K-ras mutations appear in the premalignant phase of both microsatellite stable and unstable endometrial carcinogenesis

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Abstract
Aims—Sequential events of endometrial tumorigenesis can be studied by comparison of genetic lesions seen in normal, premalignant, and malignant tissues. The distribution of k-ras mutations in microsatellite stable and unstable premalignant lesions was studied to determine whether this gene is implicated in both tumorigenic pathways.
Methods—K-ras mutations were analysed by polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) and direct sequencing in matched endometrial normal, premalignant (atypical hyperplasias), and adenocarcinoma tissues from individual patients. Identification of precancers solely by their appearance as atypical endometrial hyperplasias is very subjective; therefore, in addition to histopathological assessment, we performed molecular testing (non-random X inactivation or clonal altered microsatellites) for an expected feature of precancers—that is, monoclonality .
Results—Equivalent K-ras mutation frequencies were seen in microsatellite stable (six of 33) and unstable (three of 23) cancers. In both types, K-ras mutation in monoclonal precancers usually corresponded to a change from normal to an equivocal (two of 12) or hyperplastic (10 of 12) histology. Divergent K-ras genotypes among multiple neoplastic tissues of individual patients (two of six patients) are exceptions explained either by multicentric premalignant disease, or acquisition of K-ras mutation late in neoplastic progression.
Conclusions—K-ras mutation occurs in both premalignant microsatellite stable and unstable endometrial neoplasia, sometimes before acquisition of features readily diagnostic as atypical endometrial hyperplasia.

(*J Clin Pathol: Mol Pathol* 1999;**52**:257–262)

Endometrial carcinogenesis is a multistep process, the intermediate stages of which can be discerned as physically distinctive precursor lesions. These interim stages, if accessible for genetic analysis, can provide insights into the sequence of events leading to endometrial adenocarcinoma: especially if normal, premalignant, and malignant tissues are studied in individual patients. Many of the common genetic changes seen in endometrial cancers, such as K-ras mutation[1 2] and microsatellite instability,[1 2] have been observed in endometrial hyperplasias, often equated with putative endometrial precancers. However, the pathological diagnosis of atypical endometrial hyperplasia is notoriously hard to reproduce,[3 4] and despite efforts to standardise criteria and nomenclature, complicates attempts to understand the genetics of premalignant endometrial disease. The recent demonstration that some atypical endometrial hyperplasias are monoclonal[1 2 5 6] suggests that premalignant hyperplasias are neoplasms that differ from their malignant sequelae in the ability to invade adjacent tissues. As might be anticipated, some monoclonal endometrial putative precancers have contentious histologies,[2] but their role as precancers is reinforced by the demonstration that they are progenitors of malignant tissues.[1] For these reasons, we and others have developed and extensively
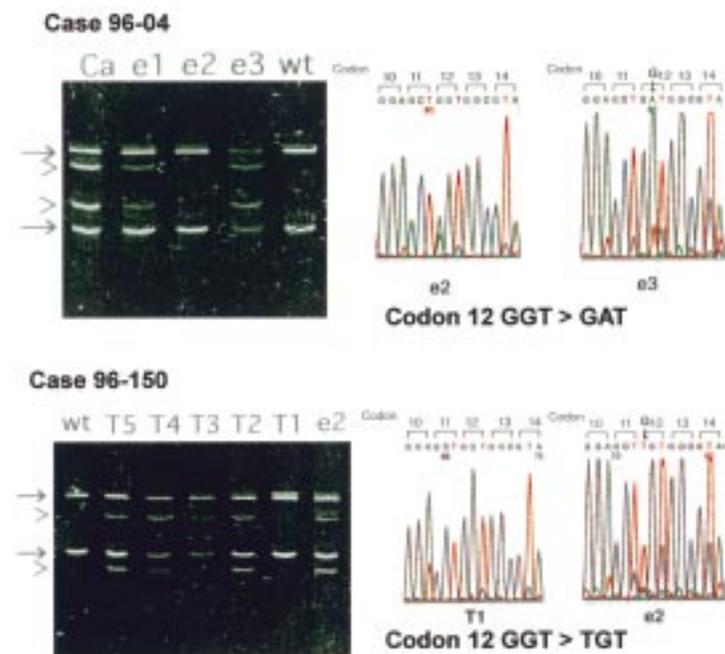

