Differential mRNA Expression and Detection of Alternatively Spliced Forms of VEGFA in different Neuroblastoma Cell Lines

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ABSTRACT

Neuroblastoma is a malignant, solid tumor that develops from neural crest cells and usually occurs in children younger than 5 years. The neuroblastoma cell lines exhibit 3 phenotypes – N (Neuroblastic), S (Substrate adherent) and I (Intermediate). I type cells are the most malignant. The mRNA expression of VEGFA was studied in these cell lines. VEGFA is a signaling protein, a member of Platelet Derived Growth Factor (PDGF), associated with angiogenesis and vasculogenesis. Angiogenesis plays an important role in tumor development. Differential expression of VEGFA mRNA in a few neuroblastoma cell lines was found. Two transcript variants were expressed in all four cell lines. Transcript variant four and six were detected. No

significant differences in the expression of these two transcript variants were found in the N and S cell lines. However, the levels of transcript variant six appeared to be higher in both I type cell lines studied.

INTRODUCTION

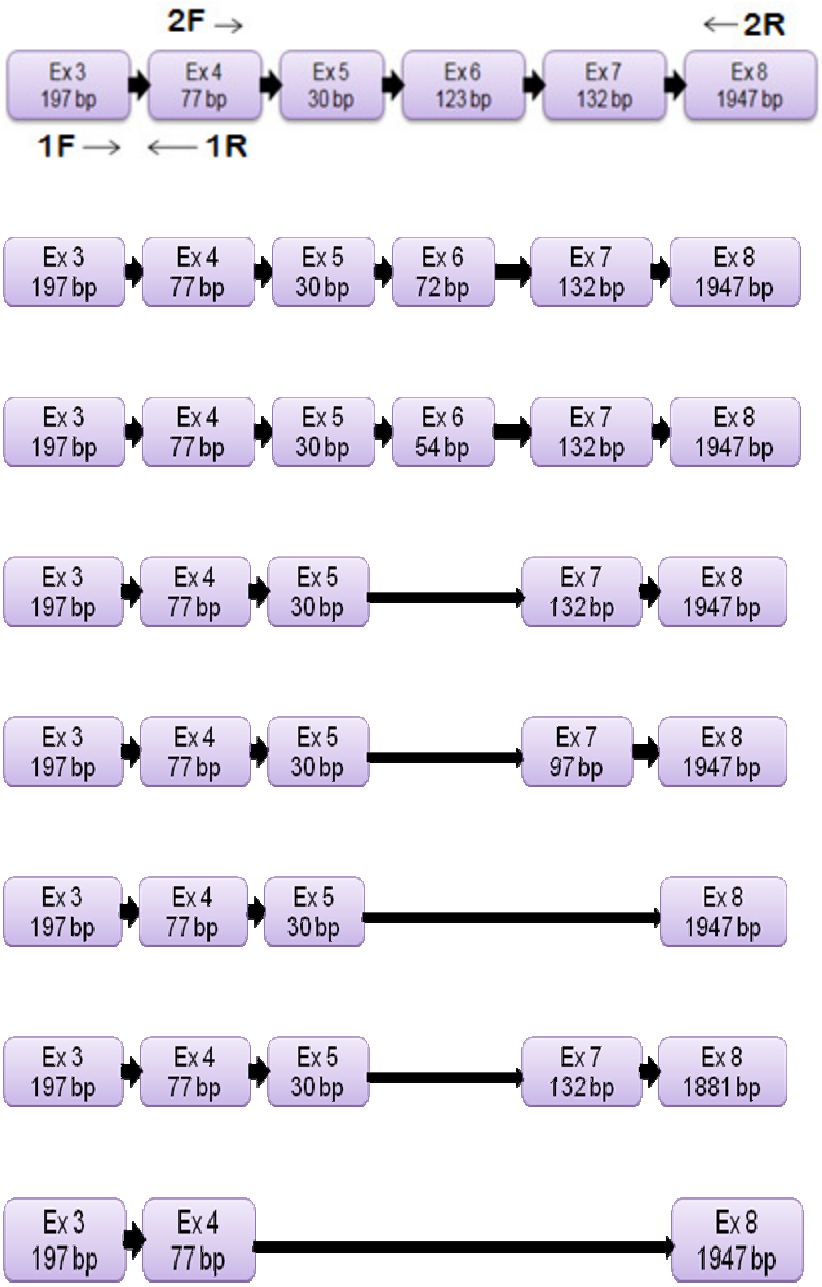
Neuroblastoma is a malignant, solid tumor that develops from embryonic neural crest cells found in several areas of the body (most commonly originating in and around adrenal glands). It usually occurs in children younger than five years and sometimes forms before a child is born. It is slightly more common in males. [5] There are three phenotypes of neuroblastoma cell lines. They are N (neuroblastic), S (substarte adhesive) and I (intermediate).[4] I type cells are precursor cells because N type cells and S type cells are thought to be derived from I type cells. I type cells share properties of both N and S cell lines. I type cells are the most malignant cells followed by N type cells. S type cells are considered non malignant.

