Expression of NCAM1 and CDH2 mRNA and the Presence of Alternatively Spliced NCAM1 mRNA in Neuroblastoma Cell Lines

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Abstract

Neuroblastoma comprises approximately 9% of all childhood cancers. The neuroblastoma population is comprised of three cell types that can exhibit a neuroblastic (N), a flat or substrate adherent (S), or an intermediate (I) phenotype. The expression levels of Neural Cell Adhesion Molecule 1 (NCAM1) and N-cadherin (CDH2) mRNA were studied by RT-PCR in these cell lines. NCAM1 is a cell-adhesion glycoprotein from the immunoglobulin superfamily. CDH2 is calcium dependent glycoprotein that functions in the adherens junctions of the cell. NCAM1 mRNA exhibited evidence of differential expression in the three different cell lines with lowest expression in the S cell line and the highest in the N cell line. There were two isoforms of NCAM1 mRNA expressed in all three cell lines encoded by transcript variants one and two. Sequencing confirmed that transcript variant two was missing the 30bp exon 9. There were no significant differences observed in expression levels of the CDH2 between the three cell lines.

Introduction

Neuroblastoma makes up approximately 9% of all childhood cancers, occurring once out of every 8,000 live births (Thiele, 1998). In children under 5 years old, it typically presents as tumors in the abdomen, commonly with involvement of the sympathetic ganglia of the paraspinal region or the adrenal gland. In infants less than one year old, the tumors are generally in the thoracic region. Neuroblastoma has the highest rate of spontaneous regression of all tumors, where widely disseminated tumors disappear with minimal treatment.

Neuroblastoma cytogentics include two common aberrations – the loss of the short arm of chromosome 1 (1p31-term) and the amplification of the n-myc gene in chromosome 2p23-23. Chromosome 1p houses several punitive tumor suppressors and its monosomy is common in cancers of the neural crest (Thiele, 1998). N-myc deletions are commonly seen histologically either as double minutes (DM) - circular extrachromosomal DNA that lacks centromeres or telomeres and consists of numerous repeats of n-myc, or as homogenously staining regions (HSR) - lengths of uniform banding in a chromosome consisting of m-myc repeats. Both are markers for poor prognosis. Aberrations in chromosome 17 are also common.

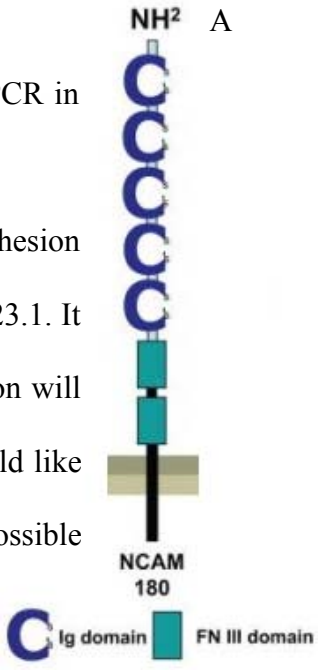
Neuroblastoma is thought to arise from the postganglionic sympathetic neuroblasts of the neural crest (Thiele, 1998). The neuroblastoma population is comprised of three cell types that can exhibit a neuroblastic (N), a flat or substrate adherent (S), or an intermediate (I) phenotype. The N line cells are small, weakly substrate adherent, and positive for neurofilament proteins. They grow as clumps of cells (pseudoganglia) with small cytoplasmic extensions (neurites) and show neurosecretory granules under the electron microscope (Ross and Spengler, 2007). The N-line representative, KCN-83n, is a subline of the SMS-KCN line, which was taken from a 11 month old boy with an adrenal tumor with metastases to the lymph nodes, bone and bone

marrow (Thiele, 1998). It has a 1pdel and a DM n-myc amplification. The S cell lines are usually teardrop shaped cells, positive for vimentin, with dense core granules that are strongly substrate adherent and form a contact-inhibited monolayer in culture (Ross and Spengler, 2007). The S-line representative, SH-EP1, is a subline of the SK-N-SH line, which was taken from a 4 year old girl with tumors in the thorax and metastasis to the bone marrow (Thiele, 1998). It has a single copy of n-myc. The I cell line lines shows characteristics of both the N and S lines and has been shown to most closely resemble a cancer stem cell, in fact, under the right conditions, I cells can be induced to differentiate into an S or N cell (Ross and Spengler, 2007). The I-line representative, BE(2)-C, is a subline of the SK-N-BE(2) line, which was taken from a 26 month old boy with tumors in the bone marrow (Thiele, 1998). It has a 1pdel, t(1p), an HSR n-myc amplification and a translocation t(3p21;17q21).