Figure 1   K-ras mutation detection by polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) and direct sequencing. Representative results from a non-radioactive SSCP–PCR K-ras mutational screen are shown alongside sequence waveforms confirming the associated primary sequence. In the ethidium bromide stained SSCP polyacrylamide gels (left) arrows denote the positions of sense and antisense strands of the wild-type (wt) sequence, and aberrant bands (mutant sequence) are indicated by an arrowhead. Histology and clonal analysis results for case 96-04 are shown in fig 2, and those for case 96-150 in fig 3. DNA was isolated from paraffin wax embedded tissues and the K-ras codon 12 region was amplified and PCR products resolved on an SSCP gel as described. Aberrant PCR products were sequenced by dye terminator cycle sequencing with results shown on the right.

Table 1   Distribution of mutations in 56 endometrial adenocarcinomas

Tumour feature	n	K-ras mutations	p53 mutations
Histological grade			
Grade I	39	6	0
Grade II	6	1	0
Grade III	11	2	3
MI	23	3	1
MS	33	6	2

MI, microsatellite instability; MS, microsatellite stability.

applied[1 5 7 8] polymerase chain reaction (PCR) based clonality assays as a means of identifying monoclonal putative endometrial precancers, irrespective of their histological appearance.

Approximately 17–23%[9–13] of sporadic endometrial cancers have microsatellite instability (MI), acquired in the precancerous stages.[1 2] Evidence that MI tumours have accelerated mutation rates in non-repeat sequences,[14 15] and the observation of frequent mutations, such as p53 and K-ras,[16 17] shared between microsatellite stable (MS) and MI tumours raises the intriguing possibility that although MI is the most visible manifestation of the MI phenotype, its primary effect in promoting tumorigenesis might be an indirect one, modulated by acceleration of mutations at non-repetitive targets. Although MI and MS endometrial cancers have approximately equivalent frequencies of some mutations, such as K-ras,[17] the 10q23 linked gene PTEN, which is mutated in 34–50%[18 19] of endometrial cancers, is implicated more often in MI than MS cancers.[18]

We have assembled a series of MI and MS endometrial adenocarcinomas, and studied K-ras and p53 mutations in associated malignant, premalignant, and normal endometrial tissues.

Methods
CASE SELECTION
One hundred and fifty eight archival paraffin wax embedded hysterectomy specimens were identified from the division of women's and perinatal pathology at Brigham and Women's Hospital, Boston, MA, after meeting the following criteria: (1) histologically confirmed endometrioid-type endometrial adenocarcinoma was present in the hysterectomy; (2) areas of histologically non-malignant endometrium were present in the hysterectomy; and (3) paraffin wax blocks were available. Cases suitable for clonal analysis were identified by testing normal (myometrial) tissues for a heterozygous genotype at the X-linked marker used for X inactivation studies (human androgen receptor gene; HUMARA) and malignant (carcinoma) tissues for presence of MI. Thirty three MS cases and all 23 MI cases were accepted for complete analysis as follows.

DNA ISOLATION AND CLONAL ANALYSIS
DNA was isolated from 7 μm thick paraffin wax embedded sections by selective ultraviolet irradiation, proteinase digestion, organic extraction, and ethanol precipitation, as described previously.[2] Paired tumour/normal DNAs from each case were screened with primers for two tetranucleotide repeat (T3.1 and T5.2) loci found previously to contain novel alleles in most (100%) MI endometrial adenocarcinomas.[1] Those tumours with at least one novel allele in the screen underwent an expanded testing with a panel of 10 microsatellites. Tumours with novel alleles in two or more of 10 studied microsatellites were scored as MI, and the full 10 loci studied in a similar manner in DNAs isolated from non-malignant areas of the endometrium. Non-malignant areas were scored as monoclonal if at least one novel allele was clonally present relative to normal reference myometrium. The 10 sets of PCR primers and their loci listed below by locus/primer/laboratory identifier were amplified and resolved under conditions described previously[1]: D1S518/GATA7C01/T1.2; D2S1384/GATA52A04/T2.4; D2S1399/GGAA20G04/T2.3; D3S2387/GATA22G12/T3.1; D3S2459/GATA68D03/T3.4; D4S1627/GATA7D01/T4.1; D5S1505/GATA62A04/T5.2; D5S816/GATA2H09/T5.3; D8S1132/GATA26E03/T8.2; and D21S1435/GATA49E01/T21.1.

