# Nutraceutical Treatment Produces Alternative VEGFA Isoforms in Cancer Cells

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## Abstract

Vascular endothelial growth factor (VEGFA) is a secreted protein that is involved in normal and pathological angiogenesis. It is known to play a role both in the growth and metastasis of tumors and the development of macular degeneration. Multiple isoforms of VEGFA have been described, and it is known that the majority of alternative splicing occurs in exon regions four through eight. Because nutraceutical compounds have been shown to alter splicing patterns in other transcripts that give rise to alternate protein isoforms, we tested their effects on VEGFA production in the colorectal cancer cell line Caco-2. RT-PCR was performed on mRNA from treated and untreated cells. Cytosolic protein extracts from treated and untreated cells were subject to immunoblotting using a polyclonal anti-VEGFA antibody. It was discovered that nutraceutical compound #54 shifted the ratio of transcripts to favor production of the Δ6,7 transcript over the Δ6 form. This result was supported by immunoblots which show reduction of the 40 kDa isoform, which corresponds to the predicted molecular weight of the translated Δ6 transcript. Therefore, this preliminary study suggests that nutraceuticals can alter splicing in VEGFA, and that compound #54 has the potential to contribute to metastases of tumors, since it increases the prevalence of the most diffusible isoform.

**Key Words**: VEGFA, tumorigenesis, alternative splicing

## Introduction

Vascular endothelial growth factor (VEGFA) is a secreted growth factor which is involved in embryonic angiogenesis, tissue repair following exercise or injury, and pathological angiogenesis[1]. Importantly, VEGFA has been implicated in a wide variety of metastases of solid tumors as well as the development of macular degeneration. A growing tumor can only grow to about two millimeters in surface area before hypoxia restricts further growth[2]. Metastatic tumors overcome this limitation by secreting VEGFA in an autocrine loop, which results in neovascularization and subsequent delivery of oxygen to the tumor interior by newly formed blood vessels[3]. Similarly, VEGFA plays a role in age-related macular degeneration. As aging progresses, the retinal tissue is exposed to oxidative damage, which leads to a shift in the equilibrium of PEDF (suppressor of angiogenesis) to VEGFA, favoring VEGFA and leading to the choroidal angiogenesis characteristic of macular degeneration[4].

The VEGFA gene includes eight exons which code for the functional domains of the VEGFA protein (Fig. 1). Numerous VEGFA protein isoforms have been described, and these isoforms are produced by alternative splicing, post-translational modifications, and alternative translational start codons[5,6]. There is evidence which suggests that the way in which the VEGFA transcript is spliced modulates the angiogenic potential of the resulting protein[7]. Studies have also determined that isoforms of different lengths play different roles in the development of pathological angiogenesis and metastasis of tumors. One such study found that longer isoforms are primarily cell-associated, and serve as a VEGFA reservoir that can be liberated to diffuse upon dissolution of the extracellular matrix, while smaller isoforms are diffusible[8]. A study of

colon tissue from patients with metastatic colorectal cancer documented that 75% of patients whose colon tumors had metastasized to the liver showed expression of one of the smaller diffusible forms ($VEGF_{121}$), as well both of the extracellular matrix (ECM)-associated forms ($VEGF_{169}$, $VEGF_{189}$). All of the patient tissues surveyed showed expression of $VEGF_{121}$[9]. Note that the subscript number of VEGFA isoforms, by convention, represents the number of amino acids in that isoform.

Extensive research on proteins whose splicing pattern modulates their function has been conducted. The potential to shift splicing events has therapeutic implications for cancers whose metastatic agents rely on alternative splicing. Many pharmaceutical drugs specifically target alternative splicing mechanisms which contribute to cancer[10,11]. A recent study has shown that

