Distinct K-ras Mutation Pattern Characterizes Signet Ring Cell Colorectal Carcinoma

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ABSTRACT

Purpose: Signet ring cell colorectal carcinoma (SRCCC) represents a unique, infrequent, and highly malignant variant of colorectal cancer. To understand the pathogenesis of SRCCC, we investigated its molecular abnormalities and compared them with those of the usual type of colorectal adenocarcinoma.

Experimental Design: Microdissected archival paraffin-embedded tissue from 16 SRCCCs and 27 non-SRCCCs was used to determine the frequency and pattern of mutation at codons 12, 13, and 61 of K-ras. A subset of tumors was examined for TP53 mutations at exons 5–8 and allele loss and genetic instability using seven microsatellite and two mononucleotide markers.

Results: Comparable data on TP53 mutation, allele loss, and microsatellite instability were found between SRCCC and non-SRCCC. However, SRCCCs demonstrated a distinct pattern of K-ras mutation with a significantly lower frequency of mutations at codons 12 and 13 (13% versus 48%, P = 0.02) as compared with the non-SRCCCs. Four cases (25%) of SRCCC demonstrated the same A:T transversion at the third base position of K-ras codon 61 (CAA to CAT; Gln to His). No such mutation was detected in non-SRCCCs or in the 30 gastric and 4 urinary bladder signet ring cell carcinomas examined.

Conclusions: Our findings suggest that a distinct pattern of K-ras mutation is present in SRCCC, including a specific codon 61 mutation that has rarely been reported in human neoplasms.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in the United States (1). Virtually 98% of colorectal cancers are adenocarcinomas (2). Mucinous and signet ring cell carcinomas are variants of adenocarcinomas with prominent mucus secretion (2). In the mucinous adenocarcinoma, the mucus is primarily extracellular, whereas in the signet ring cell carcinoma, the mucus is intracellular (3). The reported incidences for SRCCC4 are extremely low, ranging from 0.2% to 1% (4–8). The prognosis for SRCC patients is poor, with only a few patients alive more than 1 year after diagnosis (5, 6). Thus, SRCCC represents a unique, infrequent, and highly malignant variant of colorectal cancer.

Considerable progress has been made toward defining the constellation of molecular abnormalities that underlie the development of colorectal tumors (9). A multistep genetic model of colorectal tumorigenesis, which relies on the assumption that most carcinomas arise from pre-existing adenomas, has been proposed. This model includes mutations in a number of oncogenes (the ras gene family, β-catenin, neu, and so forth) and tumor suppressor genes (TP53, APC, DPC4/SMAD4, DCC, MCC, and so forth) in a relative orderly fashion (2, 9). Mutations in the ras gene family (K-ras, H-ras, and N-ras) can be identified in about 40% of colorectal cancers (10). The vast majority of ras mutations are present at codons 12 and 13 of K-ras, with about 80% of the ras mutations present at codon 12, and about 15% of the ras mutations present at codon 13 (10). A very small fraction of ras gene mutations are present at codon 61 of K-ras (9, 11, 12) and codons 12, 13, and 61 of N-ras (9, 13). The biological basis for the specific spectrum of ras mutations seen in colorectal tumors is not well understood. The timing of K-ras mutations with respect to different stages of colorectal tumorigenesis is of some interest (2, 9). The frequency of K-ras mutation increases in colorectal adenomas according to the size and degree of dysplasia (14).

Despite enormous progress, much work lies ahead before we will have a fully developed picture of the pathogenesis of colorectal cancer. The relative significance to the cancer cell phenotype of each of the various inherited and somatic mutations has not been defined. It seems most likely that the specific constellation of genetic alterations present in the tumor cell may determine its phenotype and may be responsible in part for the biological and clinical heterogeneity seen in colorectal cancer. To understand the pathogenesis of SRCCC, we investigated the genetic abnormalities of this infrequent type of colorectal tumor,
especially the K-ras mutation profile, and compared the findings with those of the non-SRCCCs.

MATERIALS AND METHODS

Case Selection. Archival, formalin-fixed, paraffin-embedded material from 16 surgically resected SRCCCs was obtained for molecular analysis. SRCCC is characterized by neoplastic cells with an eccentric crescent-shaped nucleus pushed by the intracytoplasmic mucus against the cell wall (Fig. 1). Because some signet ring cells can be detected in mucinous colorectal carcinomas, they were defined as signet ring cell carcinoma when this cell population represented >50% of the tumor cells. We compared the findings from the SRCCCs with those from 27 tubular surgically resected colorectal adenocarcinomas. We refer to this group as the non-SRCCCs. They were 20 moderately differentiated and 7 poorly differentiated tubular adenocarcinomas. Whereas all colorectal cases were analyzed for K-ras mutations, subsets of the SRCCC (n = 10) and non-SRCCC adenocarcinoma (n = 18) cases were examined for TP53 mutations and microsatellite abnormalities (LOH and genetic instability) at four chromosomal regions. In addition, 30 gastric and 4 urinary bladder archival signet ring carcinomas were examined for K-ras mutation at codon 61.

