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Original Paper

The Clinicopathological Significance of *K-RAS* Point Mutation and Gene Amplification in Endometrial Cancer

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The aim of this study was to examine the prevalence and clinicopathological significance of *K-RAS* oncogene activation in endometrial carcinoma and atypical hyperplasia. We analysed *K-RAS* point mutation and gene amplification in 55 endometrial carcinomas using polymerase chain reaction associated with restriction fragment length polymorphism and genomic differential polymerase chain reaction. Point mutations at codon 12 of *K-RAS* oncogene were identified in 8 of 55 (14.5%) tumour specimens. In addition, we were unable to detect any *K-RAS* gene amplification in any of the endometrial carcinomas studied. No correlation was found between *K-RAS* gene mutation and age at onset, histological subtype, grade of differentiation, clinical stage or current patient status. We conclude that *K-RAS* mutation is a relatively common event in endometrial carcinomas, but with no clear prognostic value. © **1997 Elsevier Science Ltd.**

Key words: endometrial carcinoma, atypical hyperplasia, polymerase chain reaction, K-ras, point mutation, gene amplification

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INTRODUCTION

NORMAL CELLULAR genes also called proto-oncogenes can be activated by different processes and trigger an uncontrolled growth of cells. The correlation between the activation of these genes and the apppearance of cancer has already been established in several human tumours, as reviewed by De Vita and associates [1].

Carcinoma of the uterine endometrium is the most frequently diagnosed gynaecological malignancy in the Western world with approximately 40 000 new cases reported annually in the United States [2], but causes only 13% of all gynaecological cancer deaths. This is mainly due to early diagnosis. Approximately 78% of all uterine cancer is diagnosed while it is still restricted to the uterus[2, 3]. However, carcinoma of the endometrium may spread along the uterine cavity to the cervix, penetrate the uterine wall or spread through the fallopian tubes. Once disseminated, it is as lethal as ovarian cancer [4].

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Nevertheless, when compared to other neoplasia, the involvement of activated transforming genes in the development of endometrial cancer is still poorly understood. Inactivation of the tumour suppressor gene *P53* by deletion and point mutation in 10–20% of advanced endometrial neoplasia have been detected [5]. In addition, the *C-ERBB-2/NEU* proto-oncogene is overexpressed in approximately 10% of advanced stage endometrial cancers and its expression correlates with the presence of intraperitoneal metastatic disease [6]. In addition, an abnormal *C-ERBB-2/NEU* gene dosage has been described in approximately 14% of endometrial neoplasms [7]. Epidermal growth factor receptor (EGFR) overexpression has also been correlated with poor prognosis of the disease [8]. EGFR gene amplification has not been detected in any case [7]. Finally, *FGF-3/INT-2* gene amplification, a marker of the 11q13 amplicon involving the cyclin D1 gene, has been correlated with advanced stage of disease [7].

A significant event in endometrial tumorogenesis is *K-RAS* oncogene mutation. The ras family of G proteins (N, H, K-ras) are thought to play a critical role in the regulation of cellular proliferation and, in this sense, mutations of the *K-RAS* gene have been implicated in the development of

numerous human malignancies [9]. In many types of cancers, the *K-RAS* proto-oncogene has been found to have undergone point mutation in codon 12, 13 or 61, which alter the ras products by abolishing their intrinsic GTPase activity and decreasing their interaction with the GTPase-activating protein (termed 'ras-GAP'), resulting in a constitutive stimulation of autonomous growth and contributing to neoplastic development [10]. In a few cases, an alternative pathway of carcinogenesis due to *K-RAS* amplification has been detected [11, 12].

The ras proteins are expressed in normal endometrium, and levels may be upregulated in some endometrial cancers [13]. Moreover, mutations in *RAS* genes are present in immortalised endometrial carcinoma cell lines [14]. The mutation in codon 12 of *K-RAS* has, in fact, been found in 10–30% of endometrial carcinomas [15–20]. Some studies have indicated that mutations in codon 12 of *K-RAS* are an independent, unfavourable prognostic factor [15], whereas others have not found this correlation [19, 20]. However, many such studies have been conducted in Japan, where the epidemiological and clinical characteristics of endometrial carcinoma differ from those in the United States and Europe. A higher prevalence of *K-RAS* mutations in endometrial carcinomas in Japan (27%) compared with the United States (11%) has been indicated [19]. Moreover, despite the accumulated data about the implication of *K-RAS* gene activation in endometrial cancer, mutation of codon 12 of *K-RAS* in endometrial carcinomas in European patients has not been analysed in any report. In addition, the status of *K-RAS* amplification has not been analysed in any of the studies mentioned.

