Loss of Heterozygosity at the Human RAP1A/Krev-1 Locus Is a Rare Event in Colorectal Tumors

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ABSTRACT

Kirsten-ras-revertant-1 (Krev-1/RaplA) is a recently identified tumor suppressor gene which induces flat revertants when introduced into a variety of ras-transformed cell lines in vitro. Since 47% of colorectal carcinomas have transforming mutations in ras protooncogenes, and since Krev-1 is expressed at high levels in normal colonic mucosa, we hypothesized that inactivation at the Krev-1 locus may be necessary for transformation of colonic cells. Loss of heterozygosity is a common method of inactivation of tumor suppressor genes in colorectal tumors. Therefore, we analyzed loss of heterozygosity in 52 patients with sporadic colorectal cancer. Because Krev-1 had no previously described polymorphisms, we first identified a BclI restriction fragment length polymorphism which showed 40% heterozygosity in 50 unrelated individuals. However, only one tumor from 18 informative patients showed allelic loss at the Krev-1 locus. This suggests that loss of heterozygosity is not a common mechanism of inactivation at the Krev-1 locus in colorectal cancer. However, the results do not exclude a role for Krev-1 in the etiology of this neoplasm because inactivation may occur by other mechanisms.

INTRODUCTION

One approach to understanding the molecular basis of cancer in recent years has been the study of loss of heterozygosity in order to identify genomic regions which may contain tumor suppressor genes. It is thought that frequent allelic loss in a specific chromosomal region reflects the inactivation, by chromosome or gene deletion, of one allele of a tumor suppressor gene located within this region. Furthermore, in many cases in which the candidate tumor suppressor gene has been cloned, it has been shown that the homologous allele has been inactivated by another mechanism, most often a point mutation (1, 2).

The tumor that has been studied most intensively in this way is adenocarcinoma of the colorectum (3). Because of the paucity of cloned tumor suppressor genes, most studies of loss of heterozygosity have used clones (of both coding genes and anonymous loci) chosen for their chromosome location and polymorphism information content value, rather than their function as suppressor genes. Since small deletions of the critical region where the suppressor gene is located may not be detected by syntenic clones, this inevitably leads to false-negative results.

Fearon and Vogelstein (4) proposed a model for colorectal carcinogenesis which involves the accumulation of mutations, most commonly, specific loss of the adenomatous polyposis coli, p53, and DCC genes on chromosome 5, 17, and 18, respectively, as well as hypomethylation and ras point mutations in the progression from normal epithelium to metastasis. Mutations of a ras gene are found in 47% of colorectal tumors, and 88% of these occur in c-Ki-ras (5). These mutations, which occur at specific positions within the ras genes, give it the ability to transform cultured cell lines (6). However, this transformation can be reversed by a tumor suppressor gene, Krev-1, the complementary DNA of which induces flat revertants when introduced into a variety of ras-transformed cultured cell lines (7–9). There is a correlation between levels of Krev-1 expression and suppression of transformed cells, and high levels of expression appear to be necessary for suppression (9).

Krev-1 encodes a GTP-binding protein, also known as rap1A (10) or smg-p21 (11), of molecular weight 21,000. It shares 50% amino acid homology with the products of the ras protooncogenes. Homology is especially high in three essential regions: the GTP-binding site, the effector-binding site, and the COOH-terminal acylation site (12). It is likely that Krev-1 exerts its effects through competitive inhibition with ras gene products of guanosine triphosphatase activating protein-mediated ras-GTPase activity (13, 14). Human Krev-1/RaplA has been mapped to 1p12-p13 (15), and it is noteworthy that structural and numeric abnormalities of the short arm of chromosome 1 occur in many tumors, including those of the colorectum (16). The only chromosomes that are more often involved in colorectal tumors are chromosomes 17 and 18 (16).

We therefore hypothesized that, since 50% of colorectal tumors carry activated ras genes, and since Krev-1 is capable of suppressing ras-activated cells when expressed at high levels (as it is in colonic cells (9)), transformation of colorectal cells by ras mutations may require inactivation of Krev-1. One method of inactivating suppressor genes is gene deletion. This commonly occurs in colorectal tumors and can be detected by identification of allelic loss. We therefore analyzed loss of heterozygosity at the Krev-1 locus in 52 colorectal tumors. Since no RFLPs have been described for Krev-1, it was necessary that we first identify a suitable RFLP with which to study loss of heterozygosity. In addition, because chromosome 1 deletions are frequently observed at the karyotypic level (16), we analyzed syntenic loci to determine whether other regions of this chromosome frequently undergo allelic loss.

