Differential Expression of Neurotrophic Typosine Kinase Receptor A (TrkA) in Two Human Neuroblastoma Cell Lines

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Abstract

Human neuroblastoma is the most common extracranial solid tumor in the pediatric population. Some neuroblastoma cell lines express neurotrophic tyrosine kinase receptor A (TrkA), which binds nerve growth factor, and promotes the differentiation of neuronal progenitor cells, immature neurons and other cells. Tissue specific expression of TrkA in two different cell lines, LA1-55N and LA1-5S was studied. RT-PCR and western bolt anaylsis demonstrated TrkA expression in LA1-5S cell lines and no expression in LA1-55N lines. This result was somewhat unexpected because LA1-5S is a schwannian/ melanoblastic precursor, not a neuronal precursor.

Introduction

Human neuroblastoma is the most common extracranial solid tumor in the pediatric population. Tumors arise from the developing neural crest along its migratory pathways and can be characterized clinically by their location, age at diagnosis, metastases and degree of cellular maturation and heterogeneity (1). Studies have shown that there are three different cell types found in neuroblastoma (2). These three different cell types have been characterized into I-type stem cells, N-type neuroblastic/ neuroendocrine precursors, and S-type schwannian/ melanoblastic precursors (1). The I-type cells show features common to both N and S type cells. The N-type cells adhere tightly to other cells forming structures like

pseudoganglia whereas the S type cells adhere tightly to the substrate. The presence of these cell phenotypes are often used for the prognosis of the disease (9). The I-type and N-type cell lines are malignant but the S-type cell lines are not malignant.

Numerous genetic and biological features have been studied in an effort to improve the understanding of neuroblastoma behavior and to identify markers that can improve the prognosis of patients with this cancer. One of these biological features that is being studied is the expression of the neurotrophic tyrosine kinase receptor (Trk) family which is a family of molecules that bind nerve growth factors. Nerve growth factor has different effects on cells depending on the type. For neurons and neuroblastoma cells it plays a role in their differentiation (10).

The Trk family of receptors consists of three different members, TrkA, TrkB and TrkC (3). Analysis of the Trk family indicates that these receptors bind neurotrophins differentially. TrkA is the preferred receptor for nerve growth factor, TrkB for brain derived neurotrophic factor and neurotrophin 4/5, and TrkC for neurotrophin 3 (4).

Differential expression of various gene products has been studied in different neuroblastoma lines to try to determine if there is a correlation between gene expression and growth (7, 8). Neuroblastoma cells that express high levels of TrkA have been shown to have a more favorable prognosis than tumors with low levels of TrkA (5,6). Because of the ability of these receptors to bind nerve growth factors and play a role in cell growth differentiation and programmed cell death of neurons in both the peripheral and central nervous system (4), the differential expression of TrkA in two different neuroblastoma cell lines, LA1-55N and LA1- 5S has been investigated. An unexpected result was obtained, where in the LA1-5S cell line, a schwannian/ melanoblastic precursor, showed expression of TrkA. This was

unexpected because TrkA is a neuronal marker, and LA1-5S showed expression of this gene even though it is not a neuronal precursor.

Materials and Methods

RNA samples and Cell lines

Cell lines LA1-55N and LA1- 5S were generously provided by Dr. Robert Ross. The cells were cultured in MEM with 10% FCS, 10 ug/ml penicillin, 10 ug/ml of streptomycin and were incubated at 37º C, with 5% CO_2 and humidity.