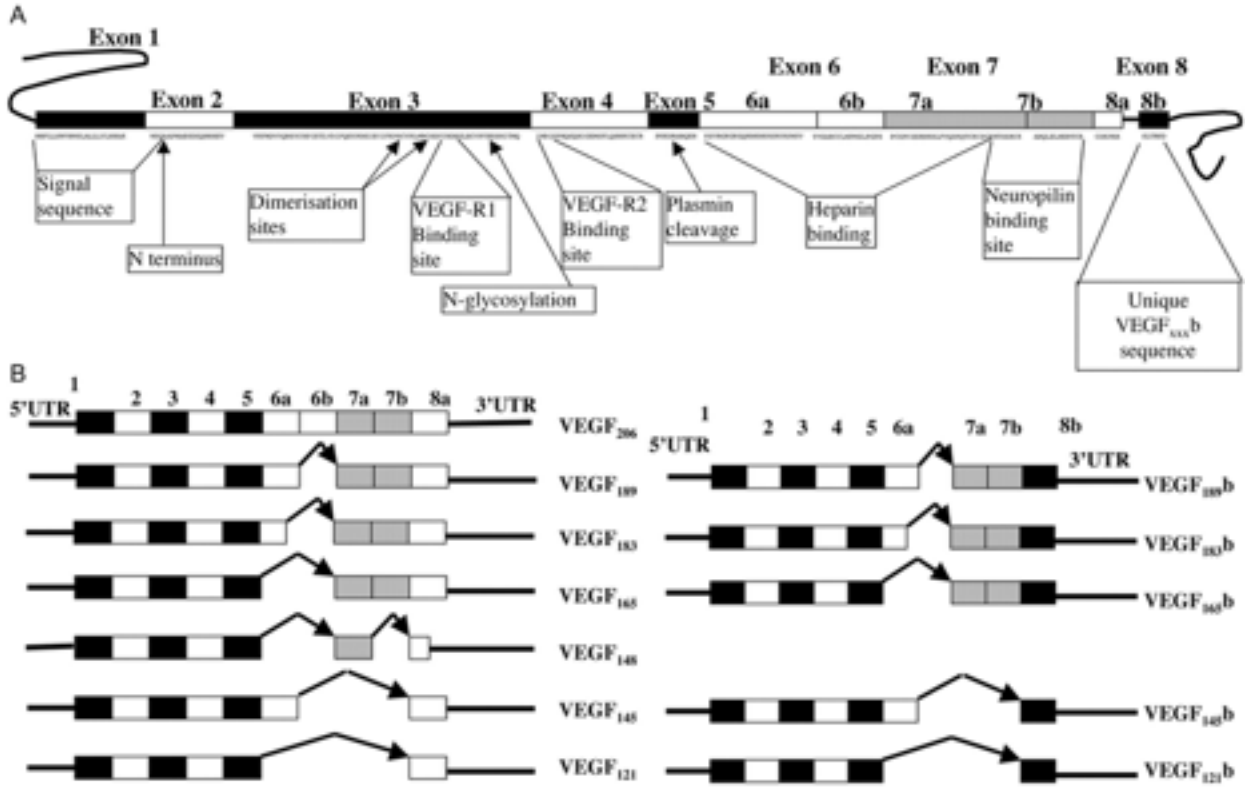


**Figure 1.** Structure of VEGFA gene and some examples of alternative splice forms. Image adapted from Nowak, D. et al. (2008).

nutraceutical agents, or pharmacologically relevant chemicals ingested as part of the diet, can change mRNA splicing patterns to favor one splice form over another[12,]. Because VEGFA's angiogenic potential and diffusibility are greatly impacted by the manner in which it is spliced, the research question for this study was: *can nutraceutical agents change the splicing of the VEGFA transcript in cancer cells?* It was hypothesized that nutraceutical agents which have been shown to be effective in altering splice patters for other transcripts would also impact the splicing of VEGFA.

## Methods and Materials

Immortalized colorectal cancer cells (Caco-2), kindly provided by S. Anderson, were treated with a variety of nutraceutical agents. Controls in all experiments were untreated Caco-2 cells grown at the same time and under the same conditions as treated groups. To determine if nutraceuticals impact alternative splicing, mRNA was purified from treated and untreated cells using the RNeasy® Plus Mini Kit (QIAGEN, Germany). Intron-spanning primer pairs were developed to span exons four through the 3' UTR just after exon eight (Fig. 2). RT-PCR was performed using the One-Step RT-PCR kit per the manufacturer's instructions (QIAGEN, Germany). PCR products were run on a 1% agarose gel and visualized with ethidium bromide on a UV illuminator (Carestream, USA). Band intensities for each treatment were quantified and compared using ImageJ (NIH, Bethesda, MD, USA). PCR products were purified using the QIAQuick Gel Extraction Kit (QIAGEN, Germany) and sent for sequencing (GENEWIZ, NJ, USA). Recovered sequences were BLAST against NCBI's database, and alternative transcripts were identified.

Replicate nutraceutical treatments were applied to new Caco-2 cells. Cytosolic proteins were extracted from treated and untreated cells and were run on a 10% Bis-Tris SDS-PAGE gel (Life Technologies, MA, USA). Proteins were transferred to nitrocellulose membrane and probed using a polyclonal rabbit anti-VEGFA antibody (Abcam, Cambridge, UK). Protein isoforms were recorded and corroborated with the RT-PCR results.