In addition, *K-RAS* mutations have been identified in endometrial hyperplasias, which suggests the *K-RAS* mutation may be a relatively early event in endometrial tumorigenesis. Mutations may be found more frequently as the severity of hyperplasia increases [19].

In view of these inconclusive data, we analysed mutation of codon 12 of *K-RAS* and its gene copy number in fresh surgical specimens of endometrial carcinoma and endometrial hyperplasias from European patients using polymerase chain reaction associated with restriction fragment length polymorphism (PCR–RFLP) [21] and the genomic differential polymerase chain reaction [22], two techniques with rapid and non-radioactive procedures, which are useful as a diagnostic tool.

PATIENTS AND METHODS

Patient population and tissue specimens

Fifty-five primary endometrial tumours and 5 atypical hyperplasias, collected at the time from the surgery of the Department of Obstetrics and Gynaecology at Hospital Vall d'Hebrón of Barcelona, were evaluated in this study. The protocol was previously approved by the Institutional Review Board and informed consent was obtained from all the patients involved in the study. Each tissue sample was stored at −80°C until analysed. Serial paraffin sections were stained with a haemotoxylin and eosin for light microscopic study at the Department of Pathology in the same hospital. None of the patients had received radiation therapy or hormonal treatment prior to surgery. Their ages ranged from 45 to 86 years. Only 3 patients were premenopausal and the remaining 52 were postmenopausal. The stage distribution of the 55 patients according to the International Federation of Gynecology and Obstetrics (FIGO) staging system was Stage Ia (5 cases), Stage Ib (10 cases, Stage Ic (6 cases), Stage IIa (19 cases), Stage IIb (5 cases), Stage IIIa (7 cases), Stage IIIb (1 case) and Stage IIIc (2 cases). Histologically, 39 of the 55 patients had endometrioid-type carcinomas, 11 had adenocarcinomas with squamous differentiation, 2 had papillary serous carcinomas and 3 had clear cell carcinomas. 30 were well-differentiated (G1), 15 were moderately differentiated (G2) and 10 were poorly differentiated (G3). Among all surgically collected endometrial carcinomas, the median follow-up was 22 months and the current status of the patients was alive without evidence of disease in 48 cases, alive with disease in 1 case, death non-related to disease in 1 case and dead from disease in 5 cases.

Finally, an additional diagnosed set of 5 atypical hyperplasias was included. All five showed glandular cytological atypia sufficient for inclusion in an atypical endometrial hyperplasia category, but did not have features of carcinoma such as large, solid areas of epithelium and cribiforming. The initial manifestation of all cases of atypical hyperplasia was abnormal vaginal bleeding and the patients range in age from 38 to 50 years.

High molecular weight DNA was extracted from each specimen by proteinase K and phenol/chloroform treatment [23].

Detection of K-RAS *codon 12 mutations by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP)*

Activated codon 12 *KI-RAS* genes were identified by designed PCR–RFLP analysis according to Jiang and associates [21], using a 5′ end primer that contains a cytosine substitution at the first position of codon 11. This substitution creates an Mva I restriction enzyme site that overlaps the first two nucleotides of codon 12. The 3′ primer also contains a control Mva I site. The primers were as follows: for K-ras 5′, 5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′ and for K-ras 3′,5′-TCA AAG AAT GGT CCT GCA CC-3′. DNA was amplified using a PTC-100 TM Programmable Thermal Controller (M. J. Research, Watertown, Massachusetts, U.S.A.). 1 μg of each target DNA sample was subjected to the polymerase chain reaction using Linus DNA Amplification Kit (Linus, Madrid, Spain) in a PCR volume of 50 μl. Initially the samples were heated for 5 min at 94°C followed by 30 times of a cycle consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Extension of the final cycle for each sample was extended to 7 min. Thereafter, 15 μl aliquots of the PCR reaction product were digested with the restriction enzyme Mva I at 37°C overnight and electrophoresed through a 2% agarose gel. The results were analysed after ethidium bromide staining and UV translumination. Placental DNA and RWP-2 cell line DNA were used as negative and positive controls for K-ras codon 12 mutation, respectively. The appearance of a single 128 bp fragment after Mva I digestion indicates the presence of wild-type *K-RAS* gene, while the appearance of two bands of 128 and 157 bp indicates the presence of a mutant *K-RAS* gene.

Detection of K-RAS gene amplification by the genomic differential polymerase chain reaction

The technique was performed as described by Frye and associates [22] with few modifications by our group [24].

