American Journal of Pathology, Vol. 146, No. 1, January 1995

Mutations of the Ki-*ras* Oncogene in Carcinoma of the Endometrium

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Mutations of the Ki-ras oncogene in endometrial carcinoma have been reported in Japan, but the prevalence and clinical significance of such mutations in the United States remains unclear. DNA extracted from paraffin sections of 112 carcinomas of the endometrium was amplified by the polymerase chain reaction with mismatched primers that generated a BstNI recognition site with the wild-type codon 12. Loss of this recognition site indicating Ki-ras codon 12 mutations was observed in 13 tumors (11.6%), including 11 endometrioid carcinomas, one undifferentiated carcinoma, and one carcinosarcoma. None of 17 papillary serous-clear cell carcinomas contained Ki-ras codon 12 mutations. These mutations were confirmed and characterized by direct sequencing. We found no evidence of correlation of the presence of Ki-ras mutations with stage, grade, depth of invasion, or clinical outcome. Our results indicate that Ki-ras oncogene mutations in carcinoma of the endometrium may be less prevalent in the United States than in Japan. (Am J Pathol 1995, 146:182–188)

Mutations of the Ki-*ras* oncogene have been implicated in the development of numerous human malignancies,[1] particularly colorectal carcinoma.[2] The frequent occurrence of carcinoma of the endometrium in patients with hereditary nonpolyposis colorectal cancer[3] provided an indication that these two neoplasms share common genetic pathways. The demonstration of other genetic alterations common to carcinomas of colon and endometrium[4] therefore raises the possibility that mutations in Ki-*ras* may be important in the development of endometrial carcinoma as well. Such mutations, particularly in codon 12, have in fact been reported in endometrial carcinomas.[5–9] Many such studies have been conducted in Japan, however, where the epidemiological and clinical characteristics of endometrial carcinoma differ from those in the United States.[10,11] One comparative study has indeed indicated a higher prevalence of Ki-*ras* mutations in endometrial carcinomas in Japan (23%) compared with the United States (12%).[12]

The prognostic significance as well as prevalence of Ki-*ras* mutations occurring in endometrial carcinoma in the United States remains uncertain. Some studies have indicated that mutations in codon 12 of Ki-*ras* are an independent, unfavorable prognostic factor,[9] whereas others have demonstrated the opposite.[12] Analyses of endometrial carcinoma that rely on high molecular weight DNA are hindered by the number of fresh specimens available for study.[5] This has particularly limited the analysis of the less common, more aggressive nonestrogen related carcinomas, including tumors with papillary serous or clear cell characteristics, making it difficult to compare this group with typical endometrial adenocarcinomas. We analyzed DNA from 112 archival formalin-fixed paraffin-embedded endometrial carcinomas, including typical "endometrioid" adenocarcinomas, carcinomas with papillary serous or clear cell features, carcinosarcomas, and undifferentiated carcinomas. Using polymerase chain reaction (PCR), we identified mutations in codon 12 of Ki-*ras* using an "amplified created restriction sites" (ACRS) method[13] and then confirmed and characterized these mutations with direct sequencing of the PCR products. We analyzed our results to determine the prevalence of Ki-*ras* mutations in our midwest United States population and whether such alterations could be correlated with histopathological or clinical parameters.

Supported in part by grants to TSF from the Harris Foundation and the American Cancer Society (IRG-40-33 to the University of Michigan Cancer Center), and by a grant to RFC from the Union Bank of Switzerland made possible by an anonymous donor.

Accepted for publication October 5, 1994.

