Mechanisms of Inactivation of the Receptor Tyrosine Kinase EPHB2 in Colorectal Tumors

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Abstract

The receptor tyrosine kinase *EPHB2* has recently been shown to be a direct transcriptional target of TCF/β-catenin. Premalignant lesions of the colon express high levels of EPHB2 but the expression of this kinase is reduced or lost in most colorectal carcinomas. In addition, inactivation of *EPHB2* has been shown to accelerate tumorigenesis initiated by *APC* mutation in the colon and rectum. In this study, we investigated the molecular mechanisms responsible for the inactivation of *EPHB2* in colorectal tumors. We show here the presence of mutations in repetitive sequences in exon 17 of *EPHB2* in 6 of 29 adenomas with microsatellite instability (MSI), and 101 of 246 MSI carcinomas (21% and 41%, respectively). Moreover, we found *EPHB2* promoter hypermethylation in 54 of the 101 colorectal tumors studied (53%). Importantly, EPHB2 expression was restored after treatment of *EPHB2*-methylated colon cancer cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. In conclusion, in this study, we elucidate the molecular mechanisms of inactivation of *EPHB2* and show for the first time the high incidence of frameshift mutations in MSI colorectal tumors and aberrant methylation of the regulatory sequences of this important tumor suppressor gene. (Cancer Res 2005; 65(22): 10170-3)

Introduction

The great majority of colorectal tumors display a constitutive up-regulation of TCF/β-catenin transcriptional activity, most commonly caused by mutations in the tumor suppressor gene *APC* (1). Increased activity of the TCF/β-catenin pathway is therefore a hallmark of colorectal cancer. This complex transcriptionally up-regulates key genes that are important in the development of these tumors. However, potentially oncogenic changes are often counterbalanced by additional effects of these alterations that abrogate a possible growth advantage. For example, deregulation and amplification of the transcription factor c-MYC is one of the most common events in colorectal tumors. Although c-MYC can promote cell growth by regulating the expression levels of multiple cell cycle regulators, it can also induce apoptosis through the modulation of proapoptotic genes such as *BAX* (2). The receptor tyrosine kinase EPHB2 has recently been shown to be a direct transcriptional target of TCF/β-catenin and premalignant lesions of the colon express high levels of expression of this kinase (3–5). However, EPHB2 expression is reduced in colonic carcinomas and low levels are correlated with tumor progression (5). In addition, inactivation of EPHB2 has been shown to accelerate tumorigenesis initiated by *APC* mutations in the colon and rectum of APC$^{Min/+}$ mice (5), demonstrating that *EPHB2* is an important tumor suppressor in the large intestine. Therefore, despite being up-regulated by TCF/β-catenin signaling, inactivation of EPHB2 seems to be an important requirement in the progression of colorectal tumors. However, it is currently not known how EPHB2 activity is lost during tumor progression.

Approximately 15% of the tumors of the colon and the rectum display a microsatellite unstable phenotype (MSI). This is observed as frequent insertions and deletions within short repetitive sequences known as microsatellites. Mutations within coding regions of the target genes result in frameshifts that can disrupt protein function. Mutations that confer a growth advantage to the cells are selected and can be found in a significant percentage of colorectal tumors with an MSI phenotype. Genes frequently targeted by these mutation include those involved in molecular mechanisms important in the development of colorectal tumors, including the transforming growth factor-β pathway, Wnt signaling, and DNA damage repair and apoptosis pathways (6). Because loss of EPHB2 activity is an important step in tumor progression, and because this receptor contains an A9 track in exon 17 that could be a target for mutation in MSI tumors, we screened this region for alterations in MSI tumor cell lines as well as in primary adenomas and carcinomas with MSI.

