Infrequent Detection of Germline Allele-Specific Expression of TGFBR1 in Lymphoblasts and Tissues of Colon Cancer Patients

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Abstract
Recently, germline allele-specific expression (ASE) of the gene encoding for transforming growth factor-β type I receptor (TGFBR1) has been proposed to be a major risk factor for cancer predisposition in the colon. Germline ASE results in a lowered expression of one of the TGFBR1 alleles (>1.5-fold), and was shown to occur in ~20% of informative familial and sporadic colorectal cancer (CRC) cases. In the present study, using the highly quantitative pyrosequencing technique, we estimated the frequency of ASE in TGFBR1 in a cohort of affected individuals from familial clusters of advanced colon neoplasias (cancers and adenomas with high-grade dysplasia), and also from a cohort of individuals with sporadic CRCs. Cases were considered positive for the presence of ASE if demonstrating an allelic expression ratio <0.67 or >1.5. Using RNA derived from lymphoblastoid cell lines, we find that of 46 informative Caucasian advanced colon neoplasia cases with a family history, only 2 individuals display a modest ASE, with allelic ratios of 1.65 and 1.73, respectively. Given that ASE of TGFBR1, if present, would likely be more pronounced in the colon compared with other tissues, we additionally determined the allelic ratios of TGFBR1 in the RNA derived from normal-appearing colonic mucosa of sporadic CRC cases. We, however, found no evidence of ASE in any of 44 informative sporadic sporadic cases analyzed. Taken together, we find that germline ASE of TGFBR1, as assayed in lymphoblastoid and colon epithelial cells of colon cancer patients, is a relatively rare event. [Cancer Res 2009;69(12):4959–61]

Introduction
Colon cancer is one of the major leading causes of cancer-related deaths in developed nations. Although most of the colorectal cancer (CRC) cases are sporadic in nature, up to 30% of the cases show familial clustering of the disease (1). Two well-characterized, highly penetrant inherited syndromes, familial adenomatous polyposis and hereditary nonpolyposis CRC, together account for ~3% of all CRC cases (1). However, in 20% to 25% of additional colon cancer cases with a family history, the underlying inherited genetic risk factors for cancer predisposition remain largely unknown (1).

The transforming growth factor-β (TGF-β) signaling pathway is a key tumor suppressor pathway that is commonly targeted for inactivation in colon cancers, with one-third of colon cancers acquiring somatic mutations in TGF-β type II receptor (2), and many of the remaining colon cancers acquiring somatic mutations and deletions in the downstream signaling proteins, SMAD elements (3). Although genetic aberrations in the TGF-β type I receptor (TGFBR1) have not been identified thus far in colon cancer, a recent study reported that peripheral blood leukocytes from ~20% of informative sporadic and nonsyndromic familial CRC cases show reduced expression of the TGFBR1 gene owing to a lowered expression one of the TGFBR1 alleles (4). This differential allele expression, called germline allele-specific expression (ASE), was suggested to be dominantly inherited and proposed to confer a substantially increased risk for CRC (4). To verify the extent of ASE of TGFBR1 in colon cancer, we used a panel of 5 single nucleotide polymorphisms (SNP) in the 3' untranslated region (UTR) region of the TGFBR1 gene to determine the allele expression ratio in a group of familial and sporadic CRC cases. Using DNA and RNA from lymphoblastoid cells and from colonic mucosa cells, this study finds that germline ASE of TGFBR1 is less common in colon neoplasia than originally proposed (4).

Materials and Methods

Patients

Familial colon neoplasia cases. For this study, colon neoplasia kindreds were obtained from the Colon Neoplasia Sibling Study cohort that were each selected to have at least one pair of siblings in which both individuals had by age 65 y been diagnosed with either a colon cancer or an adenoma with high grade dysplasia (5). A total of 102 of these affected siblings (98% Caucasian, 2% African American) and 44 disease-free familial member controls (98% Caucasian, 2% African American) were chosen based on availability of EBV-transformed lymphocyte cell lines. The average age at diagnosis for the CRC cases was 51.2 y, and the average age of having documented a clean colonoscopy for the unaffected family members was 55.4 y. All advanced colon neoplasms arising in the Colon Neoplasia Sibling Study cohort were microsatellite stable, with no evidence for familial adenomatous polyposis or hereditary nonpolyposis CRC, as described earlier (5). The familial cases and controls included 53% and 61% females, respectively.