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Materials and Methods

Case Selection

108 consecutive primary endometrial carcinomas from between 1988 and 1992 were identified from the computerized database of the University of Michigan Department of Pathology, to which were added six additional papillary serous carcinomas from 1993 and 1994. (Two carcinomas were subsequently excluded after failure of PCR amplification, presumably due to degradation of DNA in the fixed tissue). The racial composition of the study group consisted of 101 Caucasian patients and seven African-American patients, and four patients whose race was unknown to the investigators. Hematoxylin and eosin (H&E) stained slides from each case were reviewed for verification of tumor cell type, depth of invasion, and grade. Tumors were classified in accordance with the International Society of Gynecological Pathologists as typical glandular "endometrioid" adenocarcinoma, papillary serous-clear cell carcinoma (joined because of the frequent concurrence of both patterns), carcinosarcomas (classified as carcinomas in accordance with the findings of several recent investigations[14,15]) and undifferentiated carcinomas. Stage was assigned according to the surgical criteria of the International Federation of Gynecology and Obstetrics (FIGO).[16] Clinical outcome was tabulated for each patient for whom it was known at least 12 months after surgery.

DNA Extraction

Based on review of H&E-stained slides, two paraffin tissue blocks, one each of neoplastic endometrial and non-neoplastic tissue (usually uninvolved lymph nodes, occasionally uninvolved ovarian or cervical tissue) were selected from each case. Using a standard microtome with disposable blades, tissue sections of 4 μ thickness were cut from each block and placed on plain glass slides. Non-neoplastic tissue was selectively removed from unstained sections of carcinoma under a dissecting microscope using a serial H&E-stained section as a template guide. One tissue section from each case was transferred with a disposable razor blade to 100 μl of xylene in a 1.7-ml non-siliconized microcentrifuge tube. An equal volume of 100% ethanol was added, and the samples were pelleted (10 minutes in a countertop microcentrifuge), dried under vacuum, and digested overnight in 100 μl of 50 mmol/L TRIS pH 8.3 with 200 ng/μl proteinase K at 37 C. Samples were then boiled for 8 minutes, cooled on ice, and spun 5 minutes to pellet out debris. DNA extraction was performed in batches of 10 to 14 samples in which each batch included a reagent-only negative control.

PCR for Analysis of Mutations in Ki-ras Codon 12

Screening for a mutation in codon 12 of Ki-*ras* was performed using the ACRS method.[13] A 151-base pair (bp) segment of the Ki-*ras* gene was amplified using a pair of oligonucleotide primers in which the "upstream" primer was deliberately mismatched three bases from its 3′ end in order to generate a *Bst*NI restriction enzyme site (CCWGG, in which W can be either an A or T). In the resulting PCR product, the first three nucleotides (CCT) in this restriction site are derived from the 3′ end of the upstream oligonucleotide primer, and the last two (GG) are contributed by the first two bases of the wild-type codon 12 (Figure 1). Oligonucleotide primers were designed to anneal at a common temperature and to prevent primer dimers.[17] Each PCR was performed with 20 μl of crude DNA (⅕ of the extracted material from each slide) in a final 50-μl reaction mix contained 200 μmol/L of each dNTP, 2.5 ng/μl (~380 nmol/L) of each primer, one unit of Taq polymerase (Promega Biotec, Madison, WI), and 1× Taq polymerase buffer (with 1.5 mmol/L $MgCl_2$ supplied with the enzyme). PCR was performed with an initial 4-minute denaturation step at 94 C coupled to a repeating cycle of 1 minute at 94 C (denaturation), 2 minutes at 57 C (annealing), and 2 minutes at 72 C (extension) for 35 cycles, followed by a 7-minute "completion" step at 72 C.

Five units of *Bst*NI (New England Biolabs, Beverly, MA) were added to 10 μl of PCR-amplified product using buffer provided with the enzyme in a 20 μl volume at 60 C for at least 4 hours. 18 to 20 μl of PCR product were electrophoresed through gels consisting of 1.5% NuSeive (FMC Bioproducts, Rockland, ME) and 1.5% agarose (Gibco-BRL, Grand Island, NY) with 0.5 μg/ml ethidium bromide and were visualized with ultraviolet illumination. In the absence of a mutation in Ki-*ras* codon 12, digestion with *Bst*NI yielded a fragment of 129 bp (and another of 22 bp, too small to be visualized on an agarose gel). Mutation of the 12th codon of Ki-*ras* resulted in loss of this recognition site. In a carcinoma in which one copy of Ki-*ras* contained a mutation in codon 12 and the other was wild-type, both cut (129 bp) and uncut (151 bp) bands were apparent on an agarose gel (Figure 1).