Primers

Primers specific to TrkA (MN_002529) were designed and ordered from Invitrogen Life Technologies. Two different sets of primers were used. The first set of primers only recognized the first and second isoforms of TrkA. The forward primer, located in exon 1 (LB-5) was 5' GCGCAGAGAACCTGACTGAGCT 3', which is homologous to nucleotides 248-269 of the cDNA, and the reverse primer matched exon 6, (LB-6) was 5' ACGTCGTCCCCCACATCCA 3' which was homologous to nucleotides 670 to 688 of the cDNA (11). These primers were predicted to generat a product of 442 base pairs from cDNA and if amplification were to occur from genomic DNA, a product of ~ 2Kb. The second set of primers that was used recognized all three isoforms of TrkA. The forward primer, located in exon 11 (LB- 7) was 5' TGCCTGCCTCTTCCTTTCTA 3', which is homologous to nucleotides 1337 to 1356 of the cDNA, and the reverse primer, located in exon 12 (LB- 8) was 5' GTGGTGAACACAGGCATCAC 3', which matched nucleotides 1549 to 1568 of the cDNA (12). These primers were expected to generate a cDNA product of 230 base pairs and if amplification were to occur from genomic DNA, a product of ~ 750 base pairs. Primers specific

to GAPDH were also generated (NM_002046). The forward primer was homologous to nucleotides 100 to119 of the cDNA, and the reverse primers matched nucleotides 308 to 327. The primers were expected to generate a product of ~225bp from cDNA and if amplification were to occur from genomic DNA, a product ~1.9 Kb. The dried primers were resuspended in deionized water and diluted to a concentration of 10 pmoles/ul.

Antibodies

The anti-TrkA antibody was obtained from Zymed Laboratories Inc. The antibody was generated against the extracellular N-terminal region of the gene, and recognizes all three of the TrkA isoforms. The anti-GAPDH antibody was obtained from Ambion and the anti mouse-IgG was obtained from Promega.

RNA Extraction

RNA was isolated using the Ambion RNAqueous phenol free total RNA isolation kit according to the manufacture's protocol with minor modifications. The RNA was then brought to a concentration of 20 ng/ul and stored at -80º C.

RT-PCR

RT-PCR was performed on the RNAs prepared from different neuroblastoma cell lines using the Qiagen One Step RT-PCR kit. Before setting up the RT-PCR reactions, a master mix was prepared containing 5X buffer, dNTPs, either TrkA or GAPDH primers (10pmoles/ul), enzyme mix and RNAase free deionized water. Equal volumes of master mix were added to RT-PCR reactions and then template RNA (1ug/ul) was added making the total reaction volume 15ul. Tubes were placed in a Thermal Cycler which was set up using the following protocol: RT—50°C for 30min, 95°C for 15mins, PCR reaction—94°C for 30sec, 56°C for 30sec, 72°C for 30 sec. Amplification of TrkA, was done at 50 cycles and the amplification of GAPDH was done at

26 cycles. Final extension was carried out at 72°C for 5 minutes followed by a hold period at 4°C. After completion of the RT-PCR, 2 ul of loading dye was added to the reaction tubes, and 8 ul of this material was loaded in a 1% agrose gel and electrophoresed at 140V.

PCR

Before setting up the PCR reactions, a master mix was prepared containing 5X buffer, dNTPs, TrkA primers (10pmoles/ul), enzyme mix and RNAase free deionized water. Equal volumes of master mix were added to PCR reactions and then template DNA (1ug/ul) was added making the total reaction volume 15ul. Tubes were placed in a Thermal Cycler which was set up using the following protocol:94°C for 15mins, PCR reaction—94°C for 30sec, 56°C for 30sec, 72°C for 30 sec. For the amplification of TrkA products, the amplification step was done for 50 cycles. Final extension was carried out at 72°C for 2 minutes followed by a hold period at 4°C.

RT-PCR/ PCR product purification

The TrkA from the LA1-5S cell line was purified using Qiagen's Purification kit as per the manufacturer's instructions with minor modifications.

Gel Extraction

Products from RT-PCR were extracted from the gel using the Qiagen rapid gel extraction kit following the instructions provided by the manufacturer with minor modifications.

