wild-type ECCC patients. Women with a *KRAS* mutated ECCC were preferentially in a stage III-IV according to the International Federation of Gynecology and Obstetrics classification. In detail, 4 mutations at codon 12 were identified—2 were the transition (c.35G>A) p.G12D and 2 were the transversion (c.35G>T) p.G12V—in 4 different samples (Fig. 4D and E).

Finally, 2 synchronous mutations affecting *PIK3CA* and *KRAS* genes, respectively, were found in a single patient who harbored the mutations p.Q546K (c.1636C>A, *PIK3CA*) and p.G12D (c.35G>A, *KRAS*). Interestingly, we observed a mutual exclusivity of *KRAS* and *PIK3CA* mutations with the MMR defects detected by immunohistochemistry.

4. Discussion

The molecular background of endometrial cancer, over time, has been intensely investigated. Several mutations have been identified, mainly in endometrioid, serous, and mucinous carcinomas [7-12]. After numerous molecular studies, often

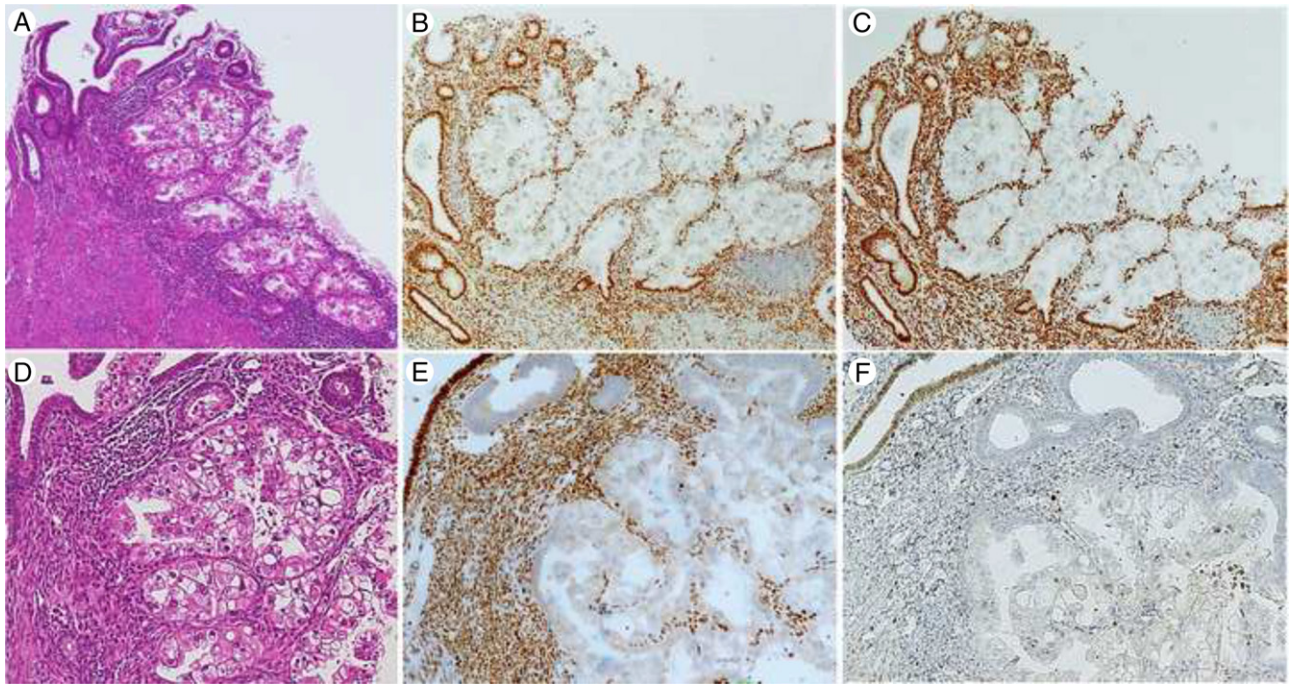

Fig. 1 A, Low magnification showing a superficial focus of clear cell endometrial carcinoma. As shown in the picture, this case was confined to the endometrium without evidence of