EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA RECEPTORS IN NEUROBLASTOMA CELL LINES WITH DIFFERENT TUMORGENICITIES

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ABSTRACT

Transforming Growth Factor Beta (TGFβ), ubiquitously expressed during embryonic development and in most adult tissues, is a potent growth inhibitor for both tumor and non-tumor cells. TGFβ exerts its biological effects by binding cell surface receptors; TGFβRI, TGFβRII and TGFβRIII, initiating a signaling cascade, ultimately activating several transcription factors that regulate genes involved in the cell cycle. Disruption of the TGFβ signaling pathway is observed in several human cancers, either by downregulation of either the ligand itself or its receptor expression. Neuroblastoma is derived from the arrested differentiation of neural crest progenitor cells. Recent studies have indicated that growth, differentiation and apoptosis in neuroblastoma cells, is strongly regulated and mediated by TGFβ via the increased expression of TGFβ receptors. This project investigated the expression levels of the TGFβ receptor genes, between I type (SK-N-ER) and S type (SH-EP1) neuroblastoma cell lines which exhibit differences in their invasiveness properties. The RT-PCR analysis suggested a decreased expression of TGFβ receptors II and III in the SK-N-ER cell line as compared to SH-EP1 cell line, under identical incubation conditions. There was no significant difference in the expression of TGFβRI observed. Based on the obtained data, it cannot be concluded that, the decreased expression of TGFβRII and RIII mRNA in the SK-N-ER neuroblastoma cells has a direct correlation with its invasiveness. There are other factors that may also affect the invasiveness of these cell lines and hence further studies are needed.

INTRODUCTION

Transforming Growth Factor Beta-1 (TGFβ1) which is ubiquitously expressed during embryonic development and in most adult tissues, regulates proliferation, differentiation, and apoptosis. TGFβ belongs to a super family of cytokines that include activins/inhibins, bone morphogenetic proteins (BMPs), growth differentiation factors, and a few other related proteins. Mice with TGFβ receptors knocked out have various abnormalities in development, regulation of immune response, vessel formation, wound healing, and hematopoiesis (1).

One of the effects of TGF β is potent growth inhibition of both tumor and non-tumor mammary epithelial cells. TGFβ induces growth inhibition by arresting cells in the G1 phase of the cell cycle, which in some circumstances may lead to terminal differentiation or induction of apoptosis (2).

TGFβ exerts its biological effects by binding to high-affinity cell surface receptors. There are three ubiquitously expressed TGFβ receptors: type I, type II, and type III (TGFβRI, TGFβRII and TGFβRIII, respectively). TGFβRIII is a membrane proteoglycan that has a short cytoplasmic tail and lacks any consensus signaling motif (3, 4). TGFβRIII functions as a co-receptor to increase ligand binding to TGFβRII. (5). The RI and RII receptors belong to a family of serine/threonine kinase receptors. RII binds to TGFβ in the absence of RI but requires RI to transduce signals, whereas RI doesn't bind to TGFβ directly.

According to the proposed model for TGFβ receptor system, TGFβRII auto-phosphorylates itself upon ligand binding and recruits TGFβRI and trans-phosphorylates it (6, 7). Activated TGFβRI then phosphorylates a specific subset of SMAD proteins, which subsequently translocate into the nucleus. Once in the nucleus, SMAD proteins form functional transcription complexes in association with DNA binding factors, coactivators, or corepressors (Fig.1) (2).

TGFβRI is 503 amino acids in length and its gene is localized to chromosome 9q22. TGFβRII is 592 amino acids in length and its gene is localized to chromosome 3p22. TGFβRIII is 851 amino acids in length and its gene is localized to chromosome 1p33-p32.

