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Early Mutational Activation of the c-Ki-*ras* Oncogene in Endometrial Carcinoma[1]

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ABSTRACT

Endometrial carcinoma is theorized to arise from a series of somatic mutations which alter benign endometrium to progressively less differentiated histological lesions. One genetic alteration implicated in the carcinogenesis of endometrial cancer is the mutational activation of the c-Ki-*ras* oncogene. This study characterizes the frequency and the topographical distribution of activated c-Ki-*ras* alleles in endometrial carcinoma. Sixty formalin-fixed, paraffin-embedded endometrial cancer specimens were screened for point mutations at codons 12 and 13 of the c-Ki-*ras* oncogene by polymerase chain reaction and allelic specific oligomer dot-blot hybridization. c-Ki-*ras* mutations were identified in nine of 60 (15%) tumor specimens. Five cases resulted in G to A transitions, three in G to T transversions, and one in a G to C transversion. These nine mutant tumors were analyzed by selective UV radiation fractionation and polymerase chain reaction for the presence of activated c-Ki-*ras* alleles in cell populations of various histological phenotype. In eight tumors, c-Ki-*ras* mutations were uniformly present in the carcinoma cells. One tumor exhibited heterogeneous mutational activation, with mutant c-Ki-*ras* alleles detected in only grade 2 carcinoma cells but not grade 1 carcinoma cells. c-Ki-*ras* mutations were present in adjacent hyperplasia with atypia but absent from hyperplasia without atypia. With rare exception, c-Ki-*ras* activation appears to be an early oncogenic event since it is homogeneously present in premalignant and malignant endometrial tissues.

INTRODUCTION

Endometrial carcinoma is the most common gynecological malignancies in the United States with approximately 40,000 new cases diagnosed annually. It is thought to arise through a series of somatic mutations which alter benign epithelium to progressively less differentiated histological lesions: simple, complex, or atypical hyperplasia to carcinoma. Considerable histological intratumor heterogeneity may be present. Nonmalignant and premalignant endometrial proliferations may be present alone or concomitantly with specimens of frankly invasive endometrial carcinoma (1). While less is known about the genetic mutations responsible for endometrial tumorigenesis, recent studies have detected the mutational activation of the c-Ki-*ras* oncogene in 10–37% of endometrial cancers (2–9). c-Ki-*ras* mutations are also present in 6–16% of endometrial hyperplasias, suggesting that a *ras* mutation may be one of the earliest oncogenic events in endometrial cancer (4, 8, 9). However, the possibility that c-Ki-*ras* mutations arise later in endometrial tumor progression cannot be eliminated.

The possible role and timing of the activation of the c-Ki-*ras* oncogene in endometrial carcinogenesis can be analyzed by determining the topographic distribution of the mutant allele with respect to the specific histological phenotypes within a single tumor. A method using SURF[3] followed by PCR can analyze specific cell populations present on a microscope section and localize genetic alterations to relatively homogeneous cell groups, thereby directly correlating phenotype with genotype (10). Utilizing the SURF method, we examined the diverse phenotypes commonly present in endometrial cancer specimens for activated c-Ki-*ras* alleles.

MATERIALS AND METHODS

Tissues and Clinical Data. Sixty endometrial carcinoma samples were selected from patients who underwent surgery at Los Angeles County-University of Southern California Medical Center between July 1, 1987, and March 31, 1993. Patients were identified from review of statistics from the University of Southern California Department of Obstetrics and Gynecology and represent all cases of endometrial cancer primarily treated by surgery during the study period. Information concerning patient demographics, stage, patient status, and months of follow-up was abstracted from hospital charts. Review of hematoxylin and eosin-stained slides of all 60 cases was performed by two authors (J. F. and B. D.) to confirm the diagnosis of endometrial carcinoma. Endometroid adenocarcinomas were further subclassified according to their degree of histological differentiation into three grades using the current International Federation of Gynecology and Obstetrics classification. Papillary serous adenocarcinomas were not graded. All patients were surgically staged according to the current International Federation of Gynecology and Obstetrics system (11).

