# Variants in the Netrin-1 Receptor UNC5C Prevent Apoptosis and Increase Risk of Familial Colorectal Cancer

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**BACKGROUND & AIMS:** Expression of the netrin-1 dependence receptor UNC5C is reduced in many colorectal tumors; mice with the *UNC5C* mutations have increased progression of intestinal tumors. We investigated whether specific variants in *UNC5C* increase risk of colorectal cancer (CRC). **METHODS:** We analyzed the sequence of *UNC5C* in blood samples from 1801 patients with CRC and 4152 controls from 3 cohorts (France, United States, and Finland). Almost all cases from France and the United States had familial CRC; of the Finnish cases, 92 of 984 were familial. We analyzed whether CRC segregates with the *UNC5C* variant A628K in 3 families with histories of CRC. We also performed haplotype analysis to determine the origin of this variant. **RESULTS:** Of 817 patients with familial CRC, 14 had 1 of 4 different, unreported missense variants in *UNC5C*. The variants p.Asp353Asn (encodes D353N), p.Arg603Cys (encodes R603C), and p.Gln630Glu (encodes Q630E) did not occur significantly more often in cases than controls. The variant p.Ala628Lys (A628K) was detected in 3 families in the French cohort (odds ratio, 8.8; Wald's 95% confidence interval, 1.47–52.93; $P = .03$) and in 2 families in the US cohort (odds ratio, 1.9; $P = .6$) but was not detected in the Finnish cohort; UNC5C A628K segregated with CRC in families. Three families with A628K had a 109-kilobase identical haplotype that spanned most of *UNC5C*, indicating recent origin of this variant in white subjects (14 generations; 95% confidence interval, 6–36 generations). Transfection of HEK293T cells with UNC5C-A628K significantly reduced apoptosis compared with wild-type UNC5C, measured in an assay of active caspase-3. **CONCLUSIONS: Inherited mutations in *UNC5C* prevent apoptosis and increase risk of CRC.**

*Keywords:* Colon Cancer; Tumor Suppression; Tumorigenesis; Neoplasm; UNC5H3.

The commonly accepted scheme for transmembrane receptor–mediated signal transduction relies on the fact that the receptor becomes active on ligand binding. However, over the past decade, a new functional family of receptors has been identified. These receptors, named dependence receptors, share the ability to be active in the absence of their respective ligand and in this setting trigger apoptosis. Such receptors thus create cellular states of dependence on their respective ligands.[1,2] The prototypical dependence receptors are the netrin-1 receptors. Netrin-1, a diffusible laminin-related protein, has been shown to play a major role in the control of neuronal navigation during the development of the nervous system by interacting with its main receptors, DCC (Deleted in Colorectal Cancer)[3–5] and UNC5H.[6,7] However, netrin-1 has rapidly emerged as a multifunctional protein implicated in multiple functions beyond the brain.[8,9] We and others have shown that a large part of its activity is associated with the fact that netrin-1 regulates endothelial and epithelial cell survival by inhibiting the proapoptotic activity of the dependence receptors DCC and UNC5H, that is, UNC5H1, UNC5H2, UNC5H3, and UNC5H4, also called UNC5A,B,C,D-.[9–15]

The proapoptotic activity of these unbound receptors has been suggested to act as a mechanism to eliminate tumor cells that would develop in settings of ligand unavailability, such as proliferation of tumor cells in an environment with constant and limited ligand presence or migration of metastatic tumor cells in tissues with no or low ligand expression. This hypothesis fits with the observation that the *DCC* and *UNC5H* genes are downregulated in tumors, hence suggesting that the loss of expression of these genes represents a selective advantage for tumor development.[9,16–18] Along this line, it has been shown in different animal models that the balance between netrin-1 and its receptors is important in the reg-

***Abbreviations used in this paper:*** **CI, confidence interval; CRC, colorectal cancer; OR, odds ratio; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.**


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ulation of cancer progression. On the one hand, a gain of netrin-1 has been shown to block tumor cell death in vitro and to be associated with intestinal tumor initiation and progression in mice.[19,20] On the other hand, inactivation of *UNC5C* (*UNC5H3/rcm*) in mice is associated with intestinal tumor progression,[21] thus demonstrating per se that UNC5C acts as a tumor suppressor.

Based on these observations, we speculated that genetic abnormalities of *UNC5C* might be involved in human colorectal carcinogenesis. To identify germline mutations in the human *UNC5C* gene that may increase predisposition to cancer, we analyzed genomic DNA from human blood samples of unrelated individuals with family histories of colorectal cancer (CRC). We report here the detection of several missense mutations, one of which leads to significant loss of proapoptotic activity of UNC5C and cosegregates with the disease.