The expression levels of mRNA encoding two proteins were studied by RT-PCR in these cell lines: Neural Cell Adhesion Molecule (NCAM1) and N-cadherin (CDH2).

Neural Cell Adhesion Molecule 1, also CD56, is a calcium independent cell-adhesion glycoprotein from the immunoglobulin (Ig) superfamily, located on chromosome 11q23.1. It binds homophillically in a cis and trans fashion. Its surface prevalence and distribution will greatly modify its binding kinetics (Cunningham et al, 1987). NCAM1 has five Ig-fold like domains and two fibronectin type III domains (Fig 1A). It has numerous possible posttranslational modifications that affect its expression and activity, including the addition of asparagine-linked oligosaccharides, phosphorylation of serine and

A



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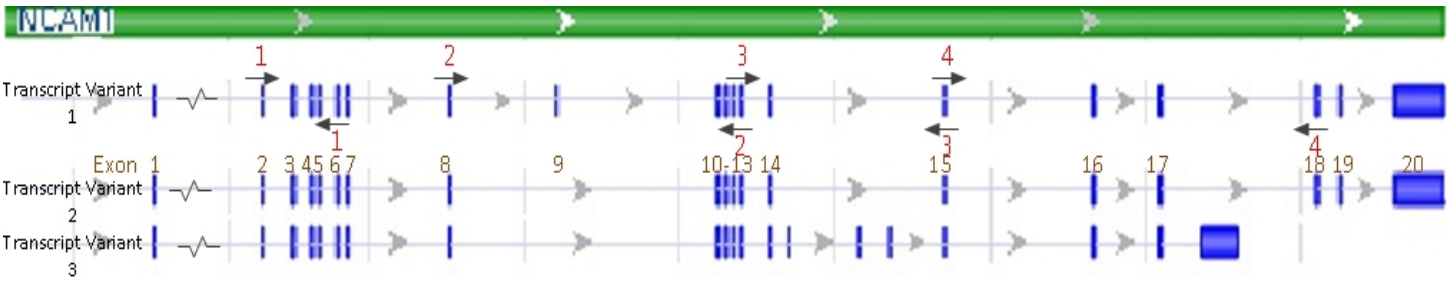


Fig 1 A-Graphical representation of NCAM 180kDa showing different domains (Frenette and Wagner 1996)
B- Comparison of the three transcript variants of NCAM1 and the placement of primers(GenBank)

threonine residues in the cytoplasmic domains, and fatty acid acylation (Cunningham et al, 1987). The most regulated and perhaps the most functionally significant modification is the additions of α-2,8-linked polysialic acid (PSA) by sialyltransferase to asparagine-linked oligosaccharides in the central portion of the protein. This anionic carbohydrate will cause PSA-NCAMs to repel each other and their inhibit binding.

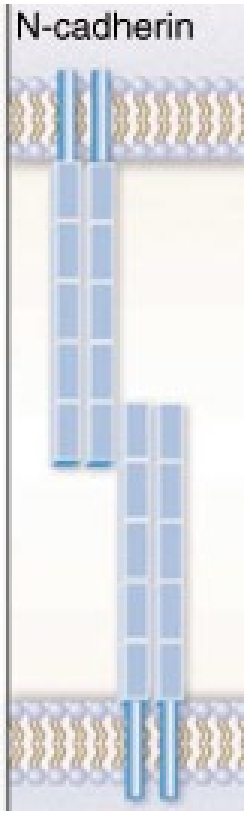
NCAM1 has three observed transcript variants (TV) (Fig 1B). Transcript variant 1 makes an 180kDa protein with a long cytoplasmic domain and contains 20 exons. Transcript variant 2 makes a 140kDa protein with a short cytoplasmic domain; it is missing the 30bp exon 9 and thus contains 19 exons. Transcript variant 3 makes a 120kDa glycophosphatidylinositol (GPI)-anchored protein which is missing exons 9, 18, 19, 20 but has three additional exons between exons 14 and 15, and an early polyadenylylation with a novel exon 20, resulting in a transcript with 20 exons (Phimister et al, 1991). Shifts in expression are recorded from the adult 120 kDa isoform to the embryonic 140 and 180 kDa isoforms in various tumors (Cavallaro and Christofori, 2001).