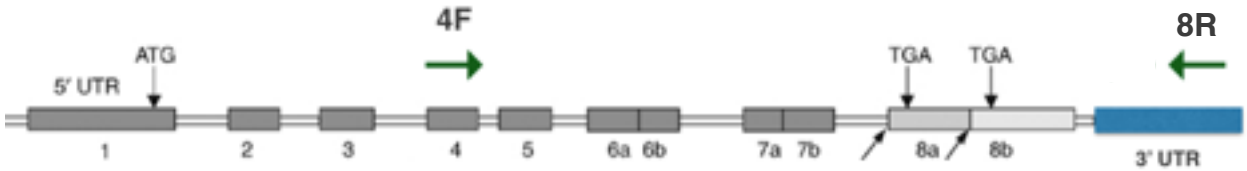


**Figure 2.** VEGFA transcript showing regions in which primer pairs were developed for RT-PCR analysis.

## Results

RT-PCR experiments showed that one nutraceutical agent, compound #54, altered the relative abundance of the two alternative transcripts compared to untreated control cells. For untreated cells, there was relatively more of the upper band (~580 bp) than the lower band (~430 bp). Only treatment #54 altered the relative abundance so that the Δ6,7, 430 bp band was relatively less intense than the Δ6, 580 bp band. The bands were quantified in ImageJ to corroborate what was seen with the unaided eye. The quantification showed that most nutraceutical treatments did not alter the ratio of Δ6 to Δ6,7 band compared to untreated cells. Most of the treatments had a consistent ratio in which the ratio of Δ6 to Δ6,7 was slightly greater than 1:1. Compound #54, however, flipped the ratio to favor the Δ6 band over the Δ6,7 band,

producing a Δ6:Δ6,7 ratio of 0.67 (Fig. 3a). When these bands were cut out of the gel, purified, and sent for sequencing, the BLAST results of the sequences showed that the 580 bp band corresponds to a known transcript which lacks exon six ($VEGF_{169}$; Δ6), while the 430 bp band corresponds to a transcript which lacks both exons six and seven ($VEGF_{121}$; Δ6,7) (Fig 3b).

An immunoblot was performed to see what effect nutraceutical #54 might have at the protein level. A blot probed with a rabbit polyclonal anti-VEGFA antibody showed that this treatment reduces the amount of the 40 kDa isoform compared to untreated cells (Fig. 4). The predicted molecular weight of the Δ6 transcript is 40.7 kDa; this observation is consistent with the RT-PCR results which show a reduction in the amount of the Δ6 transcript compared to the Δ6,7 transcript.

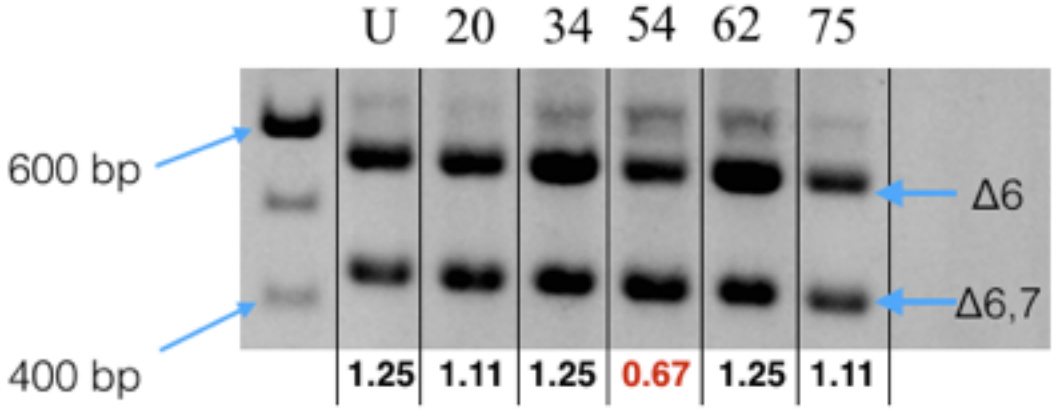


**Figure 3a.** RT-PCR products run on 1% agarose gel. Only nutraceutical compound #54 alters the relative ratio of Δ6: Δ6,7 bands to favor the Δ6,7 transcript.