DNA from normal myometrium of all patients was amplified at the HUMARA trinucleotide repeat locus using primers

Table 2   DNA from non-malignant endometrium (non-CA) of patients with K-ras mutated tumours

Case number	MS/ MI	K-ras mutation	Non-CA mutation	Non-CA tissue	Non-CA diagnosis	Non-CA clonality
94-154	MS	12 GGT→GTT	+	a	Atypical hyperplasia	Monoclonal
96-04	MS	12 GGT→GAT	+	E1	Atypical hyperplasia	Polyclonal
			wt	E2	Normal	Polyclonal
			+	E3	Atypical hyperplasia	Monoclonal
96-06	MS	12 GGT→GTT	wt	E1	Atypical hyperplasia	Monoclonal
			+	E2 (prior)	Insufficient tissue	Monoclonal
			+	E3 (prior)	Atypical hyperplasia	Monoclonal
96-12	MI	12 GGT→GTT	wt	E1	Reactive	Polyclonal*
96-41	MS	12 GGT→GTT	+	E1	Atypical hyperplasia	Monoclonal
			+	E2	Atypical hyperplasia	Monoclonal
			+	E3 (prior)	Atypical hyperplasia	Monoclonal
96-51	MS	12 GGT→GAT	wt	E1	Atypical hyperplasia	Monoclonal
			+	E2	Unclassified	Monoclonal
			wt	E3 (prior)	Atypical hyperplasia	Monoclonal
96-67	MS	12 GGT→GTT	wt	E1	Normal	Polyclonal
96-100	MI	12 GGT→GTT	wt	E1	Normal	Polyclonal*
96-150	MI	12 GGT→TGT	wt	E1	Normal	Polyclonal*
			+	E2	Atypical hyperplasia	Monoclonal*
			wt	E3	Reactive	Polyclonal*

"Prior" designations refer to pre-hysterectomy curettings, whereas remaining non-malignant tissues were harvested from the hysterectomy specimen. Non-CA mutation refers to the genotype of the non-malignnt tissue and is shown as as either wild-type (wt) or with the mutation seen in the corresponding cancer (+).
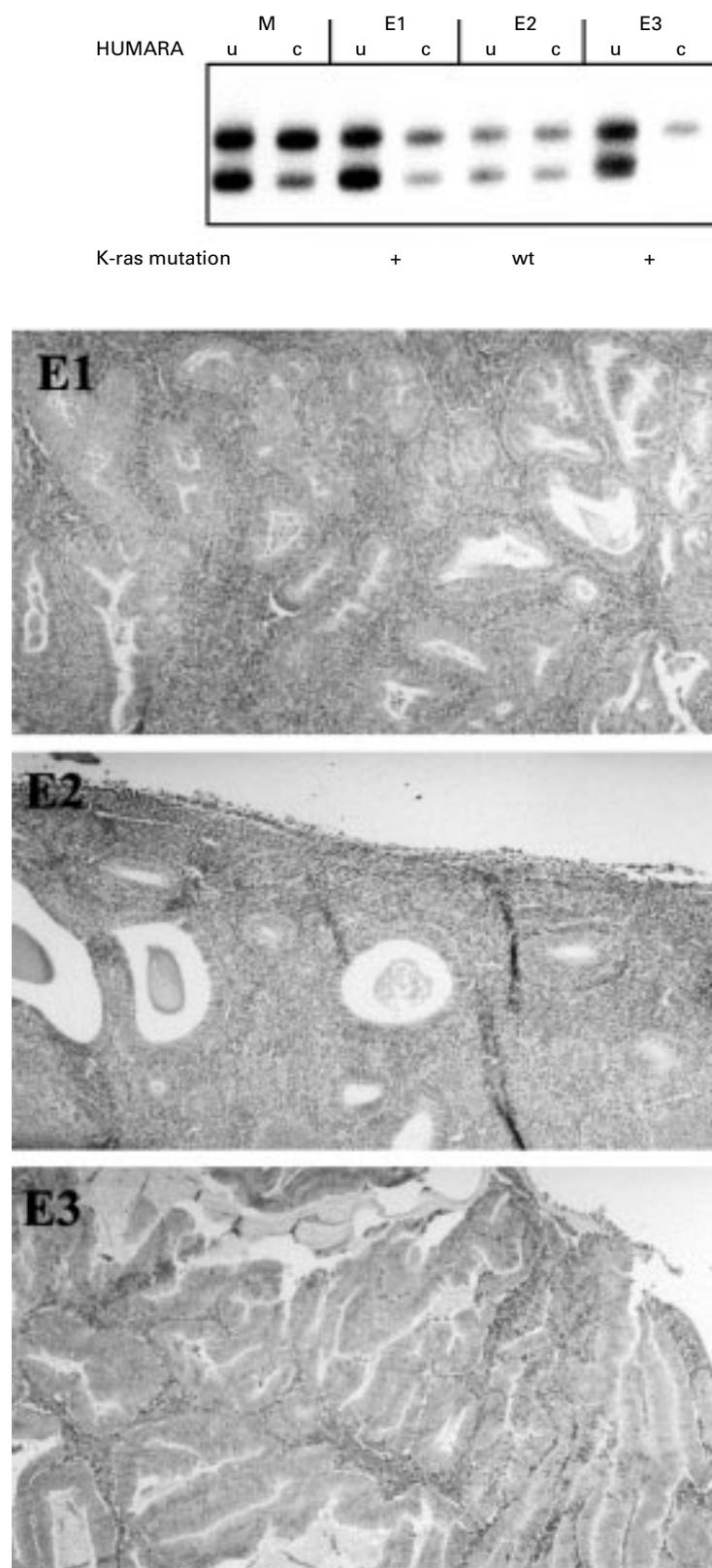MI, microsatellite instability; MS, microsatellite stability.