Vascular Endothelial Growth Factor (VEGF) is a sub family of growth factors, to be specific, Platelet Derived Growth Factors. The members of this

subfamily are VEGFA, VEGFB, VEGFC, VEGFD and Placenta Growth Factor (PlGF). The most important member is VEGFA. VEGFA is a signaling protein, associated with angiogenesis and vasculogenesis. It is also referred to as Vascular Permeability Factor (VPF) for its vascular permeability enhancing activities.

VEGFB makes vascular endothelial cells pump free fatty acids from the blood stream into nearby muscles and adipose tissue. Thus VEGFB has a critical biological function in stimulating fatty acid transport through endothelial cells of the vasculature.[9] Like VEGFA, VEGFC and VEGFD stimulates cell functions through selective binding and phosphorylation of tyrosine kinase receptors followed by activation of downstream signaling molecules. However, they all bind to different receptors. VEGFC binds to flt4 receptors while VEGFD binds to flk1 and flt4 receptors. VEGFA binds to flt1 and flk1 receptors. VEGFC also plays a role in lymph angiogenesis.

VEGFA is a disulfide linked homodimer but it is also found as a heterodimer with Placenta Growth Factor (PGF), homolog of VEGFA. VEGFA gene is located on the short arm of chromosome 12. It has 8 exons and 8 transcript variants.



TV	Expected Product Length with VEGFA primers 2F/2R (bp)
1	414
2	363
3	345
4	291
5	256
6	159
7	225
8	129

MATERIALS AND METHODS

CELL LINES

The cell lines, LAI-5S (S phenotype), LAI-55N (N phenotype), JMN (I phenotype) and Be2C (I phenotype) were generously provided by Dr. Robert Ross. They were cultured in DMEM/F 12 medium supplemented with 10% Fetal Bovine Serum (FBS) at 37° C.

RNA EXTRACTION

Total RNA was extracted using RNeasy® Plus Mini Kit (QIAGEN) from the cell lysates using manufacturer's protocol with minor modifications.

PRIMERS

Gene	Primer	Primer Sequence 5' → 3'
VEGFA	**1F**	GATGTCTATCAGCGCAGCTACTGC
(NM_003376)	**1R**	GGAAGCTCATCTCTCCTATGTGC
VEGFA	**2F**	GAGGAGAGATGAGCTTCCTACAGC
(NM_003376)	**2R**	CAGTCTTTCCTGGTGAGAGATCTG
GAPDH	**Fwd**	GAAGGTGAAGGTCGGAGT
(NM_002046)	**Rev**	GAAGATGGTGATGGGATTTC

RT-PCR

RT-PCR was performed using QIAGEN® one-step RT-PCR kit following manufacturer's protocol. 20 ng of RNA from each cell line was amplified in final reaction mixture of 25 µl volume. Each reaction mixture consisted 5µl 5×RT buffer, 1µl 10mM dNTPs, 1µl enzyme mix, 1µl 10pmol/µl forward primer, 1µl 10pmol/µl reverse primer, 4µl 5ng/µl RNA and 15µl ddH_2O. The reaction conditions for RT-PCR were as follows:

One cycle was run, first at 50°C for 30 minutes and then at 95° for next 15 minutes. Then 34 cycles at 94°C for 30 seconds, then 57°C for next 30 seconds, then 72°C for following 30 seconds. The final extension was carried out at 72°C for 10 minutes followed by a final hold at 4°C. The RT-PCR for VEGFA was optimized to 34 cycles. GAPDH was used as a loading control.

GEL ELECTROPHORESIS

RT-PCR products were analyzed on 1% agarose gel. 2.5µl of loading dye was added to each RT-PCR product. 5µl from each product was then loaded on agarose gel containing ethidium bromide and electrophoresis was then carried out at 150 V for 45 minutes. The products were finally visualized under UV trans-illuminator.

PCR PURIFICATION, GEL EXTRACTION & SEQUENCING

From each primer pair, the characteristic PCR products were PCR purified using QIAquick® PCR Purification Kit (QIAGEN) or QIAquick Gel Extraction Kit (QIAGEN). These purified products were then sent out for sequencing to Genwiz Inc.