DNA Isolation. DNA was extracted from 60 formalin-fixed paraffin-embedded endometrial cancer tissues using standard procedures as previously described (10).

PCR and Allelic Specific Oligomer Dot-Blot Hybridization. DNA samples were analyzed for point mutations at codons 12 and 13 of the c-Ki-*ras* oncogene by PCR using 44 cycles and allelic specific oligomer dot-blot hybridization (12). The topographic distributions of the mutant c-Ki-*ras* alleles in the nine positive cases were determined using SURF (10). Multiple sections from different paraffin blocks were sampled in order to analyze all possible phenotypes. At least two mirror sections from each block were analyzed independently in order to verify the topographic distribution of the mutations. Under the microscope, cells of a specific phenotype were identified and carefully dotted by an ink-filled disposable pipet tip (Microloader; Eppendorf, Hamburg, Germany), which was mounted on a micromanipulator. Typically, 10–25 dots were placed per slide. The ink was placed directly over approximately 100–250 cells of the selective phenotype. The desired phenotype of the dotted cell groups was estimated to comprise at least 70% of all cells, and normal or hyperplastic cell groups were not contaminated with malignant cells. Slides were placed tissue side down on a UV transilluminator for 3 h, inactivating the DNA of all unprotected areas. Each dotted area was cut from the plastic slide, and its DNA was extracted into 30-μl of a Tris-HCl-EDTA, proteinase K solution. Approximately 12 μl of this solution were used for the c-Ki-*ras* PCR. A similarly sized unprotected tissue region from the same slide was analyzed in parallel as a UV inactivation control and was consistently negative for any PCR products.

Statistics. Fisher's exact test was used to evaluate statistical correlation between c-Ki-*ras* mutation and histology, grade, stage, and age. The log rank test was used to evaluate statistical correlation between c-Ki-*ras* mutation and patient survival. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Point mutations were detected in nine of 60 (15%) endometrial adenocarcinomas. There were five G to A transitions, three G to T transversions, and one G to C transversion. Seven mutations were identified in codon 12 and two in codon 13.

The clinicopathological parameters of all endometrial carcinoma cases are presented in Table 1. The mean age of the 60 patients was

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[3] The abbreviations used are: SURF, selective UV radiation fractionation; PCR, polymerase chain reaction.

Table 1 *c-Ki-ras oncogene in endometrial cancer: clinicopathological parameters*

Cases	No. examined	*ras* mutations
Total	60	9
Histology		
Endometroid	54	9
Papillary serous	6	0
Grade		
1	28	5
2	13	1
3	13	3
[a]	6	0
Stage		
I	36	6
II	3	0
III	11	3
IV	9	0
Unstaged	1	0
Age (yr)		
<40	9	0
41–50	8	0
51–60	24	5
>60	19	4
Race		
Latina	39	7
Afro-American	6	0
Caucasian	9	2
Asian	6	0

[a] Papillary serous tumors were not graded.

54 years (range, 21–83 years). The predominant race of our population was Latina (65% of the patients), followed by Caucasian (15%), Afro-American (10%), and Asian (10%). Endometroid adenocarcinoma made up 90% of the cancers sampled. Sixty % of the patients (36 of 60) had stage I lesions, 5% (3 of 60) had stage II lesions, 18% (11 of 60) had stage III lesions, 15% (9 of 60) had stage IV lesions, and 2% (1 of 60) had unstaged disease.

The clinicopathological parameters and specific c-Ki-*ras* mutation of each tumor with *ras* mutation are summarized in Table 2. All tumors with c-Ki-*ras* mutations were of the endometroid type. Although most of the endometrial carcinomas with mutant c-Ki-*ras* alleles were grade 1, stage I endometrial carcinomas and tended to have <50% myometrial invasion or no invasion, this difference was not significant. c-Ki-*ras* mutations were significantly more frequent in women older than 50 years ($P = 0.049$). No significant difference in patient survival between the two groups was determined ($P = 0.998$).