Figure 2 K-ras mutation corresponds to acquisition of a hyperplastic phenotype. Three endometrial tissues from a single patient (case 96-04) illustrate a range of endometrial histologies from normal inactive endometrium with a wild-type K-ras genotype (E2) to two unambiguous atypical endometrial hyperplasias (E1 and E3) with codon 12 K-ras mutations identical to those seen in the accompanying carcinoma. Haematoxylin and eosin stained photomicrographs below. K-ras codon 12 sequencing results in the upper panel of fig 1 show a codon 12 GGT→GAT transition. Abundant contaminating polyclonal stromal cells in tissue E1 (only one third of the tissue was epithelium) prevented the HUMARA assay from detecting the presumably monoclonal precancer, not a problem in the more densely packed glandular lesion of E3, which was demonstrably monoclonal. Clonality assay results shown in upper panel are polymerase chain reaction products of HUMARA gene amplified with (cut (c)) or without (uncut (u)) HhaI predigestion. Control polyclonal endometrium from the same patient is also shown (M).

AR-a/b.[5] Alleles resolved by a minimum of 3 mm on non-denaturing polyacrylamide gel electophoresis (PAGE) were judged to be informative for X inactivation analysis. Informative cases underwent full X inactivation analysis of DNAs selectively isolated from the tumour and representative areas of other endometrial types present. HUMARA PCR (primers AR-a/b[5]) of HhaI predigested and undigested matched myometrial/endometrial DNAs was performed in the presence of ^{32}P-TTP, substituting 7-deaza-2'-dGTP for dGTP during amplification,[8] and products resolved by non-denaturing 8% PAGE followed by autoradiography. Scoring of paired normal/tumour results was based on visual assessment of band patterns validated previously in a study comparing results of quantitative and visual analysis of HUMARA data.[2 7]