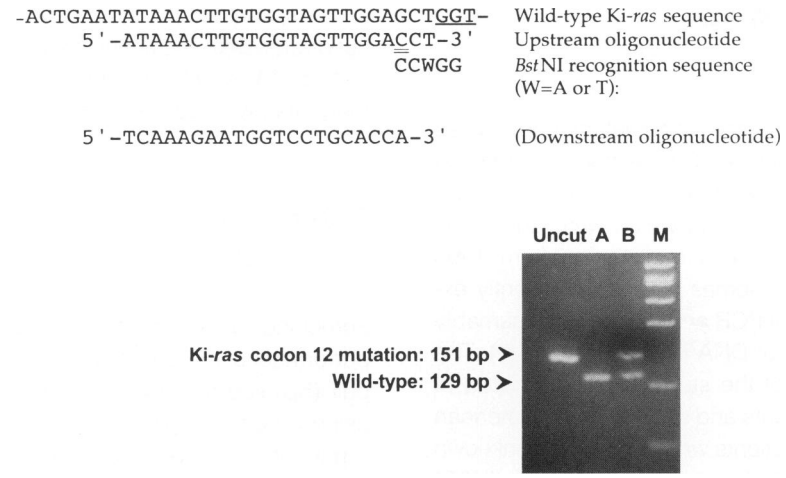

Figure 1. *Screening for mutations in Codon 12 of Ki-ras using ACRS. (Top) Oligonucleotide primers used in PCR-ACRS analysis. A* BstNI *site is introduced into the PCR product by a deliberate mismatch three bases from the 3' end of the upstream oligonucleotide primer (double-underlined) in order to generate a* BstNI *restriction enzyme site with the wild-type codon 12. A mutation in either of the first two bases of codon 12 of the Ki-*ras *gene (underlined) results in loss of this restriction recognition site. The downstream oligonucleotide primer is also shown. (Bottom) Demonstration of loss of created* BstNI *site indicating Ki-*ras *codon 12 mutation. PCR products derived from the wild-type Ki-*ras *codon 12 are entirely digested by* BstNI*, as in tumor A. Mutation one copy of Ki-*ras *codon 12 is indicated by an uncut (151-bp) band, as in tumor B. Undigested PCR product ("uncut") and a molecular weight size marker PhiX174-HaeIII ("M") are shown. PCR product from tumors containing evidence of a Ki-*ras *mutation by this method were subsequently sequenced for confirmation.*

*Confirmation and Characterization of Ki-*ras *Codon 12 Mutations by Automated Sequence Determination*

The presence of Ki-*ras* codon 12 mutations was confirmed by automated sequencing by the University of Michigan's DNA Sequencing Core Facility using an automated fluorescent sequencer (Applied Biosystems, Inc., Foster City, CA), which required that the sequence of the PCR product containing the mutant sequence be the predominant moiety. Because the PCR products consisted of both mutated and wild-type codon 12 sequences, a second PCR was performed using 5 μl of *Bst*NI-digested PCR product as a template with the same primers and conditions as above. Because the wild-type PCR product had been enzymatically cleaved it could not serve as a template for logarithmic amplification, so that the PCR product containing the mutation was selectively amplified. Unincorporated nucleotides and primers were subsequently removed by filtration through an Ultrafree-MC 30,000 filter (Millipore, Bedford, MA) and the sequence of the PCR product was determined using an internal primer 5′-CTCTATTGTTGGATCATATT-3′. Because of the proximity of the mutation to the 5′ end of the "sense" strand of the PCR product, only the nonsense strand was sequenced. As controls, four (undigested) PCR products from tumors that demonstrated complete digestion with *Bst*NI were sequenced to confirm the absence of mutations in codon 12.