Sialylated NCAM1 (PSA-NCAM1) has been shown to be involved in patterning during development. It is highly prevalent in the developing brain, but in the adult brain is only present in the olfactory bulb, the hypothalamus, and the hippocampus - areas that require synaptic plasticity and continual structural reorganization. In fact, PSA-NCAM1 has been shown to be upregulated at the synapse during learning, where it will facilitate the release, repositioning and expansion of the synaptic complex. NCAM1 also functions in neuron-neuron adhesion, neurite fasciculation, neuronal pathfinding, and neurite outgrowth (also with the addition of PSA) (Mirnics et al, 2005).

Mice null for NCAM1 exhibit reduced brain size, especially in the olfactory lobes, late-onset learning defects, hypoplastic corticospinal tract, abnormally distributed anterior pituitary cell types, and morphological and functional defects of neuromuscular junctions (Singh et al, 2005).

NCAM1 expression has been shown to be dependent on nerve growth factor (NGF) stimulation via the neurotrophin receptor p75. p75 is a substrate for the protease presenilin 1 (PS1) which will cleave the intracellular domain of p75 allowing it to translocate to the nucleus where it will increase NCAM transcription (Mirnics et al, 2005).

N-cadherin (CDH2) is a classic cadherin - a calcium dependent glycoprotein that functions in the adherens junctions in the cell. It is located on chromosome 18q11.2, contains 16 exons, and has no known splice variants. It meditates strictly homotypic adhesion first in a cis-dimerization then a trans tetramer adhering two cells with a so called "cadherin zipper" (Fig 2). CDH2 contains five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail where it interacts β-catenin, γ-catenin and $p120^{ctn}$ to form a cytoplasmic cell adhesion complex where it links to the actin cytoskeleton(Christofori, 2003).



N-Cadherin is essential in synaptic buton - dendritic spine association. It also functions in gastrulation and the establishment of left-right asymmetry. It is described as a mesenchymal cadherin as it tends to be expressed in migratory cells, as opposed to the epithelial cadherins. In fact, a cadherin switch mechanism has been proposed that would explain metastasis as the switching from a pro-adhesive E-cadherin to a pro-migratory N-cadherin expression (Cavallaro and Christofori, 2001).

Fig 2 Graphical representation of N-Cadherin binding (Frenette and Wagner 1996)

NCAM1 and CDH2 are both highly expressed in the advancing axonal growth cones and the cellular substrates the cone contacts (Doherty and Walsh, 1996). NCAM1 and CDH2 can associate with and stimulate fibroblast growth factor receptors (FGFRs), inducing signal transduction cascades that affect neurite outgrowth, cell adhesion, motility, and invasion. CDH2 prevents the internalization of ligand bound FGFR increasing signal while NCAM1 directly activates FGFR bay assembling a a classical signaling complex, including FRS-2, c-Src, cortactin and phospholipase Cγ (Doherty and Walsh, 1996).

NCAM1 and CDH2 are intertwined with neuronal attachment, growth, and migration so a differential expression between the three cell lines is expected.

Materials and Methods

Neuroblastoma Cell Lines

KCN-83n, BE(2)-C and SH-EP1 cells lines were provided by Leleesha Samaraweera of the Laboratory of Neurobiology, Department of Biological Sciences, Fordham University. The cell lines were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum at 37 ℃.

Primers

Four pairs of primers specific for the reported mRNA sequence of NCAM1 (GenBank Accession Number NM_181351.3) were designed (Fig 1B). Primer pair one (F1 5' GATTCCTCCTCCACCCTCAC 3', R1 5' CAATATTCTGCCTGGCCTGGATG 3') amplified exons 3-6. Primer pair two (F2 5' CATCACCTGGAGGACTTCTACC 3', R2 5' CCAAGGACTCCTGCCCAATG 3') amplified exons 8-12. Primer pair three (F3 5' CATTGGGCAGGAGTCCTTGG 3', R3 5' CGTCATCCTGCTTGATCAGG 3') amplified

exons 12-16. Primer pair four (F4 5' ACCTGATCAAGCAGGATGACG 3', R4 5' CTCCTTGGACTCATCTTTCG 3') amplified exons 16-19.

Three pairs of primers specific for the reported mRNA sequence of CDH2 (GenBank Accession Number NM_001792.3) were designed (Fig 3). Primer pair one (F1 5' GTAGAGGCTTCTGGTGAAATCG 3', R1 5' CAAGTTGATTGGAGGGATGAC 3') amplified exons 2-4. Primer pair two (F2 5' CAGAATCGTGTCTCAGGCTC 3', R2 5' GATTCTGTACACTGCGTTCC 3') amplified exons 7-9. Primer pair three (F3 5' CAGATGTGGACAGGATTGTGG 3', R3 5' GAGCCACTGCCTTCATAGTC 3') amplified exons 13-16.