K-ras AND p53 MUTATION ANALYSIS

Tumour DNA was screened for p53 and K-ras mutations by non-radioactive single strand conformation polymorphism (cold SSCP), followed by confirmatory direct sequencing. Exons 5, 6, 7, and 8 of the p53 gene and exon 1 of the K-ras gene were amplified individually by PCR using published primer sequences.[20 21] Cold SSCP was performed as described previously.[22] A mixture consisting of 5 µl of PCR product (equivalent to 20–200 ng of DNA), 0.2 µl 1 M methylmercury hydroxide, 3 µl of loading buffer (15% ficoll of Mr 40 000, 0.25% bromophenol blue, and 0.25% xylene cyanol), and 13.6 µl of 1× TBE (Tris/borate/ EDTA) buffer was prepared to yield a total volume of 20 µl. This mixture was heated to 90°C for four minutes to denature double stranded DNA and then plunged into ice before loading. The entire 20 µl was loaded on to 18% polyacrylamide TBE gels and run at 25°C for exon 8 of p53 and exon 1 of K-ras, at 10°C for exon 7 of p53, and at 35°C for exons 5 and 6 of p53. The gels were stained with ethidium bromide. Samples that showed one or more band migrated apart from the wild-type bands by cold SSCP analysis were analysed further by direct sequencing. The variant bands were selected from the gels and reamplified. PCR products were gel purified by electrophoresis on 10% polyacrylamide TBE gels and sequences were determined by direct sequencing using the dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, California, USA) and a model 310 genetic analyser (Perkin-Elmer). DNA isolated from non-cancerous endometrial tissues in those 11 patients with confirmed mutations was then isolated, and tested for the presence of mutations by cold SSCP analysis and subsequently confirmed by direct sequencing. Mutation results were correlated with histopathology and the clonal growth pattern of the sampled area.

Results

The distribution of K-ras mutations (fig 1) was similar among all grades of endometrial adenocarcinomas (table 1), with an overall rate of 16% (nine of 56) comparable to the 12–25%
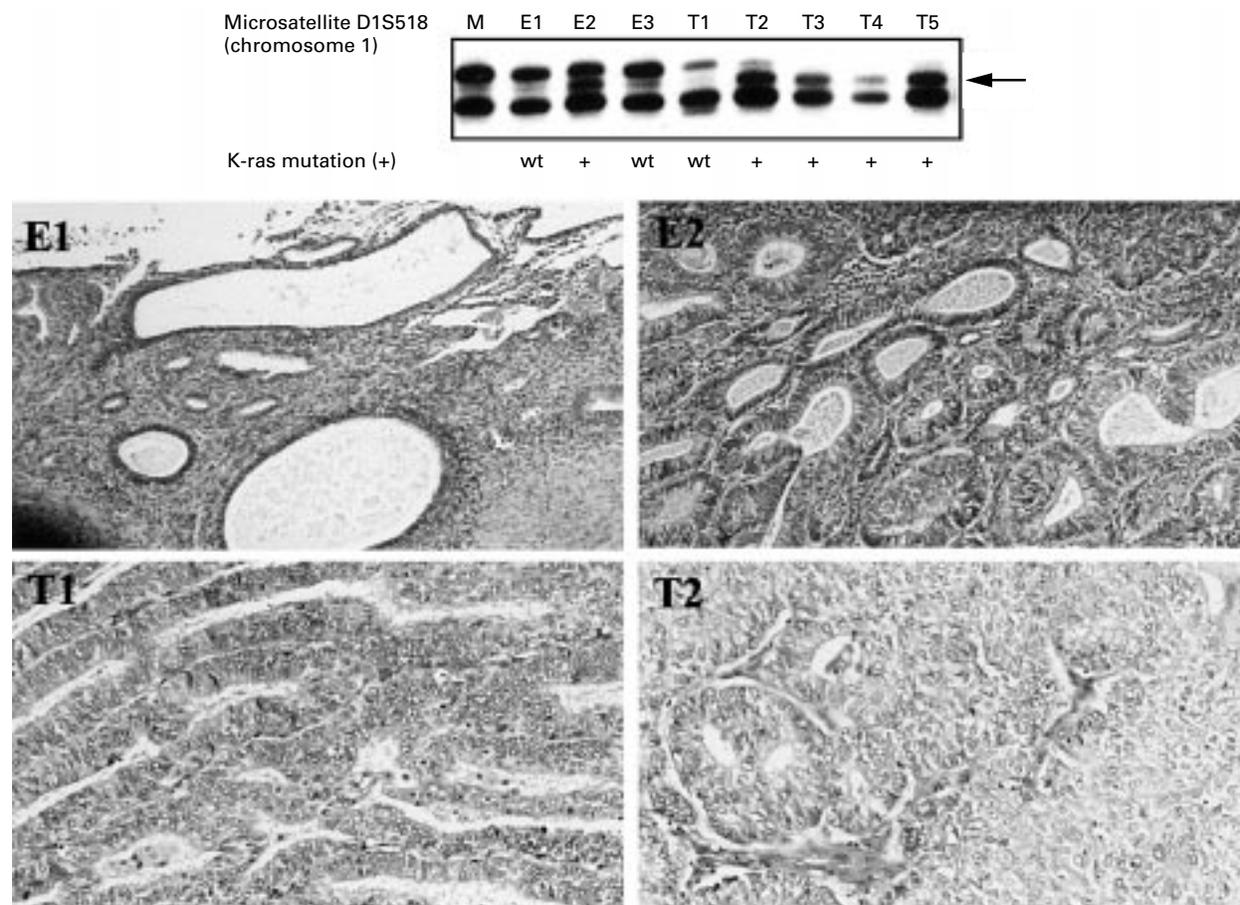
Figure 3   K-ras heterogeneity in microsatellite unstable neoplastic endometrium. Heterogeneity of K-ras genotypes among endometrial tissues segregates in case 96-150 into genotypic clusters defined by tetranucleotide microsatellite markers, such as D1S518 on chromosome 1 (upper panel), and presence or absence of K-ras mutation (fig 1, lower panel). Constitutive heterozygous alleles seen in normal myometrium (M) are retained in most lesional tissues, which vary in the appearance of a novel allele indicated by the arrow. Normal atrophic (E1 and E3) endometrium in case 96-150 had no structurally altered microsatellites, unlike histologically malignant (T1–5) and hyperplastic (E2) tissues. Monoclonality in neoplastic tissues E2 and T1–5 (T1 had novel alleles at other loci not shown) is apparent by the clonal propagation of discrete molecular weight novel microsatellites not present in source polyclonal tissues (arrow). Detailed microsatellite allelotyping[1] showed that K-ras mutant tissues (E2 and T2–5) shared multiple novel microsatellites not seen in the one area of tumour with a wild-type K-ras genotype (T1). Either tumour T1 is an independent clone, or it diverged from other neoplastic tissues very early in neoplastic progression.

