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Genetic Instability of Microsatellites in Endometrial Carcinoma

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Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is characterized by a familial predisposition to colorectal carcinoma and extracolonic cancers of the gastrointestinal, urological, and female reproductive tracts, notably the endometrium. A genetic locus for HPNCC was recently determined by linkage analysis to exist on chromosome 2p; both sporadic and HNPCC-associated colorectal carcinomas exhibit a "replication error" phenotype, characterized by instability of dinucleotide repeat sequences throughout the genome. Here, we address the hypothesis that the replication error phenotype would be evident in some fraction of sporadic endometrial carcinomas or in those associated with HNPCC. Microsatellite instability was observed in 17% of sporadic endometrial carcinomas and in 75% of those tumors associated with HNPCC. These data indicate that the HNPCC gene is also involved in heritable and somatic forms of endometrial carcinoma.

Introduction

HNPCC,[2] also referred to previously as cancer family syndrome (1) and Lynch syndrome II (2), is one of at least three major forms of hereditary colorectal cancer (3). Minimal criteria for identifying HN-PCC kindreds include histologically verified colorectal cancer in three closely interrelated persons in two generations, at least one of which was diagnosed before the age of 50 (4). Unlike familial adenomatous polyposis, for which the gene has now been cloned (5, 6), diffuse polyposis of the colon is absent in HNPCC. In contrast to Lynch syndrome I, which is characterized by predisposition to colorectal carcinoma only, increased rates of extracolonic cancers, especially of the gastrointestinal, upper urological, and female reproductive tracts, are evident in most HNPCC families (3). Among these extracolonic cancers, endometrial carcinoma is reported to occur most frequently and to cosegregate with colon cancer in HNPCC kindreds (7–9).

Proof of a genetic basis for HNPCC was recently provided through genetic linkage analysis; the *HNPCC* locus is closely linked to the anonymous microsatellite marker *D2S123* on chromosome 2p, with tentative assignment to 2p15–16 (10). Associated with most of the familial tumors (11) and with a lesser percentage of sporadic colorectal carcinomas (11–13) is the unusual feature of somatic microsatellite instability, manifested by alterations in the electrophoretic mobility of $(CA)_n$ dinucleotide repeat fragments. This instability is suggestive of DNA replication error and is presumed to result from the chromosome 2p gene defect, although proof of this relationship must await cloning and characterization of the HNPCC gene. Similarly, unstable trinucleotide repeats have recently been demonstrated to represent the cause of at least four other genetic diseases (14); in contrast to HNPCC, however, these heritable unstable trinucleotide

DNA elements disrupt the disease gene itself and do not appear to occur at multiple sites throughout the genome of an affected individual.

In this study, we addressed the hypothesis that the dinucleotide repeat replication error phenotype would be evident in some fraction of sporadic endometrial carcinomas or those associated with HNPCC. The presence of this tumor type, together with colorectal carcinoma, was considered to represent definitive manifestation of the disorder in HNPCC families studied in the chromosome 2p linkage analysis (10). Thirty-six cases of sporadic endometrial carcinoma, as well as endometrial carcinomas from four independent HNPCC families, were examined for evidence of microsatellite instability.

Materials and Methods

Sporadic endometrial carcinomas and corresponding normal tissues were obtained from 36 patients treated at the Duke University Medical Center from 1988 to 1992. This tumor series was representative for grade, stage, invasion, histological subtype, metastatic status, ploidy, and clinical outcomes. The 72 normal and tumor tissue samples consisted of an approximately equal number of fresh frozen biopsy specimens and formalin-fixed, paraffin-embedded material. Our procedures for the isolation of genomic DNA from both types of tissue samples have been described previously in detail (15). Ploidy analysis of these tumors was performed by computerized image analysis of Feulgen-stained cells as described (16). Endometrial carcinomas from HNPCC families were obtained through the Creighton University/Hereditary Cancer Institute colon cancer family resource. These cases consisted entirely of archival formalin-fixed, paraffin-embedded material, and DNA was isolated as above.