The gene GAPDH was used as a loading control for this study (F 5' GAAGGTGAAGGTCGGAGT 3', R 5'GAAGATGGTGATGGGATTTC 3') expecting to yield a product of 226 bp.

RNA extraction

Total RNA was extracted from the cell lysates using the RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions with minor modifications. The RNA was eluded twice with 50µl of dH_2O.

RT-PCR

RT-PCR was performed using QIAGEN® One-Step RT-PCR Kit following the instructions. GAPDH was used as the loading control. Twenty-five nanograms of RNA were amplified in 25µl RT-PCR reactions (5µl 5×RT buffer, 1µl 10mM dNTPs, 1µl enzyme mix, 1µl 10pmol/µl forward primer, 1µl 10pmol/µl reverse primer, 2.5µl 10ng/µl RNA and 13.5µl dH_2O). Temperature cycles are as follows: one cycle of 50℃ for 30min and 95℃ for 15min, 30 cycles of

94℃ for 30sec, 56℃ for 30sec, and 72℃ for 30sec, followed by a final extension of 72℃ for 10min followed by a final hold at 4°C.

Agarose Gel Electrophoresis

5µl of loading dye was added to each RT-PCR product. 5µl of each product was then added to a 1% agarose gel containing ethidium bromide, and electrophoresis was performed at 125V for 1 hour. PCR products were then visualized in a UV trans-illuminator (BioRad).

PCR Purification, Gel Extraction and Sequencing

Representative PCR reactions from each primer pair were purified using the QIAquick® PCR Purification Kit (QIAGEN) and interesting bands were cut out and purified using the QIAquick Gel Extraction Kit (QIAGEN). The samples were then sent out for sequencing by Genewiz Inc. The results were compared by BLAST to ensure proper product was formed.

Results

RT-PCR using specific primers (Fig 1b, 3) was performed on mRNA isolated from three neuroblastoma cell lines - KCN-83n, SH-EP1, and BE(2)-C, to study the expression of NCAM1 and CDH2. GAPDH was used a loading control, to monitor the quality and amount of RNA in the samples. The PCR reactions were then analyzed on a 1% agarose gel (Fig 4,5). NCAM1 shows differential expression with the greatest band intensity in the N cell line and the least in the S cell line. It also shows the expression of two splice variants in primer pair 2. The sequencing confirmed it was from the removal of exon 9 (Fig 6) in transcript variant 2. Transcript variant 3 was ruled out due to a lack of band in primer pair 3. There were no significant differences in the band intensities for CDH2 between the three cell lines. All products were confirmed by sequencing and all experiments were replicated at least three times.