Microdissection and DNA Extraction. Serial 5-μm sections were cut from archival, formalin-fixed, paraffin-embedded tissue. All slides were stained with H&E, and one of the slides was coverslipped. The coverslipped slide was used as a guide to localize regions of interest for microdissection for the other slide. Microdissection and DNA extraction were performed as described previously from noncoverslipped H&E-stained slides (15). Precisely identified areas of stromal lymphocytes and invasive colorectal carcinoma were microdissected under microscopic visualization.

K-ras Mutation Analysis. For detection of K-ras mutations, we used a designed RFLP method (Fig. 2), using nested PCR methodology, followed by sequencing, as described previously (16, 17). Using the designed RFLP method, we screened for mutations in codons 12, 13, and 61 of K-ras. For this first step, amplification of DNA corresponding to exons 1 and 2 of K-ras was performed by nested PCR methods. The first PCR product was used as template for the second PCR reaction. In the latter, inner mismatched primers were used to introduce a new restriction site into the PCR product derived from wild-type allele. The second PCR products thus obtained were digested using corresponding restriction enzymes. The digested products were electrophoresed and visualized under UV light after ethidium bromide staining. Wild-type alleles were digested and yielded a smaller product than mutant forms. Both bands could be recognized after electrophoresis. K-ras mutations discovered in this fashion were confirmed by direct sequencing of both strands of the PCR product from the screening step, as described previously (16).

TP53 Mutation and Microsatellite Analyses. We examined samples for mutations in exons 5–8 of TP53 using nested PCR methodology and single-strand conformational polymorphism analysis followed by sequencing of both strands of abnormal bands, as described previously (18). A corresponding positive control was included in the analyses for each exon.

Microdissected tumor tissue was used for LOH analysis at four chromosomal regions frequently deleted in colorectal cancer, using six polymorphic microsatellite markers located at the following genes or chromosomal regions: 3p14.2-APC gene (D3S1234 and D3S1234); 5q21-APC gene (LNSCA); 13q12-RB gene (dinucleotide CA repeat); and 17p13-TP53 gene (TP53 dinucleotide CA and pentanucleotide repeat). Primers sequences were obtained from Genome Database. Because we used DNA extracted from archival paraffin-embedded tissues, the amplicon size was restricted to less than 250 bp. A two-round PCR strategy (multiplex PCR followed by uniplex PCR) was used to amplify each marker, as described previously (15). In addition, for genetic instability analysis, mononucleotide markers BAT-25 and BAT-26 were examined, as described previously (19). The final product was separated on a 6% denaturing polyacrylamide gel and subjected to autoradiography. LOH was scored by visual detection of complete absence of one allele of informative cases.

Data Analysis. Statistical analysis was performed using Fisher’s exact test. P < 0.05 was considered significant.

RESULTS

K-ras Mutation. We screened for the presence of mutations in codons 12, 13, and 61 in microdissected tumor tissue from 16 SRCCCs and compared the results with those obtained from 27 non-SRCCCs. All microdissected materials provided suitable template for PCR reactions. SRCCCs demonstrated a low frequency of mutations at codon 12 (2 of 16 SRCCCs;
13%), and no mutation at codon 13 was detected (Table 1). However, they demonstrated a higher frequency of mutations at codon 61 (4 of 16 SRCCCs; 25%), and the identical point mutation at the third base of codon 61 was detected in those four SRCCCs, with CAA (Gln) to CAT (His) substitution (Fig. 2). In all experiments, the codon 61 mutation was confirmed by performing three independent experiments using neoplastic cells obtained from different areas of the tumor, and sequencing was performed using forward and reverse primers in replicate experiments. In each case, the codon 61 mutation was confirmed by performing three independent experiments using neoplastic cells obtained from different areas of the tumor, and sequencing was performed using forward and reverse primers in replicate experiments. In all experiments, the same results were obtained. Whereas non-SRCCC K-\textit{ras} mutation frequencies at codons 12 (11 of 27; 41%) and 13 (2 of 27; 7%) were similar to those reported previously (10), no mutation at codon 61 was detected in that tumor type. The differences in the frequencies of mutations at exon 1 (codons 12 and 13) and exon 2 (codon 61) of K-\textit{ras} between SRCCCs and non-SRCCCs were statistically significant (\(P = 0.02\) and 0.01, respectively; Table 1). To investigate whether the CAA (Gln) to CAT (His) substitution at codon 61 was specific to signet ring cell histological phenotype, we screened 30 gastric and 4 urinary bladder signet ring carcinomas for this particular point mutation, and no such base substitution was detected.