Ligation and Transformation

The purified RT-PCR products were ligated into pGEMT vectors (Promega, Madison, WI). JM109 cells were then transformed with the vector and allowed to incubate overnight in media containing luria broth, ampicillin, Xgal and IPTG. Colonies believed to contain the insert were randomly chosen and analyzed to confirm the presence of plasmids containing the insert using the Quiagen miniprep analysis protocol. Colonies confirmed to contain the insert were then

inoculated in 5 ml of luria broth containing 5 ul of ampicillin (10 ug/ul). Samples were then incubated overnight at 37º C shaking at 220 rpm. DNA from inoculated samples was then isolated and purified using the Quiagen rapid plasmid miniprep protocol with minor modifications.

Sequencing

the purified PCR product or the purified products from cloning were sequenced by the Sanger's dideoxy method. These products were sequenced using the AmpliCycle Sequencing Kit, [α- ^{33}P] ATP, and either SP6 and T7 primers (for cloning products) or LB-5 and LB-6 primers (for PCR products). 6ul of this mixture was added to four PCR tubes each containing either 2ul of ddGTP, ddATP, ddTTP or ddCTP. All the tubes were overlaid with 12 ul mineral oil and placed in an Applied Biosystems Thermal Cycler. The sequence program used was as follows: 94°C for 3 min followed by 35 cycles of 94°C for 30sec, 58°C for 30sec, 72°C for 1min followed by a hold period at 4°C. After completion of the reaction, 4ul of stop solution was added to each reaction tube. The products were electrophoresed on a denaturing polyacrylamide gel and the nucleotide sequence was visualized by autoradiography.

Sequence alignment

In order to confirm the results from the sequencing reaction showed homology to human TrkA gene and not other genes, the sequences were compared with previously published TrkA cDNA MN_002529). Sequences were read into a computer from autoradiographs and aligned using MacVector 6.5.3.

Protein Purification

Cells were washed twice with PBS, detached from a flask by EDTA-trypsin, and then washed again with PBS. The cultures were centrifuged to pellet down the cells, and all media and

trypsin was removed and a lysis buffer (25mM Tris-phosphate, 2mM DTT, 1mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 1% TritonX-100) was added to pellet at a volume of 100 uL per 1x 10^5 cells. The proteins were then stored at -80º C. A Bio-Rad Protein Assay kit was used to analyze protein concentration according to the manufacture's protocall; bovine serum albumin was used for the standard curve.