The procedure involves co-amplification of a target and a single-copy reference gene in the same reaction tube. With genomic differential PCR there is an increase of PCR product from a target gene with abnormal gene dosage, compared to a decrease of the single copy reference gene. 1 μg of each target DNSA sample was subjected to the polymer-

Table 1. *Clinical and pathological features of endometrial carcinomas with* K-RAS *codon* 12 *mutation and gene amplification study*

						K-RAS	
Patient number	Age	Histology	FIGO Stage	Cellular grade	Follow-up (months) and status	Mutation	Amplification
1	67	E	IA	G1	41, NED	no	no
2	82	E	IA	G1	21, NED	no	no
3	82	E	IA	G1	12, NED	yes	no
4	72	E	IA	G1	17, NED	no	no
5	69	E	IA	G1	04, NED	no	no
6	51	E	IB	G1	30, NED	no	no
7	62	E	IB	G1	31, NED	no	no
8	63	E	IB	G1	15, NED	no	no
9	73	A	IB	G1	12, NED	no	no
10	63	C	IB	G1	31, NED	yes	no
11	67	E	IB	G1	12, NED	no	no
12	63	E	IB	G2	35, NED	yes	no
13	60	E	IB	G2	44, NED	no	no
14	53	A	IB	G2	24, NED	no	no
15	62	E	IB	G2	37, NED	no	no
16	62	E	IC	G1	26, NED	no	no
17	61	E	IC	G1	42, NED	no	no
18	70	E	IC	G1	30, NED	yes	no
19	57	E	IC	G2	24, NED	no	no
20	68	A	IC	G3	06, NED	no	no
21	66	E	IC	G3	25, NED	no	no
22	56	E	IIA	G1	34, NED	no	no
23	74	E	IIA	G1	12, NED	no	no
24	73	E	IIA	G1	12, NED	no	no
25	52	A	IIA	G1	38, NED	no	no
26	45	E	IIA	G1	12, NED	no	no
27	56	E	IIA	G1	12, NED	yes	no
28	63	A	IIA	G1	36, NED	no	no
29	62	E	IIA	G1	46, NED	no	no
30	73	E	IIA	G1	12, NED	no	no
31	75	E	IIA	G1	45, NED	no	no
32	65	E	IIA	G1	45, NED	no	no
33	70	A	IIA	G2	12, NED	no	no
34	56	E	IIA	G2	25, NED	no	no
35	55	C	IIA	G2	12, NED	yes	no
36	67	E	IIA	G2	12, NED	no	no
37	56	A	IIA	G2	39, NED	no	no
38	60	E	IIA	G2	45, NED	no	no
39	71	E	IIA	G3	36, DND	no	no
40	71	E	IIA	G3	28, NED	no	no
41	77	E	IIB	G1	03, NED	no	no
42	72	E	IIB	G1	20, DOD	no	no
43	68	E	IIB	G2	10, AWD	no	no
44	86	A	IIB	G2	19, NED	no	no
45	48	A	IIB	G3	15, NED	no	no
46	50	A	IIIA	G1	12, NED	no	no
47	56	E	IIIA	G1	15, DOD	no	no
48	73	P	IIIA	G1	13, DOD	no	no
49	66	A	IIIA	G2	36, NED	no	no
50	74	E	IIIA	G3	11,NED	yes	no
51	55	E	IIIA	G3	27, NED	yes	no
52	75	E	IIIA	G3	34, NED	no	no
53	65	C	IIIB	G3	04, DOD	no	no
54	62	E	IIIC	G2	38, NED	no	no
55	64	P	IIIC	G3	04, DOD	no	no

E, endometrioid-type adenocarcinoma; P, papillary serous carcinoma; C, clear cell carcinoma; A, adenocarcinoma with squamous differentiation; NED, no evidence of disease; AWD, alive with disease; DOD, death of disease; DND, death not related to disease.