Sporadic CRC cases. A random sample of 96 CRC cases (74% Caucasian, 21% African American, and 5% unknown ethnicity) with no family history of colon cancer were accrued under an Institutional Review Board–approved protocol at the Case Medical Center. The average age at diagnosis for the sporadic CRC cases was 69 y, and included 51% females.
DNA/RNA Extraction and cDNA Synthesis
DNA and RNA from the familial CRC cases and controls were extracted from lymphoblastoid cell lines as described earlier (5). DNA and RNA from the sporadic CRC cases were extracted from the normal-appearing mucosal layer of the colon as described earlier (6). Total RNA was treated with DNase (TURBO DNA-free kit; Ambion) before cDNA synthesis (Invitrogen). Reverse transcriptase–negative control reactions were performed in parallel on the DNase-treated RNAs for each sample.

PCR Primers and Conditions
Five SNPs, rs868, rs334348, rs334349, rs420549, and rs1590, located in the TGFBR1 3' UTR, were used for quantifying individual allele expression. Three of these SNPs (rs334348, rs334349, and rs1590) were selected from the group characterized by Valle and colleagues (4), with two additional SNPs also added to increase the number of informative cases that could be analyzed for ASE. The range of minor allele frequencies for these 5 SNPs was 9% to 38%.7

PCR and sequencing primers for the analysis were designed using the PSQ Assay Design software (Biotage). One of the PCR primer pair for each SNP was biotinylated at the 5'-end, and purified using high performance liquid chromatography. The sequences are as follows. rs868: For 5'tcagttcatggcgtgatt3'; Rev 5'gtcgtctgaaaggaaagcaag3'. Seq 5'gtttgattttggcaggtg3'; rs334348: For 5'bctgctctcggtctccacct3'; Rev 5'tgaacataggtgtgagcaag3'. Seq 5'taaacttttcaagccactgtt3'; rs334349: For 5'bctgctctcggtctccacct3'; Rev 5'gcgtctgaaaggaaagcaag3'; rs420549: For 5'atctctctcctccttccat3'; Rev 5'gaactttttctttctcct3'; rs1590: For 5'tataggtctgggtcgtgatt3'; Rev 5'bctgctctcggtctccacct3'. Seq 5'gaagttctgggtcgtgatt3'. The PCR conditions for DNA included 50 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s. For RNA, similar PCR conditions as above were used except that the reaction was terminated at 35 cycles. For each SNP, the PCR reactions for DNA and RNA were performed in triplicates in a 50 μl reaction volume using AmpliTaq Gold polymerase (Applied Biosystems). In addition, PCR reactions were performed on the reverse transcriptase–negative DNase-treated RNA samples as an internal control.

Pyrosequencing and Allele Quantitation
After PCR, the DNA and RNA amplification products were sequenced using the sequencing primers listed above on a PyroMark MD pyrosequencing instrument (Biotage) as per the manufacturer’s instructions, and the frequencies of individual alleles for each SNP were obtained using the PyroMark MD software package (Biotage). The ratio of the common versus rare allele frequency in the RNA was then calculated, and normalized to their respective ratio in the DNA using the formula: Allele Expression Ratio = RNA level of (common allele expression/rare allele expression) / genomic DNA level of (common allele expression/rare allele expression). Samples were considered positive for ASE if the median value of this ratio as determined among all informative SNPs was <0.67 or >1.5, as suggested by the earlier study (4). As a quality control for this method, we also assessed ASE for these five SNPs in mixtures of varying proportions of DNA and RNA from two individuals who were homozygous for opposite SNP alleles in TGFBR1 3' UTR. Pyrosequencing yielded a ratio of allele specific expression of these mixtures that was ≥97% of the predicted ratio (data not shown).