MATERIALS AND METHODS

Samples. Tumor tissue was removed during surgery from 52 patients with sporadic colorectal neoplasms and macroscopically dissected from surrounding normal tissue. Corresponding normal tissue was obtained predominantly from normal colonic mucosa (n = 30) with the balance from peripheral blood leukocytes (n = 15) and Epstein Barr virus-transformed lymphoblastoid cell lines (n = 7). In addition, peripheral blood leukocytes were obtained from members of 4 two-generation families to demonstrate Mendelian inheritance of the Krev-1 RFLP.

Morphometry. Of the 52 tumor samples, 12 were examined by a pathologist unaware of the loss of heterozygosity results using computerized morphometry with Image Feature Measurement and Graphics Tablet (Dapple Systems, Inc.) to determine the percentage of tumor tissue in each.

Southern Analysis. Colonic tissues were homogenized with a Poly-
tron, and high molecular weight DNA was extracted from these, as well as buffy coat and cell line samples, by an adaptation of the high salt protein precipitation method of Miller et al. (17). DNA was digested with restriction endonucleases in the presence of 4 mM spermidine, electrophoresed in 0.8% agarose for 16-64 h, and alkali blotted onto charged nylon membranes (18). The membranes were incubated with whole plasmid, which had been radioactively labeled by random priming, at 65°C for 16 h. The filters were washed at high stringency in 0.5 × standard sodium citrate for 15 min at 65°C and then exposed to Kodak XAR film for 1-5 days. The probes used were: A336 for D1S47 (19), pLBK8B/ES' for ALPL (20), L-myc (21), pKrev-1 (8), 3021E1 for SPTA1 (22), pAT3 (23) for AT3, and phRnES1.9 for REN (24).

**RESULTS**

**Krev-1 RFLP Search.** Eleven Southern filters of germline DNA were prepared, with 9 unrelated individuals per blot, using the following enzymes: BclI, BglII, MspI, TaqI, PstI, EcoRI, SacI, HindII, Rsal, HindIII, and PvuII. Hybridization with pKrev-1 revealed only one putative RFLP, with BclI. This was confirmed by demonstrating Mendelian inheritance of the variant in 4 informative meioses by Southern analysis of DNAs from 4 two-generation families (Fig. 1). The polymorphism shows two variable bands (allele 1, 6.6 kilobases; allele 2, 6.2 kilobases), in addition to two constant bands (12.0 and 5.0 kilobases). The frequencies of alleles 1 and 2 in 50 individuals tested (all of whom had sporadic colorectal cancer) were 0.64 and 0.36, respectively.

**Loss of Heterozygosity at the Krev-1 Locus in Colorectal Cancer.** In order to detect loss of heterozygosity at the Krev-1 locus, filters were made from BclI-digested paired samples of tumor and germline DNA from each of the 52 patients. Hybridization with pKrev-1 showed that loss of heterozygosity occurred in only one of 18 tumors from informative patients (Table 1; Fig. 2D). This patient (patient 13) had a sigmoid tumor with multiple metastases.

**Loss of Heterozygosity at Other Chromosome 1 Loci.** Since the majority of the patients were not informative for Krev-1, but deletions of chromosome 1 are frequently observed karyotypically in colorectal tumors, other chromosome 1 loci were examined to determine whether syntenic loci had undergone loss of heterozygosity. If this were so, we planned to determine whether the smallest region of overlap of the deletions (the "critical region") contained Krev-1 or was located elsewhere on chromosome 1. Therefore, more filters were made using paired DNA (which had been digested with the appropriate restriction endonucleases) from these 52 patients, and these were hybridized to probes from different regions of chromosome 1. Only low levels of loss of heterozygosity were observed with all the probes: 1 of 15 informative patients for D1S47 at 1p36, 2 of 17 for ALPL at 1p36.1-p34, 2 of 20 for MYCL1 at 1p32, 3 of 29 for the MspI SPTA1 RLFP, 2 of 22 for the PvuII SPTA1 RLFP at 1q21, 0 of 14 for AT3 at 1q23-q25.1, and 1 of 16 for REN at 1q32-42 (Table 1; Fig. 2). Overall, 10 of 50 (20%) tumors, informative for one or more probe, showed loss of heterozygosity. Eight patients had partial deletions of chromosome 1 (patients 2, 9, 21, 26, 28, 42, 46, and 52), including two (patients 26 and 28) who appeared to have interstitial deletions. The remaining two patients with loss of heterozygosity may have had total deletions of chromosome 1, but since only one (patient 13) or two (patient 25) markers were informative, this is uncertain.