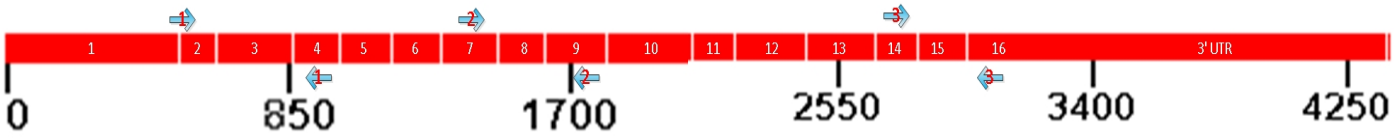


Fig 3 CDH2 primer placement

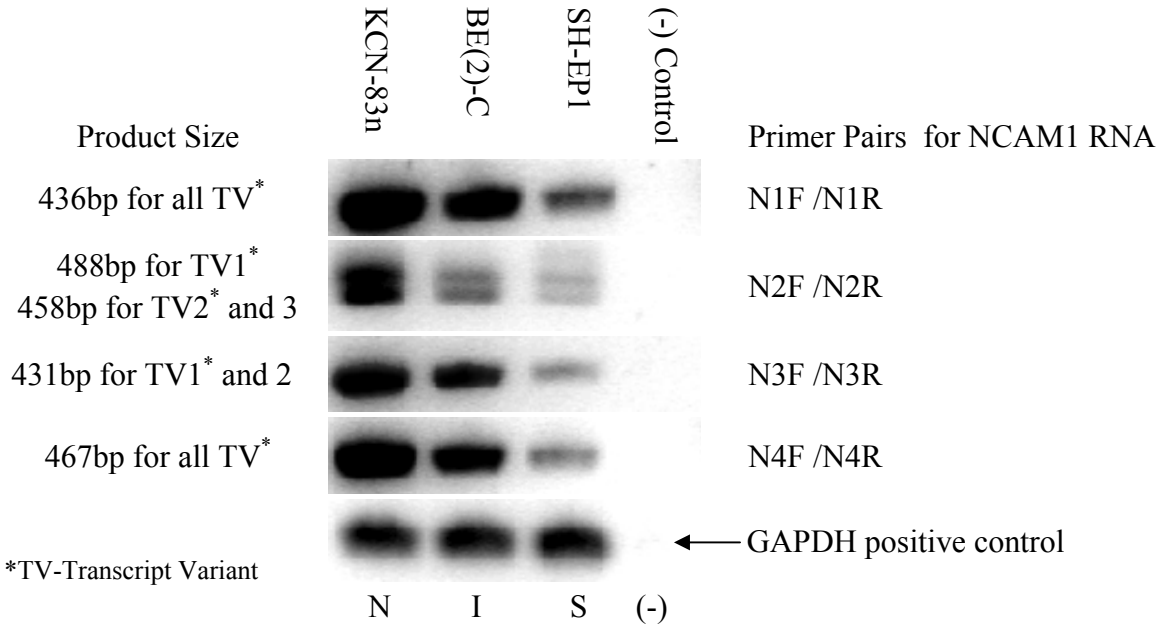


Fig 4 RT- PCR results of NCAM1

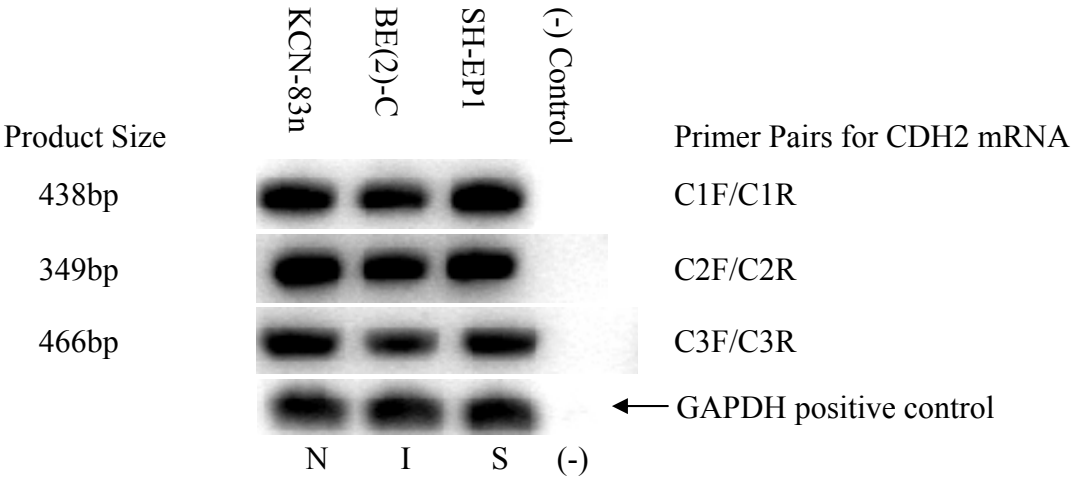


Fig 5 RT-PCR results of CDH2

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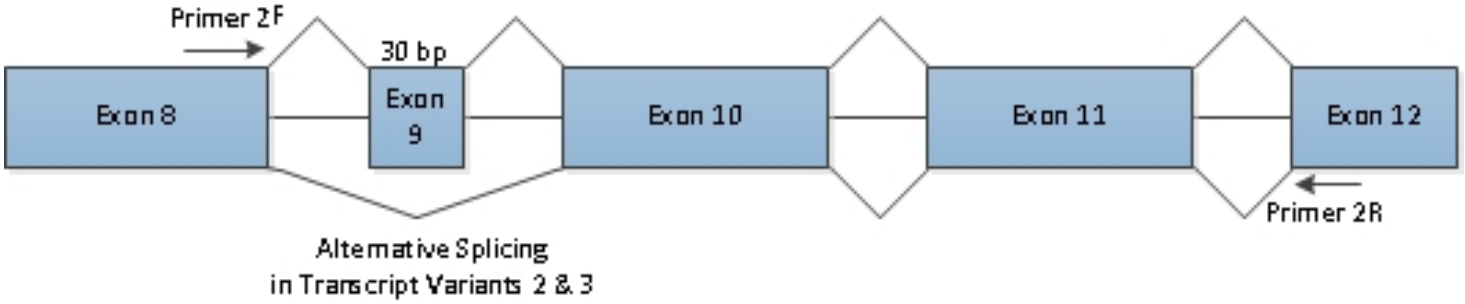

