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The Association of DCC mRNA Alternative Splicing with Colorectal Cancer

Natalie Graham

Department of Molecular, Cellular, and Developmental Biology

University of Colorado Boulder

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Thesis Advisor:

Dr. Zhe Chen, MCDB

Thesis Committee:

Dr. Zhe Chen, MCDB

Dr. Kevin Jones, MCDB

Dr. Jennifer Kugel, CHEM

Abstract

In as many as 70% of colorectal cancer cell (CRC) lines, there is a deletion of a chromosomal region, 18q21, which contains the Deleted in Colorectal Carcinoma (DCC) gene (Mehlen & Fearon, 2004). In adult cells, this single transmembrane receptor plays a role in both cell proliferation and cell death, thereby making it a promising candidate gene for the pathogenesis of colorectal cancer. It has been observed that alternative splicing of the DCC can affect its activity and that alternative splicing of DCC can be disrupted in cancer (Leggere et al., 2016; Reale et al., 1994). In this experiment, we sought to determine the association of alternative splicing of the DCC with colorectal cancer in cells without the deletion of the 18q21 region. By extracting RNA from 35 CRC cell lines and performing RT-PCR, we observed levels of the two DCC isoforms compared to normal adult colon cells. In this way, we determined that 29 of 35 CRC cell lines had altered splice patterns, indicating the alternative splicing may be related to colorectal cancer. These differences likely arose from genomic mutations in the Nova2 binding site, though additional research would be necessary to confirm this conjecture. Further research into this subject could shed light on the causes of colorectal cancer as well as perhaps one day aiding in diagnosis.

Introduction

According to the National Institute for Health, 4.4% of men and women will be diagnosed with colorectal cancer (CRC) during their lifetime (Surveillance, 2013). Cancer can arise from many types of mutations, but colorectal cancer cells often experience a deletion in the chromosome region 18q21, which encompasses the DCC (Deleted in Colorectal Carcinoma) gene (Grady, 2013). The DCC gene codes for a single transmembrane receptor, which binds the Netrin-1 ligand (Schluten, et al., 2016).

Though the DCC receptor is best known for its association with axon development, it also can play a role in tumorigenesis (Castets, 2011). In development, DCC and Netrin-1 together direct axonal growth. In mature tissues, DCC can regulate both cell growth and cell death. When the DCC receptor is no longer bound to Netrin-1, apoptosis is triggered, controlling growth and division (Schluten, et al., 2016). However, when bound to Netrin-1, DCC can promote proliferation (Mehlen and Tauszig-Delamasure, 2014). As the gene's name suggests, colorectal tumor cells often have a deletion of the DCC gene, with as many as 70% having a deleted allele (Mehlen & Fearon, 2004). However, colorectal cancer can still arise without a deletion in this region.

Recent studies in the field of axon guidance have shown that DCC activity can be regulated through its alternative splicing. Misregulation of DCC splicing at exon17 can disrupt the gene function without affecting the overall gene level (Leggere et al., 2016). Alternative splicing in other areas of the gene has also been reported to be disrupted in some colorectal cancer cells, though the implications of these disruptions are still unknown (McCullough, 2013; Huerta et al., 2001). Therefore, this experiment aims to investigate whether alteration in DCC splicing could also contribute to tumor development. We hypothesized that misregulation of alternative splicing would affect DCC's role in cell death and cell proliferation, thereby promoting tumorigenesis.

This experiment will expand upon previous work to investigate the mRNA alternative splicing of the DCC gene in CRC cell lines. In doing so, we hope to determine their correlation with colorectal cancer. The information provided by the results of this study will contribute further insight into the pathogenesis of colorectal cancer.

Materials and methods

Growing of cell lines

Frozen human colorectal cancer cell lines acquired from University of Colorado at Anschutz Medical School were thawed and grown in 10" dishes with growth medium. Cells were grown to confluency on two plates for each cell line. For one plate from each cell line, cells were removed from the plate using a chelating EDTA solution followed by trypsin. These cells were frozen at -80°C and kept for future use while the other half were used for RNA/DNA extraction.