Seventy-one dinucleotide repeat markers, representative of all nonacrocentric chromosome arms, were utilized to a variable extent in an allelotype analysis of endometrial carcinoma. The 36 tumor cases described above were studied most thoroughly, with regard to the number of markers examined per normal/tumor pair. The PCR primer sequences for these markers were generally obtained from the Genome Data Base, Welch Medical Library, Johns Hopkins University. For most markers, PCR reactions consisted of the following: 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleotide triphosphate, 1 μM concentrations of each primer, 0.5 unit AmpliTaq polymerase (Perkin-Elmer/Cetus), and 10–30 ng genomic DNA in a volume of 20 μl. One primer was end-labeled with $[\gamma\text{-}^{32}P]ATP$ by polynucleotide kinase, using the KinAce-It kit (Stratagene), and column-purified prior to the PCR reaction. Thirty cycles were performed, consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a 7 min extension at 72°C, using a Perkin-Elmer 9600 GenAmp PCR System. Primer sequences and reaction conditions for the DCC marker are described elsewhere (17).

The PCR products were processed by diluting 1:1 with a loading buffer consisting of 98% formamide, 10 mM EDTA (pH 8.0), 0.02% xylene cyanol FF, and 0.02% bromophenol blue and then denatured for 2 min at 90°C. Typically, 5 μl of this solution were subjected to electrophoresis in 6% polyacrylamide gels containing 8.3 M urea for 2–3 h at 70 W. The gels were fixed in 10% methanol/10% acetic acid, dried, and exposed to X-ray film at −80°C.

Analysis of the VNTR marker *D1S80* was performed on all of the normal/tumor DNA pairs exhibiting microsatellite instability using the Forensic DNA Amplification Reagent Set (Cetus), following the protocol supplied by the manufacturer. The PCR products were subjected to electrophoresis in a 1.5% Sea-Kem GTG agarose gel containing 0.2 μg/ml of ethidium bromide and then photographed under a UV light source with a Poloroid camera and Wratten 23A filter.
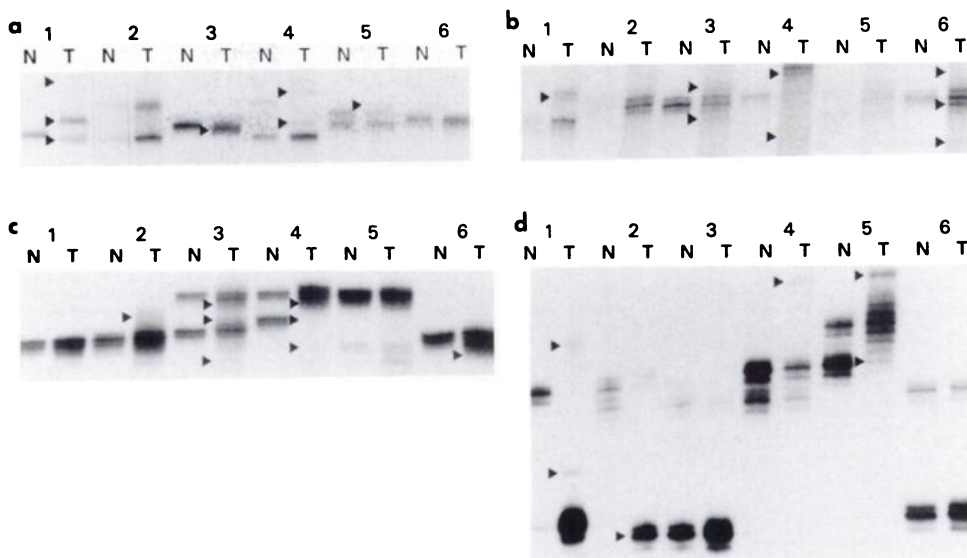
Fig. 1. Examples of microsatellite instability in sporadic endometrial carcinomas. Genomic DNA samples from paired normal and tumor tissues were subjected to PCR amplification using the dinucleotide repeat markers (a) D2S119, (b) D10S197, (c) D11S904, and (d) DCC. Arrows, alterations in the electrophoretic mobility of PCR products from tumor compared to normal tissue DNA. N, normal; T, tumor.

