Virchows Arch (2014) 465:193–198
DOI 10.1007/s00428-014-1599-1

ORIGINAL ARTICLE

Mutational status of *KRAS*, *NRAS,* and *BRAF* in primary clear cell ovarian carcinoma

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Received: 21 February 2014 / Revised: 31 March 2014 / Accepted: 22 May 2014 / Published online: 3 June 2014

Abstract Ovarian clear cell carcinoma (OCCC) is a subtype of epithelial ovarian cancer with characteristic biological features and aggressive clinical behavior. OCCCs show a pattern of gene mutations different from other type I ovarian malignancies, notably a higher frequency of *PIK3CA* mutations. In low grade serous ovarian cancer, *KRAS* and *BRAF* mutations are frequent, but little data are available on the mutational status of these genes in OCCCs. To clarify this issue, we designed a clinicopathological study with the aim to establish the incidence of *KRAS*, *NRAS*, and *BRAF* hot spot mutations in OCCC. Between December 2006 and June 2012, 22 patients with a proven diagnosis of OCCC were admitted to our Institutions. In all cases, final diagnosis was established according to FIGO and WHO criteria. All women received complete surgical staging. The PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of *KRAS*, *NRAS*, and BRAF hot spot regions on 2.5-μm sections of formalin-fixed paraffin-embedded tissue from primary OCCC. Pyrosequencing analysis of *KRAS*, *NRAS,* and *BRAF* hot spot regions revealed the presence of mutations only at codon 12 in exon 2 of *KRAS* in 3 of 22 (14 %) cases. We found no mutations in the hot spot regions of *NRAF* (exons 2, 3, 4) or *BRAF* (exon 15). The median age of women with a *KRAS* mutated OCCC was 74 years. These OCCC were unilateral FIGO stage IA lesions in two cases associated with foci of endometriosis. We conclude that in 14 % of OCCCs, a *KRAS* mutation occurs in codon 2 exon 2. *NRAS* and *BRAF* mutations were not found.

Keywords Clear-cell ovarian carcinoma · *KRAS* · *NRAS* · *BRAF* · Hot spot mutations

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Introduction

Epithelial ovarian cancer (EOC) accounts for approximately 90 % of primary malignant ovarian tumors. It is a heterogeneous tumor category, classified in serous, mucinous, endometrioid, and clear cell histotypes, each with specific molecular characteristics and clinical outcome [1].

This classical histopathological classification of EOC has been recently revised, and currently, two broad categories are distinguished. Type I EOC (including low-grade serous, mucinous, endometrioid, and clear cell carcinoma) are confined to the ovary at diagnosis, grow slowly and are chemoresistant. Type II EOC (high-grade serous, undifferentiated carcinomas, carcinosarcomas) typically show poor histological differentiation, early extra-ovarian spread and respond well to platinum-based chemotherapy [2, 3]. Type I tumors often harbor somatic mutations of genes encoding protein kinases including KRAS, BRAF, PI3KCA, and ERRB2, along with other signaling molecules, such as CTNNB1 and PTEN. In contrast, Type II tumors generally lack these mutations but are

characterized by chromosomal instability and high frequency of *TP53* mutations [4–8].

In this complex scenario, ovarian clear cell carcinoma (OCCC) is considered an entity distinct from the above cited type I EOCs, due to its specific biological behavior. Recent findings have demonstrated that OCCCs typically show a higher frequency of *PIK3CA* mutations [9], which suggests that aberrations in telomere biology may play an important role in the pathogenesis of OCCC [10]. On the other hand, *KRAS* and *BRAF* mutations have been recognized as a frequent event in low grade serous ovarian cancer, but only few data are currently available on the mutational status of these genes in OCCCs. For these reasons, we designed a clinicopathological study with the aim to evaluate the incidence of *KRAS, NRAS,* and *BRAF* hot spot mutations in a consecutive single Institution series of patients with OCCC.

Material and methods

Patients

Between December 2006 and June 2012, 22 patients with a proven diagnosis of OCCC were admitted to the Gynecologic Oncology Unit of the Catholic University of the Sacred Heart. In all cases, histological diagnosis was established at the Department of Pathology of our Institution after an extensive and careful evaluation of tumor specimens by an experienced gynecological pathologist. In all cases, a final diagnosis of primary clear cell carcinoma of the ovary was made according to FIGO and WHO criteria [11]. All women received complete surgical staging, including the following: peritoneal washing, total hysterectomy, bilateral salpingo-oophorectomy, total omentectomy, peritoneal biopses, and pelvic/para-aortic lymphadenectomy up to the renal vessels. Patients gave written informed consent for clinical data to be collected and analyzed for research purposes. The study was approved by the local institutional review board.

Molecular analysis

The PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of *KRAS*, *NRAS*, and *BRAF* hot spot regions on 2.5-μm sections of formalin-fixed paraffin-embedded tissue from primary OCCC.