**TP53 Mutation and Microsatellite Abnormalities.**

\textit{TP53} mutations were detected in 4 of 10 (40%) and 9 of 18 (50%) SRCCCs and non-SRCCCs, respectively (Table 1). In SRCCCs, mutations were detected in exon 5 (codon 142, CT to ATT, Leu to Ile), exon 7 (codon 242, TGC to TAC, Cys to Tyr; codon 246, ATG to ATA, Met to Ile), and exon 8 (codon 267, CGG to AGC, Arg to Ser). The pattern of \textit{TP53} mutation found in non-SRCCCs was similar to the one reported previously (data not shown; Ref. 20).

Although the number of cases analyzed was relatively small, SRCCCs demonstrated a higher frequency of allelic loss at 3\textit{p14.2-FHIT} and a lower incidence of losses at 17\textit{p13-TP53} (\(P = 0.04\)) and 5\textit{q21-APC-MCC} (\(P = 0.04\)) as detected in 4 of 16 SRCCCs and 0 of 10 non-SRCCCs, respectively (Table 1). Although mutations at codon 12 and 13 and by the presence of a relatively frequent (25%) and distinct point mutation at the third base of codon 61 (CAA to CAT) in all four positive SRCCCs, respectively, these were statistically significant (\(P < 0.01\), Table 1). To investigate whether the CAA (Gln) to CAT (His) substitution at codon 61 was specific to signet ring cell histological phenotype, we screened 30 gastric and 4 urinary bladder signet ring carcinomas for this particular point mutation, and no such base substitution was detected.

**Table 1** Summary of molecular abnormalities in signet ring cell and usual types of colorectal carcinomas

<table>
<thead>
<tr>
<th>Molecular abnormality</th>
<th>SRCCC</th>
<th>Non-SRCCC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K-\textit{ras} gene mutations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (N)</td>
<td>16</td>
<td>27</td>
<td>43</td>
</tr>
<tr>
<td>Exon 1</td>
<td>2 (13%)</td>
<td>13 (48%)</td>
<td>15 (35%)</td>
</tr>
<tr>
<td>Codon 12</td>
<td>2</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Codon 13</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Exon 2</td>
<td>4 (25%)</td>
<td>0*</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>Codon 61</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>\textit{TP53} gene mutations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (N)</td>
<td>10</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Mutations</td>
<td>4 (40%)</td>
<td>9 (50%)</td>
<td>13 (42%)</td>
</tr>
<tr>
<td><strong>LOH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (N)</td>
<td>10</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>5\textit{q21-APC/MCC}</td>
<td>2/10 (20%)</td>
<td>7/15 (47%)</td>
<td>9/25 (36%)</td>
</tr>
<tr>
<td>17\textit{p13-TP53}</td>
<td>2/10 (20%)</td>
<td>9/14 (64%)</td>
<td>11/24 (46%)</td>
</tr>
<tr>
<td>3\textit{p14.2-FHIT}</td>
<td>5/8 (63%)</td>
<td>6/13 (46%)</td>
<td>11/21 (52%)</td>
</tr>
<tr>
<td>13\textit{q-RB}</td>
<td>3/8 (38%)</td>
<td>4/12 (33%)</td>
<td>7/20 (35%)</td>
</tr>
<tr>
<td><strong>MSI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (N)</td>
<td>10</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>BAT-25/BAT-26 (any)</td>
<td>4 (40%)</td>
<td>2 (11%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td>Low (&lt;2 markers)</td>
<td>7 (70%)</td>
<td>15 (83%)</td>
<td>22 (79%)</td>
</tr>
<tr>
<td>High (&gt;2 markers)</td>
<td>3 (30%)</td>
<td>3 (17%)</td>
<td>6 (21%)</td>
</tr>
</tbody>
</table>

*\(P = 0.02\), *\(P = 0.01\), *All mutations detected were CAA \to CAT (Gln-His).
\(P = 0.04\).