Disruption of the TGFβ/SMAD signaling pathway by mutation has been observed in several types of human cancer. TGFβRII is inactivated by mutation in a majority of colon and gastric cancers (2). In breast cancer, TGFβ has been suggested to play a dual role. It acts as a tumor suppressor in early stages of the disease when it inhibits the outgrowth of carcinomas *in*

situ via its antiproliferative functions (2, 7, and 8). In later stages of the disease, TGFβ is believed to promote tumor progression, in part by enhancing tumor cell motility and invasiveness and the capacity to form metastases (2, 9). In some breast cancer cell lines, limited expression of TGFβRII has been correlated with the lack of TGFβ responsiveness. Stable expression of TGFβRII in such cell lines can restore TGFβ induced growth inhibition, indicating that all other signaling components are functional (2, 10). In prostate cancer cell lines, TGFβ has been shown to inhibit cellular proliferation in vitro (3). There is a downregulation of TGFβRII in several prostrate cancers which is associated with resistance to TGFβ mediated cell growth inhibition (4).

Neuroblastoma, one of the most common pediatric cancers, develops from the neural crest during early childhood (11). A defining characteristic of neuroblastoma is the existence of three distinct cell phenotypes in the tumor: N-type neuroblastic/neuroendocrine precursors, I-type stem cells, and S-type Schwannian or substrate-adherent cells (12, 13). Neuroblastomas are derived from the arrested differentiation of neural crest sympathoadrenal progenitor cells (13).

Recent studies have revealed that growth, differentiation, and programmed cell death of the developing neural crest-derived cells are strongly regulated by growth factors and their receptors. TGFβ and members of the TGFβ super family are important factors controlling early embryonic neural development. It is believed that TGFβ mediates both the growth inhibitory response and retinoic acid mediated differentiation in neuroblastoma cells, through the upregulation of TGFβ release as well as the increased expression of TGFβ receptors. It has been shown that perturbation of the signal transduction pathway of TGFβ induces the escape of cells from negative growth control, which may be an important step in the progression of tumors (11, 14).

In some studies with neuroblastoma cell lines it has been seen that TGFβRII over expression activates the TGFβ signal transduction pathway, reverting the neuroblastoma cell's neoplastic phenotype resulting in a reduction of proliferation rate and the induction of terminal maturation (11).

The purpose of this project was to characterize the expression level differences the TGFβ receptor genes in I type and S type neuroblastoma cell line. These two types of neuroblastoma cells exhibit difference not only in their phenotypes but also their tumorgenicity and ability to metastasize. SK-N-ER is an I – type neuroblastoma, which is a malignant neural crest stem cell, whereas SH-EP1 is S-type, exhibiting substrate adherence and is non-tumorigenic. TGFβ receptors are down regulated in several cancers in a stage dependent manner. It is therefore important to see if there is a down regulation of these in the invasive neuroblastoma cell line as compared to the non-invasive adherent type.