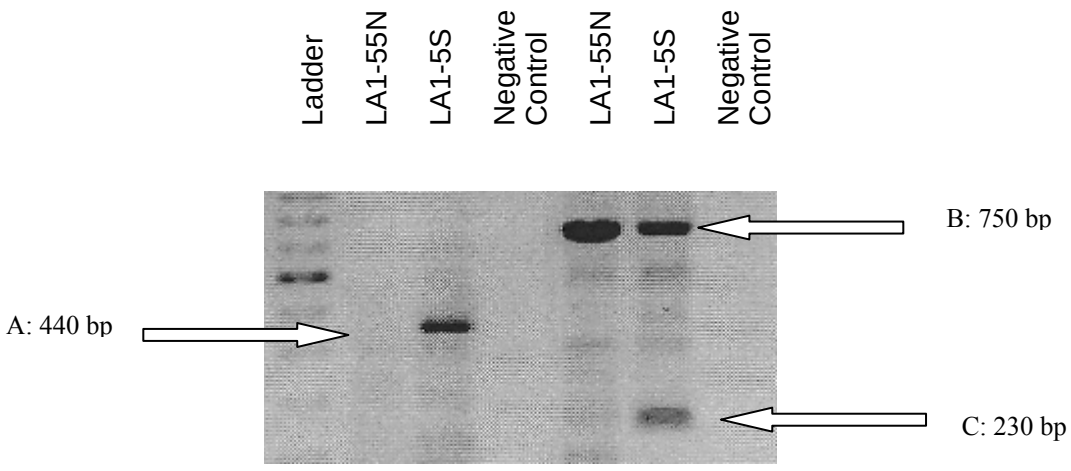
Western Blotting

Protein extracts from LA1-55N and LA1-5S cell lines were diluted in a 1:1 volume ration with 2X SDS sample buffer, denatured by boiling and run on an 8% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane using the Bio Rad Trans Cell Blot. The transfer was run at 100 volts at 4º C. When the proteins were successfully transferred to the nitrocellulose membrane, the membrane was removed from the sandwich apparatus and placed into 5% nonfat dry milk for one hour at room temperature. The membrane was then cut based on molecular weight distribution, and the top half placed in a dish containing TBST buffer and the Anti-TrkA (.5 mg/ml from Zymed) monoclonal antibody. The bottom half was placed in a dish containing TBST and anti-GAPDH (4 ug/ul; 1:2000 dilution in TBST) (Ambion) antibody. Both were left overnight at 4º C. Both parts of the nitrocellulose membrane were then washed 3 times in TBST buffer and placed in a dish containing TBST buffer and the Anti-mouse IgG (1 ug/ul; 1:4000 dilution in TBST) (Promega) antibody where it remained for 2 hours at room temperature. The nitrocellulose membrane was then washed again with TBST and placed in Western Blue substrate (Promega) with high agitation at room temperature until the staining appeared. The reaction was terminated by washing the membrane in tap water.

Results

To study TrkA expression in the two different cell lines, LA1-55N and LA1-5S, RT-PCR was performed on the RNA from these two cell lines. GAPDH was used to determine if the RNA loaded on to the gel was equal. Figure 1a uses both sets of primers that were designed to preferentially express the TrkA cDNA sequence by using a sequence in exons that are separated by introns. For the first set of primers, PCR product A was the predicted size of 440 bp, but is only present in the LA1-5S cell line. For the second set of primers, PCR product B had a size of 750 bp and was expressed in both cell lines. PCR product C was the predicted size of 230 bp and is only present in the LA1-5S cell line. The variation in product expression in the two different cell types is most likely due to differential expression of the product since the same amount of template RNA was loaded in all the RT-PCR reactions (Figure 1b). Because the results were not what was expected, an internal control was used to make sure the RNA samples were correct. Platelet derived growth factor beta was used as an S-type marker (17) (data not shown). The results were consistent with the previous data, and it was concluded that the samples were not switched.

a)



b)

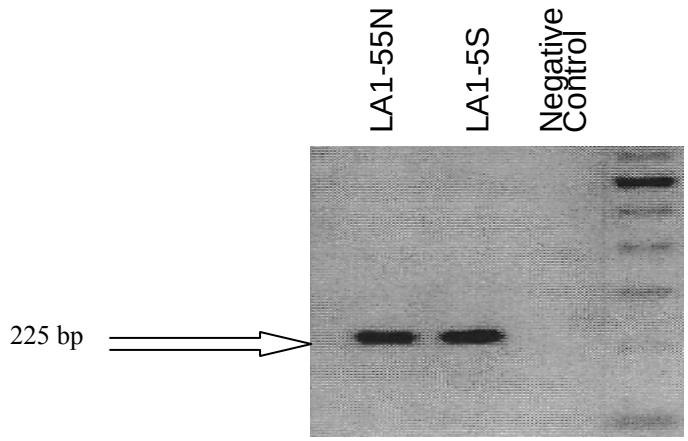
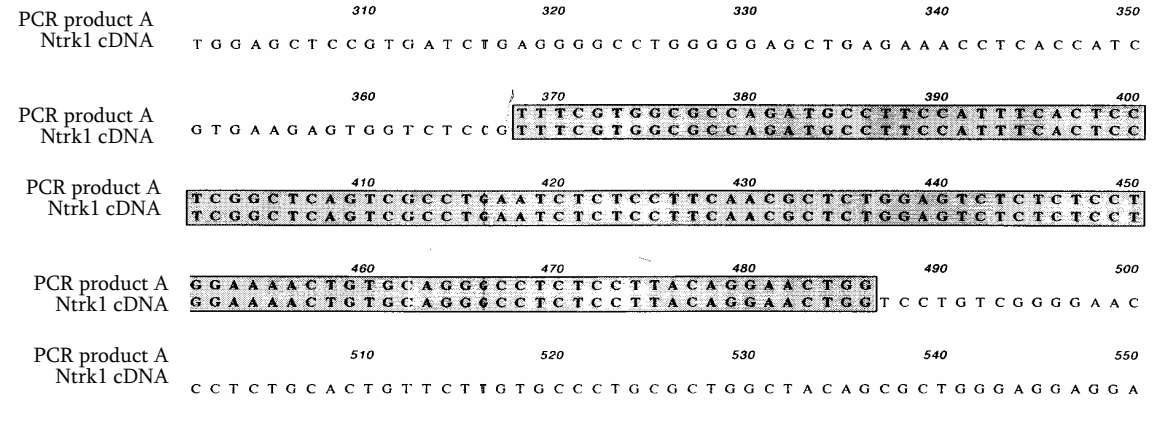