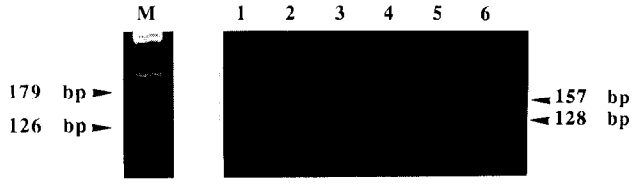

Figure 1. *K-RAS* point mutations in codon 12 analysed by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP). Electrophoretic separation of digested PCR fragments on a 2% agarose gel. Lane M, molecular weight marker (pGEM/Hinf I, Rsa I and Sin I); Lanes 1, 3 and 4, endometrial carcinoma samples with *K-RAS* point mutation (cases 10, 12 and 27); Lanes 2, 5 and 6, endometrial carcinoma samples without *K-RAS* point mutation (cases 3, 26 and 37).

ase chain reaction using the Linus DNA Amplification Kit (Linus, Madrid, Spain). Primers for the target gene, *K-RAS*, and the single copy reference gene, interferon-gamma (*INF-γ*), were added simultaneously to the same PCR tube. The primers were as follows: for *K-RAS* 5′, 5′-ATG ACT GAA TAT AAA CTT GT-3′ and for *K-RAS* 3′, 5′-CTC TAT TGT TGG ATC ATA T-3′; for *INF-γ* 5′, 5′-TCT TTT CTT TCC CGA TAG GT-3′ and for *INF-γ* 3′, 3′-CTG GGA TGC TCT TCG ACC TC-5′. Initially the samples were heated for 5 min at 94°C followed by 30 times of a cycle consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Extension of the final cycle for each sample was extended to 7 min. Placental DNA was used as negative control of gene amplification. After PCR, 10 μl of each sample were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed using Polaroid 667 (Polaroid Corporation, Cambridge, Massachusetts, U.S.A.).

Statistical analysis

The odds ratio (OR) and 95% confidence intervals (CI) were calculated as a measure of the association between *K-RAS* mutations and clinical parameters. The StatXact-Turbo statistical package was used to obtain exact *P*-values.

RESULTS

Clinical and histopathological data are correlated with the results of *K-RAS* oncogene codon 12 mutation and gene amplification analysis in Table 1.

Analysis of mutations in K-RAS *codon* 12

55 endometrial carcinomas were screened for the presence of *K-RAS* codon 12 mutations using the PCR–RFLP technique. We detected the *K-RAS* codon 12 mutation in 8 of 55 (14.5%) patients with endometrial carcinoma. A representative sample of PCR–RFLP analysis is shown in Figure 1. Table 1 summarises all of the relevant data concerning the patients included in the study. Of the 8 tumours with *K-RAS* codon 12 mutations, 6 were endometrioid carcinomas and 2 clear cell carcinomas; 4 were G1, 2 G2 and 2 G3; one was Stage IA, two Stage IB, one Stage IC, two Stage IIA and two Stage IIIA. The median follow-up of these tumours was 21 months and all the patients are alive without evidence of disease. As shown in Table 2, *K-RAS* mutations did not show a statistically significant association with the clinicopathological parameters (age at diagnosis, tumour histology, F.I.G.O. stage, cellular grade and clinical status) analysed ($P > 0.05$). None of the 5 atypical hyperplasias showed *K-RAS* codon 12 mutation.

Analysis of K-RAS *gene amplification*

We investigated the presence of amplification of the *K-RAS* gene in the same 55 endometrial carcinoma specimens

Table 2. Distribution of K-RAS *mutations according to clinicopathological characteristics in endometrial carcinoma*

Parameters	Number of cases	Cases with *K-RAS* mutations (%)	*P*-value
Overall	55	8 (14.5%)	
Age at diagnosis (years)			NS
<53	5	0 (0%)	
54–59	8	3 (37.5%)	
>60	42	5 (11.9%)	
Histology			NS
Endometrioid	39	6 (15.4%)	
Adenoacanthoma	11	0 (0%)	
Clear cell carcinoma	3	2 (66.7%)	
Papillary serous carcinoma	2	0 (0%)	
F.I.G.O. Stage			NS
I	21	4 (19%)	
II	24	2 (8.3%)	
III	10	2 (20%)	
Cellular grade			NS
G1	30	6 (20%)	
G2	15	2 (13.3%)	
G3	10	2 (20%)	
Clinical status			NS
No evidence of disease >12 months after surgery	43	7 (16.3%)	
<12 months after surgery or death of intercurrent disease	6	1 (16.7%)	
Recurrent carcinoma (alive)	1	0 (0%)	
Death of disease	5	0 (0%)	

NS, not significant ($P > 0.05$).