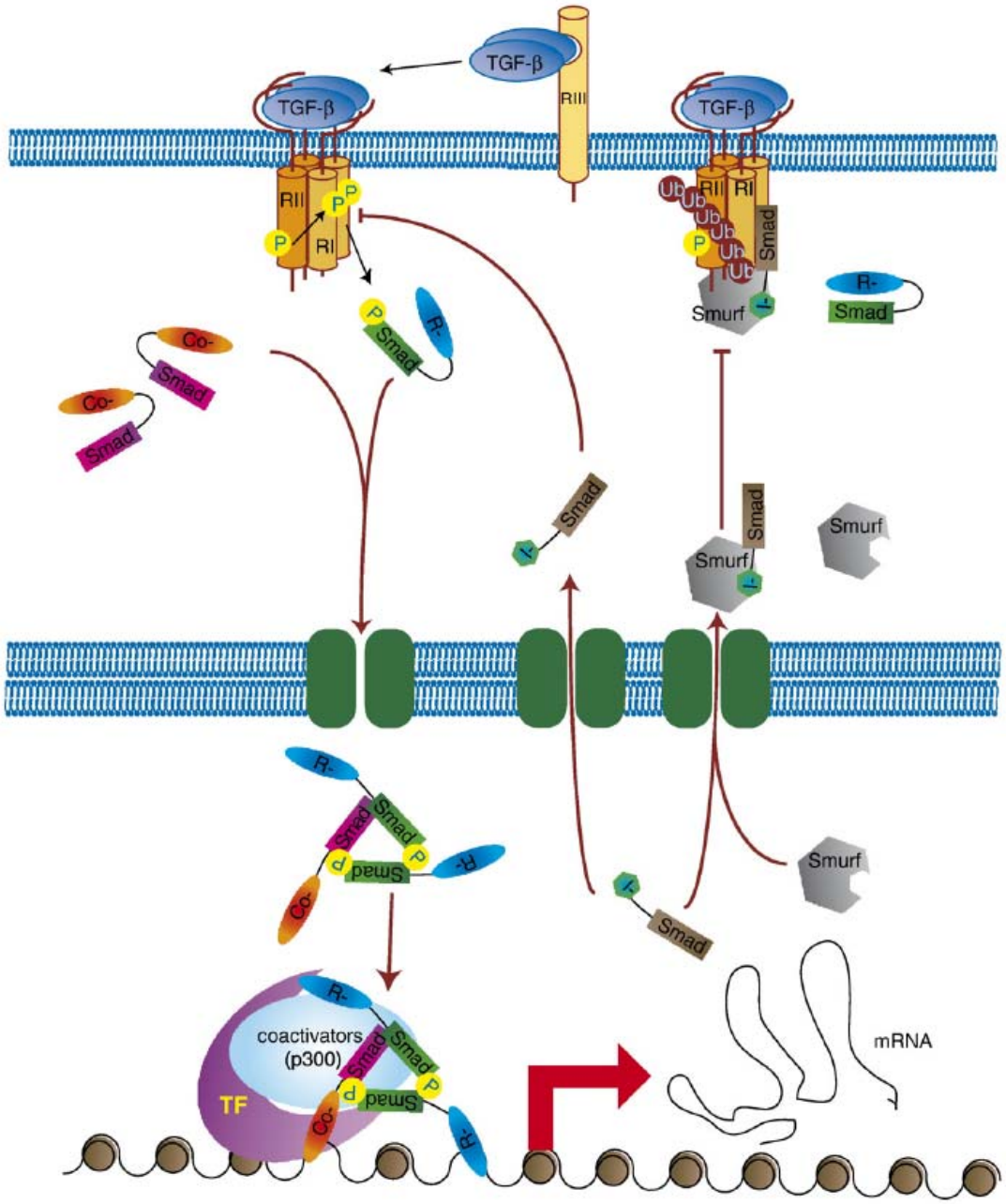

Figure 1. TGF-β signaling by Smads. TGF-β bound to TGFβRII/TGFβRI receptors on cell surface. TGFβRII phosphorylates TGFβRI, which then phosphorylates Smads that move to the nucleus and form complexes. These Smad complexes bind to DNA and regulate transcription (6).

MATERIALS AND METHODS

Cell Lines

SK-N-ER and SH-EP1, cells were kindly provided by Dr. Robert Ross. The cell lines were cultured in MEM with 10% fetal calf serum, 10 μg/mL penicillin and 10 μg/mL streptomycin at 37°C, with 5% CO2 and humidity.

Table 1. Neuroblastoma cell lines studied in this project

Cell Line	Phenotype
SK-N-ER	Invasive
SH-EP1	Substrate adherent

RNA Extraction

Total RNA was extracted from SK-N-ER and SH-EP1 cell lines using the RNAqueous kit from Ambion. The RNA concentration in each sample was quantified using a spectrophotometer (260 nm). The RNA was diluted serially with RNAse free de-ionized water (1:50 and 1:10) and then stored along with the undiluted stock at -80°c.

Primer Design

Primers were designed for each of the TGFβ receptor genes in regions spanning exons of each of the genes as follows and ordered from Integrated DNA Technologies, Inc.