Statistical Analysis

The significance of differences in the frequency of Ki-*ras* mutations with regard to clinical and pathological parameters was determined by Fisher's exact test and the χ^2 test, performed on a Macintosh Centris 650 using Statview version 4.01 (Abacus Concepts, Berkeley, CA). The presence of a mutation in codon 12 of Ki-*ras* was tested for significant association with tumor cell type, FIGO grade, depth of invasion, age at diagnosis, stage (stage I *versus* II–IV), and clinical outcome. A Kaplan-Meier curve was plotted as a function of recurrence for patients whose tumors did or did not demonstrate a Ki-*ras* codon 12 mutation.

Results

Clinical and histopathological data are correlated with the results of Ki-*ras* codon 12 mutation analysis in Table 1.

Histology

The 112 primary endometrial carcinomas used for analysis included 87 cases of typical glandular "endometrioid" adenocarcinoma, 17 tumors with papillary serous or clear cell features (or both), three carcinosarcomas, and five undifferentiated carcinomas.

Table 1. *Clinical and Pathological Features of Endometrial Carcinomas with Ki-ras Codon 12 Mutations*

Tumor type	Months since surgery (follow-up/ recurrence)	Status	Age	Stage	Grade	Depth of invasion	Ki-*ras* codon 12 GGT (glycine)
Endometrioid	19	NED*	79	I	3	outer 3rd	GAT (aspartic acid)
Endometrioid	19	NED	66	I	2	outer 3rd	AGT (serine)
Endometrioid	26	NED	52	II	1	inner 3rd	GAT (aspartic acid)
Endometrioid	26	NED	41	I	2	middle 3rd	GAT (aspartic acid)
Endometrioid	28	NED	60	I	1	none	GCT (alanine)
Endometrioid	30	unknown	58	II	3	outer 3rd	GAT (aspartic acid)
Endometrioid	34	NED	70	I	1	inner 3rd	GAT (aspartic acid)
Endometrioid	36	NED	50	I	1	inner 3rd	TGT (cysteine)
Endometrioid	37	NED	65	I	1	inner 3rd	GCT (alanine)
Endometrioid	44	AWD†	61	I	2	inner 3rd	TGT (cysteine)
Endometrioid	61	NED	72	I	2	none	GTT (valine)
Undifferentiated	2	DOD‡	54	I		outer 3rd	TGT (cysteine)
Carcinosarcoma	39	NED	67	I		inner 3rd	GCT (alanine)

*No evidence of disease.
†Alive with disease.
‡Dead of disease.

Clinical Information and Patient Course

The average age at the time of diagnosis ranged from 31 to 87 years (average 62.5 years). After exclusion of one patient who died of intercurrent disease, clinical outcome at least 12 months after surgery was known for 91 patients. Of these, one patient was alive with recurrent carcinoma, 22 had died of carcinoma, and 68 were alive with no evidence of disease (from 14 to 69 months following surgery, average 34.2 months). The clinical and pathological features of the cases analyzed are summarized in Table 2.

*Analysis of Mutations in Ki-*ras *Codon 12*

PCR-ACRS analysis demonstrated the presence of a Ki-*ras* codon 12 mutation in 13 of 112 (11.6%) of the carcinomas analyzed. Ki-*ras* codon 12 mutations were present in 11 of 87 (12.6%) endometrioid carcinomas but none of the 17 papillary serous-clear cell carcinomas. This difference did not achieve statistical significance. Ki-*ras* codon 12 mutations were also present in one each of five undifferentiated carcinomas and three carcinosarcomas. All of the samples containing a mutation also demonstrated a wild-type PCR band of equal intensity at 129 bp, consistent with mutation in only one copy of Ki-*ras*. When characterized by sequencing four cases contained mutations in the first position, normally occupied by a guanine residue (thymine in three cases and adenine in one). Nine cases were mutated in the second position, also normally occupied by a guanine (thymine in one case, adenine in five cases, and cytosine in three cases). No case contained mutations in more than one base of codon 12. Sequencing of the PCR product from four control cases that had demonstrated complete digestion with *Bst*NI confirmed the absence of mutations. Clinical and pathological details of the 13 cases with mutations of Ki-*ras* codon 12 are summarized in Table 1.