In brief, all the slides were reviewed by a pathologist to evaluate the percentage of cancer cells (>50 % tumor cells was the base line requirement) before performing manual dissection of the tumor to avoid areas with tumor necrosis or few neoplastic cells. From these samples, DNA was extracted using QIAamp MinElute spin columns (Qiagen, GmbH, Hilden, Germany), according to the manufacturer's protocol and the sequence of interest was amplified by PCR (Veriti 96 well Fast Thermal Cycler, Applied Biosystems Inc., Foster City CA). Using therascreen KRAS, *NRAS* and *BRAF* Pyro Kits CE (Qiagen GmbH, Hilden, Germany), all hot spot regions were analyzed and samples with a potential low-level mutation were reexamined in duplicates to arrive at a specificity of 0.98 and sensitivity of 0.99 [12–14]. The PyroMark ™ Q24 software (Qiagen, GmbH, Hilden, Germany) was used for data analysis.

Results

Genomic profiling was conducted on a consecutive series of 22 women with OCCC. The clinicopathological characteristics of the study patients are summarized in Table 1. Our series confirms the trend toward presentation of OCCC at an early stage, with only 27 % of patients showing late stage disease at diagnosis. Furthermore, only one patient had bilateral ovarian lesions, and in 18 % of patients, OCCC occurred along with foci of endometriosis, an endometriotic ovarian cyst in one patient and pelvic endometrial lesions in another patient (Table 1).

Pyrosequencing analysis of *KRAS*, NRAS, and *BRAF* hot spot regions revealed the presence of mutations only at codon 12, exon 2, of *KRAS* in 3 (14 %) cases. The following mutations were found: p.G12V (gly12→val12), p.G12A (gly12→ala12), and p.G12S (gly12→cys12) (Table 2). Pyrogram traces demonstrating three hot spot *KRAS* mutations at codon 12 of exon 2 are presented in Fig. 1. No mutations in

Table 1 Clinico-pathological characteristics of the study series

Characteristics	All cases Number (%)	Patients without KRAS mutations Number (%)	Patients with KRAS mutations Number (%)
All cases	22	19 (86.4)	3 (13.6)
Age, median (range), years	50 (29–75)	49 (29–71)	74 (46–75)
FIGO stage			
I–II	16 (72.7)	13 (68.4)	3 (100)
III–IV	6 (27.3)	6 (31.6)	0
Unilateral ovarian lesion			
Yes	21 (95.5)	18 (94.7)	3 (100)
No	1 (4.5)	1 (5.3)	0
Dimension of ovarian lesion, median (range), mm	120 (20–180)	110 (20–180)	120 (110–150)
Occurrence on endometriotic foci			
No	18 (81.8)	17 (89.5)	1 (33.3)
Yes	4 (18.2)	2 (10.5)	2 (66.7)

Table 2 Histological features of patients with ovarian clear cell ovarian carcinoma, according with the specific KRAS mutations

	Histological features	Mutation	Amino acid
1	OCCC with tubulocystic pattern	GGT→GTT	gly12→val12
2	OCCC with tubulocystic pattern	GGT→GCT	gly12→ala12
3	OCCC with papillary pattern	GGT→AGT	gly12→ser12

the hot spot regions of *NRAF* (exons 2, 3, 4) and *BRAF* (exon 15) genes were found. Hematoxylin/eosin stained histological images of the three *KRAS* mutated cases are presented in Fig. 2. Two of the *KRAS* mutated OCCC showed a tubulocystic pattern, and the remaining case a papillary pattern. The median maximum diameter of *KRAS* mutated OCCC was 110 mm. The median age was 74 years compared to 49 years in *KRAS* wild type OCCC. All women with a