Table 2. Primers Used in RT-PCR

Gene	NCBI Acc #	Exon	Nucleotide	Primer sequence 5'→ 3'	RT PCR Prod (bp)	Genomic DNA (bp)
TGFβRI	NM_004612.2	6	1054-73	GCCAGCCATTGCTCATAGAG	278	1874
		7	1310-31	CACCAATGGAACATCGTCGAGC		
TGFβRII	NM_001024847.1	5	1602-21	GCTCCAATATCCTCGTGAAG	240	1907
		6	1830-49	CACTGCATTACAGCGAGATG		
TGFβRIII	NM_003243.2	9	1440-61	CTATTCCTCCTGAGCTACGGAT	423	851
		10	1880-1900	GGAGTTATAGTAGACCACACC		
GAPDH*	NM_002046	2, 3	127-146	AACGGATTTGGTCGTATTGG	225	2100
		4,5	335-54	TTTGGAGGGATCTCGCTCCT		

*Primers amplifying a region of GAPDH were ordered from Invitrogen Life Technologies and were used as a control.

RT-PCR

RT-PCR was performed on the total RNA samples prepared from the two neuroblastoma cell lines using the Qiagen OneStep RT-PCR kit as per the manufacturer's protocol. For each set of reactions, a master mix was prepared using 3 μL of 5x RT buffer, 0.6 μL of dNTP's, 0.6 μL of enzyme mix. 20ng of RNA was used per reaction, RNAse free de-ionized water was used to make up the volume of each reaction to 15 μL. Approximately 20 μL of mineral oil was added to each PCR reaction mix to prevent evaporation during RT-PCR run. The tubes were placed in a thermal cycler and RT PCR was carried out for all the three TGFβ receptor genes & GAPDH for both neuroblastoma cell lines according to the following protocol: One cycle of 50°c for 30mins (RT), 94°C for 15 min, 38 cycles of 94°C for 30 sec. (denaturation), 58°C for 30 sec.(annealing), 72°C for 30 sec. (extension), followed by one cycle of 72°c for 7 mins (Final extension). The same protocol was also followed for all the three genes & GAPDH at 50 cycles for both the neuroblastoma cell lines. The RT-PCR products were analyzed on a 1% agarose gel.

RT-PCR Product Purification

PCR products obtained were purified from both the cell lines, using Marligen's Purification Kit as per the manufacturer's instructions. The purified products were then quantified using a spectrophotometer (260 nm).

Sequencing

The purified RT-PCR products were quantified then and approximately 80ng/ul of each product along with 8pM of the appropriate primers were sent to Genewiz Inc. for DNA sequencing analysis.

RESULTS

The expression of the three TGFβ receptor genes was studied via RT-PCR in two different neuroblastoma cell lines, SK-N-ER and SH-EP1. The cycle number was optimized to 38 to see the differential expression TGFβ receptor genes in the above cell lines. RT-PCR was also carried out for 50 cycles to see whether these cell lines express TGFβR genes, at low levels. Twenty nanograms of Total RNA from each cell line was used for RT-PCR and the products were analyzed on a 1% agarose gel. As an external control to normalize the variations of RNA quantities within the samples, GAPDH was amplified from equal amounts of RNA in the two cell lines by RT-PCR using GAPDH primers as described in the materials and methods. The RT-PCR products generated using primers for the various TGFβR genes were from the respective mRNAs and not from genomic DNA as amplification from the genomic DNA would have generated a much larger size bands (refer table 2).

There was no significant difference in gene expression for TGFβRI between SK-N-ER and SH-EP1 neuroblastoma cell lines (Fig.2). TGFβRII and TGFβRIII exhibited differential expression between the two cell lines. The mRNA for both the genes was expressed at a higher level in the non-invasive S type, SH-EP1 cell line as compared to the invasive SK-N-ER neuroblastoma cell line (Fig. 3 and 4).