B

BLAST Partial Alignment of Gel Purified RT-PCR Products of the Top Band of Primer 2

```
Homo sapiens neural cell adhesion molecule 1 (NCAM1), transcript variant 1, mRNA
Score =  680 bits (368),  Expect = 0.0
 Identities = 370/371 (99%), Gaps = 0/371 (0%)
 Strand=Plus/Minus
Query  310   TCTTCAGGGTCAGCGACGACACACGGGCATGGCTACGCACCACCATGTGCCCATCCAGAG  369
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1490  TCTTCAGGGTCAGCGACGACACACGGGCATGGCTACGCACCACCATGTGCCCATCCAGAG  1431
                                  Exon 10
Query  370   TCTCTTGTTTCTCTGGTCGAGTCCACGAAGCCTTTTCTTCGCTGCTGATGT  421
              |||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1430  TCTCTTGCTTCTCTGGTCGAGTCCACGAAGCCTTTTCTTCGCTGCTGATGT  1380
                          Exon 9                  Exon 8
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BLAST Partial Alignment of Gel Purified RT-PCR Products of Bottom Band of Primer 2

```
Homo sapiens neural cell adhesion molecule 1 (NCAM1), transcript variant 2, mRNA
 Score =  754 bits (408),  Expect = 0.0
 Identities = 413/416 (99%), Gaps = 1/416 (0%)
 Strand=Plus/Minus
Query  303   ACTGGATGCTCTTCAGGGTCAGCGACGACACACGGGCATGGCTACGCACCACCATGTGCC  362
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1469  ACTGGATGCTCTTCAGGGTCAGCGACGACACACGGGCATGGCTACGCACCACCATGTGCC  1410

Query  363   CATCCAGAGTCTTTTCTTCGCTGCTGATGTTCCGGGTAGAAGTCCTCCAGGGATG  418
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1409  CATCCAGAGTCTTTTCTTCGCTGCTGATGTTCCGGGTAGAAGTCCTCCAGGGATG  1354
               Exon 10                        Exon 8
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Figure 6 Alternative Splicing in NCAM1 A – schematic of alternative splicing of NCAM1 B- BLAST results of cut out bands in primer pair N2F/R showing the splicing out of exon 9 in the bottom band

Discussion

NCAM1 showed evidence for differential expression between the different cell lines with lowest expression in the S cell line and the highest in the N cell line. As NCAM is used for neurite adhesion and growth, it is logical that the N line, which produces the most neurites, would express the highest levels of this transcript. There were also two isoforms expressed in all three cell lines encoded by transcript variants one and two. Transcript variant 2 is missing the 30bp exon 9 and thus has a shorter cytoplasmic tail, the significance of with is unknown. It appears that transcript variant 3 is not synthesized in these cells as there was no RT-PCR product that reflected its presence. This is in accordance with previous studies that showed a return to the embryonic 180 and 120 kDa forms of NCAM from the adult 140-kDa form in cancer cells (Cavallaro and Christofori, 2001). Further analysis of additional cell lines would be required to substantiate the reduced presence of NCAM1 in S cell lines and establish that expression has shifted from the adult form to the embryonic form, preferably with a non-tumor cell as a control.

There were no significant differences observed in expression levels of CDH2 between the three cell lines. This is a surprising result as the S cell lines, while very adherent to the substrate, are not very adherent to each other, which is the purpose of cadherins. N cells are very adherent to each other forming pseudoganglia in culture and thus should have higher expression. I cells the most invasive of the three should also have higher levels of CDH2 levels as it is associated with metastasis or pro-migratory phenotype (Christofori, 2003). As there is sufficient circumstantial evidence that the protein would have differential expression further studies with different cell lines, I cells which have been induced to differentiate, or perhaps comparisons with other cadherins should be done before it can be conclusively stated that there is no differences in expression.

Acknowledgments

I would like to thank Dr. Berish Rubin for his guidance. I would also like to thank Leleesha

Samarawera and Bo Liu for their unflagging patience in spite of what we put them through.

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