Tumor Necrosis Factor-α Allelic Frequency and Chromosome 6 Allelic Imbalance in Patients with Colorectal Cancer

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ABSTRACT

The human tumor necrosis factor (TNF) locus is located on chromosome 6p21.3 and contains at least five polymorphic microsatellites. In this study, we compared the allelic frequencies derived from 50 normal controls to 64 patients with colorectal cancer at one of these loci, TNFa. No differences in allelic frequencies were observed between these two groups (P = 0.47). However, sequencing of the TNFa PCR product revealed two populations of TNFa alleles; alleles with the expected DNA sequence (i.e., the expected number of AC/GT repeats) and alleles that contained 8-bp deletions adjacent to the microsatellite repeat. In addition, we also examined paired normal and tumor DNA from the colorectal cancer group for microsatellite alterations at the TNFa locus, including allelic loss of heterozygosity and microsatellite instability. Of the 64 tumors examined, 13 (20%) demonstrated microsatellite instability, and 14 (42%) of 33 informative cases demonstrated allelic imbalance. Analysis of 10 additional chromosome 6 loci for allelic loss showed that at least one chromosome 6p marker, 23 (47%) of 49 informative cases exhibited allelic imbalance, with at least one chromosome 6p marker, 23 (47%) of 49 with at least one 6q marker, and 29 (59%) of 49 with at least one marker on chromosome 6. Examination of tumors for the minimal region of deletion overlap suggests the presence of tumor suppressor genes on both 6p and 6q.

INTRODUCTION

CRC is one of the leading causes of cancer mortality in the United States, affecting nearly 160,000 individuals annually (1). This tumor system has provided a particularly useful model for studying the molecular genetic changes that occur in the progression from normal tissue to adenoma and finally to carcinoma. In CRC, a number of studies have suggested that the process of tumorigenesis proceeds through a series of genetic alterations, which include dominantly acting oncogenes, recessive acting tumor suppressor genes (2, 3), and, more recently, mutator genes (4). Tumor suppressor genes include the APC gene on chromosome 5q (5–8), p53 on chromosome 17p (9), and DCC on chromosome 18q (10). Although these loci have been of much focus, it is clear that other chromosomes harbor additional tumor suppressor genes (3), which may be important in the pathogenesis of CRC.

Recently, Campbell et al. (11) reported a significant difference in allelic frequency within the TNFa locus, the most polymorphic of the TNF locus microsatellites, in patients with CRC compared with normal, healthy controls. The most striking finding of this study was an α3 allele frequency of almost 30% in the CRC group with a complete absence in the normal control group. The role of the TNF locus in colorectal carcinoma, however, is unknown. The human TNF locus consists of two tandemly arranged and closely linked genes, TNFα and lymphotixin (12). Five polymorphic microsatellites and an NcoI RFLP have been identified within the TNF locus (chromosome 6p21.3) as described previously (13–15). These polymorphic markers have been used in both TNF and HLA haplotype studies (16, 17) and in a number of studies exploring the association of various TNF alleles with a variety of disease states (18, 19).

In this study, we compared TNFa allelic frequencies derived from 50 normal controls to 64 patients diagnosed with CRC. Although no differences were detected between these two populations, a high frequency of allelic imbalance was noted at this locus, suggesting the presence of a tumor suppressor gene on this chromosome. In an effort to extend this observation and localize candidate tumor suppressor genes further, we analyzed paired normal and tumor DNA for allelic imbalance at 10 other loci on chromosome 6. Our data suggest the presence of at least two sites on chromosome 6 that may harbor additional tumor suppressor genes important in the pathogenesis of CRC. Finally, we report the identification of two populations of TNFa alleles: alleles with the expected DNA sequence; and alleles that contain an 8-bp deletion adjacent to the AC/GT repeat.

MATERIALS AND METHODS

Tissue Samples. All protocols were approved by the Mayo Institutional Review Board. CRC tissue was obtained from 64 patients who underwent resection for CRC at the Mayo Clinic. Of the 64 patients with CRC, 15 were identified previously as having tumor MIN (20). Harvested tissue was frozen immediately at −70°C and stored until the time of DNA extraction.