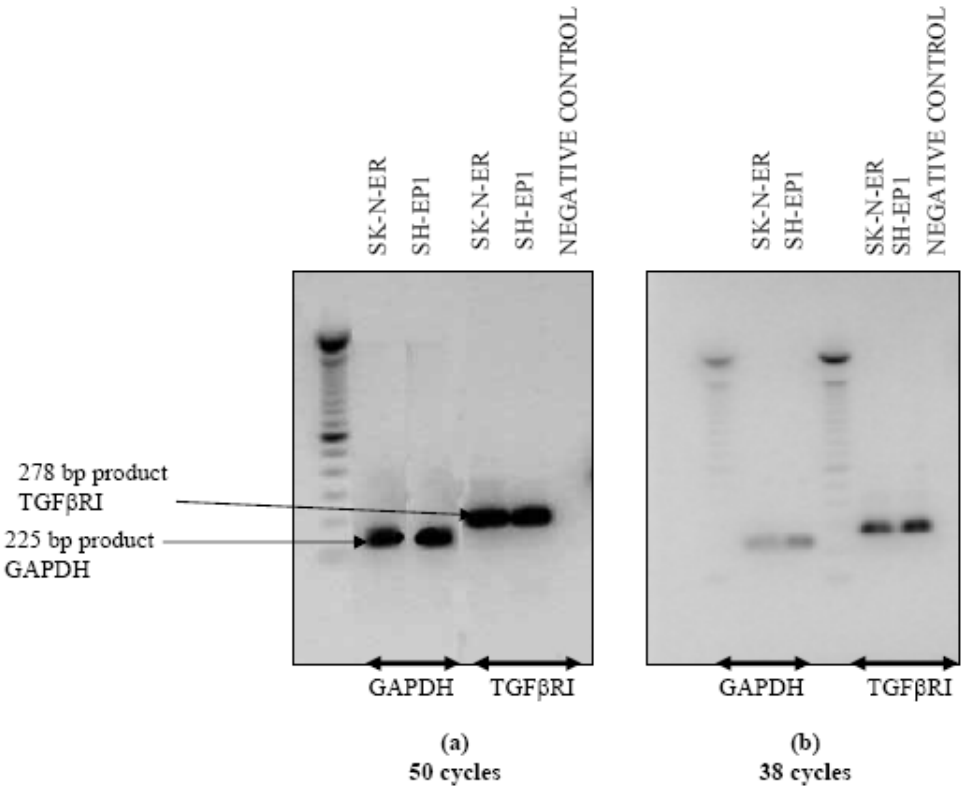

Figure 2. RT-PCR analysis of TGFβRI transcript levels in SK-N-ER and SH-EP1 cell lines. RT-PCR products generated from RNA isolated from each of the cell lines, amplified by primers recognizing GAPDH and TGFβRI (a), (b).