myometrial invasion. By immunohistochemistry, neoplastic cells showed negative staining for estrogen (B) and progesterone (C) receptors, which stained exclusively non-neoplastic endometrium (shown on the left side of both pictures). D, Higher magnification of the same case showing the classic clear cell morphology. Immunohistochemical analysis in this case demonstrated homogenous loss of MSH6 (E) and PMS2 (F) in tumor cell nuclei with positive control staining in the surrounding endometrial stroma, lymphocytes, and a normal endometrium.

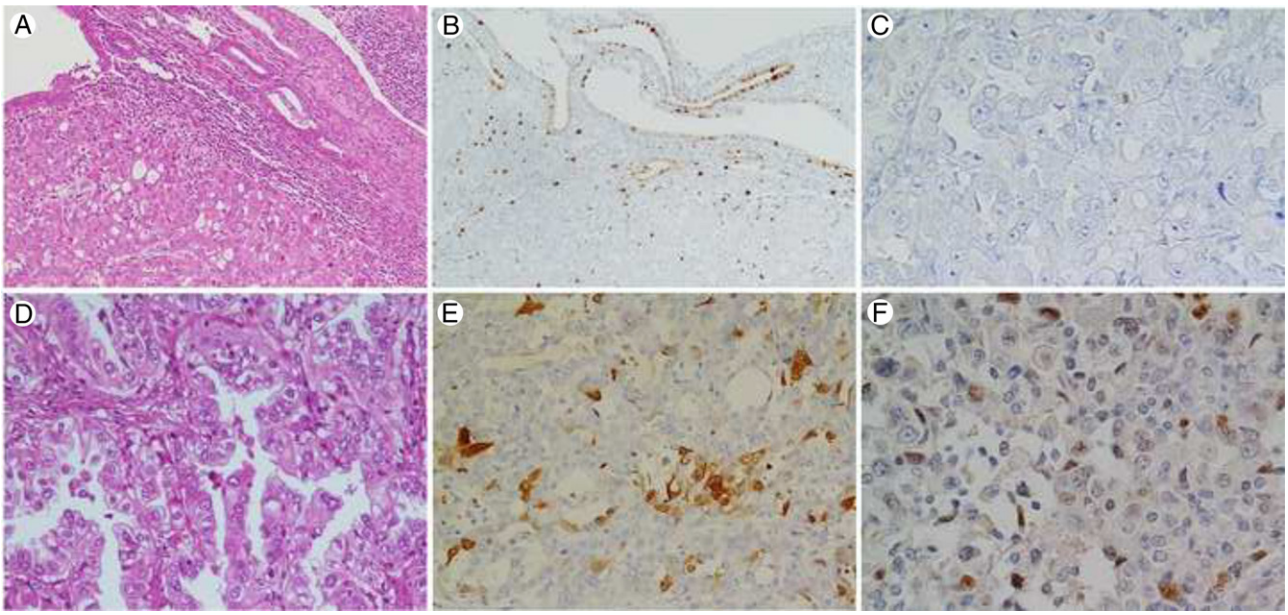

Fig. 2 A, Low magnification showing another case of clear cell endometrial carcinoma with a solid and glandular architecture. By immunohistochemistry, neoplastic cells showed dual loss of MSH6 (B) and PMS2 (C) in tumor cell nuclei with positive control staining in the surrounding endometrial stroma, lymphocytes, and normal endometrium. D, Higher magnification demonstrating a glandular architecture with the presence of eosinophilic and clear neoplastic cells. E, Tumor cells showed a focal (patchy) staining for p16. F, Immunohistochemistry for p53 revealed a wild-type pattern of staining.