Results and Discussion
ASE of TGFBR1 is rare in familial colon neoplasia. The recent study by Valle and colleagues (4) reports that ASE of TGFBR1 occurs in ~20% of the informative familial colon cancers. Although most cases of ASE showed an imbalance in allele expression ratio of <2-fold, individuals with ASE show a marked deregulation in the downstream TGF-β signaling in four of the four cases tested (4). To determine the prevalence of ASE in an independent group of familial colon neoplasia cases, we assayed for the presence of ASE of TGFBR1 in lymphoblastoid cells from a well-characterized cohort of familial colon neoplasia cases (5). Of the 102 familial cases and 44 familial controls selected, 46 and 17 individuals, respectively, were heterozygous for informative TGFBR1 SNPs. These 46 familial cases included 20 individuals with colon carcinoma, and 26 individuals with advanced colon adenomas that showed high-grade dysplasia (carcinoma in situ; ref. 5). As defined by the earlier report, samples were considered positive for ASE if the allele ratio is >1.5-fold (4). Based on the above criteria, we found that of the 46 informative familial cases, only 2 individuals showed ASE with allele ratios slightly above the cutoff value (allele ratios, 1.65 and 1.73, respectively), 1 of whom was affected by colon cancer, and the other by an advanced adenoma with high grade dysplasia (Fig. 1). These two cases belonged to two different families. Thus, ASE as assayed in this cohort was infrequent [4.3% of cases, 95% confidence interval (CI), 0.5–14.8%], and did not account for a substantial number of the familial colon neoplasia cases that we investigated.

One intriguing observation regarding ASE is that the TGFBR1 gene on chromosome 9q22.33 lies in the middle of a genomic region in which we and others have previously found linkage to risk of familial colon neoplasia (5, 7). Furthermore, Valle and colleagues (4) observed that within four families that they studied, ASE seemed to segregate as a dominant genetic trait inherited in consort with markers of high risk TGFBR1 alleles. Of the 46 familial cases that we studied, 31 cases (derived from 22 families) were from sibships showing excess sharing of genomic markers across the 9q22.3-31.2 interval, consistent with disease in these families being linked to a 9q22.3-31.2 pathogenic allele (5). Both of the individuals in whom we detected ASE were from families belonging to this 9q22.3-31.2 linked cohort (5). However, even among these 9q linked families, ASE was an uncommon finding, accounting for only 2 of 31 cases (6.5%; 95% CI, 0.8–21.4%) and 2 of 22 families (9.1%; 95% CI, 1.1–29.2%). Thus, we conclude that ASE of TGFBR1 is unlikely to be the major driver of linkage of some colon neoplasia families to the 9q22.2-31.2 region.

Absence of TGFBR1 ASE in sporadic colon cancers. In addition to familial colon cancers, ASE was also found to occur in up to 20% of informative sporadic CRCs included in the initial study that defined this phenomenon (4). We did not have available a collection of lymphoblastoid cells from individuals with sporadic CRCs. However, we reasoned that if ASE of TGFBR1 were a driver of sporadic colon cancers, the lower expression of TGFBR1 allele frequency in these cancers would be consistent with disease in sporadic colon cancers. Accordingly, we determined the frequency of ASE in normal colon mucosa resections from a group of 96 sporadic CRC cases. As per the analysis of ASE in lymphoblastoid cells, we used RNA and DNA extracted from the normal-appearing colonic mucosa of these CRC cases for subsequent pyrosequencing analysis. Of the 96 sporadic CRCs, 44 were heterozygous for informative TGFBR1 SNPs. Pyrosequencing analysis revealed that none of these 44 informative sporadic cases show allele ratios >1.5-fold in their colonic mucosa (Fig. 1). These results further suggest that ASE of TGFBR1 is infrequently found in individuals with sporadic colon cancers. In summary, our current findings in a Caucasian-dominated study population did not find TGFBR1 ASE as being a common inherited risk factor for colon cancer. Of the total 90 colon neoplasia cases we studied, only 2 showed evidence for ASE. This was true despite enriching our study with 31 cases of familial


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Figure 1. Analysis of TGFBR1 allele ratios in familial and sporadic colon neoplasia. Shown in the figure is the allele expression ratio of TGFBR1 for each sample as determined in lymphoblastoid cells from affected individuals from colon neoplasia kindreds (familial cases), lymphoblastoid cells from unaffected members of colon neoplasia kindreds (familial controls), and normal colon mucosa from individuals with sporadic CRCs (sporadic CRCs). Samples with ratios of <0.67 or >1.5 cutoff values (indicated by horizontal dashed lines) were considered positive for ASE. The solid lines within each scatter plot indicate the mean and SE values. The triangles and spheres within the familial cases data set indicate individuals with colon cancers and colon adenomas with high-grade dysplasia, respectively.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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