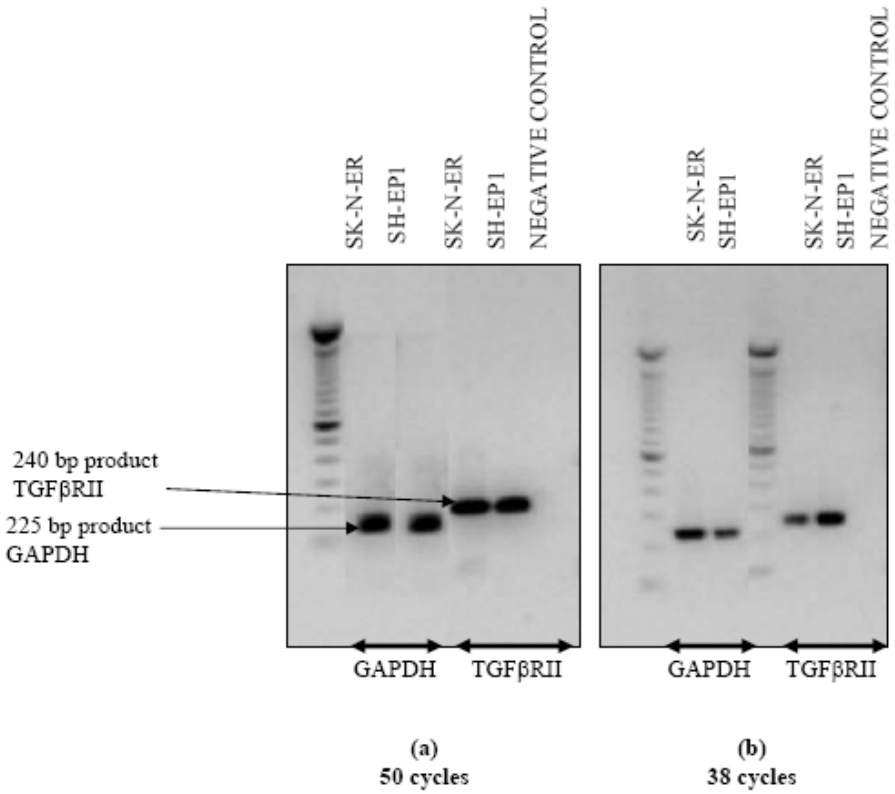

Figure 3. RT-PCR analysis of TGFβRII transcript levels in SK-N-ER and SH-EP1 cell lines. RT-PCR products generated from RNA isolated from each of the cell lines, amplified by primers recognizing GAPDH and TGFβRII (a), (b).

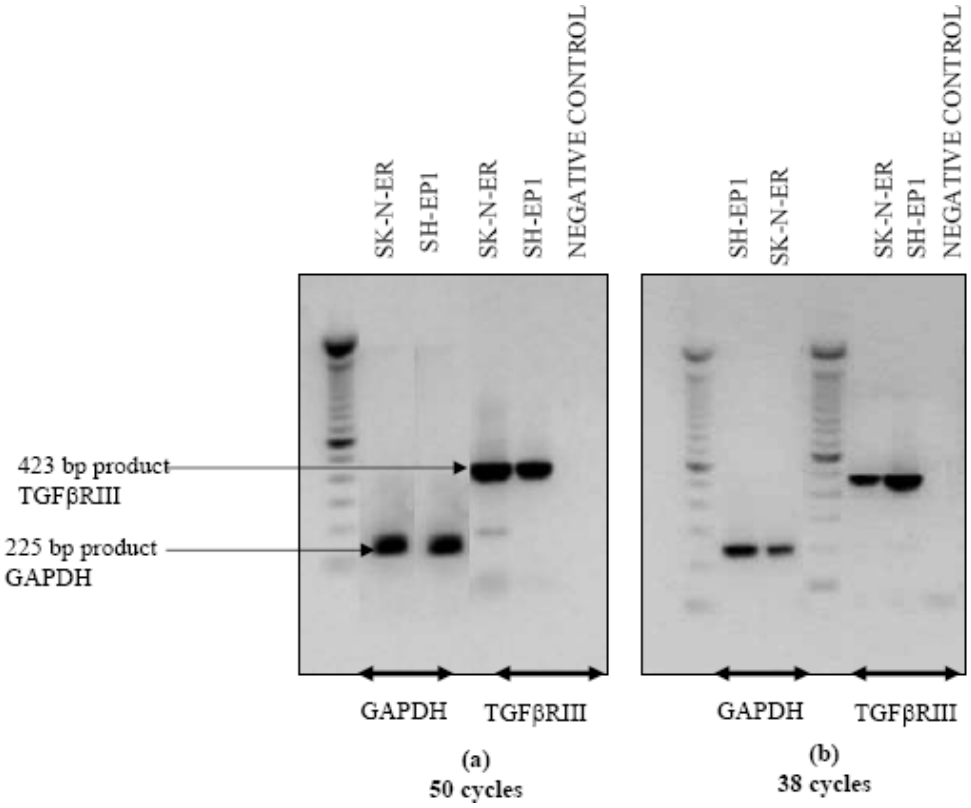

Figure 4. RT-PCR analysis of TGFβRIII transcript levels in SK-N-ER and SH-EP1 cell lines. RT-PCR products generated from RNA isolated from each of the cell lines, amplified by primers recognizing GAPDH and TGFβRIII (a), (b).