## Patients and Methods

### *Individuals and Clinical Samples*

We obtained genomic DNA from blood from 1801 individuals with CRC (984 from the Department of Medical Genetics at the University of Helsinki [Helsinki, Finland], 489 from the Division of Human Genetics at The Ohio State University [Columbus, OH], 179 from the Centre Leon Bérard-HEH [Lyon, France], 60 from the CHU Strasbourg [Strasbourg, France], and 10 from the previously described Calvados ColoREctal Family [CCREF] cohort[28]), representing unrelated families. Institutional review board approval and informed consent for cancer gene predisposition research was obtained for all patients. Almost all families fulfill the criteria of revised Bethesda guidelines[29] or had at least 2 cases of CRC (except families 2 and 9, which had one case of CRC associated with another type of cancer). Family 10 is an exception because the proband had kidney cancer at 37 years of age and developed colon polyps at 43 years of age but was the only living member of the family with 4 cases of CRC. The samples from Finland are mostly sporadic CRC but included 92 familial cases.[30,31] Cases of familial polyposis or hamartomatous polyposis syndromes or with mismatch repair gene mutations were excluded.

The tumors of probands have been checked for a mismatch repair gene deficiency (ie, *MLH1*, *MSH2*, and *MSH6*).[32] All the individuals from Ohio had microsatellite stable tumors. Additionally, for the probands of families 4, 7, 9, 11, 12, and 14, the presence of the 3 mismatch repair proteins was verified by immunohistochemistry. All the individuals from the Molecular Oncology Unit at Centre Léon Bérard (probands of families 1–3, 10, and 13) have been sequenced for *MLH1*, *MSH2*, and MSH6 (exons and intron/exon junctions), and no mutations were detected.

The control samples were obtained after approval by the appropriate institutional review boards. Every donor signed an approved consent form for genetic studies. The control cohorts were derived as follows. The 1416 individuals from the metropolitan region of Columbus, Ohio, belonged to a series of randomly chosen volunteers representing the same geographic, ethnic, and social background as the patients with CRC from Ohio.[33] The 1031 individuals from the Calvados ColoREctal Family (CCREF) study[28] were randomly derived relatives of probands with CRC. The 780 anonymous blood donors were from the Etablissement Français du Sang Rhône-Alpes.[34] Because one of the probands with the A628K mutation was of Portuguese ethnicity, 100 randomly chosen Portuguese controls were also studied (a kind gift of Professor J. Sequeiros, University of Porto, Porto, Portugal). The 825 Finnish anonymous blood donors were obtained from the Finnish Red Cross Blood Transfusion Service. All the blood donors were healthy and cancer-free at the time the blood was drawn.

Conditional maximum likelihood estimate of the odds ratio (OR), associated 95% confidence interval (CI), and *P* values were obtained with Fisher exact text.

### UNC5C *DNA Sequencing and Haplotype Analysis*

Genomic DNA (50 ng) was amplified by polymerase chain reaction (PCR) using primers encompassing all the exons of *UNC5C* with HotStarTaq (Qiagen, Valencia, CA) or AmpliTaq Gold DNA Polymerase (Applied Biosystems, Life Technologies, Grand Island, NY). PCR products were screened using endo-1 to detect heteroduplex[35] or by single-strand conformation polymorphism gel electrophoresis[36] or High Resolution Melting (Roche Applied Science, Basel, Switzerland)[37] and then sequenced in both directions by Cogenics (Grenoble, France) or GATC Biotech (Konstanz, Germany) or by using the ABI Prism BigDye Terminator Cycle Sequencing Kit version 3.1 and the Applied Biosystems 3730 DNA Analyzer (PE Applied Biosystems, Foster City, CA). Single nucleotide polymorphism (SNP) markers were designed with HapMap and University of California, Santa Cruz databases, and microsatellite markers were designed by using Repeat Masker (http://www.repeatmasker.org) for haplotype and loss of heterozygosity analysis. All primers and PCR conditions are available on request.

### *Loss of Heterozygosity Assessment and UNC5C Promoter Methylation Analysis*

Microsatellites and SNPs were used as markers to compare normal (blood) and tumor tissues using the SNaPshot (PE Applied Biosystems) technique as described previously[38] or by using fluorescently labeled primers for the microsatellites.[39] All primers and PCR conditions are available on request.

The UNC5C promoter region, amplified from bisulfite-modified DNA by 4 different PCRs, was cloned into a TA vector (pCR2.1; Invitrogen, Carlsbad, CA). Ten positive recombinants were isolated and sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing Kit version 3.1 and the Applied Biosystems 3730 DNA Analyzer (PE Applied Biosystems).

### *Plasmid Constructs*

pcDNA3-UNC5C-HA containing human UNC5C full-length coding sequence was used as a template for insertion of the different mutant alleles using the Quick change strategy (Stratagene, La Jolla, CA). All primers are available on request.

### *Cell Line, Transfection Procedure, and Cell Death Assays*

Transient transfections of human embryonic kidney cells (HEK293T) were performed using Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). For detection of DNA fragmentation, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed with 300 U/mL TUNEL enzyme and 6 $\mu$mol/L biotinylated deoxyuridine triphosphate (Roche Diagnostics, Mannheim, Germany) and detected with avidine coupled Cy3. Caspase-3 activity was measured using Caspase-3/