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Consistent absence of BRAF mutations in cervical and endometrial cancer despite KRAS mutation status

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Abstract

Background. Mutational activation of KRAS and BRAF proto-oncogenes contributes to the development of many human cancers. Current research on gynecological cancer and specifically in cervical and endometrial cancer is focused on the mechanisms of their mutational activation.

Objectives. In view of the paucity of data on their mutation frequency and the status of BRAF in these two types of gynecological cancer, we performed a systematic molecular study in 114 clinically and histologically well-defined malignant tumors of uterine cervix and endometrium and correlated the mutation status of KRAS and BRAF with the age at diagnosis and with tumor grade, stage or histological type.

Methods. Direct sequence analysis of the PCR products of KRAS and BRAF genes was used to screen for known activating mutations.

Results. In 67 cases of endometrial cancer, six KRAS mutations (8.9%) were found, four at codon 12 (5.9%) and two at codon 13 (2.9%), while no mutation was detected at codon 61. Most of the mutations occurred in surgical stage I and in the endometrioid adenocarcinoma subtype. We also detected three KRAS point mutations (6.3%) in the 47 cervical cancer samples, two at codon 12 (4.2%) and one at codon 13 (2.1%), while there was no mutation at codon 61. On the contrary, no mutation was identified in BRAF exon 15 for either endometrial or cervical cancer samples at position V600, which represents the most frequently mutated site of BRAF in human cancer. There was no association between KRAS mutations with either histological type, tumor grade or stage. Interestingly, however, KRAS mutation status in endometrial cancer was strongly associated with increased age at diagnosis ($P < 0.001$).

Conclusions. Our data document (a) the absence of BRAF mutations in cervical and endometrial cancer, despite the mutation status of KRAS, (b) suggest that KRAS mutations reflect an early event in endometrial carcinogenesis and (c) imply that BRAF activation is involving alternative pathways in these two types of cancer.

Keywords: KRAS; BRAF; Cervical cancer; Endometrial cancer

Introduction

Gynecological cancer, and particularly cervical and endometrial carcinomas, like colorectal carcinomas, represents one of the multi-stage models of human carcinogenesis [1]. Although the introduction of novel high throughput functional genomic approaches, such as the DNA microarray technology, has offered so far invaluable insights for most types of gynecological cancer [2], the parallel study of individual key components of cellular transformation still provides additional clues on the operating cellular networks in cancer. To this end, part of the research on endometrial and cervical cancer has focused on the mechanisms of mutational activation of KRAS and BRAF proto-oncogenes [3–5].

The RAS-RAF-MEK-ERK-MAP kinase signaling pathway is pivotal for the control of cell proliferation and differentiation [4–6]. Mutations in genes encoding primarily the RAS and to a

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lesser extent the RAF components of the pathway result in constitutive aberrant gene expression and contribute to the development of many human cancers [3–8].

About 30% of human cancers contain mutations in one of the three closely related members of the RAS gene family, i.e. KRAS, NRAS and HRAS [1,9]. KRAS, being the most frequently mutated member, is activated by point mutations within the 'hot-spot' regions primarily at codons 12 and 13 and at a lower frequency, at codon 61. Activating mutations of the BRAF gene have been recently identified in a variety of human cancers, including colorectal [1,4,5,8], thyroid [10] and ovarian–mainly serous–carcinomas [11–14], and primarily in malignant melanomas, with a high frequency of nearly 70% [8,15–19]. Approximately 90% of the mutations detected contain a single transversion in exon 15 at position T1799A [20], previously designated as T1796A [8] and corresponding to an amino acid substitution of glutamate for valine at residue 600, i.e. V600E [20], previously designated as V599E [8]. BRAF is a member of the RAF family genes (including ARAF, BRAF, and RAF1 or CRAF) and encodes a serine–threonine kinase of the MAPK pathway, which transduces regulatory signals from RAS to MEK1/2. A mutant version of BRAF harboring the V600E mutation has a ten-fold higher basal kinase activity compared to the wild type [8,20].