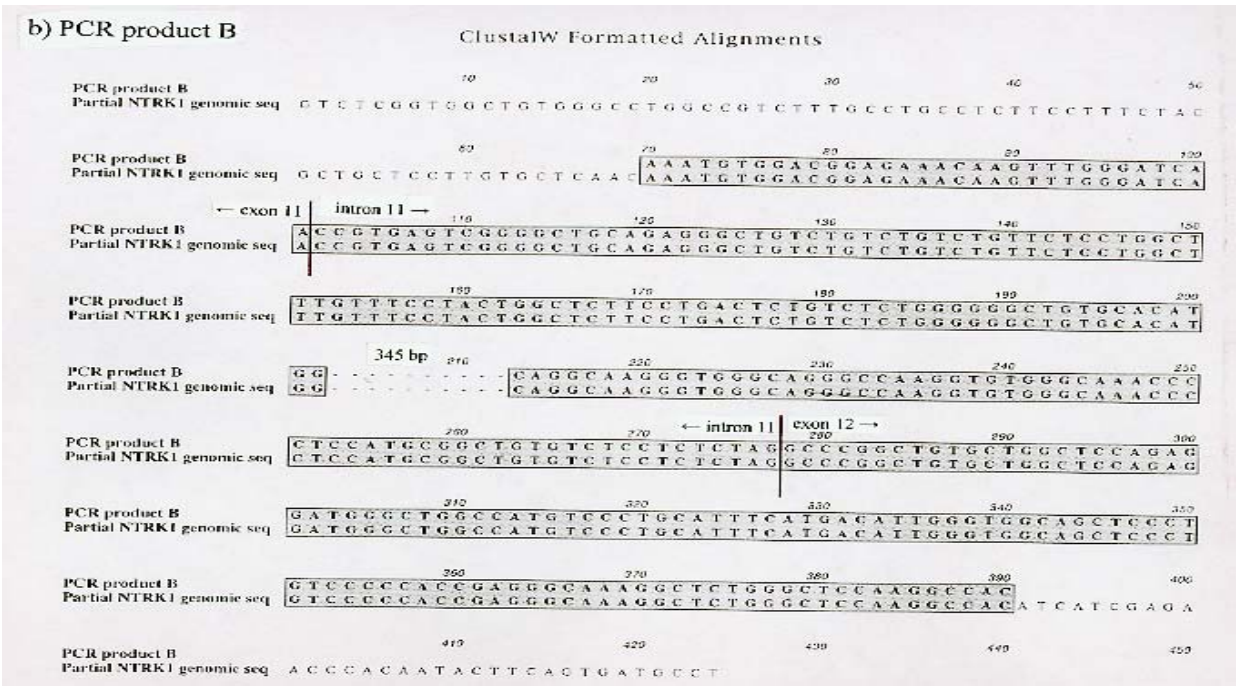
Figure 1: a) RT-PCR examining TrkA in LA1-55N and LA1-5S, using primer set 1 (248-688, exon 1 and exon 6, NM_002529, first three lanes) and primer set 2 (1337-1567, exon 11 and exon 12, NM_002529, last three lanes). 8 ul of the product was loaded onto the 1% Agarose gel. b) RT-PCR examining GAPDH mRNA (100-327, NM_002046) in LA1-55N and LA1-5S used as a RNA loading control. Amplification was preformed for 26 cycles and 8 ul of the product was loaded onto the gel.