DNA Extraction. Tissue processing and DNA extraction were performed as described previously (21). Briefly, tissue was first mounted and examined microscopically with hematoxylin and eosin-stained cryosections. Microdissection of the specimen was performed then for removal of normal tissue to assure that tumor tissue contained >70% cancer cells. Tumor DNA was obtained from multiple 10-μm-thick cryosections, whereas paired noncancerous DNA was obtained from either normal mucosa or peripheral blood leukocytes. DNA was also extracted from peripheral blood leukocytes obtained from 50 normal, healthy controls (blood bank donors).

Microsatellite Analysis. The AC/GT repeat at the TNFa locus (chromosome 6p21.3) was analyzed by PCR with use of primers IR-2 and IR-4 described by Niedospasov et al. (14). In addition, normal and tumor pairs were analyzed for microsatellite alterations at TNFa and 10 other loci (Research Genetics, Huntsville, AL). These loci are described in more detail in Table I. PCR and gel electrophoresis were performed essentially as described by Thibodeau et al. (20). Allelic imbalance was determined by comparing the intensities of alleles observed for the match tumor and normal DNA. Autoradiographs of normal and tumor pairs were examined visually by two independent reviewers (R. H. and S. N. T.). Normal and tumor pairs that were not obvious for allelic imbalance or retention of heterozygosity were quantitated using either densitometry (NIH Image version 1.47) or with the use of a 445 Si phosphomager (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant (version 4.1) software package. Allelic imbalance was considered to be present when the relative intensity of the two alleles in tumor DNA differed from the relative intensity in normal DNA by a factor of at least 1.4 (22).

TNFa Sequencing. Primers IR-2 and IR-4 were used to create the template by PCR. The IR-2 primer was used then as the sequencing primer with the use of the fmol sequencing kit (Promega, Madison, WI).

Statistical Analysis. Allelic frequencies at the TNFa locus in normal individuals and patients with CRC were compared using Fisher’s exact test as implemented in StatXact (23). This was a single global test for the 2 × 12 table of allele counts; the exact method was used because of the small counts.

Allelic imbalance at each of the loci was assessed for associations with a number of clinical and pathological parameters, including site of the tumor (proximal or distal), sex and age of the subject, and Duke’s stage of the tumor. Fisher’s exact test was used to assess the association between allelic imbalance.
CHROMOSOME 6 ALLELIC IMBALANCE IN COLORECTAL CARCINOMA

Table 1 Allelic imbalance on chromosome 6 in sporadic CRC

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Allelic imbalance/informative cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D6S477</td>
<td>6p24-pter</td>
<td>15/33 (45)</td>
</tr>
<tr>
<td>D6S259</td>
<td>6p23</td>
<td>13/36 (36)</td>
</tr>
<tr>
<td>D6S258</td>
<td>6p21.3-p22.3</td>
<td>15/33 (45)</td>
</tr>
<tr>
<td>TNFa</td>
<td>6p21.3</td>
<td>14/33 (42)</td>
</tr>
<tr>
<td>D6S271</td>
<td>6p21.1-p21.2</td>
<td>17/42 (40)</td>
</tr>
<tr>
<td>D6S257</td>
<td>6p11-p12</td>
<td>13/42 (31)</td>
</tr>
<tr>
<td>D6S251</td>
<td>6q14-q16.2</td>
<td>13/36 (36)</td>
</tr>
<tr>
<td>D6S252</td>
<td>6q21-q22.1</td>
<td>12/35 (34)</td>
</tr>
<tr>
<td>D6S305</td>
<td>6q25-q27</td>
<td>16/40 (40)</td>
</tr>
<tr>
<td>D6S254</td>
<td>6q25-q27</td>
<td>13/32 (41)</td>
</tr>
<tr>
<td>D6S503</td>
<td>6q27-qter</td>
<td>14/34 (41)</td>
</tr>
</tbody>
</table>

for each of the markers with both site and sex. The Mantel-Haenszel χ² test for
linear trend (24) was used to determine whether there was a trend in the proportions of allelic loss over the increasing levels of stage. The ages at the
time of surgery of the subjects whose tumors showed allelic imbalance were
compared with those of subjects whose tumors did not show allelic imbalance
by the Wilcoxon rank sum test. Analyses were performed using the SAS
software (25).