Hypermethylation of cytosines located within CpG islands in the promoter of tumor suppressor genes is emerging as an important mechanism of gene silencing in both microsatellite stable (MSS) and unstable colorectal tumors, and has been reported to disrupt important pathways in colorectal tumorigenesis, including the *TP53* pathway (*p14ARF*), the WNT signaling pathway (*APC*, E-cadherin), DNA repair (*MGMT, hMLH1, BRCA1*), apoptosis (*DAPK*), and metastasis (E-cadherin, *TIMP3*; ref. 7). We identified a CpG island spanning the proximal *EPHB2* promoter and the first exon, and investigated the possible contribution of aberrant methylation of this region in the regulation of *EPHB2* expression.
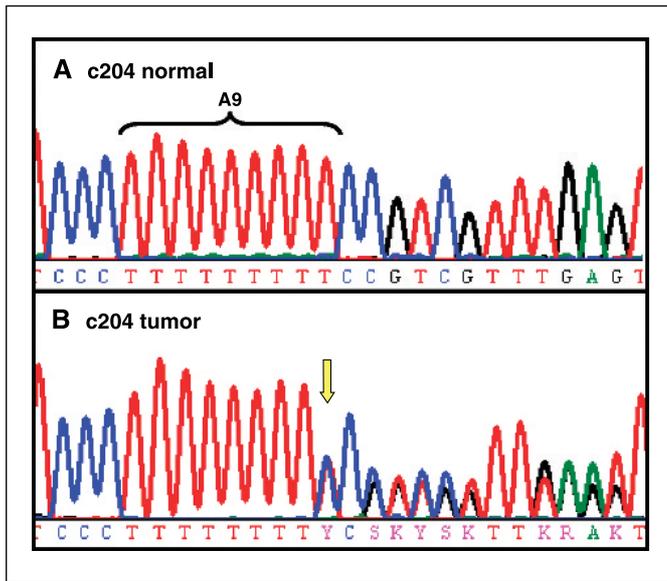
Figure 1. *EPHB2* mutations in MSI colorectal tumors. *A,* fragment of the genomic sequence of *EPHB2* exon 17 in a normal mucosa sample showing the A9 repeat (reverse sequence). *B,* genomic sequence in a microsatellite unstable colonic tumor from the same patient showing deletion of one A (*yellow arrow*).

In this study, we investigated the mechanisms of inactivation of EPHB2 in colorectal tumors and found that microsatellite unstable tumors have frequent mutations in the A9 repeat in exon 17 of *EPHB2*. Moreover, a CpG island in the proximal promoter region of this gene was hypermethylated in most of the colorectal tumors studied.

Materials and Methods

Cell lines and clinical samples. The cell lines used in this study were obtained and maintained as previously described (8). A total of 246 MSI colorectal carcinomas collected at medical institutions in Spain, Finland, Germany, and Japan were used in this study. The MSI status of these tumors was characterized as previously described (9–12). The 41 MSS colorectal tumors used for DNA hypermethylation analysis were collected at collaborating medical institutions in Spain and Finland. The series of 29 MSI adenomas from patients with hereditary nonpolyposis colorectal cancer used in this study has previously been described (9). Informed consent for genetic analysis of the tumor samples was obtained from each patient, according to the Human Investigations and Ethical Committee–approved research proposal in the corresponding Institution.

EPHB2 mutation screening. The A9 repeat in exon 17 of the larger EPHB2 transcript (accession no., NM_017449) and flanking genomic DNA sequence were PCR-amplified in the 246 MSI tumor samples entered in this study (primer sequence and PCR conditions available upon request). Mutation screening in the amplified PCR fragments was done by direct automated sequencing (ABI 3100 capillary sequencer), fragment analysis (GeneScan Software, Applied Biosystems, Foster City, CA) and/or single-stranded conformational polymorphism, as previously described (9, 13, 14).

Methylation-specific PCR. DNA methylation status of an *EPHB2* promoter-associated CpG island (from −537 to +836) was determined in colorectal tumor samples and cell lines included in the study by bisulfite conversion of unmethylated, but not methylated, cytosine to uracil as previously described (15). PCR reactions using primers specific for either the methylated or the modified unmethylated DNA (methylation-specific