The topographical distributions of the mutant c-Ki-*ras* alleles in nine endometrial specimens were determined by SURF analysis. The presence or absence of the c-Ki-*ras* mutation in the specific histological regions of each mutant tumor is summarized in Fig. 1. Areas of hyperplasia without atypia were present in four of nine cases. Sections of hyperplasia with atypia were present in three of nine cases. c-Ki-*ras* mutations were detected only in areas of simple and complex hyperplasia with cytological atypia and in adenocarcinoma cells. c-Ki-*ras* mutations were consistently absent in benign epithelium and in simple and complex hyperplasias without atypia. In case one, the mutant allele was absent in tissues of simple hyperplasia without atypia but homogeneously present in the immediately adjacent tissues of simple hyperplasia with atypia, complex hyperplasia with atypia, and adenocarcinoma (Fig. 2). c-Ki-*ras* mutations were uniformly present in all areas of adenocarcinoma in eight of nine cases. Case 9 possessed two distinct cell phenotypes: grade 1 adenocarcinoma cells with squamous differentiation and grade 2 adenocarcinoma cells. Both phenotypes demonstrated myometrial invasion. The less differentiated cell type made up approximately 50% of the tumor and uniformly contained the c-Ki-*ras* mutation, while in the grade 1 areas of the tumor, the c-Ki-*ras* mutation was uniformly absent (Fig. 3).

DISCUSSION

Multiple mutations appear necessary for malignant transformation. Tumor progression has been best documented in colorectal neoplasms, with well-defined premalignant (adenoma) and malignant stages. In colorectal tumors, mutations appear to be sequentially acquired in association with the premalignant stages of tumor progression (13). Similarly, endometrial carcinoma appears to arise from a series of well-characterized progressive histological changes. Less is known about the genetic alterations necessary for endometrial carcinogenesis. The c-Ki-*ras* locus has been studied extensively in endometrial cancers. Point mutations at this locus are present in 10–37% of endometrial cancers (2–9). A similar frequency of mutation (15%) was observed in this study.

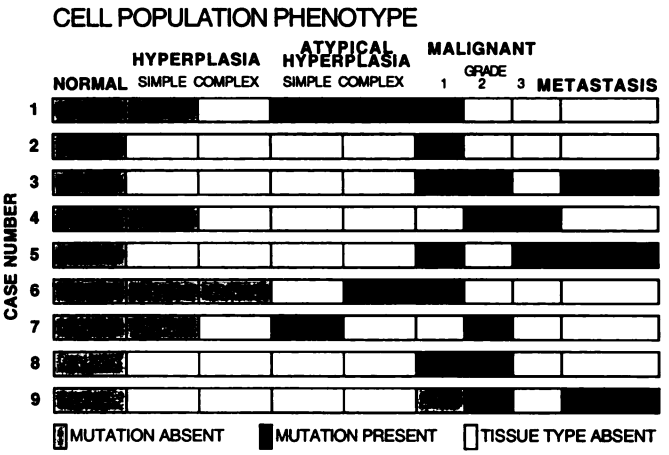
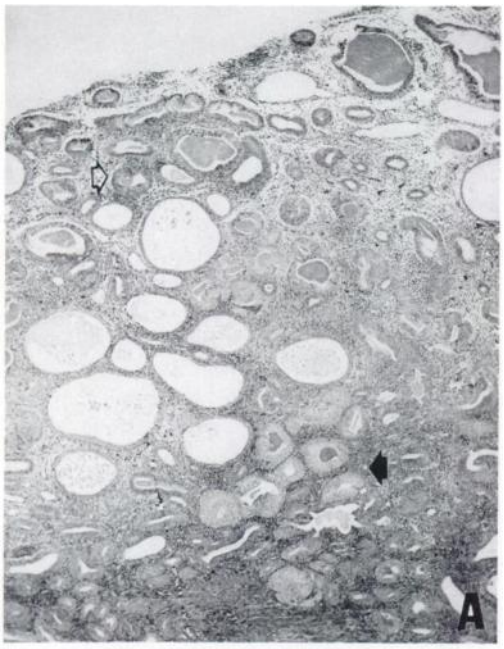