reported by others[23–26]. K-ras mutation rates did not change with the appearance of MI (table 1).

Nine patients with K-ras mutant cancers had non-malignant areas of endometrioid tissue available as discrete foci (n = 15) within the cancer bearing hysterectomies or biopsy samples (n = 4) obtained in a two month window preceding hysterectomy. Table 2 shows the clonal composition, K-ras mutation status, and histopathological appearance of these tissues.

p53 mutations were seen in only three of 56 cancers studied, neither of which had coexisting K-ras mutations. These three patients were not informative in evaluating premalignant lesions, because the only non-malignant endometrium present was polyclonal, histologically normal endometrium with a wild-type p53 genotype.

Discussion

Microsatellite instability had no influence on the rates of K-ras mutation in cancers (table 1), and appeared in the premalignant phases of both MI and MS pathways (table 2). These findings are similar to those of most other groups,[16 17] although isolated reports have suggested enrichment for K-ras mutations in MI endometrial adenocarcinomas. K-ras mutation might play a similar role in the premalignant phases of endometrial carcinoma, irrespective of the presence or absence of MI.

After exclusion of contentious or subdiagnostic histologies, acquisition of K-ras mutation usually corresponds to a change in morphology from a non-hyperplastic to a hyperplastic type (fig 2). In patients with K-ras mutant cancers, all six associated polyclonal areas with non-hyperplastic morphologies had wild-type K-ras. In contrast, all 10 regions with mutant K-ras genotypes had a histopathology that was either hyperplastic or non-diagnostic. Aggregated data from three independent studies[24–26] support the idea that K-ras mutation occurs in the premalignant phase, because endometrial precancers selected by an atypical hyperplasia histology have an overall rate of K-ras mutation of 21% (13 of 63) compared with a cancer K-ras mutation rate of 20% (41 of 201).