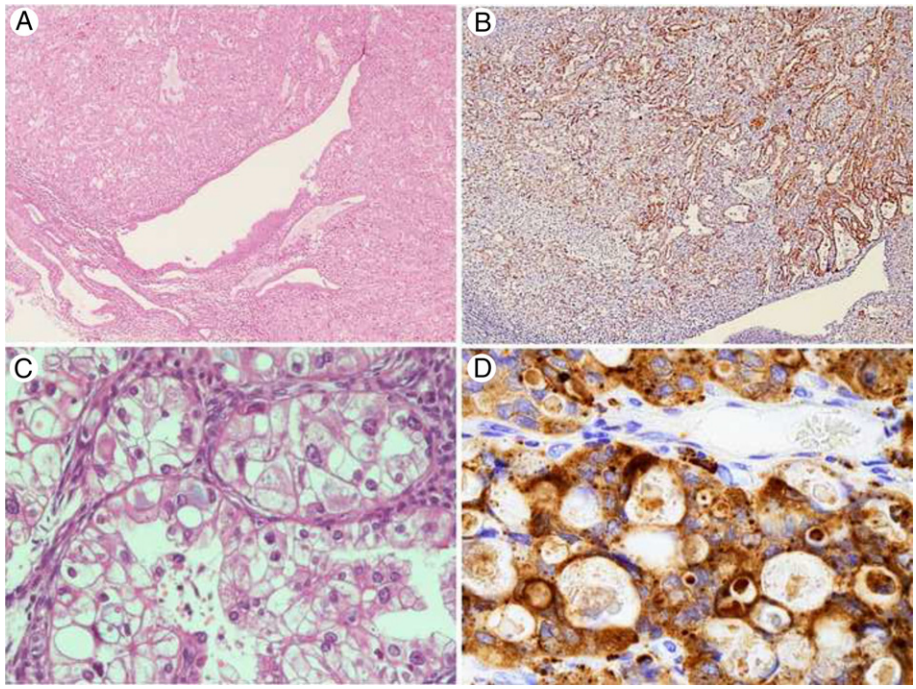

Fig. 3 A, Low magnification (original magnification ×4) showing another case of clear cell endometrial carcinoma with a solid and glandular architecture. B, By immunohistochemistry, neoplastic cells showed positive staining for p504S. C, Higher magnification (×40) of the case presented in panel A demonstrating clear cell features of neoplastic cells. D, Immunohistochemistry for Napsin A revealed positive cytoplasmic staining in neoplastic cells.

performed only on small series of endometrial cancers, in 2013, The Cancer Genome Atlas published the first large-scale whole-exome in-depth molecular analysis defining the existence of 4 molecular categories: (1) ultramutated (POLE mutated), (2) hypermutated secondary to MSI, (3) low copy number, and (4) high copy number (serous-like) [10]. In this study, around 40% of endometrial cancers showed defective DNA mismatch repair, which can be related to genetic or epigenetic