RESULTS

TNFa Allelic Frequencies. We compared the TNFa allelic frequencies derived from 50 normal controls to 64 patients with CRC
(Table 2). Unlike previously reported results, no statistical difference
in allelic frequencies was observed between the control and the CRC
groups (P = 0.47).

The TNFa locus has 13 known alleles, each separated by one
dinucleotide repeat (14). Based on a study of TNFa allelic frequencies
in four European populations (26), it seemed likely that our patient
population did not have the a1 allele. To confirm this observation, we
sequenced the TNFa PCR product in one individual who was ho-
mozygous for the a11 allele and two individuals who were homozy-
gous for the a2 allele. As expected, the individual believed to be
homozygous for the a11 allele had (AC/GT)16 repeats (14). Thus, the
allele assignment made for the data shown in Table 2 seem to be
consistent with previously published results. However, both individ-
uals believed to be homozygous for the a2 allele had 8-bp deletions
just upstream of the AC/GT repeats and, thus, contained four more
repeats than expected (Fig. 1). To determine the frequency and dis-
tribution of the 8-bp deletion, the TNFa PCR products from 27
controls and 33 individuals with CRC were sequenced, for a total of
120 chromosomes examined. In general, alleles a2—a6 contained the
8-bp deletion, whereas alleles a7—a13 lacked this deletion and con-
tained the expected number of repeats. All patients that were either
homozygous or heterozygous for alleles a2—a6 (n = 26; 52 chromo-
somes) were homozygous for the 8-bp deletion. On the other hand, all
but two patients homozygous or heterozygous for alleles a7—a13
lacked the 8-bp deletion (n = 14; 28 chromosomes). One of these
individuals was homozygous for the a7 allele but heterozygous for the
8-bp deletion (phase unknown). Finally, although it was not
possible to determine the phase, all but one heterozygote with one of
the alleles in the a2—a6 range and the other in the a7—a13 range were
heterozygous for the 8-bp deletion (n = 20; 40 chromosomes). The

single exception was a patient who was heterozygous a5/a10 but
homozygous for the 8-bp deletion.

Chromosome 6 Allelic Imbalance. In addition to determining the
allelic frequencies in normal DNA, paired normal and tumor DNA
from the CRC group were also analyzed for other microsatellite
alterations at the TNFa locus, including loss of heterozygosity (or
allelic imbalance) and MIN. Of the 64 normal and tumor pairs
examined, 14 (42%) of 33 informative cases demonstrated allelic
imbalance, whereas 13 (20%) of 64 tumors demonstrated MIN. Because
allelic imbalance and MIN were observed commonly at the
TNFa locus, paired normal and tumor DNA from the CRC group
were analyzed for microsatellite alterations at 10 additional microsat-
ellites localized to chromosome 6 (Table 1). Typical examples of
allelic imbalance and MIN are shown in Fig. 2. As expected, those 15
tumors identified previously as having tumor MIN (20) continued to
display this phenotype with each of the markers used. In fact, of the
165 analyses performed on these 15 tumors (15 tumors × 11 mark-
ers), only 23 failed to demonstrate MIN. For each of these 15 tumors,

Table 2 TNFa allelic frequencies derived from 50 normal controls and 64 patients with CRC

<table>
<thead>
<tr>
<th>Allele</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.30</td>
<td>0.00</td>
<td>0.09</td>
<td>0.05</td>
<td>0.19</td>
<td>0.09</td>
<td>0.01</td>
<td>0.01</td>
<td>0.10</td>
<td>0.15</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>CRC</td>
<td>0.00</td>
<td>0.37</td>
<td>0.01</td>
<td>0.07</td>
<td>0.04</td>
<td>0.10</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.15</td>
<td>0.18</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Fig. 1. Sequence analysis of the TNFa locus from individuals who are homozygous
a10/a10 and homogenous for the intact sequence (A), heterozygous a2a7 and heterozy-
gous for the 8-bp deletion (B), and homozygous a2a2 and homozygous for the 8-bp
deletion (C).
no Al MIN
change

CHROMOSOME 6 ALLELIC IMBALANCE IN COLORECTAL CARCINOMA (proximal versus distal). On the other hand, a trend toward higher frequencies of allelic imbalance was detected when progressing from Duke's stages A–D for some of the chromosome 6 loci (D6S305 and D6S503; P = 0.03 and 0.053, respectively), and for markers on chromosome 6p as a group (imbalances of 50, 32, 58, and 64% for Duke's stages A–D, respectively; P = 0.10). In general, patients with Duke's D disease tended to have a higher frequency of allelic imbalance at loci on chromosome 6.