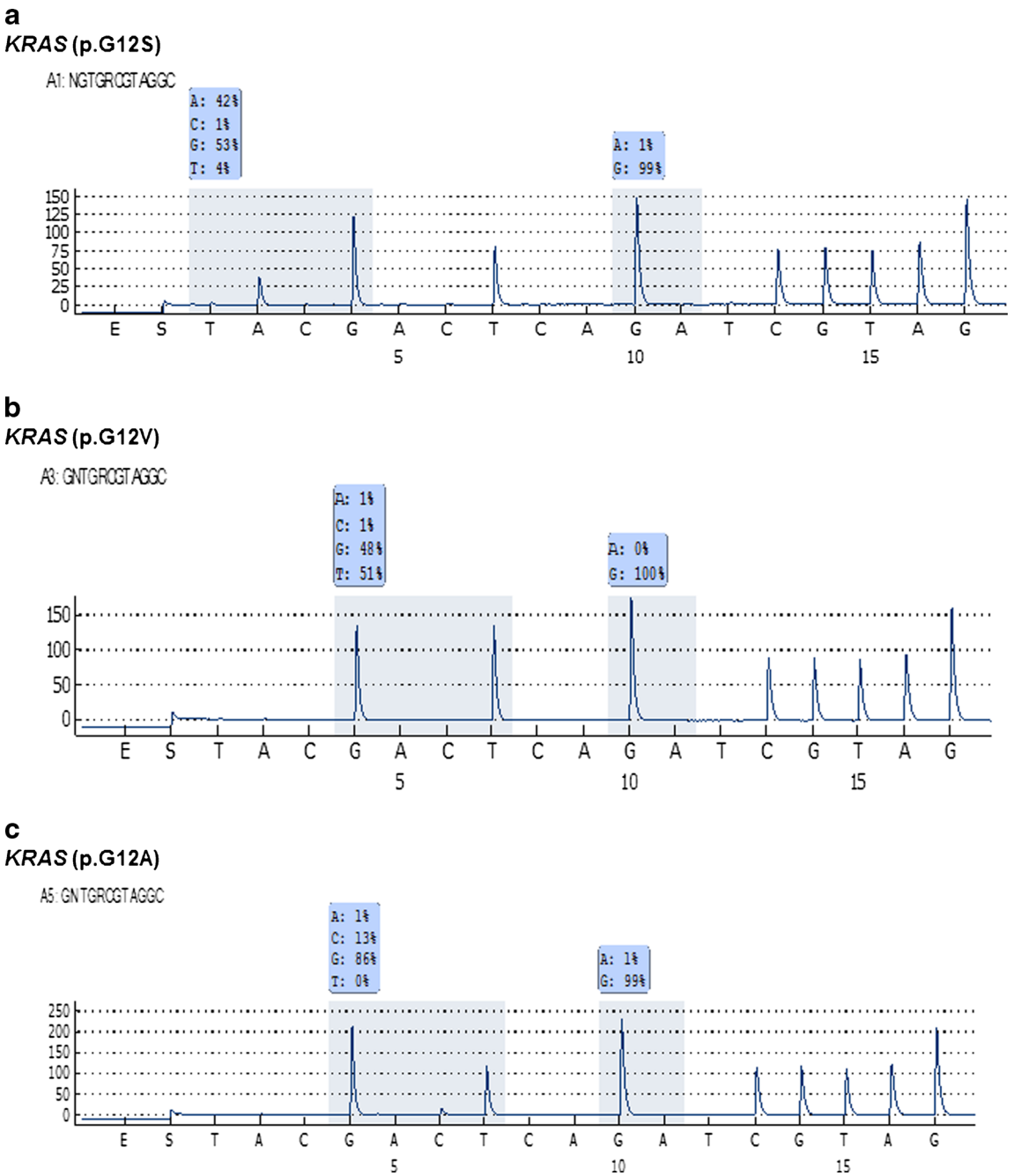

Fig. 1 Representative pyrosequencing mutation analysis results, showing **a** p.G12S (gly12→ser12), **b** p.G12V (gly12→val12), and **c** p.G12A (gly12→ala12) mutations in codon 12, exon 2 of *KRAS*