RNA/DNA Extraction

Using Qiazol (Qiagen, Hilden, Germany), cells were removed from the plate and cell membranes disrupted. RNA and DNA were extracted per the manufacturer's protocol. The nucleic acids were re-suspended in elution buffer.

RT-PCR

Using the Maxima RT reagent, (Thermofisher, Waltham, MA), cDNA was generated from the mRNA using poly(T) and random hexamer primers. The elongation step was performed at 55°C for 60 minutes. Using the cDNA template generated from RT, PCR was performed to amplify the cDNAs specifically from the exon16/17 alternative region of the DCC gene. Additionally, PCR was performed to amplify Nova2 and GAPDH. Gene specific primers were as follows: DCC: 5'TTAACAATGCCGGAGAAGGAGT3' and 5'GGTTTGAGGCCTGTTGCTGTGTAA3'; Nova2: 5'CGACAGAGCCAAGCAGGCCA and ACGGTCACCACGCGCTCTTG3'; GAPDH: 5'AGGGCTGCTTTTAACTCTGGT3' and 5'CCCCACTTGATTTTGGAGGGA3'. Thermocycler conditions were set so that the initial condition was 95°C for ten minutes, followed by 30 cycles. Each cycle consisted fifteen seconds

at 95°C, fifteen seconds at 60°C, and then twenty-five seconds at 72°C. After the PCR was completed, the results were visualized using gel electrophoresis.

Cloning

PCR products were purified using a gel purification kit (Biobasic, Markham, Ontario). After isolating the desired RT-PCR product, the product was then cloned into a pJET 1.2/blunt vector. A ligation reaction was assembled according to the blunt cloning protocol for the Fermentas CloneJET PCR cloning kit (Waltham, MA). The ligation mixture was transformed into competent *E. coli* cells. A control plate with cells transformed without PCR product was also grown for reference. Single bacterial colonies were grown in LB broth with ampicillin overnight at 37°C. The plasmid DNA was purified with a miniprep kit (Biobasic, Markham, Ontario). The plasmid was then Sanger sequenced to confirm the identity of the isolated cDNA products.

Results

We sought to characterize the expression of DCC in CRC cell lines. We first extracted RNA from 35 CRC cell lines. We then used RT-PCR with gene-specific primers to amplify cDNAs from the exon 16/17 region of the DCC gene. Splicing of the DCC generates two alternative products, Dcclong and Dccshort. Dcclong is the most prevalent isoform present in wild type colon cells (Reale et al., 1994). PCR was semiquantitative so that differences in expression levels could be visualized. We observed different expression levels of these two isoforms in these CRC cell lines (Figure 1). After Sanger sequencing, we confirmed that the two products are indeed Dcclong and Dccshort. Cell lines CL-34, LOVO, SNU-1235, SW48, and WiDr exhibit a normal splicing pattern. Others, such as HCA7, LS180, and MDST8, express higher levels of Dccshort.

Most lines appear to express both isoforms at comparable levels. Certain lines also exhibit more aberrant splicing patterns, such as MDST8, which has a few other minor products, confirmed by sequencing to have a partial intronic sequence. 293T is a common cell line used as a positive control. Most (29/35) of these cell lines exhibit an abnormal splicing pattern, suggesting that there may be a correlation with aberrant alternative splicing of the DCC and colorectal cancer.