There was no evidence of significant association of Ki-*ras* mutation with FIGO grade, depth of invasion, age at diagnosis, stage, or clinical outcome (whether analyzed in aggregate or separated by tumor cell type). A Kaplan-Meier curve did not demonstrate any significant difference between patients whose neoplasms contained or did not contain a mutation in Ki-*ras* codon 12 (not shown).

Discussion

Ki-*ras* is a member of the *ras* family of oncogenes that encode closely related proteins that act as signal transducers within eukaryotic cells.[18] It was recently discovered that the Ki-*ras* gene product regulates a mitogen-activated protein kinase cascade by interacting with another oncogene product, Raf.[19] Although point mutations in codon 12 of the *ras* genes may be the most frequent oncogene mutation identified in human tumors, the prevalence of such alterations varies with tumor type. Ki-*ras* mutations have been reported in 75 to 95% of pancreatic adenocarcinomas, 40 to 47% of colon adenocarcinomas, and 33% of pulmonary adenocarcinomas.[1] Mutations in the *ras* gene family have been reported less frequently in gynecological malignancies, and mostly occur in codon 12 of Ki-*ras* rather than codons 13 or 61 of Ki-*ras*[7,20–23] or codons 12, 13, or 61 of Ha-*ras* or N-*ras*,[7,8,23,24] with the exception of squamous cell carcinomas of the cervix in which mutations in Ha-*ras* have been reported in 11%.[25]

Table 2. *Correlation of Clinical and Pathological features of Endometrial Carcinomas Analyzed for Ki-*ras *Codon 12 Mutations*

Parameters	Number of cases	Cases with Ki-*ras* mutations (percentage of cases to left)
Overall	112	13 (11.6%)
Type		
Endometrioid	87	11 (12.6%)
Papillary Serous/Clear Cell	17	0 (0%)
Carcinosarcoma	3	1 (33%)
Undifferentiated	5	1 (20%)
FIGO grade (endometrioid)		
1	54	6 (11%)
2	17	3 (18%)
3	16	2 (13%)
Depth of invasion		
Unknown	2	0
None	22	2 (9%)
Inner third	39	6 (15%)
Middle third	21	1 (5%)
Outer third	28	4 (14%)
Surgical stage		
Unknown	5	0
I	72	10 (13.9%)
II	12	2 (17%)
III	16	1 (6%)
IV	6	0 (0%)
Age at diagnosis (years)		
<40	3	0 (0%)
41–50	12	2 (17%)
51–60	25	4 (16%)
61–70	44	5 (11%)
>70	28	2 (7%)
Clinical Status:		
<12 months after surgery, dead of intercurrent disease, or unknown	21	1 (5%)
No evidence of disease >12 months following surgery	68	10 (14.7%)
Recurrent carcinoma (alive)	1	1 (50%)
Dead of disease	22	1 (5%)

We identified Ki-*ras* codon 12 mutations in only 11.6% of our cases of endometrial adenocarcinoma, in contrast to recent studies from Japan, which report such alterations in a range from 22 to 34% of their samples.[8,23] Given that our patient population was predominantly Caucasian (and included no Asian patients), differences between these observations and our own may reflect true differences between patient populations in the United States and Japan. Such differences include the rarity of endometrial carcinoma in the Japanese population compared with the United States, with an incidence of 2 to 3/100,000 (women) in Japan compared with 15 to 25/100,000 in the United States.[9] Histopathological differences exist as well, with endometrial tumors in Japan having a tendency toward higher grade, myometrial and vascular invasion, and higher incidence of malignant adenosquamous carcinoma.[10] In fact, a recent study[12] specifically comparing Ki-*ras* codon 12 mutations in Japanese and American patients found mutations in 5 of 41 (12%) tumors from American patients compared with 10 of 43 (23%) from Japanese patients. The differences in incidence of Ki-*ras* mutations identified in endometrial adenocarcinomas suggest a biological basis for the observed differences in the incidence and histopathology of endometrial carcinoma between these two countries.