**Morphometry.** Twelve tumors were examined with computerized morphometry. The percentage of tumor cells ranged from 30 to 88% with a mean of 63.5%. Eight of the tumors had at least 59% tumor cells and showed allelic loss on chromosome 1 or on 18 (data not shown). There were two tumors, both from the proximal colon, which had 68 and 59% tumor cells but showed no allelic loss on chromosomes 1 or 18. The remaining two tumors (patients 19 and 29) contained only 43 and 30% tumor cells, respectively, and showed no loss on either chromosome 1 or 18.

**DISCUSSION**

Having identified a BclI RFLP of the Krev-1 gene, we examined loss of heterozygosity in 52 colorectal tumors. Only one of 18 tumors from patients who were heterozygous for this RFLP showed loss of an allele. Furthermore, RFLP analysis of another 7 loci on chromosome 1 showed that deletions were also rare events at all of these (Table 1). One possible explanation for the low level of loss of heterozygosity observed could be excessive contamination in the tumor specimen with non-tumor cells. The tumors in this study were fractionated mac-
### Table 1 Tumor position, presence of metastases, and loss of heterozygosity data for markers on chromosome 1 in 10 patients with colorectal cancer

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Tumor position</th>
<th>Metastases</th>
<th>Probe Locus</th>
<th>Map</th>
<th>Enzyme</th>
<th>A336</th>
<th>pLBK8B/ES'</th>
<th>L-myc</th>
<th>pKrev-1</th>
<th>3021E1</th>
<th>3021E1</th>
<th>pAT3</th>
<th>phRnES1.9</th>
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<tr>
<td>2</td>
<td>Rectum</td>
<td>Yes</td>
<td>--</td>
<td>1p36</td>
<td>TaqI</td>
<td>NI</td>
<td>No loss</td>
<td>NI</td>
<td>No loss</td>
<td>NI</td>
<td>No loss</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>9</td>
<td>Sigmoid</td>
<td>No</td>
<td>--</td>
<td>1p36</td>
<td>p36.1-p34</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
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<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>13</td>
<td>Sigmoid</td>
<td>Yes</td>
<td>--</td>
<td>1p32</td>
<td>EcoRI</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>21</td>
<td>Caecum</td>
<td>No</td>
<td>--</td>
<td>1q12</td>
<td>BclI</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>--</td>
<td>NI</td>
<td>NI</td>
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<tr>
<td>25</td>
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<td>No</td>
<td>--</td>
<td>1q21</td>
<td>MspI</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>NI</td>
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<tr>
<td>26</td>
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<td>No</td>
<td>--</td>
<td>1q23-q25.1</td>
<td>BclI</td>
<td>NI</td>
<td>Loss</td>
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<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
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<tr>
<td>46</td>
<td>Rectum</td>
<td>Yes</td>
<td>--</td>
<td>1q32 or 42</td>
<td>HindIII</td>
<td>NI</td>
<td>Loss</td>
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<tr>
<td>52</td>
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<td>--</td>
<td>1q32</td>
<td>Not informative</td>
<td>NI</td>
<td>Loss</td>
<td>NI</td>
<td>Loss</td>
<td>--</td>
<td>No LOH</td>
<td>NI</td>
<td>No LOH</td>
</tr>
</tbody>
</table>

| Total patients tested | 24 | 52 | 51 | 50 | 52 | 52 | 35 | 50 |
| No. not informative   | 9  | 35 | 31 | 32 | 23 | 30 | 21 | 34 |
| No. informative       | 15 | 17 | 20 | 18 | 29 | 22 | 14 | 16 |
| No. with LOH          | 1  | 2  | 2  | 1  | 3  | 2  | 0  | 1  |
| % with LOH            | 6.7% | 11.8% | 10% | 5.6% | 10.3% | 9.1% | 0% | 6.3% |

*LOH, loss of heterozygosity; NI, not informative; --, genotyping not done.