**DISCUSSION**

Our findings indicate that a distinct pattern of K-\textit{ras} mutations is involved in the pathogenesis of SRCCC compared with those of the usual type of tubular colorectal adenocarcinomas. SRCCCs are characterized by a significantly lower frequency [13% (SRCCC) versus 48% (non-SRCCC); \(P = 0.02\)] of K-\textit{ras} mutations at codon 12 and 13 and by the presence of a relatively frequent (25%) and distinct point mutation at the third base of codon 61 (CAA to CAT transversion, producing a Gln to His amino acid change). This distinct codon 61 mutation was not detected in any of the non-SRCCCs examined. Contamination or artifactual results were ruled out by the finding of the same codon 61 mutation in three independent experiments, using neoplastic cells obtained from different areas of the tumor, in all four positive SRCCCs. There is only one report on the genetic abnormalities of SRCCC (21) that also detected a lower
frequency of mutations at codon 12 and 13 of K-ras in this tumor type (11%) compared with those of the non-SRCCC (39%). However, our study is the first report on mutation analysis on codon 61 of K-ras in this infrequent type of colorectal cancer. Few studies on K-ras mutation of colorectal cancer have included codon 61 analysis, and a very small fraction (estimated as <5%) of K-ras mutations are present at this codon (9, 11, 12). Thus, our finding that 4 of 16 SRCCCs had this particular mutation is of major interest. Because no database for ras gene mutations is available, we searched for K-ras mutations at codon 61 on MEDLINE. We concluded that our A to T transversion at codon 61 is an extremely rare mutation detected in human neoplasms, and only one report involved a colorectal carcinoma sample. Interestingly, this sample corresponded to a human colorectal cancer-derived cell line (KC-1) established from a mucinous adenocarcinoma of colon (12), also characterized by prominent mucus secretion.

SRCCC is considered a tumor arising in flat colonic mucosa and not following the adenoma-carcinoma sequence (22). Colorectal cancers of non-SRCCC type also arising in flat mucosa have been characterized as colorectal neoplasms with a somehow different pattern of molecular abnormalities, including a relatively low frequency of K-ras mutations at codon 12 (23). However, no mutation data on codon 61 have been reported in colorectal carcinomas arising in flat mucosa to date.

Signet ring cell carcinoma phenotype has been described in other organs, including the stomach, gallbladder, urinary bladder, prostate, and lung (24, 25). To address the question of whether our A to T transversion at codon 61 of K-ras could be a mutation associated with this specific tumor phenotype, we examined its presence in 30 gastric and 4 urinary bladder signet ring carcinomas available to us, and no mutation was detected. Although ras has been the focus of intense research activity over the past two decades, the exact function of cellular ras proteins is far from completely understood. It has been established that ras mediates important cellular processes such as proliferation, survival, and differentiation; however, the exact contribution of the H, N, and K isofoms is not clear (26). The major hot spots for activating ras mutations are all located in the phosphate-binding domain of the protein (27). ras mutations found at residues 12, 13, and 61 decrease the intrinsic rate of GTP hydrolysis by ras and make the molecule significantly less sensitive to GAP-stimulated GTP hydrolysis (28). The identification of a large number of K-ras mutations at A:T bp of the second base of codon 61 in various neoplasms chemically induced in mice (29, 30) suggests that adenosine sites at codon 61 of K-ras may represent a critical site for the interaction of the metabolic intermediates with DNA to form adenine adducts and the generation of apurinic sites. Further misreplication at these sites may represent a critical step in the initiation of the carcinogenic process.

Although we analyzed a relatively small number of cases, the frequency and pattern of TP53 mutations were similar between SRCCCs and non-SRCCCs (40% versus 50%, respectively). A relatively lower frequency of p53 protein immunostaining overexpression has been reported previously on SRCCCs (29%) compared with non-SRCCCs (56%; Ref. 21). However, no mutation analysis of TP53 on SRCCCs has been published previously. A significantly lower frequency of 17p13 (TP53 locus) allelic loss was detected in our SRCCC cases compared with non-SRCCCs, suggesting that less frequent genetic abnormalities may involve this tumor suppressor gene in SRCCC. No other significant differences were detected in the pattern of allelic loss between both groups of colorectal carcinomas.

SRCCCs demonstrated a lower incidence of MSI, an expression of a mutator phenotype in colorectal cancer. Although mucinous colorectal cancers, including SRCCCs, have been reported as overrepresented in patients with mismatch repair gene inactivation in hereditary non-polyposis colorectal cancer, our cases did not show a high incidence of the high-grade MSI that characterizes this phenomenon (31).

In summary, we detected a distinct pattern of K-ras mutation in SRCCCs, including a particular codon 61 mutation, which has rarely been reported in human neoplasms. Although the significance of this particular mutation to the cancer signet ring cell phenotype needs to be further investigated, our finding may help to elucidate the pathogenesis of this highly malignant colorectal cancer type. Because K-ras mutations at codon 12 have been suggested as a useful marker for early detection of colon cancer, the finding of a colon cancer variant, such as SRCCC, that has a different pattern of K-ras mutations needs to be known.

REFERENCES


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