KRAS mutations have been shown to occur in 14–24% of endometrial carcinomas [21–23], suggesting that a KRAS mutation may play an important role in some cases of endometrial cancers. On the contrary, the data on the KRAS mutation status during carcinogenesis of the cervix are divergent. Several studies have suggested that mutational activation of the KRAS gene is involved in the disease [24–26]. Studies by other groups, however, do not confirm these findings [27–29]. As stated above, information on BRAF activation is limited in tumors other than melanomas, lung, thyroid, ovarian or colorectal cancers [3–8]. Regarding gynecological cancer, other than ovarian cancer, a study by Sasaki et al. [18], in six endometrial cancer cell lines (KLE, HEC1-A, AN3CA, RL95-2, SK-UT-1B and Ishikawa 3H 12) showed no mutations in the BRAF gene, while recently Mutch et al. [21] reported a single case of BRAF intron 11 alteration (which conceivably may reflect a polymorphism) in endometrial cancer series. Additionally, no mutations were detected by Davies et al. [8] or by Cohen et al. [10] in a small series of cell lines or clinical samples of cervical cancers.

Furthermore, it has been widely documented that mutations affecting KRAS and BRAF both in human cancers and in chemically induced mouse liver tumors [30] seem to be mutually exclusive [1,3,5,21], suggesting the operation of alternative activating pathways for BRAF. The latter was further corroborated recently in gene-targeted studies where BRAF was found to be dispensable for KRAS-mediated oncogenesis [3].

In view of the paucity of extensive studies regarding the status of BRAF mutations in cervical and endometrial cancer and to (a) further assess the individual effect of the KRAS and BRAF mutations in these two types of cancer and (b) to test whether their mutations are mutually exclusive, as in other cancers, we performed a systematic molecular study in clinically and histologically well-defined malignant tumors of uterine cervix and endometrium and correlated the mutation status of KRAS and BRAF with the age at diagnosis and with tumor grade, stage or histological type.

To this end, we screened a large group of primary endometrial and cervical cancers for the KRAS codon 12, 13 and 61 mutations and for the BRAF V600E point mutation, using PCR and direct sequencing techniques [16].

Materials and methods

Clinical specimens, classification and extraction of genomic DNA

Specimens from patients and normal control subjects were collected in and retrieved from the Departments of Pathology at both the University of Crete, Heraklion and the Alexandra Hospital, University of Athens, Athens. All samples were formalin-fixed, paraffin-embedded and genomic DNA was extracted using the QIAamp extraction DNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. Concentration of genomic DNA was assessed by a GenQuant spectrophotometer (Pharmacia LKB Biotechnology Inc., Piscataway, New Jersey). A total of 67 endometrial cancer samples, 47 cervical cancer samples and 17 control samples of normal endometrial and cervical tissue were evaluated, classified according to the staging system of FIGO and the histological classification system of WHO. For the grading of endometrial cancers, the FIGO system was applied, while for cervical cancers, a modification of the original Broders system was used [31]. The clinical and pathological features of the 114 patients are summarized in Table 1.

PCR analysis and determination of the KRAS and BRAF mutations

The individual point mutations of the KRAS and BRAF genes were documented by employing three gene-specific oligonucleotide primer pairs, designed to specifically amplify by PCR the regions of the KRAS gene harboring codons 12–13 and 61 [32] and the region of exon 15 of the BRAF gene, encompassing codon 600 [8,16], followed by direct sequencing. The sequences for the three PCR primer pairs were as follows: (a) KRAS 12–13 forward 5′ GTGTGACATGTTCTAATATAGTCA 3′ and KRAS 12–13 reverse 5′ TTACTGGTGCAGGACCATTC 3′, generating a 122 bp fragment; (b) KRAS 61 forward 5′ TCAAGTCCTTTGCCCATTTT 3′ and KRAS 61 reverse 5′ GTCTTTGCTAATGCCATGCAT 3′, generating a 179 bp fragment; and (c) BRAF 15 forward 5′ TGCTCTGATAGGAAAATGAGATC 3′ and BRAF 15 reverse 5′ CTGAGATGCTGCTGAGTTACTAG 3′, generating a 119 bp fragment. The PCR reaction was carried out in a total volume of 30 μl in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Waltham, Massachusetts). The PCR reaction mixture contained 3 μl 10× Reaction Buffer, 1 μl dNTP (10 mM of each dNTP), 1 μl forward primer (25 pmol), 1 μl reverse primer (25 pmol), 1.8 μl $MgCl_2$ (25 mM), 1 μl Taq DNA Polymerase (5 units/μl) and 5 ng/ml DNA template. Amplification was carried out with 5 min initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min and extension at 72°C for 1 min.