![Fig. 2. Southern analysis of paired tumor (T) and normal (N) DNA demonstrating loss of heterozygosity (LOH) in colorectal tumors. Probes used were: A, A336 for D1S47 (multiple alleles detected); B, pLBK8B/ES' for ALPL; C, L-myc for MYCL1; D, pKrev-1 for RAP1A; E, 3021E1 for Pvull RFLP of SPTA1; and F, phRnES1.9 for REN. Arrows, variable bands; *, position of lost allele; #, patient number; kb, kilobases.](image-url)
RARE ALLELIC LOSS OF Krev-1 IN COLORECTAL TUMORS

Fig. 3. Diagram of human chromosome 1 showing position of loci probed in this study (black bars) and critical region as defined by Leister et al. (30; shaded bar).

roscopically to remove any excess normal tissue. In addition, the 18 patients informative at the Krev-1 locus had been previously examined for loss of heterozygosity on chromosome 18. Of these, 11 of 15 patients (informative for at least one marker at chromosome 18q21-qter) showed allelic loss on chromosome 18. This compares well with data from Vogelstein et al. (5) and indicates that the level of stromal contamination was not too high in our tumors. In addition, morphometric analysis showed that, with two exceptions, loss of heterozygosity (on chromosome 1 or 18 or both) was detected in samples with at least 59% tumor cells. The two exceptions (patients 4 and 50), containing 59 and 68% tumor cells, respectively, came from the proximal colon. This is significant because genetic abnormalities are less common in the proximal colon: 60% of proximal tumors show no allelic loss compared with only 7% in the distal colon (25). Furthermore, cytogenetic studies suggest that abnormalities of chromosome 1 occur less frequently in proximal tumors (17%) compared with 57% in the distal colon (26).

Most other molecular studies of loss of heterozygosity on chromosome 1 in colorectal tumors have also found low levels of allelic loss, although none of these studies analyzed Krev-1 directly (3, 27-29). In contrast, Leister et al. (30), who analyzed loss of heterozygosity in 69 colorectal tumors with multiple probes for the region 1p22-1p36, found that 40% revealed allelic loss with at least one probe. The critical region appeared to be 1p35 which was found to be deleted in 26 of 28 (93%) informative patients using a probe for the D1S7 locus (Fig. 3). We did not use any probes which have been localized to Ip35 and, therefore, would not have detected small interstitial deletions of this band.

Tumorigenesis has long been thought to be a multistep process. In the colon, it probably occurs as a result of activation of oncogenes, coupled with inactivation of suppressor genes (4). We hypothesized that, since activating point mutations of ras protooncogenes are a common and significant event in the progression of colorectal cancer, the suppressor gene, Krev-1, may be concomitantly inactivated in these tumors. Indications that Krev-1 may be involved in tumorigenesis come from Cline et al. (31) who showed severe decreases in expression of Krev-1 in a fibrosarcoma and a salivary gland adenocarcinoma compared with their corresponding normal tissue. However, the fact that we did not detect loss of heterozygosity at the Krev-1 locus does not rule out its involvement. Inactivation could occur through other mechanisms, including point mutation, methylation of promoter sequences, or rearrangement (4, 7). Point mutations at the guanine-binding site, the effector-binding domain, and the acylation site have all been shown to reduce the tumor suppressor activity of Krev-1 in vitro (7). Rearrangements of Krev-1 may result from small translocations which have previously been described in cytogenetic studies as chromosome deletions (16). This also may account for the fact that “deletions” of chromosome 1 appear to be more common on the basis of cytogenetic analyses than have been generally found with molecular analyses.

ACKNOWLEDGMENTS

The technical assistance of M. Payne, J. Ryan, M. Southall, L. Thomas, and Z-A. Walsh is gratefully acknowledged. We thank M. Noda (The Institute for Physical and Chemical Research, Ibaraki, Japan) for pKm-1, J. Minna (National Cancer Institute, Bethesda, MD) for L- myc, B. Forget (Yale University School of Medicine, New Haven, CT) for 3021E1 (SPTA1), and J. Chirgwin (University of Texas Health Science Center, San Antonio, TX) for phRnES 1.9 (REN), pAT3 (AT3) and pLBK8B/ES' (ALPL) were obtained from the American Type Culture Collection and A336 (D1S47) from Collaborative Research, Inc.

REFERENCES


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