Results

Microsatellite Analysis. We utilized polymorphic dinucleotide repeat markers to determine loci subject to allelic deletion in endometrial carcinoma by assessing loss of heterozygosity using DNA from matched normal and tumor tissue pairs. After completing this allelotype analysis,[3] it was apparent that a number of the tumor DNA samples examined exhibited consistent abnormalities in the electrophoretic mobility of PCR products containing dinucleotide repeats. Of 36 thoroughly characterized tumors, 6 showed mobility shifts in tumor compared to corresponding normal DNA samples, for multiple microsatellite markers interspersed throughout the genome. These six tumor samples were studied further with additional dinucleotide repeat markers, including several that surround the HNPCC locus on chromosome 2p. Representative examples of electrophoretic mobility alterations are shown in Fig. 1 and include data derived from microsatellite markers on chromosomes 2 (D2S119), 10 (D10S197), 11 (D11S904), and 18 (DCC). A number of different types of alterations were observed, and include additional, larger alleles (Fig. 1a, case 4), expansion of an allele(s) (Fig. 1b, case 4), additional, smaller alleles (Fig. 1d, case 2), and the most common aberration, a characteristic laddering pattern of both smaller and larger alleles (Fig. 1b, case 6; Fig. 1d, case 5).

If the replication error phenotype observed in nonfamilial endometrial carcinomas is the result of a mutant chromosome 2p susceptibility gene, as implicated in sporadic and HNPCC-associated colorectal carcinomas (10–13), then this phenotype should be evident in a significant fraction of HNPCC-associated endometrial tumors. Thus, endometrial carcinomas from four members of three independent HNPCC kindreds, the pedigrees of which have been published elsewhere (18–20), were obtained and examined for microsatellite instability. Three of these cases, each from a different kindred, displayed clear evidence of dinucleotide repeat mobility shifts in tumor compared to normal DNA (Fig. 2); the fourth case exhibited identical patterns in tumor and normal DNA for eight microsatellite markers. The types of alterations in electrophoretic mobility of PCR products were similar to those described above for the sporadic tumors, and included larger alleles (Fig. 2b, case 1), smaller alleles (Fig. 2b, case 2), and a laddering pattern of smaller and larger alleles (Fig. 2a, case 1).

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[3] J. Boyd et al., manuscript in preparation.

Clinical Features. Patient histories associated with the six sporadic tumors exhibiting microsatellite instability revealed no evidence of colorectal or endometrial cancer in either previous or subsequent generations, supporting their classification as sporadic endometrial carcinomas. Clinical and histopathological features associated with these cases are listed in Table 1. All six of the tumor DNAs exhibiting microsatellite instability were from diploid or near-diploid stage I adenocarcinomas, which represent the most common form of endometrial carcinoma and are classifiable as type I (21). No correlation with grade was evident, inasmuch as tumors ranged from well to poorly differentiated. Myometrial invasion ranged from none to moderate, and all six tumors were nonmetastatic at the time of initial surgery. Seven of the 37 tumors included in the original allelotyping analysis were aneuploid and/or type II histologic variants (papillary serous or clear cell), and DNA from these tumors exhibited no evidence of microsatellite instability.

Fewer clinical data were available for the HNPCC-associated endometrial carcinoma cases but the three tumors exhibiting microsatellite instability consisted of two well differentiated adenocarcinomas and one moderately differentiated adenosquamous carcinoma metastatic to one of three lymph nodes (Table 1). The average age of diagnosis for the three HNPCC-associated endometrial cancers was 20 years younger than for the sporadic tumors (48 and 68, respectively). As for the sporadic cases, all three of these patients were currently alive with no evidence of disease.