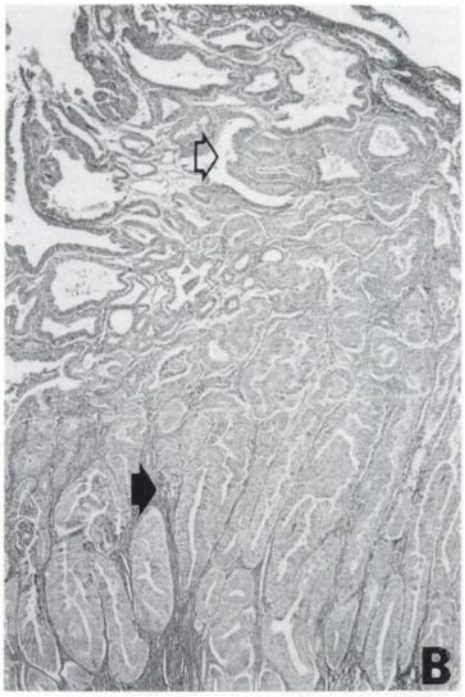
Fig. 1. Graphic representation of the topographical distributions of the c-Ki-*ras* mutations. These mutations were only detected in atypical hyperplasia and malignant cells.

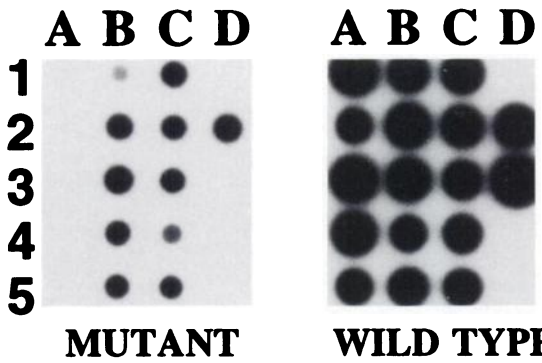
Table 2 *c-Ki-ras oncogene in endometrial cancer: clinicopathological parameters of tumors with mutations*

Case	c-Ki-*ras*	Histology	Grade	Surgical stage	Myometrial invasion (%)	Recurrence site (mo s/p surgery)	Status	Follow-up (mo)
1	G → A[a] (Asp)	Endometroid	1	IA	None	None	NED[b]	11
2	G → A[a] (Asp)	Endometroid	1	IB	<50	None	NED	20
3	G → T[a] (Val)	Endometroid	3	IIIC	>50	PAN (12)	AWD	25
4	G → A[c] (Asp)	Endometroid	3	IB	<50	None	NED	21
5	G → T[a] (Cys)	Endometroid	3	IIIC	<50	Vagina (7)	DOD	10
6	G → T[a] (Val)	Endometroid	1	IA	None	None	NED	12
7	G → C[a] (Ala)	Endometroid	1	IA	None	None	NED	46
8	G → A[c] (Asp)	Endometroid	1	IB	<50	None	NED	43
9	G → A[a] (Asp)	Endometroid	2	IIIC	<50	Liver (15)	DOD	15

[a] Codon 12.
[b] s/p, status post; PAN, periaortic nodes; NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease.
[c] Codon 13.







In endometrial hyperplasia, the precursor lesion to endometrial carcinoma, a similar frequency of activation (6–16%) has been observed. When present, the c-Ki-*ras* mutations are usually detected in atypical hyperplasia rather than simple or complex hyperplasia, suggesting that c-Ki-*ras* activation occurs before malignant transformation (4, 8, 9). Unfortunately, the natural history of premalignant lesions is variable and unpredictable. Only approximately 23% of patients with atypical hyperplasia and <2% with simple or complex hyperplasia will progress to cancer (14). Therefore, hyperplasia with c-Ki-*ras* mutations may or may not progress to cancer, and some hyperplasias without c-Ki-*ras* mutations may acquire the mutation coincident with or after malignant transformation.