A subset of patients, however, have incomplete conservation of K-ras mutation among sampled neoplastic tissues, consistent with either a multicentric origin or later acquisition
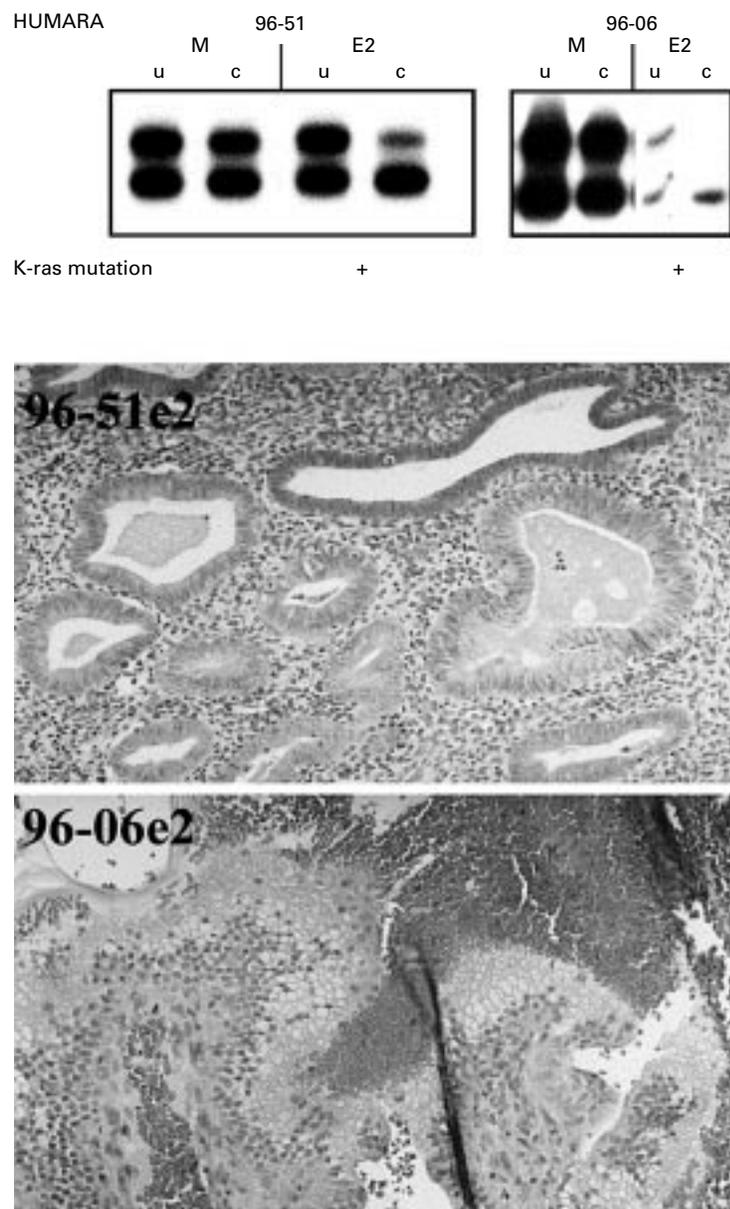
Figure 4 K-ras mutations in histologically ambiguous endometria. Two (96-06E2 and 96-51E2) endometrial tissues subdiagnostic for atypical endometrial hyperplasias (photomicrographs, lower panels) are identified as precancers based upon a monoclonal growth pattern on HUMARA assay (upper panel) and appearance of K-ras mutations identical to those seen in matching carcinomas. The non-uniform histological appearance of monoclonal putative precancers confounds efforts to standardise pathological criteria for their diagnosis, resulting in poor reproducibility of atypical hyperplasia diagnoses even among experienced pathologists. The upper panel shows HUMARA polymerase chain reaction products from undigested (u) or HhaI predigested (cut (c)) DNA in matched normal myometrium (M) and non-malignant endometrial (E) tissues. Relative skewing of signal within the two alleles of differing molecular weight is diagnostic of a monoclonal process (both E2 samples), in contrast to a retained balance of signal in polyclonal myometrium digested lanes. Uncut bands in 96-06E2 were slightly distorted by a gel artifact but are clearly of equivalent intensity, unlike the digested (c) sample.

of K-ras mutation. Only through use of matched tissues from single patients is it possible to discern this pattern. Two patients showed K-ras mutation discordance among multiple precancers (cases 96-06 and 96-51; table 2), whereas one had divergent mutant and wild-type K-ras genotypes among subregions of adenocarcinoma (case 96-150). The latter has been noted previously in detailed mapping of K-ras mutations in microdissected tumours,[24] but our use of tetranucleotide repeats as loci for

scoring of MI provided additional genetic markers to distinguish between multicentric disease and late onset mutation. In case 96-150, the K-ras mutation seen in the precancer was conserved in only four of five sampled areas of associated cancer. We mapped[1] the constellation of altered microsatellites seen in each tissue area and found that premalignant and malignant tissues sharing K-ras mutations also shared novel marker alleles not seen in the area of cancer devoid of K-ras mutation (fig 3). The finding that these K-ras mutant and non-mutant tissues are genetically divergent at loci other than K-ras favours a multicentric process, or very early divergence of premalignant lineages into multiple subgroups.