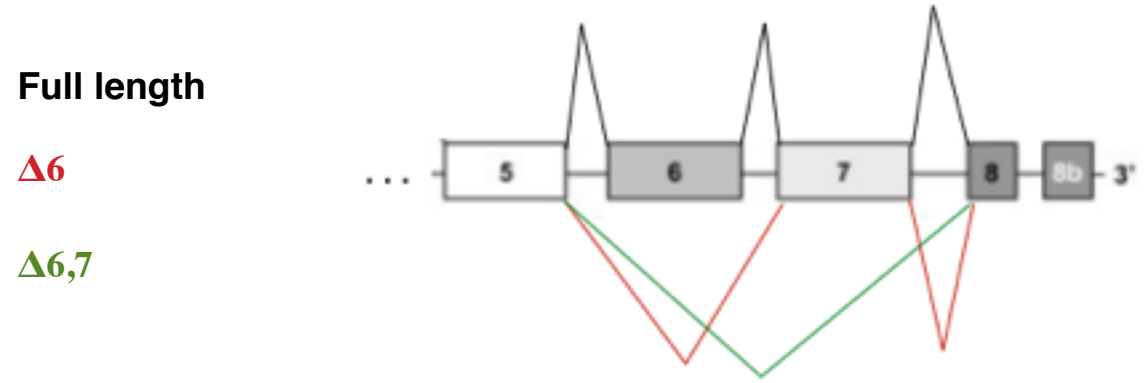


**Figure 3b**. Diagram of the alternatively spliced isoforms detected in Figure 3a. One splice form lacks exon 6, and one lacks both 6 and 7 as determined by alignment with the full length transcript and NCBI BLAST results.

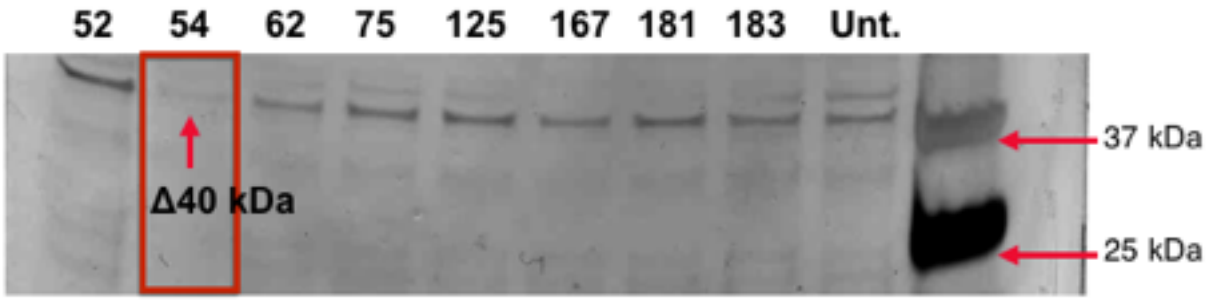


Figure 4. Immunoblot performed on protein extracted from treated and untreated Caco-2 cells. Probed with rabbit polyclonal anti-VEGFA antibody.

**Figure 4.** Immunoblot performed on protein extracted from treated and untreated Caco-2 cells. Probed with rabbit polyclonal anti-VEGFA Ab.

## Discussion

This study presents evidence that nutraceutical compounds can influence the alternative splicing of VEGFA to favor the production of certain isoforms over others. Treatment with compound #54 results in the production of relatively more of the Δ6,7 transcript compared to the Δ6 transcript. Exon six codes for a heparin-binding domain of the protein, while exon seven codes for a domain which is involved in both heparin binding and extracellular matrix (ECM) binding[13]. The protein isoform which includes exon seven, but not exon six, is $VEGF_{165,}$ the most abundant VEGF isoform. This isoform retains some heparin-binding due to inclusion of exon seven; it also interacts with the neuropilin-1 (Nrp-1) receptor, acting independently of the normal VEGF receptor (VEGFR)[14]. By contrast, the VEGFA isoform which lacks both exons six and seven, $VEGF_{121}$, lacks the heparin and ECM binding domains, but still requires heparin-like molecules to interact with the VEGFR[15]. $VEGF_{121}$ is therefore the most diffusible isoform, which can travel through the body and cause metastasis if it is secreted by tumor cells. Studies conducted *in vivo* have confirmed that transfected cells which express $VEGF_{121}$ undergo more angiogenesis than cells which express the same levels of $VEGF_{165}$, $VEGF_{189}$, or $VEGF_{206}$[16]. Currently, metastatic colorectal cancers are treated using bevacizumab, a monoclonal antibody which sequesters diffusible VEGFA[17]. The discovery of a nutraceutical which can lower endogenous diffusible VEGFA isoforms would augment such treatments. Further screens will need to be conducted to determine if such a compound exists.

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