These two possibilities can be distinguished by determining the topographic distribution of the c-Ki-*ras* mutations in carcinomas which harbor the mutation. If the mutation is acquired before the majority of tumor growth, there should be a homogeneous distribution of the mutation in the tumor. Detection of the mutation in the hyperplasia commonly adjacent to most cancers would indicate activation of the c-Ki-*ras* allele prior to malignant transformation. Conversely, a heterogenous distribution of the mutation in the tumor would indicate activation after transformation.

In the tumors with c-Ki-*ras* mutations, the mutations were confined to premalignant (atypical hyperplasia) and malignant cells and were absent from normal and simple or complex hyperplasias. The topographic distribution of the c-Ki-*ras* mutations was homogeneous throughout eight of nine cancers and their metastases. The c-Ki-*ras* mutations were also homogeneously present in the adjacent atypical hyperplasia of all three tumors with such tissues. Therefore, there is a direct genetic relationship between the premalignant and malignant endometrial epithelium and the activation of the c-Ki-*ras* locus, which likely occurred prior to clonal expansion of the atypical hyperplasias. The absence of atypical hyperplasia in the remaining tumors may indicate that malignant transformation occurred without a histologically recognizable premalignant stage. More likely, the precursor lesions were obliterated by the clonal tumor expansion. Nevertheless, the homogeneous distributions of the c-Ki-*ras* mutations in these tumors still indicated activation prior to clonal expansion. There were no similar genetic relationships between the mutant tumors and simple or complex hyperplasia without atypia, suggesting a more distant stage in carcinogenesis. This finding is consistent with the low frequency (<2%) with which patients with simple or complex hyperplasia without atypia progress to cancer.

The activation of c-Ki-*ras* appeared to occur after malignant transformation in one tumor (case 9) since it was present in only half of its mass. This tumor exhibited a similar histological dichotomy, and the mutation was homogeneously present in the grade 2 areas and the metastases and absent in all grade 1 areas. This homogeneous association of the mutation with the higher grade tumor and the metastasis suggests that the mutation arose in the lower grade portion with subsequent clonal tumor progression. A similar later onset of c-Ki-*ras* mutation and association with histologically distinct tumor regions has been previously reported in one endometrial cancer (4).

Fig. 2. *A*, photomicrograph of simple hyperplasia with atypia (*filled arrow*) and an adjacent area of simple hyperplasia without atypia (*open arrow*; case 1, hematoxylin and eosin, ×40). *B*, photomicrograph of complex hyperplasia with atypia (*open arrow*) and grade 1 adenocarcinoma (*filled arrow*; case 1, hematoxylin and eosin, ×40). *C*, dot blots of the c-Ki-*ras* products obtained from the SURF experiment of case 1. Simple hyperplasia without atypia was negative for the c-Ki-*ras* mutation (*A1–A5*), whereas simple hyperplasia with atypia was positive for the c-Ki-*ras* mutation (*B1*). Complex hyperplasia with atypia and grade 1 adenocarcinoma were positive for the c-Ki-*ras* mutation (*B2–C5*). No amplification was detected from an unprotected tissue region (*D1*), indicating complete inactivation by the UV radiation. Controls for the c-Ki-*ras* were a composite or unfractionated section (*D2*), normal DNA (*D3*), and a water blank (*D4*).