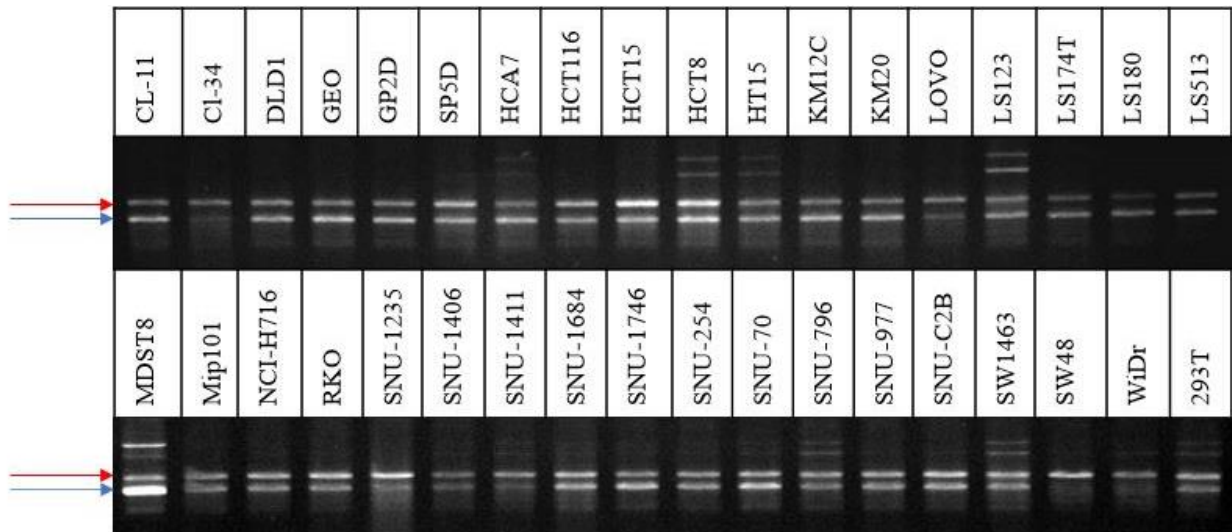


Figure 1: Dcc alternative splicing patterns in CRC cell lines. RNA was extracted from 35 CRC cell lines as well as 293T, a common cell line used here as a positive control. RT-PCR was performed using primers to amplify the exon 16/17 region in DCC. Red arrows indicate Dcclong, blue arrows indicate Dccshort. In normal adult colon cells, Dcclong is the prevalent isoform.

When examining these altered splice patterns, a question arises as to what causes these changes in expression. Previous studies have shown that Nova2 splicing factor controls DCC alternative splicing at exon17. In vivo knockout of Nova2 disrupts the splicing pattern of DCC and results in loss of Dcc activity (Leggere et al., 2016). Therefore, either expression of Nova2 or genomic mutations in the Nova2 binding site of the DCC gene could be responsible for the different DCC alternative splice patterns observed in CRC. In order to determine the cause, we performed RT-PCR on the RNA extracted from the CRC cell lines with gene-specific primers to amplify cDNAs from the Nova2 gene. PCR was semiquantitative so that expression levels could be

observed. Since Nova 2 appears to be expressed at a comparable level in most lines, the difference in Dcc splicing patterns is unlikely to result from a reduction in the splicing factor (Figure 2). It is more probable that these differences arose from genomic mutations at the Nova 2 binding site in the DCC gene.

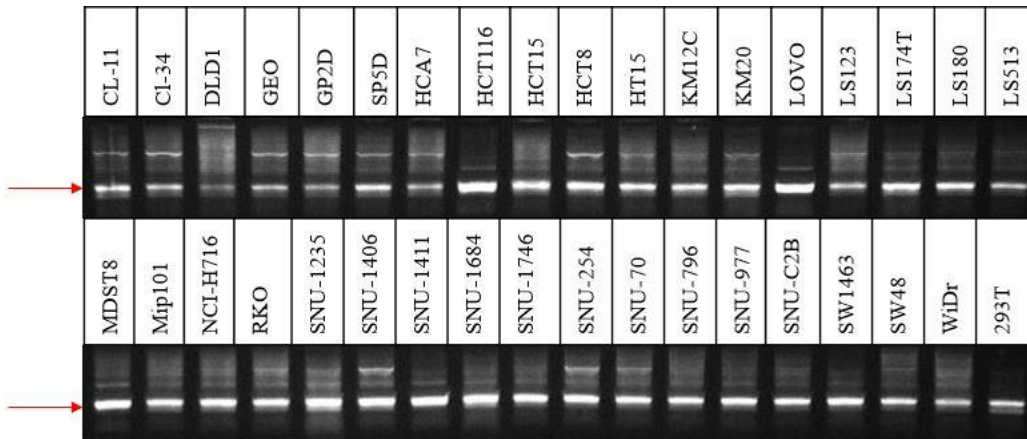


Figure 2: Nova2 expression in CRC cell lines. Using RNA extracted from the 35 CRC cell lines, RT-PCR was performed using gene-specific primers to amplify the Nova2 product. Red arrows indicate Nova2. Expression levels appear to be comparable in most lines.