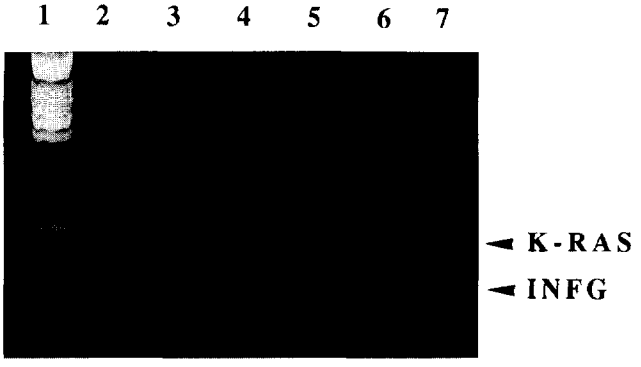

Figure 2. *K-RAS* gene amplification analysed by genomic differential PCR. Electrophoretic separation of *K-RAS* (110 bp) and single copy gene *INF-γ* (150 bp) PCR fragments on a 2% agarose gel. Lane 1, molecular weight marker (pGEM/ Hinf I, Rsa I and Sin I); Lane 2, water control; Lanes 3, 4, 5, 6 and 7, endometrial carcinoma samples (cases 3, 7, 30, 13 and 37).

described above using the genomic differential PCR technique. We were not able to detect *K-RAS* gene amplification in any of the 55 endometrial carcinomas studied. Furthermore, none of the five atypical hyperplasias showed *K-RAS* gene amplification. A representative example of genomic differential PCR analysis is shown in Figure 2.

DISCUSSION

K-RAS is a member of the *RAS* family of oncogenes that encode closely related proteins that act as signal transducers within eukaryotic cells [11]. Although point mutations in codon 12 of the *RAS* genes may be the most frequent oncogene mutation identified in human tumours, the prevalence of such alterations varies with tumour type [11]. In gynaecological malignancies, mutations in the *RAS* gene family mostly occur in codon 12 of *K-RAS* rather than codons 13 or 61 of *K-RAS* [16] or codons 12, 13 or 61 of *H-RAS* or N-RAS [16, 17, 25, 26]. In the present study, we identified *K-RAS* codon 12 mutations in 8 of 55 (14.5%) cases of endometrial carcinoma. None of the 5 atypical hyperplasias showed this mutation. In addition, none of the 55 endometrial carcinomas or 5 atypical hyperplasias showed *K-RAS* gene amplification. The frequency of codon 12 *K-RAS* mutation in our study (14.5%) is similar to the values found in endometrial cancers in U.S.A. (11%) [16, 20, 27] but below that described in endometrial cancers in Japan (27%) [15–19, 25, 26]. Despite the implication of codon 12 *K-RAS* mutation in endometrial carcinoma, its frequency has not been previously assessed in endometrial cancers in Europe. It is important to mention that striking differences exist in the incidence of endometrial cancer between the United States and Europe (approximately 20 cases per 100 000) compared with Japan (approximately 5 cases per 100 000), but the mortality from the disease is roughly equivalent in the two areas. Epidemiological studies have suggested that the higher mortality per case in Japan occurs because few Japanese women are obese and develop oestrogen-dependent well-differentiated favourable lesions. It is therefore possible that *K-RAS* mutation is associated with unfavourable prognosis [15] and a clinicopathological assessment of genetic alterations in endometrial carcinoma subtypes has been proposed [28].

The absence of *K-RAS* gene mutations in our set of atypical hyperplasias can be reasonably explained by the limited number of samples. It has been suggested that the frequency of mutations in atypical hyperplasias is similar to that seen in endometrial cancers [18, 19, 26, 27]. Due to the fact that endometrial atypical hyperplasias show monoclonal cell patterns, as well as carcinomas [29, 30] and only a minority of endometrial atypical hyperplasias contain *K-RAS* mutations, other genes must play a role in their development and progression to endometrial carcinomas.

We did not find evidence of a statistically significant correlation of *K-RAS* mutations with any clinicopathological feature (age at diagnosis, FIGO stage, grade of differentiation, histological subtype and clinical status) among the patients with endometrial carcinoma, in line with most other studies [17, 19, 20, 26], although one study did suggest that codon 12 *K-RAS* mutation was an unfavourable prognostic factor [15].

An interesting finding is the presence of *K-RAS* mutations in 2 of 3 clear cell carcinomas analysed, although it is a very small subset. Reviewing the literature, 1 of 7 endometrial clear cell carcinomas had codon 12 *K-RAS* mutation [15, 17, 26, 31] and 1 of 3 had *K-RAS* gene amplification [17]. It is important to mention that patients with endometrial clear cell carcinoma have an overall death rate significantly higher than that of patients with endometrioid type, with two-thirds of the patients relapsing outside the pelvis [32].

In conclusion, our results demonstrate the contribution of codon 12 *K-RAS* gene mutation to the development of endometrial carcinomas and indicate no evidence of prognostic significance.

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