The RT-PCR products were sent to Genewiz Inc. for sequencing to confirm their identity. The sequence data was analyzed using NCBI Blast website, and ClustalW analysis (Fig. 5, 6 and 7).

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TGFBRI                  GATATTGCTCCAAACCACAGAGTGGGAACAAAAAGGTACATGGCCCCTGAAGTTCTCGAT
278bp_PCR_Product       GATATTGCTCCAAACCACAGAGTGGGAACAAAAAGGTACATGGCCCCTGAAGTTCTCGAT
                        ************************************************************

TGFBRI                  GATTCCATAAATATGAAACATTTTGAATCCTTCAAACGTGCTGACATCTATGCAATGGGC
278bp_PCR_Product       GATTCCATAAATATGAAACATTTTGAATCCTTCAAACGTGCTGACATCTATGCAATGGGC
                        ************************************************************

TGFBRI                  TTAGTATTCTGGGAAATTGCTCGACGATGTTCCATTGGTGGA
278bp_PCR_Product       TTAGTATTCTGGGAAATTGCTCGACGATGTTCCATTGGTGGA
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Figure 5. The ClustalW alignment of the partial sequence of the 278 bp RT-PCR product amplified using TGFβRI primers with a segment of *Homo sapiens* TGFβRI mRNA sequence from NCBI (NM_004612.2).

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TGFBRII                 TGGAGTAGACATCGGTCTGCTTGAAGGACTCAACATTCTCCAAATTCATCCTGGATTCTA
240bp_PCR_Product       TGGAGTAGACATCGGTCTGCTTGAAGGACTCAACATTCTCCAAATTCATCCTGGATTCTA
                        ************************************************************

TGFBRII                 GGACTTCTGGAGCCATGTATCTTGCAGTTCCCACCTGCCCACTGTTAGCCAGGTCATCCA
240bp_PCR_Product       GGACTTCTGGAGCCATGTATCTTGCAGTTCCCACCTGCCCACNGTTAGCCAGGTCATCCA
                        ****************************************** ****************

TGFBRII                 CAGACAGAGTAGGGTCCAGACGCAGGGAAAGCCCAAAGTCACACAGGCAGCAGGTTAGGT
240bp_PCR_Product       CAGACAGAGTAGGGTCCAGACGCAGGGAAAGCCCAAAGTCACACAGGCAGCAGGTTAGGT
                        ************************************************************

TGFBRII                 CGTTCTTCACGAGGATATTGGAGC
240bp_PCR_Product       CGTTCTTCACGAGGATATTGGAGC
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Figure 6. The ClustalW alignment of the partial sequence of the 240 bp RT-PCR product amplified using TGFβRII primers with a segment of *Homo sapiens* TGFβRII mRNA sequence from NCBI (NM_001024847.1).

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TGFBRIII              CCTGCCTGCCCTGCAGAACCCGCCCATCCGGGGAGGGGAAGGCCAAAATGGAGGCCTTCC
423bp_PCR_Product     CCTGCCTGCNCTGCAGAACCCGCCCATCCGGGGAGGGGAAGGCCAAAATGGAGGCCTTCC
                      ********* **************************************************

TGFBRIII              GTTTCCTTTCCCAGATATTTCCAGGAGAGTCTGGAATGAAGAGGGAGAAGATGGGCTCCC
423bp_PCR_Product     GTTTCCTTTCCCAGATATTTCCAGGAGAGTCTGGAATGAAGAGGGAGAAGATGGGCTCCC
                      ************************************************************