Sequence analysis was also performed on the three different PCR products to confirm their identity. PCR product A shows homology to the 440bp cDNA of the TrkA gene where primer set 1 is designed (Figure 2a). Ninety eight of the base pairs were sequenced against the 440bp and was found to be an identical match (Figure 2a). PCR product B, when sequenced, was a match to the exon sequence (Figure 2 b) as well as the intron sequence of the TrkA gene. Band C was also sequenced and showed homology to the 230 bp cDNA of the TrkA gene where primer set 2 was designed (Figure 2c). Eighty one of the base pairs were sequenced against the 230 bp product and found to be an identical match as well.

a) PCR product A



b) PCR product B



c) PCR product C

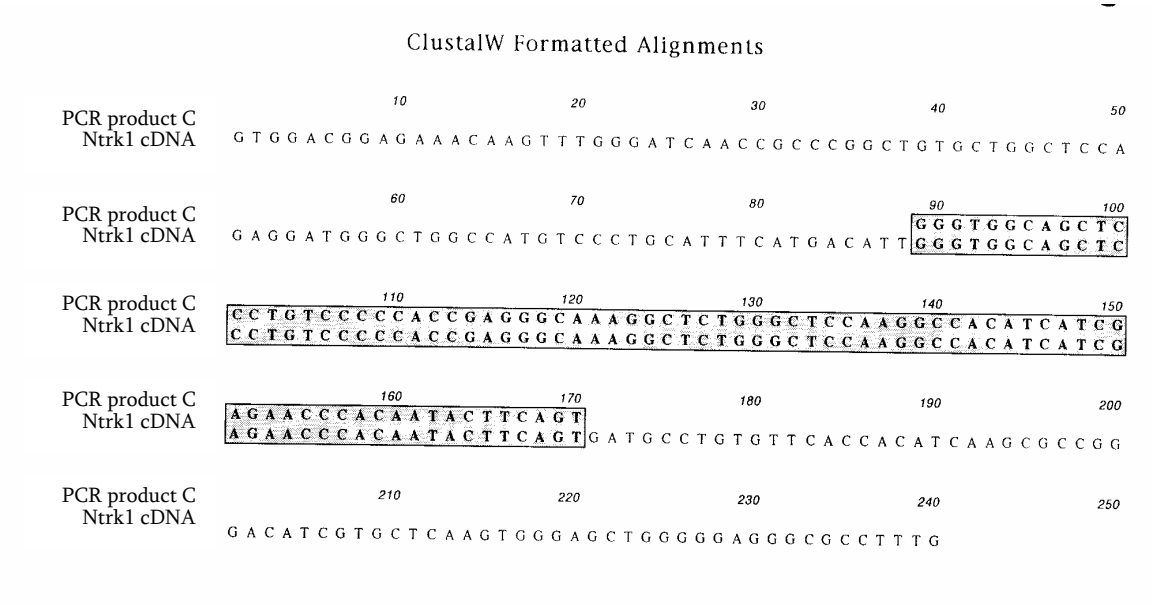


Figure 2: Sequencing generated from PCR products A, B and C was then compared to the cDNA of TrkA (MN_002529). Blast analysis was performed on PCR products A, B and C. a) PCR product A matched against the cDNA sequence of TrkA in the region where the primer set 1 was designed. b) PCR product B sequence matched the exon and intron sequence of the TrkA gene in the region where primer set 2 was designed. c) PCR product C sequence matched to the cDNA sequence of the TrkA gene, where primer set 2 is designed.

To examine the level of the TrkA protein, a western blot analysis was performed on the total protein extraction from the two different cell lines. A monoclonal antibody (Zymed) was used that recognized all three TrkA isoforms (~140 kDa protein) (Figure 3a). Based the results from the RT-PCR and sequencing, it was expected that only the LA1-5S cell line would produce the protein because the cDNA for TrkA was only found in that line. Protein expression was also determined for GAPDH which was used to confirm equal amounts of protein were loaded onto the gel (Figure 3b). An anti-GAPDH antibody (Ambion) was used to detect the presence of GAPDH. The results were as expected; only the LA1-5S cell line produced the protein.