PCR) were carried out to determine the methylation profile of each sample. Primers were designed using MethPrimer 1.1 software. *EPHB2* primers sequences for the methylated sequence were 5′-TTGTTTTTGTTAGTCGC-GTTAGAC-3′ (sense) and 5′-CAATAATCTCTCCCGACGCT-3′ (antisense), and for the unmethylated sequence 5′-TGTTTTTGTTAGTTGTGTTA-GATGT-3′ (sense) and 5′-CAACAATAATCTCTCCCAACACT-3′ (antisense). PCR amplification was done using EcoStar DNA polymerase (Ecogen, Barcelona, Spain) under the following conditions: 95°C for 10 minutes, 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C, and 4 minutes of final extension at 72°C. *In vitro* methylated DNA (CpG Genome Universal Methylated DNA; Chemicon International, Temecula, CA) was used as a positive control for methylated alleles, whereas DNA from normal lymphocytes and normal colon tissues were used as negative controls. Each PCR product was directly loaded onto 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Western blotting. SW620 cultures were treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine for 72 hours (0, 2, 5, or 10 μmol/L). Twenty micrograms of total protein (radioimmunoprecipitation assay buffer lysates) were fractionated in 8% SDS-polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ), blocked with 10% nonfat milk for 1 hour and then probed overnight at 4°C with a 1:100 dilution of anti-EPHB2 primary antibody (Stratagene, La Jolla, CA). Membranes were washed thrice with washing buffer (PBS with 0.1% Tween 20) and then probed with a peroxidase-conjugated secondary antibody for 1 hour (1:2,000; Boehringer Mannheim, Indianapolis, IN). After washing thrice with washing buffer, the signal was detected using enhanced chemiluminescence plus (Amersham) and a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The membranes were then stripped and reprobed with an anti-β-actin antibody (clone AC74, 1:1,000; Sigma, St. Louis, MO). The signal from the β-actin probe was used as a loading control.

Results and Discussion

Frequent *EPHB2* mutations in microsatellite instability tumors. The longer EPHB2 transcript contains an A9 track in exon 17 that could be a target for frameshift mutations in colorectal tumors with MSI. To investigate the possible mechanisms of EPHB2 inactivation in MSI tumors, we screened for mutations in the genomic region corresponding to exon 17 in a panel of 24 MSI colorectal cancer cell lines. Nine of these lines (37.5%) had a 1 bp deletion in the A9 repeat in exon 17. To further investigate the incidence of mutations in this repeat, we used a series of 246 primary MSI colorectal tumors. Frameshift

Table 1. Type of *EPHB2* mutations found in MSI tumors

Mutation	Mutation frequency	Effects on protein
A9→A8	94 of 101 (93%)	protein extension (26 amino acid)
A9→A8 and A9→A7	3 of 101 (3%)	protein extension (26 amino acid)/ protein truncation
A9→A10	2 of 101 (2%)	protein truncation
T6→T5	2 of 101 (2%)	protein extension (26 amino acid)

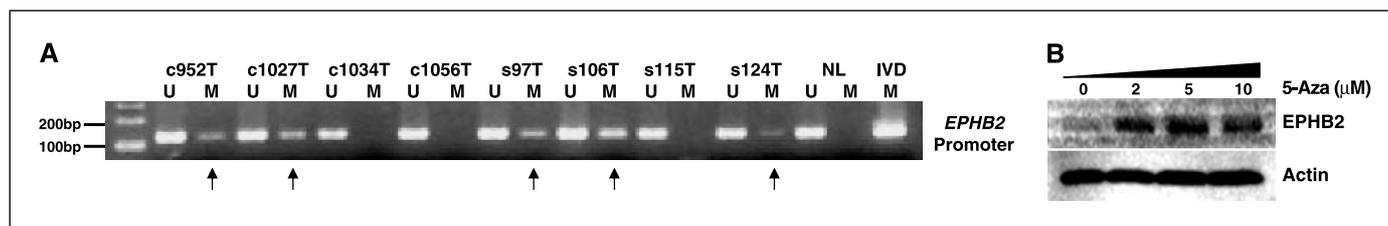NOTE: A total of 246 MSI tumors were studied and 101 mutations found in exon 17 of *EPHB2*.

Figure 2. *EPHB2* promoter hypermethylation in colorectal tumors. Primers specific for the bisulfite-transformed methylated or unmethylated sequence were used to PCR-amplify DNA samples from a series of 101 colorectal tumors (*U*, unmethylated; *M*, methylated). *A,* representative cases of tumors with and without EPHB2 hypermethylation. *Arrows,* tumors with *EPHB2* promoter hypermethylation. *B,* as shown by Western blotting, treatment of SW620 cells with the indicated concentration of 5-aza-2′-deoxycytidine for 72 hours restored EPHB2 protein expression.

mutations were found in 41% of these tumors (101 of 246; Supplementary Table S1; Fig. 1). We analyzed the same region of *EPHB2* in DNA samples obtained from matched normal tissue from all the mutation-positive patients. No changes were found in this A9 repeat, excluding the possibility of polymorphisms in this region as well as germ line mutations in these patients. No associations were found between mutations in *EPHB2* and other clinicopathologic variables in MSI tumors (tumor stage, grade, patient sex, age, and 5-year survival; Supplementary Table S2).