Evidence that mutation in Ki-*ras* occurs early in the development of colonic adenocarcinoma comes from studies that have identified such mutations in premalignant colon adenomas as frequently as in colon carcinomas.[2] Attempts to define where in the development of endometrial neoplasia such alterations occur have yielded differing results. Enomoto et al[23] reported finding mutations in Ki-*ras* in two of 16 cases of atypical hyperplasia but not in any of six cases of complex hyperplasia without atypia nor 12 cases of simple hyperplasia, suggesting that mutations in Ki-*ras* do not occur before the development of frank malignancy. In contrast, Sasaki et al[12] found Ki-*ras* mutations in 14 of 89 cases of endometrial hyperplasia, including two cases of simple, six of complex, and six of atypical hyperplasia, suggesting that such mutations occur early in endometrial neoplasia. Too few of our endometrioid carcinomas with Ki-*ras* mutations had areas of residual hyperplasia available for analy-

Table 3. *Summary: Prevalence of Ki-*ras *Codon 12 Mutations in Histological Subtypes of Endometrial Adenocarcinoma*

Reference	Ki-*ras* Codon 12 Mutations in endometrioid carcinomas*	Ki-*ras* Codon 12 mutations in Papillary serous-clear cell carcinomas
22	7/38 (18%)	0/2
9	5/44 (11%)	1/3
8	8/36 (22%)	0/5
12	15/72 (20.8%)	0/12
23	5/16 (31%)	1/2
This study	11/87 (12.6%)	0/17
Total	51/293 (17.4%)	2/41 (5%)

*excludes adenosquamous carcinomas

sis, but the identification of other genetic alterations in endometrial hyperplasia (with and without atypia or concurrent carcinoma) is the subject of ongoing study in our laboratory.

An intriguing finding was the absence of mutations in any of the 17 papillary serous-clear cell carcinomas analyzed. Although this difference does not achieve statistical significance because of the relative infrequency of Ki-*ras* mutations in the endometrioid carcinomas, combining our results with those of several recent studies[8,9,12,22,23] (Table 3) indicates that a significant difference in the prevalence of Ki-*ras* codon 12 mutations may indeed exist between endometrioid and papillary serous-clear cell carcinomas (χ^2, $P = .0398$). Although it has been recognized that papillary serous-clear cell carcinomas are as a group histologically and clinically distinct from typical endometrioid carcinomas, the genetic and biochemical bases for these differences have not been elucidated. In conjunction with other data,[26] this study provides further characterization of the biological differences between these two types of endometrial carcinomas.

We found no evidence of any statistically significant correlation of Ki-*ras* mutations with FIGO grade or depth of invasion among the patients with endometrioid carcinoma. Similarly, among the 91 patients for whom clinical outcome was known, we found no evidence of correlation of age at diagnosis, stage, or clinical outcome with the presence of Ki-*ras* mutations. Most studies likewise have found no evidence of such a relationship,[8,12,23] although one study has implicated Ki-*ras* mutation as an unfavorable prognostic factor,[9] and another has suggested that such alterations are associated with an improved prognosis.[12] Our findings are analogous to those derived from analyses of Ki-*ras* mutations in carcinomas of colon, lung, and pancreas that have also failed to consistently demonstrate clinical significance of such genetic alterations.[1,27] Although of little prognostic significance in individual cases, the identification of Ki-*ras* mutations in carcinomas of the endometrium contributes to the understanding of the molecular mechanisms that underlie the development of this common malignancy.

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