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Methods paper

Comparative insight into expression of recombinant human VEGF111b, a newly identified anti-angiogenic isoform, in eukaryotic cell lines

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ABSTRACT

VEGF-A is a critical growth factor in tumor growth and progression. Two families of VEGF-A isoforms are produced through alternative splicing including VEGFxxx pro-angiogenic and VEGFxxxb anti-angiogenic isoforms. VEGF111b is a new member of the VEGFxxxb family that is induced by mitomycin C and doesn't express in normal conditions. The potent anti-angiogenic properties of VEGF-111b and its remarkable resistance to proteolysis make it an interesting alternative candidate for therapeutic use in all types of cancers. Here, the recombinant VEGF-111b cDNA with insertion of intronic sequence was constructed by using a class IIs restriction enzyme-based method. The recombinant pBud-VEGF111b was transfected into CHO *dhfr*$^-$ and HEK 293 cell lines which are currently the standard hosts for the production of candidate therapeutic proteins. Then, the VEGF-111b expression was evaluated in two cell lines using the Real-time PCR. The production of VEGF-111b protein was also investigated here by dot blotting. The VEGF expression was increased about 109 and 185-folds in transfected CHO-*dhfr*$^-$ and HEK 293 cells, respectively, in comparison with the un-transfected cells. Dot blotting approach confirmed that both cell lines have successfully produced the VEGF-111b protein.

1. Introduction

Angiogenesis plays critical roles in many different diseases, including cancer, retinopathies, and psoriasis (Ferrara, 2002, 2004; Ferrara et al., 2003; Nowak et al., 2008). Anti-angiogenic therapy is particularly promising for the blocking of tumor angiogenesis and tumor metastasis in cancer treatment (De Falco, 2014; Giacca and Zacchigna, 2012). It has been shown that angiogenesis is an essential regulatory process in neoplastic growth and metastasis which was mediated by different pro-angiogeneic and anti-angiogenic factors (Ferrara, 2010; Qiu et al., 2009). Vascular endothelial growth factors (VEGFs) belong to the VEGF/PDGF (platelet-derived growth factor) group of secreted polypeptides (Ladomery et al., 2007). Among all VEGFs, VEGF-A is the most potent angiogenic protein in both normal and pathological angiogenesis. The *VEGF*-A gene spans approximately 14 kbp on chromosome 6 and consists of eight exons. There are two principal VEGF-A family isoforms including the pro-angiogenic VEGF-xxx (VEGF-xxxa) and anti-angiogenic VEGF-xxxb isoforms, where xxx demonstrates the amino acid number of mature proteins (Harper and Bates, 2008; Woolard et al., 2009). These two families contain alternate six amino acids at the C terminus due to the differential selection of 3′ distal splicing site (3′ DSS) or 3′ proximal splicing site (3′ PSS) (Biselli-Chicote et al., 2012). In recent years, functional and structural features of pro-angiogenic and anti-angiogenic VEGF-A isoforms have become a center of interest because of their potent roles in health and diseases (Hilmi et al., 2012). VEGF-xxxb isoforms were first described in 2002 by Bates et al. (2002). VEGF-165b (E1-5, 7, 8b) is the most widely described VEGFxxxb isoform, while VEGF-189b (E1-E6a & E7, E8b) and VEGF-121b (E1-5, 8b) have been identified in human tissues (Harris et al., 2012; Rennel et al., 2009). It is proposed that exon 8b-encoded sequence is a determining factor in the anti-angiogenic activity of VEGFxxxb variants (Munaut et al., 2010). VEGF-111b (E1-4, 8b) is a newly identified member of the VEGF-xxxb family and its mRNA and protein expressions are induced by mitomycin C in the human ovarian cancer cells. VEGF-111b inhibits proliferation and metastasis of tumor cells by targeting VEGF-R2 downstream signaling, similar to VEGF-165b but slightly less efficient than VEGF165b. The critical anti-angiogenic properties of VEGF-111b and its remarkable resistance to proteolysis make it an interesting alternative candidate for therapeutic use in all types of cancers and retinopathies. VEGF-111b mRNA doesn't express under normal condition of healthy human cells (Delcombel et al., 2013; Gu et al., 2013). Therefore, in this study, we constructed a eukaryotic expression vector containing VEGF-111b recombinant cDNA in order to express it in human cells. Furthermore, we have used an efficient method based on class IIs restriction enzymes to improve the level of VEGF-111b expression in mammalian cell lines by inserting an intronic sequence between exons 4 and 8b.