Ninety-three percent of the mutations found in these MSI carcinomas (94 of 101) were deletions of one A in the A9 track (Fig. 1; Table 1). Two of the remaining cases had 2 bp deletions in this A9 repeat and three cases showed two mutated alleles (1 and 2 bp deletions). The remaining two cases had deletions in a T6 track 61 bp downstream of the A9 repeat in EPHB2. All the mutations found in *EPHB2* change the translational reading frame, and result in changes in the amino acid sequence of the last 35 residues and the addition of a 26–amino acid tail, or the premature truncation of the protein (Table 1). In all cases, two serine residues (S1048 and S1052) that are predicted to be phosphorylated in the wild-type protein (16), and that could regulate the activity of this kinase, are lost in the mutant *EPHB2*.

To investigate whether EPHB2 mutations are an early event in the development of MSI colorectal tumors, we used a set of 29 MSI adenomas (9). We found that 20.7% of these adenomas (6 of 29) had a mutation in the A9 repeat of *EPHB2* (Supplementary Table S1). The mutation frequency in this set of MSI adenomas (20.7%) was significantly lower than in MSI carcinomas (41%, 101 of 246; χ^2 test; $P = 0.03$). This observation is in good agreement with earlier reports showing that EPHB2 expression was reduced or lost in colorectal carcinomas, but not in adenomas (5), and further suggests that *EPHB2* inactivation may be important for the transition from adenoma to carcinoma.

Frequent hypermethylation of the *EPHB2* promoter in colorectal tumors. Cytosine hypermethylation in CpG dinucleotides in the regulatory region of tumor suppressor genes has been linked to reduced gene expression (7). Bisulfite treatment of genomic DNA allows precise analysis of methylation in a certain region by converting all nonmethylated cytosines into uracil, whereas methylated cytosines remain unchanged. PCR primers specific for the methylated or unmethylated sequence can then be used to investigate promoter hypermethylation (15).

The proximal promoter of *EPHB2* contains a CpG island spanning 1,400 bp around the transcription start site, that could be hypermethylated and thus regulate the expression of this gene. We used a series of 60 MSI and 41 MSS colorectal tumors to investigate whether hypermethylation of the *EPHB2* promoter

could be a mechanism of gene inactivation in colorectal tumors. Of the 101 tumors investigated, 54 (53.4%) showed signs of *EPHB2* promoter hypermethylation (Fig. 2*A*; Supplementary Table S1). There was no difference in the proportion of MSS and MSI tumors showing *EPHB2* promoter methylation (51.2% and 55%, respectively; χ^2 test; $P = 0.7$) and no associations were found with other clinicopathologic features (patient age, sex, 5-year overall survival, tumor stage, grade and mutations in the A9 repeat in *EPHB2* exon 17; Supplementary Table S3).

Using a panel of MSS colorectal cancer cell lines, we found evidence of promoter methylation in 5 out the 20 lines tested (25%; data not shown). To investigate whether hypermethylation of CpG islands in the *EPHB2* promoter is functionally relevant in reducing the expression levels of EPHB2, a cell line showing *EPHB2* promoter methylation (SW620) was exposed to increasing concentrations of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine for 72 hours. This treatment resulted in a substantial up-regulation of EPHB2 protein levels in SW620 cells (Fig. 2*B*), demonstrating that aberrant methylation of the *EPHB2* promoter can regulate EPHB2 expression.

Losses of large chromosomal regions are common in MSS tumors and deletions targeting important tumor suppressor genes confer a growth advantage to the cells and are clonally selected. EPHB2 is located in the short arm of chromosome 1 (1p36), one of the most commonly deleted areas in colorectal tumors (17–19). The frequent inactivation of *EPHB2* by mutation and promoter hypermethylation described in this study is in good agreement with the high frequency of genomic losses in this region, and given the recently shown function of *EPHB2* as a tumor suppressor gene in colorectal cancer (5), this gene could be an important target for at least some of these deletions.

In conclusion, in this study, we describe for the first time the mechanisms of *EPHB2* inactivation in colorectal tumors. We found frequent mutations in repetitive sequences in exon 17 in MSI adenomas and carcinomas (21% and 41%, respectively) and hypermethylation of the *EPHB2* promoter in the majority of the tumors of the colon and rectum (53%).

Acknowledgments

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