Direct sequencing

The PCR products were subsequently analyzed on a 4200 Two-Dye DNA Analysis System (LI-COR Biosciences, Lincoln, Nebraska). Both forward and reverse sequencing traces were obtained for each sample, and the ones exhibiting allelic mixture in both were classified as containing a mutation.

Data analysis

All data among groups and correlations between the clinicopathological parameters and KRAS mutation status were analyzed by employing the χ^2 test

Table 1
Clinicopathological features of the series of 114 patients with gynecological cancer

Endometrial cancer (n = 67)	No. of samples screened (%)	Cervical cancer (n = 47)	No. of samples screened (%)
Surgical stage		Clinical stage	
I	36 (54)	I	39 (83)
II	12 (18)	II	6 (13)
III	12 (18)	III	2 (4)
IV	7 (10)	IV	
Histological type		Histological type	
Endometrioid adenocarcinoma	51 (76)	Squamous cell carcinoma	28 (60)
MMMT[a] or carcinosarcoma	8 (12)	Adenocarcinoma	13 (28)
Mucinous adenocarcinoma	4 (6)	Adenosquamous carcinoma	5 (10)
Clear cell adenocarcinoma	2 (3)	Adenoid cystic carcinoma	1 (2)
Serous adenocarcinoma	1 (1.5)		
Large cell neuroendocrine carcinoma	1 (1.5)		
Histological grade		Histological grade	
G1	22 (33)	Well differentiated	4 (8)
G2	21 (31)	Moderately differentiated	28 (60)
G3	24 (36)	Poorly differentiated	15 (32)

[a] MMMT = malignant müllerian mixed tumor (malignant mesodermal mixed tumor).

or the one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered as statistically significant.

Results

Of the 67 samples of endometrial cancer, 6 were detected carrying a mutation in KRAS gene (8.9%) as shown in Table 2. Four mutations were found at codon 12 (5.9%), resulting in G12R, G12A and G12V substitutions, and two mutations (GGC → GCC) at codon 13 (2.9%), resulting in a G13A substitution, while no mutation was detected at codon 61. Most of the mutations occurred in the endometrioid adenocarcinoma subtype, as shown in Table 2. We also detected 3 samples with KRAS point mutations in the 47 cervical cancer samples tested (6.3%). Two of the mutations were at codon 12 (4.2%) resulting in G12R and G12S substitutions and one at codon 13 (2.1%) resulting in a G13D substitution (GGC → GAC). Again, no mutation at codon 61 was detected.

Contrary to the KRAS mutation status in these samples of gynecological cancer, no mutation was identified in BRAF exon 15 for either endometrial or cervical cancer samples at position V600, which represents by far, the most frequently mutated site of BRAF in human cancer [1,4,5,8,10–20]. Finally, all 17 control samples analyzed contained wild-type KRAS and BRAF genes.

Statistical analysis disclosed that KRAS mutations in endometrial or cervical cancer were not associated with either histological type, tumor grade or stage. Furthermore, to assess the onset of KRAS mutations on patient age, we compared the age distributions between patients with KRAS mutations and those without mutations. Interestingly, KRAS mutation status in endometrial cancer was strongly associated with increased