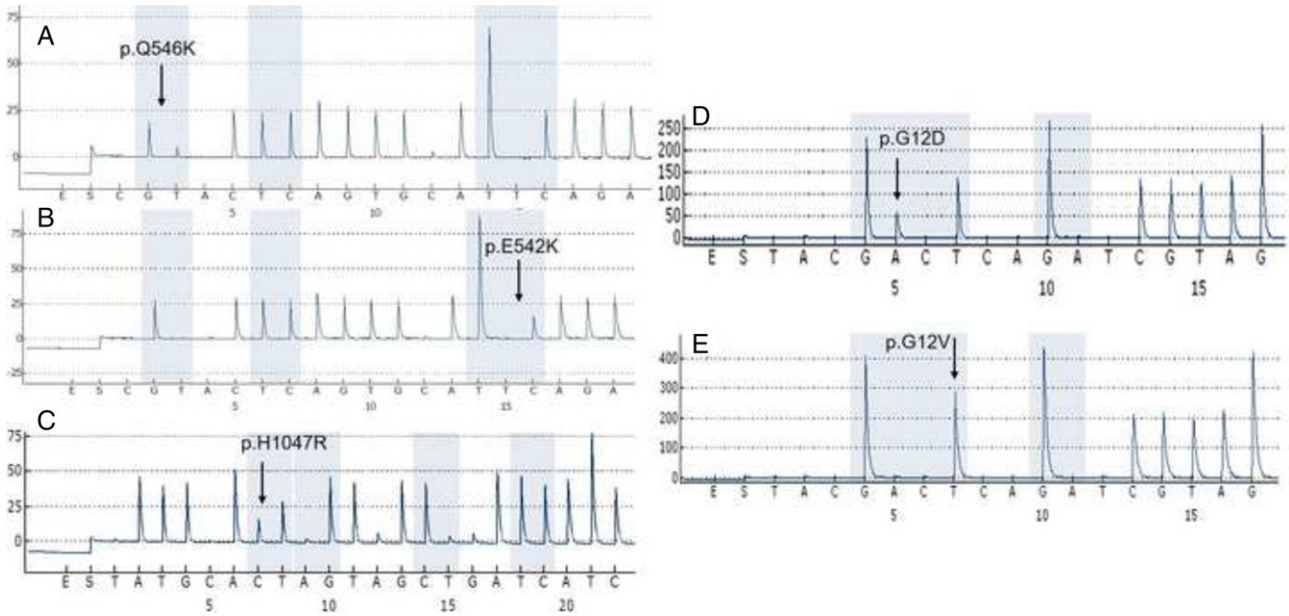

Fig. 4 Sequencing analysis of *KRAS* and *PIK3CA* genes. Pyrograms of main mutations identified in our cohort sequencing *PIK3CA* (A, p.Q546K; B, p.E542K; C, p.H1047R) and *KRAS* (D, p.G12D; E, p.G12V) genes, respectively. The points of mutations in the pyrograms are indicated by arrows.

mechanisms, through germline or somatic mutations in 1 of the MMR genes or methylation of a promoter region [9-12].

Anyway, 1 important limit of The Cancer Genome Atlas study is that it did not consider the mutational status of pure ECCC. Since then, several studies have tried to elucidate the molecular background of ECCC [9,13-18]. In all studies reported to date, *TP53* seems to be the most frequently mutated gene, exhibiting aberrant protein (p53) expression in 34% of cases [13-18]. Other frequently mutated genes include *PPP2R1A*, *PIK3CA*, *FBXW7*, *PTEN*, *KRAS*, *ARID1A*, *SPO*, and *POLE* [13-18]. In addition, the most frequently reported genomic gains include *CCNE1*, *ERBB2*, and *CEBP1,* whereas abnormal MMR protein expression has been reported in 0%-19% of cases [13-18].

Recent molecular analyses have also aimed to define a genetic profile of the clear cell phenotype and its possible pathogenetic or prognostic role both in the ovary and in the uterus.

By a microarray dataset of ovarian cancers, an OCCC signature including 437 genes has been identified [19]. Other available data include studies identifying mutations in *PIK3CA* and *PTEN* and the loss of expression of BAF250a (ARID1A binding protein) in ECCCs and OCCCs, with inactivating ARID1A mutations observed only in the ovary and not in endometrium [20-22].

Recently, Zannoni et al have reported a clinicopathological study of 22 patients with OCCC, searching for *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genetic abnormalities and reporting *KRAS* mutations at codon 12 in 13.6% of patients, *PIK3CA* mutations in exon 9 and exon 20 in 27% of patients, and the absence of mutations in the hotspot regions of *NRAS* and *BRAF* genes [23].

To better clarify this complex molecular scenario, the present study aims to provide new data on the immunohistochemistry, the genetic profile, and the MMR protein status on a large series of ECCCs.

Our immunohistochemical analysis revealed that all cases exhibited negative immunostain for ER and PR, a "patchy" immunostain for p16, and a "wild-type" staining pattern for p53. Moreover, 34 of 45 (75.5%) cases were positive for p504S, and 40 of 45 (88.8%) cases showed positive staining for Napsin A. These findings are in keeping with the previously reported studies on the immunohistochemical profile of ECCCs [4,24,25].