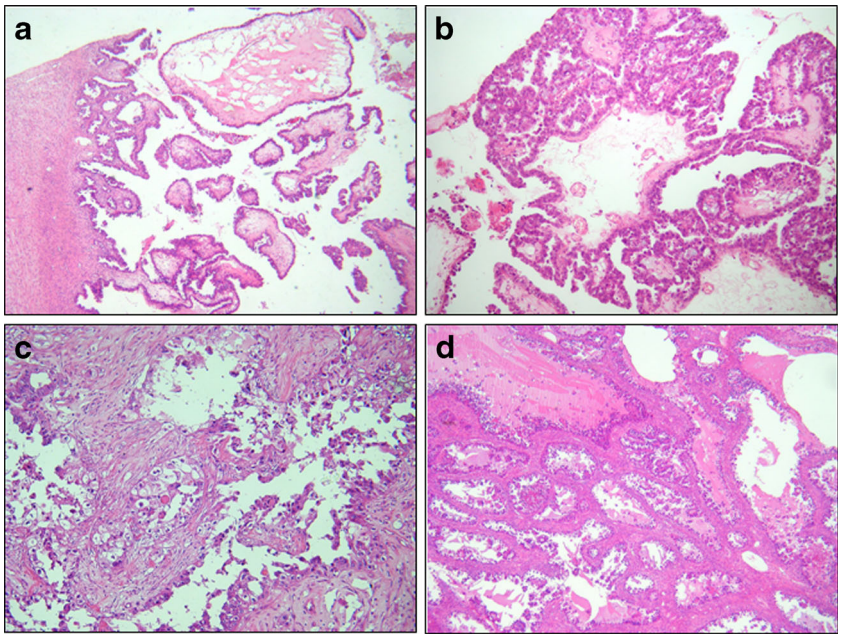

Fig. 2 Hematoxylin-eosin stained histological images of the three OCCCs with a *KRAS* mutation and of an OCCC with wild type *KRAS*. **a** OCCC with *KRAS* mutation showing a tubulocystic pattern with clearcells, hyaline globules, oxyphilic cells, and abundant eosinophilic cytoplasm (magnification=10×). **b** OCCC with *KRAS* mutation showing a papillary pattern with predominately hob-nail cells (magnification=10×). **c** OCCC with *KRAS* mutation showing a tubulocystic pattern with clear cells and hob nail cells (magnification=10×). **d** OCCC without *KRAS* mutation showing a tubulocystic pattern with clear cells and hobnail cells (magnification=10×)

KRAS mutated OCCC the tumor was unilateral, staged IA according to FIGO classification.

Discussion

OCCC accounts for 5 to 13 % of all epithelial ovarian malignancies [15]. From a clinical point of view, it usually occurs at a younger age than high-grade serous EOC [16]. It often presents as a pelvic mass confined to the ovary [17, 18], often arising in association with foci of endometriosis [17]. Moreover, the clinical behavior of OCCC is aggressive, with a low response rate to standard platinum-based chemotherapy [19–21] and poor prognosis compared to high-grade serous EOC [19].

OCCCs are generally p53 wild-type, with a low frequency of *BRCA 1* and *2* germline mutations [22–25], and a low level of chromosomal instability. OCCCs express high levels of HIF1a (hypoxia inducible factor 1 alpha), and phosphoinositide 3-kinase catalytic alpha (*PI3KCA*) mutations are found in around 40 % of cases [26–29]. The proportion of HER2/neu expressing cases of OCCC is 2.5 to 10-fold higher than that of type I and II ovarian tumors, suggesting a potential role for HER2/neu inhibitors for treatment of this tumor [30].

However, despite these interesting data, the specific pathways involved in driving the aggressive biological features of OCCC are still not completely defined [31–33]. For these reasons, we studied mutational status of *KRAS, NRAS,* and *BRAF* in OCCC. The RAS-RAF-MEK-ERK-MAP kinase pathway is often affected in human cancer. KRAS, NRAS, and BRAF are members of the RAS/RAF/MEK/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, a well-characterized signaling mechanism that mediates cellular responses to growth signals [34]. These three genes are upstream activators of the mitogen-activated protein kinase (MAPK) cascade [35]. *KRAS* gene shows a missense point mutation in 25 % of all cancers, generally located in codon 12, 13, and 61 [36]. These *KRAS* mutations lead to constitutive activation of the protein, increasing GDP/GTP exchange or decreasing GTPase activity of the protein, which ultimately increases cell proliferation [37].

Mutations of *KRAS* and *BRAF* genes in noninvasive and invasive carcinomas of the ovary have been reported previously, and *KRAS* mutations are found in around 75 % of mucinous ovarian tumors [6, 9, 35, 38–41]. Furthermore, activating mutations of *KRAS* and *BRAF* are present in over half of type I EOC and serous borderline tumors. In contrast, they are very uncommon in high-grade serous [6] and endometrioid carcinomas [40, 42–45]. As regards OCCCs, few and contrasting data have been reported until now regarding the mutational status of *KRAS* and *BRAF* [40, 46]. Jones S et al, and Auner V et al, reported *KRAS* mutations in 4.7 and 26 % of OCCCs, respectively [47, 48] while Rechsteiner M. et al failed to observe *KRAS* mutations in a large cohort of OCCCs [49].

We observed a *KRAS* gene mutation in 14 % of OCCCs which seems significantly lower than in other ovarian carcinoma subtypes, especially mucinous tumors. This frequency of *KRAS* mutation is similar to that reported for ovarian endometrioid carcinomas (7 %), which supports the hypothesis of a common origin of these two tumor types [2, 3, 6, 9, 50, 51]. Interestingly, we found KRAS mutations only in codon 12, exon 2, but not in codon 13, exon 2 nor in *NRAS* or *BRAF*.

These mutations correspond to the following amino acid substitutions: p.G12V (gly12→val12), p.G12A (gly12→ala12), and p.G12S (gly12→cys12), in agreement with other studies [12, 48, 49, 52–54]. Interestingly, the commonly described p.G12D mutation was not found in our series, which emphasizes the specific biological features of OCCCs. Finally, we confirmed the absence of *BRAF* hot spot mutations in OCCCs [40, 49, 55, 56], making *BRAF* alterations a very rare event in ovarian cancer.

In conclusion, we found a *KRAS* mutation in codon 2 exon 2 in 14 % of OCCCs. Our findings confirm that EOC, is a heterogeneous group of entities characterized by different molecular signatures.

Conflict of interest We declare that we have no conflict of interest.

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