RESULTS

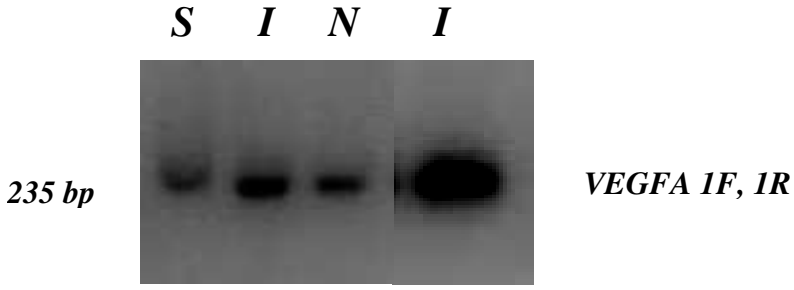


Fig 1 Primers 1F and 1R amplifies invariant section of VEGFA

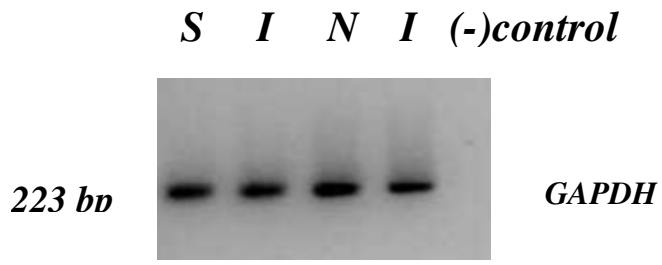


Fig 2 GAPDH was used as a loading control

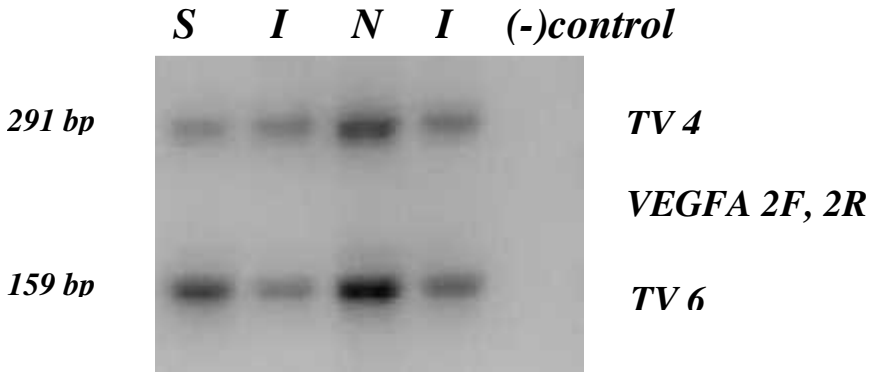


Fig 3 Detection of two alternatively spliced variants of VEGFA in all four neuroblastoma cell lines. The level of TV 4 and 6 are equivalent in both S and N cell lines studied. The level of TV 6 is relatively higher in both I type

Primer set 1F/1R amplified an invariant part of VEGFA (Fig 1). Two variants of VEGFA were detected in all four neuroblastoma cell lines with primer set 2F/2R (Fig 3). Sequencing identified the larger band as TV 4 and the smaller band as TV 6. Differential mRNA expression of VEGFA was studied in four different neuroblastoma cell lines using RT-PCR. Two TV were expressed in all neuroblastoma cell lines. No significant difference was found in

expression levels of these two transcripts in N and S cell lines. However, there was higher relative expression of the TV 6 in both I type cell lines (JMN and Be2C).

DISCUSSION

Normally, angiogenesis in adult human body occurs less frequently, usually during wound healing or during menstruation in women. But abnormally, angiogenesis plays an important role in tumor development. In tumors, insufficient oxygen activates the expression of a transcription factor, Hypoxia Inducible Factor (HIF) which then stimulates the release of VEGFA. VEGFA then binds to VEGF1 and VEGF2 receptors (VEGFR or Tyrosine kinase receptors) on endothelial cells. The receptors are then dimerized and activated triggering a tyrosine kinase pathway. This pathway contributes to endothelial cell proliferation and migration, and formation of tubular structures. These new vessels provide the tumor with additional nutrients and oxygen. It also facilitates the metastasis of tumors.

The differential mRNA expression levels of VEGFA was studied in 4 different neuroblastoma cell lines using RT-PCR. Two transcript variants

were expressed in all the neuroblastoma cell lines. There was no significant difference found in the expression levels of these two transcripts in N and S cell lines. However, there was relatively higher expression of TV 6 in both I type cell lines (JMN and Be2C).

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