TGFBRIII              TCGGCCAAAGGACCCTGTCATTCCCAGCATACAACTGTTTCCTGGTCTCAGAGAGCCAGA
423bp_PCR_Product     TCGGCCAAAGGACCCTGTCATTCCCAGCATACAACTGTTTCCTGGTCTCAGAGAGCCAGA
                      ************************************************************

TGFBRIII              AGAGGTGCAAGGGAGCGTGGATATTGCCCTGTCTGTCAAATGTGACAATGAGAAGATGAT
423bp_PCR_Product     AGAGGTGCAAGGGAGCGTGGATATTGCCCTGTCTGTCAAATGTGACAATGAGAAGATGAT
                      ************************************************************

TGFBRIII              CGTGGCTGTAGAAAAAGATTCTTTTCAGGCCAGTGGCTACTCGGGGATGGACGTCACCCT
423bp_PCR_Product     CGTGGCTGTAGAAAAAGATTCTTTTCAGGCCAGTGGCTACTCGGGGATGGACGTCACCCT
                      ************************************************************

TGFBRIII              GTTGGATCCTACCTGCAAGGCCAAGATGAATGGCACACACTTTGTTTTGGAGTCTCCTCT
423bp_PCR_Product     GTTGGATCCTACCTGCAAGGCCAAGATGAATGGCACACACTTTGTTTTGGAGTCTCCTCT
                      ************************************************************

TGFBRIII              GAATGGCTGCGGTACTCGGCCCCGGTGGTCAGCCCTTGATGGTGTGGTCTA
423bp_PCR_Product     GAATGGCTGCGGTACTCGGCCCCGGTGGTCAGCCCTTGATGGTGTGGTCTA
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Figure 7. The ClustalW alignment of the partial sequence of the 423 bp RT-PCR product amplified using TGFβRIII primers with a segment of *Homo sapiens* TGFβRIII mRNA sequence from NCBI (NM_003243.2).

DISCUSSION

The aim of this project was to investigate the mRNA expression levels of the three TGFβ receptor genes in an invasive (SK-N-ER) and a non invasive (SH-EP1) neuroblastoma cell line. The RT-PCR analysis suggested a higher expression for TGFβRII and TGFβRIII mRNA in SH-EP1, as compared to SK-N-ER neuroblastoma cell lines. There appeared to be no significant change in TGFβRI mRNA expression in the two cell lines.

The TGFβ pathway is regulated at three levels, the expression of TGFβ, expression TGFβ receptors and the expression of SMAD transcription factors. Although TGFβ is one of the most potent inhibitors of cell growth, different cancers are resistant to TGFβ, suggesting that the

developing of unresponsiveness to it, plays an important role in tumorigenesis. Tumors acquire resistance to TGFβ relatively late during malignant progression and this appears to be associated with developing invasiveness (14). This resistance is achieved by reduction in the expression of the TGFβ receptors on the cell surface. TGFβRII and RIII directly bind to TGFβ, whereas RI is phosphorylated by the RII kinase domain and isn't known to directly interact with TGFβ (2).

Consistent with results from RT-PCR (Fig. 2,3 and 4) of this study, several other investigators reported decreased expression of TGFβRII and RIII mRNA in highly invasive cancer cells (5, 11, 14). However, TGFβRI mRNA expression wasn't significantly different in the two neuroblastoma cell lines.

The ClustalW alignment, of the partial sequence of each of the three TGFβR products from RT-PCR amplified using primers specific to each receptor, confirmed that these primers amplified the desired TGFβ receptor transcript, respectively (Fig. 5, 6 and 7).

Based on the obtained data, it cannot be concluded that, the decreased expression of TGFβRII and RIII mRNA in the SK-N-ER neuroblastoma cells has a direct correlation with its invasiveness. There are other factors that may also affect the invasiveness of these cells. Due to the increasing relevance of aberrations in the TGF-β-dependent pathway in human tumorigenesis, further studies are warranted to study the level and activity of other components, of this pathway particularly SMAD transcription factors. Research in this direction could prove useful to develop molecular therapeutics targeting the TGFβ pathway.

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