Lastly, we wanted to confirm that our RNA and cDNA was of good quality. To test the purity, we performed RT-PCR on the RNA extracted from CRC cell lines using gene-specific primers for GAPDH. Since GAPDH expresses in all cells, we could use this RT-PCR result as a control to demonstrate that the RNA and cDNA had not degraded (Figure 3). All CRC cell lines expressed GAPDH. In the last lane, water, rather than cDNA, was added as a negative control.

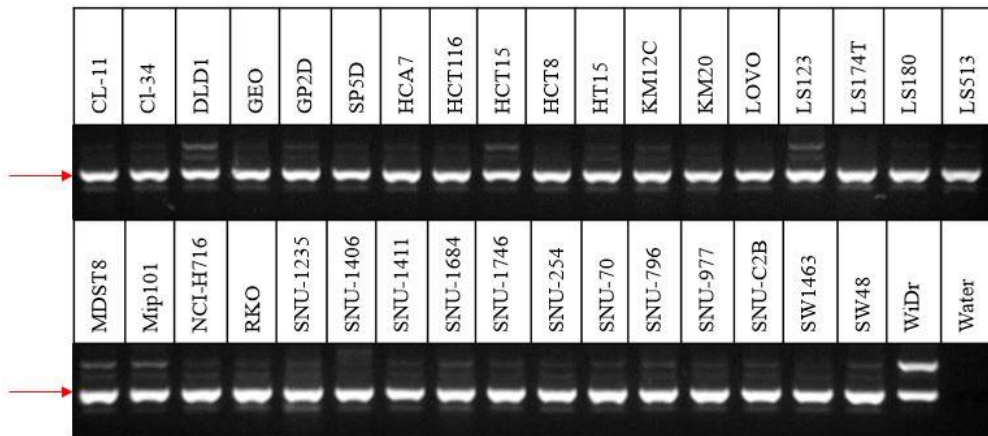


Figure 3: Expression of GAPDH in CRC cell lines. Using the RNA extracted from 35 CRC cell lines, RT-PCR was performed with gene-specific primers to amplify GAPDH. Red arrows indicate GAPDH. All cell lines express GAPDH. In the last lane, water is added as a negative control.

Discussion

Among 35 different cell lines, we observed 29 lines that show distinct splicing patterns from normal adult colorectal tissue. This indicates that disruption of DCC splicing may contribute to alteration of the gene activity. The Nova2 RT-PCR shows comparable expression between most lines, so it is likely that the differences in DCC splicing observed in our experiment is not caused by expression of Nova2. Rather, it may be caused by genomic mutations in the Nova2 binding site of the DCC.

Further research could investigate this claim by sequencing genomic DNA of these cell lines at that site. After determining any mutations at this site, their effect on the alternative splicing of DCC would have to be experimentally confirmed. Chen lab has established a splicing reporter, which includes the genomic sequence between exons 16 and 17. The alternative splicing of this reporter is consistent with the *in vivo* splicing pattern of the genomic region (Leggere et al., 2016). We would introduce the same mutations found through sequencing into the splicing reporter and determine how they would affect the expression of DCC isoforms. If mutations create changes in the splicing patterns like those observed in the CRC cell lines, it would suggest that these mutations have functional effects. In addition to this experiment, running quantitative PCR would help us ascertain the degree of changes seen each line. We have seen that aberrant alternative splicing of the DCC has some relationship to colorectal cancer, though further research is necessary to determine whether it contributes to tumorigenesis. Greater understanding of the alternative splicing of DCC could shed light on the etiology of colorectal cancer, and perhaps creating a novel diagnostic method in the future.

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