DISCUSSION

The TNF and lymphotoxin genes are arranged tandemly and map within the MHC centromeric to HLA-B and telomeric to the class III region on chromosome 6p21.3 (12). The chromosomal location and the immunomodulatory effects of these two genes has prompted much speculation about the role of the TNF locus in MHC-linked diseases. The recent identification of polymorphic markers (including microsatellites) within the TNF locus has resulted in a number of studies correlating TNF haplotypes with pathogenesis of disease (18, 19, 27, 28).

Recently, Campbell et al. (11) reported that germ line allelic frequencies for the TNFa locus were significantly different in CRC patients compared with normal controls. Most strikingly, the a3 allele had an allelic frequency of nearly 30%, whereas the a3 allele was not detected in the normal control group. TNF is well known for its cytotoxic and antitumorigenic properties. However, some properties of the TNF gene, such as enhanced angiogenesis and up-regulation of adhesion molecules, could be protumorigenic. Some malignant cell lines are known to express TNF gene products constitutively (29—31). In addition, colorectal tumors have been shown to express TNF mRNA by Northern blot analysis (32). Colorectal tumors can be infiltrated heavily with lymphocytic- and monocytic-derived cells, and these cells also can be a potential source of TNF and lymphotoxin. Monocyte tumor necrosis secretory capacity has been reported to correlate with a number of genetic markers, including HLA-DR, TNF NcoI RFLP, and TNF microsatellite alleles (18). These studies have raised the interesting possibility that the TNF locus may play a role in colorectal cancer.

To investigate the role of the TNF locus in CRC further, we compared germ line TNFa allelic frequencies derived from 50 normal

In addition to TNFa, a high frequency of allelic imbalance was also noted at most of the other loci examined. For the purposes of scoring for allelic imbalance, tumors demonstrating generalized MIN were considered uninformative. Of the 49 tumors lacking generalized MIN, the rate of allelic imbalance ranged from 31 (D6S257) to 45% (D6S258 and D6S477; Table 1). Twenty-three (47%) of 49 informative tumors demonstrated allelic imbalance with at least one chromosome 6p marker, and 23 (47%) of 49 informative tumors demonstrated allelic imbalance with at least one 6q marker. Altogether, 29 (59%) of 49 informative tumors exhibited allelic imbalance with at least one chromosome 6 locus. Fig. 3 summarizes the results obtained for the 29 tumors identified as having allelic imbalance with at least one marker on chromosome 6.

Correlation with Clinicopathological Characteristics. Although the number of cases are limited, allelic imbalance at each of the chromosome 6 loci was examined for potential associations with a number of clinical and pathological parameters. In addition to examining each of the markers individually, we also tested for associations with any allelic loss occurring on either 6p, 6q, or all of chromosome 6. For each of these analyses, no correlations were detected between allelic imbalance and age (at time of surgery), sex, or site of tumor
controls to 64 individuals diagnosed with CRC. Unlike the previous report, however, a significant difference in allelic frequencies was not detected between these two groups. Although the reason for the discrepancy is unclear, one possibility is ethnic variation. TNFα allelic frequencies have been shown to be significantly different among four European populations (26).

Of interest, our normal control and CRC populations contained a range of only 12 alleles. Because TNFα is known to have at least 13 alleles, we sequenced the TNFα PCR products derived from three individuals homozygous for TNFα alleles to confirm allele size. One individual had the expected DNA sequence, but the other two had 8-bp deletions adjacent to the microsatellite repeats and, therefore, had four more AC repeats than expected. Further sequencing revealed that the expected number of repeats within the TNFα locus was observed only in individuals with alleles a7-a13, whereas individuals with alleles a2-a6 contained primarily the 8-bp deletion sequence. Because TNFα allelic frequency often varies from one population group to another, it is likely that alleles with and without the 8-bp deletion also will vary from one population to another. This variation could create difficulty in comparing TNF haplotypes among different populations. This finding also may be important when comparing disease groups with normal control groups.