Table 2
Types of KRAS mutations by tumor histology type, grade and stage

Patient no.	Age[a] (years)	Histological type[a]	Histological grade[a]	Stage[a]	KRAS mutation		
					codon 12	codon 13	Nucleotide change
Endometrial cancer							
1	73	MMMT[b] or carcinosarcoma	G3	IB	G12R[c]		GGT → CGT
2	77	Mucinous adenocarcinoma	G1	IB	G12A		GGT → GCT
3	75	Endometrioid adenocarcinoma	G2	IC		G13A	GGC → GCC
4	68	Endometrioid adenocarcinoma	G2	IB		G13A	GGC → GCC
5	73	Endometrioid adenocarcinoma	G1	IB	G12R		GGT → CGT
6	75	Endometrioid adenocarcinoma	G2	IIIA	G12V		GGT → GTT
Cervical cancer							
1	30	Squamous cell carcinoma	PD[d]	IB1	G12R		GGT → CGT
2	49	Adenocarcinoma	PD	IIIA		G13D	GGC → GAC
3	71	Squamous cell carcinoma	MD	IB2	G12S		GGT → AGT

[a] Correlations between the clinicopathological parameters and KRAS mutation status were analyzed using the χ^2 test or the one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered as statistically significant.
[b] MMMT = malignant müllerian mixed tumor (malignant mesodermal mixed tumor).
[c] Abbreviations for the amino acid residues: G = glycine, R = arginine, A = alanine, V = valine, D = aspartic acid, S = serine.
[d] Abbreviations for the cervical cancer histological grading: PD = poorly differentiated, MD = moderately differentiated.

age at diagnosis. The 7.3 years mean age difference between patients harboring KRAS mutations (73.5 ± 3.08) and those mutation-negative cases (66.2 ± 10.53) was found to be statistically significant ($P < 0.001$).

Discussion

Our study represents a comprehensive evaluation of KRAS and BRAF mutation status in a large series of well-characterized samples of both endometrial and uterine cervical cancer, covering the entire spectrum of surgical or clinical stages, tumor grade and histological types. Previous studies have only addressed individual aspects of BRAF and/or KRAS mutations in a small number of cell lines [8,18] or clinical samples in either endometrial [21–23] or cervical cancer [10,24–29], leading to rather inconclusive results.

These data provide several new features of potential importance. First, they demonstrate that BRAF mutations in contrast to their frequent presence in ovarian cancer [8,11–14] are absent in endometrial and cervical cancer, despite the mutation status of the KRAS gene. The latter has been shown recently in endometrial cancers only, exhibiting a degree of defective DNA mismatch repair mechanisms [21] and suggesting a quite different mode of carcinogenesis from colorectal carcinomas, where BRAF is affected by a background of defective DNA repair status [1,8,21]. Second, the majority (78%) of KRAS mutations in both endometrial and cervical carcinomas were detected at surgical stage I or clinical stage I, respectively, implying that KRAS activation reflects an early event in gynecological carcinogenesis, regardless of the histological type. This feature has been observed independently also in low-grade but not in high-grade ovarian carcinomas [11–13,32], which further supports the notion that high-grade tumors are not derived from low-grade lesions, but rather represent distinct entities, following different pathways [11]. Additional studies will be needed to address the significance of the age of onset of KRAS mutations, which was documented to be statistically significant in our study, and specifically to clarify to what extent KRAS mutations reflect a characteristic of late age of onset in endometrial carcinomas.

Our findings and those of Mutch et al. [21] point to the direction that the RAS/RAF-induced transformation in gynecological cancer is mediated via distinct pathways, quite different from those of colorectal cancer, which primarily employ RAF serine–threonine kinases (c-RAF1, ARAF and BRAF) as key effectors [1,5,6,9]. Furthermore, recent experimental data [3,33,34] have provided conclusive evidence, extending this assumption, that BRAF in oncogenic transformation is not operating alone, and additional KRAS effector pathways, such as the phosphatidylinositol 3-kinase (PI3K) and the Ral guanine-nucleotide exchange factors (RalGEFs) pathways, might be also operating in certain types of cancers [3,33].

In summary, our data along with the above recent findings [21,33,34] provide the impetus for additional studies to validate the hypothesis whether BRAF activation is involving these pathways during oncogenic transformation of gynecological cancer, an issue which may eventually facilitate specific BRAF-targeted drug discovery efforts.

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