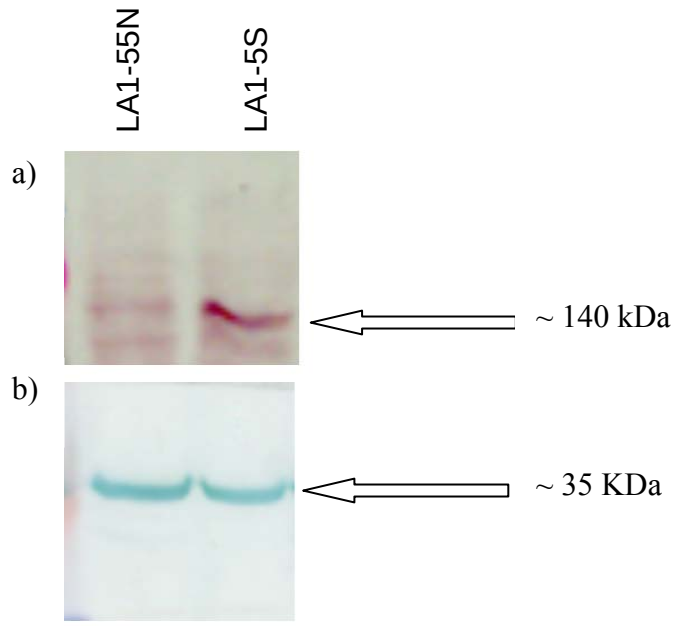


Figure 3: Western Blot analysis of the protein extracts of the LA1-55N and LA1-5S cell lines was performed using antibodies to TrkA (a) and GAPDH (b).

Discussion

Based upon the results obtained from the RT-PCR reaction (Figure 1a) and the sequencing reactions (Figure 2), the LA1-5S cell line shows higher expression of TrkA than does the LA1-55N cell line. This was a somewhat unexpected result, because LA1-5S is a schwannian/ melanoblastic precursor and TrkA is a neuronal marker, yet LA1-5S showed expression of this gene even though it is not a neuronal precursor. The LA1-5S cell line also expressed the TrkA protein, while the LA1-55N cell line did not (Figure 3a), which is consistent with the RT-PCR results.

High TrkA levels are associated with a better prognosis for people suffering from neuroblastoma (4). Therefore, it was not totally unexpected that the LA1-55N, even though it is a neuronal cell line, did not express TrkA because TrkA is generally expressed in less malignant cell lines and LA1-55N is highly tumorogenic (15, 16). It is interesting that the LA1-55N does

express the intron and exon of the TrkA gene (fig 1a) but does not express the mRNA. Based on the data from sequencing (Figure 2 b), it does not appear that the LA1-55N cell line has any mutations in the TrkA gene sequence that would cause the LA1-55N cell line not to produce mRNA. Further analysis is needed to determine if the 750 base pair band (Figure 1a) is genomic DNA or pre- RNA.

LA1-5S does show translational capabilities because proteins are produced by the cell line (Figure 3a). It is unknown, however, if the protein is functional. Further analysis on the protein is needed to determine if it has biological activity. Other researchers have found that other signal pathways influence TrkA protein expression (4, 13, 14); therefore, it may be possible that if other pathways are malfunctioning or are over expressed, TrkA expression in cells could be influenced.

Because LA1-5S expressing TrkA is counterintuitive, further research is needed to determine if this is an isolated event or if TrkA expression occurs in other S-type cell lines. Also, further investigation is required to determine if other N-type cell lines, of varying malignancy, express TrkA. Whether or not other cells exhibit the same pattern of expression as LA1-55N and LA1-5S will provide insight into the subject and offer a possible plan for determining the cause of this result.

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