In addition to determining TNFα allelic frequencies, paired normal and tumor DNA from the CRC group were also analyzed for microsatellite alterations at the TNFα locus. Forty-two percent of informative tumors exhibited allelic imbalance, and 20% of tumors demonstrated MIN at this locus. Given the high rate of allelic imbalance at the TNFα locus, we decided to investigate this observation further using 10 additional microsatellite markers localized to chromosome 6. Using these markers, the frequency of allelic imbalance ranged from 31 (D6S257) to 45% (D6S258 and D6S477) and altogether, 29 (59%) of 49 of informative tumors exhibited allelic imbalance with at least one chromosome 6 marker.

A high rate of allelic imbalance at chromosome 6 for CRC has been reported in only one other study. Using a single RFLP marker for each chromosome arm, Vogelstein et al. (3) reported that loss of heterozygosity occurred at a rate of just less than 30% for chromosome 6p and just greater than 30% for chromosome 6q. However, further examination of the markers used in this initial study suggests that both of these are localized to 6q27. Based on the 11 markers used in the current study, we confirm now the earlier finding of allelic imbalance on chromosome 6 and extend these findings to map candidate tumor suppressor genes on this chromosome further. Our data suggest the presence of multiple tumor suppressor genes on chromosome 6, with at least one localized to 6p and another to 6q. Of the 29 tumors demonstrating allelic imbalance (Fig. 3), 6 demonstrated allelic imbalance only on 6q (cases 22, 49, 11, 32, 43, and 17), and an equal number demonstrated allelic imbalance only on 6p (cases 9, 15, 39, 47, 42, and 37). Although we have an insufficient number of tumors for precise minimal deletion mapping, these data do suggest a potential region distal to D65271 on 6p and another region distal to D65305 on 6q.

In addition to CRC, several studies now suggest the possible involvement of multiple tumor suppressor genes on chromosome 6 in other tumor systems. A high rate of loss of heterozygosity for chromosome 6q has been reported in breast carcinoma (33, 34), malignant melanoma (35, 36), and ovarian cancer (37–42). A high rate of loss of heterozygosity on both chromosomes 6p and 6q has been reported for epithelial ovarian cancer (43) and small cell lung cancer (44). Additionally, studies of allelic imbalance in breast and ovarian cancer suggest at least two regions on chromosome arm 6q for putative tumor suppressor gene loci, one at 6q26-q27 and the other at 6q12-q23 (33, 38, 43).

Although there is little information on the nature of putative tumor suppressor genes on chromosome 6, one candidate is the WAF1/CIP1 gene at 6p21.2. WAF1/CIP1 is up-regulated by p53 and is an inhibitor of cyclin-dependent kinases (45). WAF1/CIP1 is not induced by mutant p53, and introduction of WAF1 cDNA into a variety of human tumor cell lines has been shown to inhibit cell growth (45). In one study, however, somatic mutations in WAF1/CIP1 were not observed in sporadic CRC, suggesting that this gene may not be a frequent site of mutation in CRC (46).

In this study, we also tested for possible associations between allelic imbalance and a number of clinical and pathological parameters. Although not statistically significant, a trend toward higher frequencies of allelic imbalance across Duke’s stages was observed, with Duke’s D demonstrating the highest frequency of allelic loss. These results suggest that these events, and the potential involvement of putative tumor suppressor genes on chromosome 6, may be late events in the progression of adenoma to carcinoma and finally to metastatic disease. Because such associations may have important clinical implications, we are now in the process of examining a larger series of patients to test these and other associations in more detail.

No significant difference was observed between normal control and CRC TNFα allelic frequencies. Two populations of TNFα alleles were identified: (a) alleles with the expected TNFα DNA sequence; and (b) alleles that contained an 8-bp deletion adjacent to the TNFα microsatellite repeats. A high rate of allelic imbalance was observed in paired normal and tumor DNA on chromosome 6 in CRC, suggesting the presence of multiple tumor suppressor genes on this chromosome.

ACKNOWLEDGMENTS

We thank Karen Erwin for her excellent secretarial assistance.

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Cancer Res 1996;56:145-149.

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