VNTR Analysis. To confirm that the normal and tumor DNA sample pairs used in this study were matched samples from the same individual, the VNTR marker D1S80 was examined in the DNA samples from the 10 individuals described above. This chromosome 1p marker consists of repeat units 16 nucleotides long, for which at least 29 different alleles determine 435 possible genotypes (22, 23). As shown in Fig. 3, all of the paired DNA samples for which tumor DNA exhibited the replication error phenotype were derived from matched tissue samples, each from different individuals demonstrating unique D1S80 genotypes. In addition, these data are consistent with the previous observation (11) that VNTR loci are not affected by the replication error phenotype.

Discussion

These data indicate that somatic microsatellite instability is a feature of a subset of endometrial carcinomas (17%). Instability is about equally prevalent in sporadic endometrial and sporadic colorectal

Fig. 2. Examples of microsatellite instability in endometrial carcinomas associated with HNPCC. Genomic DNA samples from paired normal and tumor tissues were subjected to PCR amplification using dinucleotide repeat markers (a) D10S197 and (b) D2S123. Arrows, alterations in the electrophoretic mobility of PCR products from tumor compared to normal tissue DNA. N, normal; T, tumor.
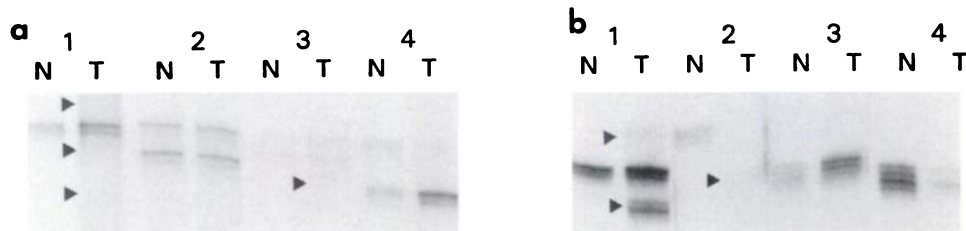

Table 1 Clinical data for endometrial tumors with microsatellite instability

Patient[a]	Age[b]	Histology	Grade[c]	Stage[d]	Invasive[e] Metastatic[f]	Ploidy[g]	Status[h]
S1	67	AC	1	IA	0/−	1.14	NED
S2	63	AC	3	IC	2/−	1.0	NED
S3	66	AC	2	IA	0/−	1.09	NED
S4	71	AC	2	IC	2/−	1.0	AWD
S5	57	AC	3	IC	2/−	1.0	NED
S6	81	AC	3	IC	2/−	0.96	NED
L1 (16)	54	AC	1	IB	1/−	NA	NED
L2 (17)	40	AS	2	NA	1/+	NA	NED
L3 (18)	50	AC	1	NA	1/−	NA	NED

[a] S, sporadic cancer; L, HNPCC-associated cancer (reference containing pedigree); AC, adenocarcinoma; AS, adenosquamous carcinoma; NA, not available.
[b] Age at diagnosis.
[c] FIGO grade: 1, well differentiated; 2, moderately differentiated; 3, poorly differentiated.
[d] Surgical stage.
[e] Myometrial invasion; 0, none; 1, inner third; 2, middle third; 3, outer third.
[f] Metastasis: +, positive; −, negative.
[g] Relative DNA content: 1.00, diploid; 2.00, tetraploid.
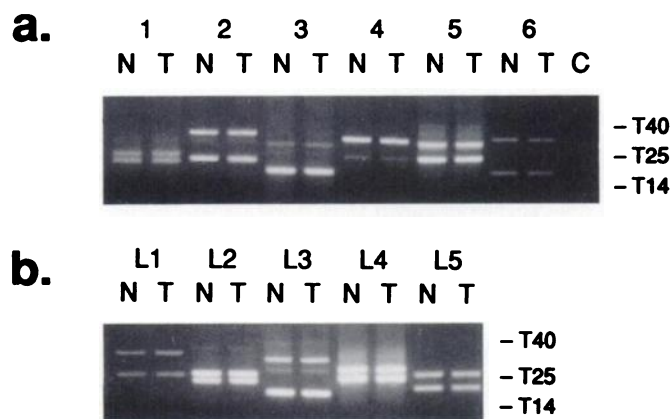[h] Patient outcome: NED, alive with no evidence of disease; AWD, alive with disease.