Regarding the molecular profile, our study revealed that only 11% of ECCCs have a *KRAS* mutation (in codon 12 exon 2), 6% of ECCCs have a *PIK3CA* mutation (in exon 9), 4% of ECCCs have a *PIK3CA* mutation (in exon 20), and 2% of ECCCs present a double mutation of *PIK3CA* and *KRAS* (respectively, in exon 9 and at codon 12 in exon 2). Finally, none of the 45 cases have *BRAF* mutations. Our data confirm that ECCC may have mutations in *PIK3CA* and *KRAS* in line with what has been previously reported, whereas the same data confirm that the ECCC does not have BRAF mutations [10,11,14-16,23]. Moreover, we did not find synchronous mutations in *KRAS* and *PICK3CA* with MMR defects detected by immunohistochemistry.

In this way, we confirm that the ECCC genetic profile remains similar to that of OCCC regarding the presence of *PIK3CA* and *KRAS* mutations and the absence of *BRAF* mutations.

Regarding microsatellite instability, 15 of 45 patients (33%) showed loss of 1 or more MMR proteins by immunohistochemistry: in detail, 7 patients showed dual loss of MSH2 and MSH6, 4 patients (8.8%) showed isolated loss of MSH6, and the remaining 4 patients showed isolated loss of PMS2. This percentage observed in our series (33%) is slightly different from that reported in the literature, which is about 20% [17,26]. Surprisingly, a recent study analyzing the molecular texture of 37 ECCCs [18] showed that no cases were MMR deficient, whereas 6 (16%) had POLE mutations. By contrast, the most commonly affected gene was *TP53* (59%, 22/37) followed by *KRAS* (13%, 2/15) and *PIK3CA* (13%, 2/15). Although the cited study claims a complete absence of MMR deficiency in ECCC, our study, similarly to other reported series of morphologically and immunohistochemically proved ECCCs, demonstrated that MMR deficiency is a possible genetic signature in this histotype, with wide variability in its frequency.

According to literature data, Müllerian carcinomas with a clear cell phenotype have been found to be more frequently associated to DNA MMR abnormalities both in the uterus and in the ovary [27,28].

In particular, MMR-deficient endometrial cancers have also been reported to show distinctive clinical and pathological features [29] such as the more prevalent lower uterine segment localization, age between 45 and 55 years, synchronous neoplasias, and rich inflammatory stroma (increased number of CD8+ tumor-infiltrating T cells).

A detailed pathological review of our series revealed no substantial morphological differences between ECCCs with and without abnormal MSI immune phenotype. The only interesting findings have been observed in the entity of immune infiltrate and in the anatomic uterine localization of neoplasia; in fact, 5 of 15 MMR-deficient cases (33%) had an intense lymphoplasmacytic inflammatory response and 2 of 15 MMR-deficient cases (13.3%) were localized in the isthmus.

After these considerations, further studies are needed to determine the real frequency of MMR defects in ECCC because the identification of the MSI phenotype may often represent the sentinel of Lynch syndrome, and therefore, cancer genetics counseling, germline testing, and careful surveillance for second primary malignancies may be suggested for these patients [30].

Moreover, the Food and Drug Administration has recently approved the use of pembrolizumab for solid tumors with MMR deficiency [31]. Therefore, the awareness that a subset of ECCCs shows MSI by immunohistochemistry may be crucial for a more personalized therapy for these patients often showing resistance to conventional chemotherapy that alternatively would potentially benefit from immune checkpoint inhibitors [26].

Finally, a more extensive analysis of the genomic features of a much larger series of clear cell carcinomas should be required to formally define the genomic landscape of this subtype and to determine whether there are molecular alterations that are unique to, or significantly enriched in, clear cell tumors compared to other subtypes.

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