Because of the lack of monoclonal precancers in the three patients with p53 mutant cancers, we are unable to comment on p53 mutations in precancers. This is unfortunate, because there has been some controversy about whether p53 mutation is[26] or is not[27 28] a feature of premalignant endometrium. Two of three endometrial cancers with a mutant p53 were MS, whereas the remaining one was MI. The 5% rate of p53 mutation in endometrial cancer is somewhat lower than the 13–23% reported previously.[29 30] In part, this could be because all the cases studied were endometrioid adenocarcinomas, thereby excluding papillary serous and clear cell subtypes, which are particularly associated with high p53 mutation rates.[31] All three p53 mutant cancers were poorly differentiated (grade III), as would be expected.[29 31]

The molecular basis of precancer initiation is increasingly being resolved as additional genes are found to have aberrant function in these tissues compared with their normal counterparts. For example, mutation of the tumour suppressor gene PTEN also occurs in the premalignant phases[32] of MS and MI endometrial tumorigenesis. A mechanistic basis for MI in endometrial neoplasia, transcriptional silencing of the hMLH1 gene through de novo CpG island methylation,[33 34] suggests that modifications of gene expression are not all explained by primary structural mutations within the coding regions of genes.

Morphological diagnosis occasionally fails to recognise precancers that are apparent either through clonality or mutational analysis. Indeed, it is the unreliability of histopathological diagnosis that prompted us to define potential precancers by their monoclonal growth patterns. Tissue E2 from case 96-06 (fig 4) is a scrap of mucinous epithelium in a previous curetting that was interpreted as inadequate for diagnosis of the endometrium. This patient had an endometrial adenocarcinoma with focal mucinous differentiation, and the non-endometrioid character of the fragment was misinterpreted as endocervical in origin. Similarly, a consistent diagnostic label was not rendered for area E2 from case 96-51 (fig 4) after review by several pathologists, yet it can be identified as a precancer because of its monoclonal growth pattern and presence of K-ras mutation.

K-ras mutation in colonic mucosa might also precede the acquisition of histological stigmata

of neoplasia,[35] confirming in that model that K-ras changes are among the earliest detectable features of neoplastic transformation, and that they might play a role in the early clinical detection of cancer risk. In addition to early acquisition, the colonic and endometrial carcinoma models both have K-ras mutations in equivalent frequencies in MS and MI tumours.[36]

PCR–SSCP can detect K-ras mutations characteristic of some precancers at a level of sensitivity that exceeds that of the HUMARA assay. This is because polyclonal stromal or epithelial cells contaminate most DNA isolates intended to sample a focal epithelial lesion. PCR–SSCP can detect a mutant K-ras allele if only 5% of all alleles are mutant[22] (T. Enomoto et al, 1998, unpublished observation), whereas the HUMARA assay requires 50–75% of tested cells to be derived from the neoplastic clone.[2 7] Area E1 from case 96-04 (fig 2) is an atypical endometrial hyperplasia with a K-ras mutation, which failed to display the skewed X inactivation pattern in the HUMARA assay characteristic of a monoclonal precancer. We believe the HUMARA determination to be an error, caused by confounding contamination with normal tissues and, in this case, some slight skewing of X inactivation in baseline polyclonal tissues, which would be expected to reduce the sensitivity of monoclonal detection even further.[7]

We conclude that K-ras mutation is an integral feature of the MI and MS endometrial carcinogenesis pathways, and in most cases onset of K-ras mutation occurs early during the premalignant phases. These K-ras mutant, monoclonal, putative precancers are histologically pathological, but vary in the extent to which they fulfill the criteria for atypical endometrial hyperplasia. Absence of K-ras mutation in normal polyclonal tissues produces a highly specific association between K-ras mutation and the onset of neoplasia, which might be useful in the recognition of the earliest stages of precancer initiation.

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