Fig. 3. Products from the PCR amplification of VNTR marker D1S80 in paired normal and tumor DNA samples from all individuals exhibiting evidence of microsatellite instability. (a) Sporadic endometrial carcinomas. Lane C, negative control PCR reaction containing water instead of DNA. (b) Endometrial carcinomas associated with HNPCC. Sample pair L1 is from a colorectal tumor patient; sample pairs L2–L5 correspond to endometrial tumor sample pairs 1–4 in Fig. 2. Right ordinate, size markers corresponding to tentative repeat unit designation (T), which ranges from 14 to 40 for this VNTR. N, normal; T, tumor.

carcinomas (10–30%) (11–13). The majority of endometrial carcinomas associated with the HNPCC syndrome also exhibit this phenotype. All tumors that exhibited microsatellite instability showed this phenotype for multiple markers throughout the genome. Generalized microsatellite instability may thus be related to human carcinogenesis at multiple tissue sites. Other tumors frequently observed in HNPCC families, such as carcinoma of the stomach, small intestine, hepatobiliary system, kidney, ureter, and ovary (8), would be likely candidates for this genetic mechanism.

Previous studies have shown that linkage of HNPCC to anonymous markers on chromosome 2p correlates with replication errors in polymorphic simple repeated sequences, suggesting that the phenotype may result from a defective gene in this region (10, 11). It will be interesting to test for linkage of chromosome 2p markers to endome-

trial cancer in families with multiple cases of specifically endometrial cancer (24, 25). Although the gene itself has yet to be cloned and characterized, the HNPCC gene product may be involved in DNA replication or repair. Germline transmission of a mutant allele of the HNPCC gene would predispose to the HNPCC syndrome, whereas somatic mutation would contribute to the development of sporadic colorectal, endometrial, and perhaps other carcinomas. The HNPCC gene does not behave as a typical tumor suppressor gene in either endometrial or colorectal cancers; as with colorectal carcinomas, no evidence was found in sporadic or HNPCC-associated endometrial carcinomas for allelic loss at any of several markers on chromosome 2p, including D2S123.

The precise mechanism through which this replication error phenotype contributes to tumorigenesis is unknown. Although generalized genomic instability is a hallmark of neoplastic transformation, the instability associated with the HNPCC gene has thus been described only in microsatellite repeats. It would seem probable, however, that altered DNA replication or repair would affect the fidelity of replication in a more generalized fashion. Dinucleotide repeats may occur within genes (e.g., DCC), possibly affecting transcription of the gene adversely. Trinucleotide repeats are also subject to replication error in tumors exhibiting the instability phenotype (11), and this type of microsatellite sequence frequently occurs within the coding regions of genes (14). Finally, dinucleotide repeat sequences are thought to play a role in chromatin structure and nucleosome placement (26), the disruption of which could also interfere with proper gene transcription.

The clinical and histopathological features of the sporadic endometrial carcinomas displaying microsatellite instability were examined for possible correlations with this phenotype. All six tumors were stage I adenocarcinomas with a diploid or near-diploid DNA content, and five of six of these patients were currently alive with no evidence of disease, as were the three patients with HNPCC-associated endometrial carcinoma. This phenotype, and presumably the chromosome 2p gene mutation, would thus appear to occur in a subclass of type I

(21) endometrial carcinomas. Mutation of *K-RAS* codon 12 is also seen in 10–20% of type I tumors (15), while *ERBB-2* overexpression and *p53* mutation are found in subclasses of type II tumors (27). Similarly, the replication error phenotype is also associated with diploid colorectal tumors and good clinical prognosis (11–13). The replication error phenotype has also been observed in premalignant colorectal adenomas (13). Since type I endometrial carcinomas are often preceded by or associated with endometrial hyperplasia, it will be of interest to examine these premalignant lesions for evidence that the chromosome 2 gene mutation, when present, is an early event in endometrial carcinogenesis.

Acknowledgments

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