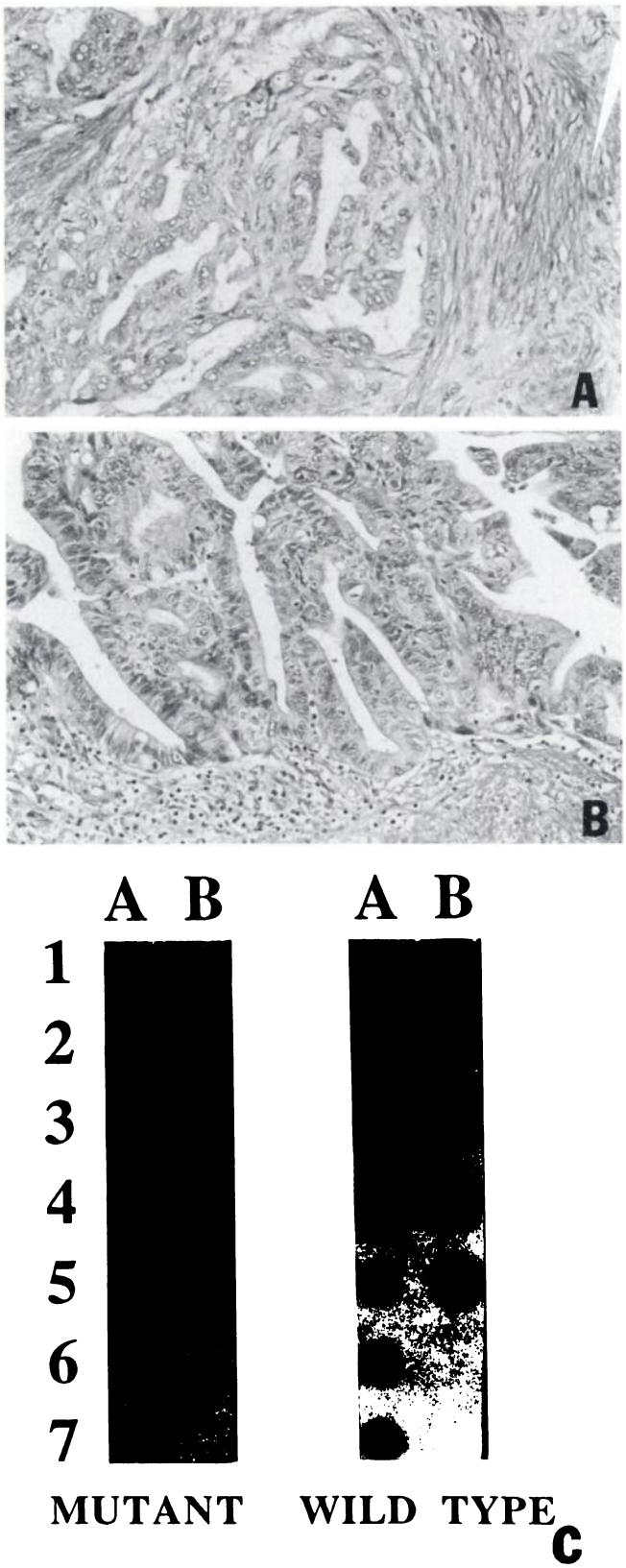


Fig. 3. *A*, photomicrograph of grade 2 adenocarcinoma (case 9, hematoxylin and eosin, ×200). *B*, photomicrograph of grade 1 adenocarcinoma (case 9, hematoxylin and eosin, ×200). *C*, dot blots of the c-Ki-*ras* products obtained from the SURF experiment of case 9. Areas of grade 2 adenocarcinoma were positive for c-Ki-*ras* mutations (*A1–A3*), whereas areas of grade 1 adenocarcinoma were negative (*A4–B3*). Controls for the c-Ki-*ras* were a composite or unfractionated section (*B4*), normal DNA (*B5*), and a water blank (*B6*).

There were no significant associations between clinical parameters and the c-Ki-*ras* mutation except for age >50 years. The clinical significance of this observation in our study is unclear; however, among patients with endometrial cancer without metastasis, age >55 years is an independent risk factor for recurrence (15). The lack of prognostic significance for the c-Ki-*ras* mutation observed in this study is in contrast to other studies which have indicated both poorer (6) or better (8) outcomes.

These results directly demonstrate that c-Ki-*ras* mutations arise prior to clonal expansion in the majority of endometrial carcinomas with this mutation. The absence of the mutation in simple or complex hyperplasia in these same tumors suggests that acquisition of this mutation is associated with histological atypia. Once activated, the mutant c-Ki-*ras* alleles are present throughout all subsequent stages of tumor progression, indicating a possible role in maintaining the malignant phenotype. Additional genetic alterations are likely responsible for other phases or phenotypes of tumor progression, and the mapping of these mutations on the same histological sections should increase our understanding of endometrial tumor evolution.

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