Abbreviations: VEGF-A, vascular endothelial growth factor; VEGFs, vascular endothelial growth factors; PDGF, platelet-derived growth factor; PlGF, placenta growth factor; *dhfr*$^-$, dihydrofolate reductase-negative.

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2. Materials and methods

2.1. Reagents and cell lines

Monoclonal anti-polyhistidine peroxidase conjugate was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). ECL Prime detection reagent was from Amersham Company. Two mammalian cell lines HEK 293 and CHO−*dhfr*$^{-}$ (dihydrofolate reductase-negative) were purchased from the Pasteur Institute, Iran. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. RNA isolation and cDNA synthesis

A 40 mg fresh malignant breast tumor tissue was used for RNA isolation. The fresh tumor tissue sample was collected from the Breast Cancer Research Center of Isfahan. This study was approved by the institutional ethics committee of the University of Isfahan. The patient was informed clearly and provided standardized written consent (Tabatabaeian and Hojati, 2013). Disruption of the tumor tissue was done using a mortar and pestle along with liquid nitrogen. The whole RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and stored in RNase-free water at 80 °C. In addition, the transfected CHO-*dhfr*$^{-}$ and HEK 293 cells were dispersed by trypsin–EDTA and centrifuged at 200 *g* for 10 min. Total RNA was extracted from the transfected HEK 293 and CHO-*dhfr*$^{-}$ cells by RNeasy® mini Kits following the manufacturer-recommended protocol. Finally, the RNA quantity and quality were confirmed by spectrophotometer and electrophoresis, respectively. After reverse transcription with hexameric random primers using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Germany), aliquots of their products were used for performing Real-time PCR.

2.3. Plasmid construction

Exons 1–4 and 8b were precisely selected for coding sequence of our recombinant VEGF-111b. As *VEGF-A* gene has a high level of expression in breast tumor; the malignant breast tumor cDNA was used for amplification of exons 1–4. For amplification of these four exons from the cDNA, two specific primers were designed using the primer design software Oligo®7. The second PCR reaction was also designed and used for amplification of an intronic sequence of *VEGF-A* gene from the human chromosomal DNA isolated from the normal blood cells (Fig. 1). Blood collection was performed from a healthy free volunteer who had referred to the Masih Daneshvari Hospital (Isfahan, Iran) for regular health checks. Blood sampling was performed based on the patient satisfaction and a signed agreement between the University of Isfahan and the Masih Daneshvari Hospital. An intronic region sequence was selected to be added to the cDNA in order to enhance its expression. The intron 4/5 of *VEGF* gene was chosen for its short length and conserved 5′ and 3′ splice site sequence. This is important to join PCR fragments including exons 1–4 and intron 4/5, and exon 8b without introducing any undesired nucleotide in order to have an appropriate splicing following the transcription. In this regard, the unique feature of *Eco*31I enzyme was used for joining these two fragments. *Eco*31I restriction enzyme cuts 4 nucleotides (NNNN) outside of its recognition site and leaves a 4-base 5′ overhang. This is why R1 and F2 primers contain the recognition site of *Eco*31I enzyme. Furthermore, the recognition sites of *Kpn*I and *Bgl*II restriction enzymes were introduced in F1 and R2 primers, respectively, in order to facilitate the ligation of

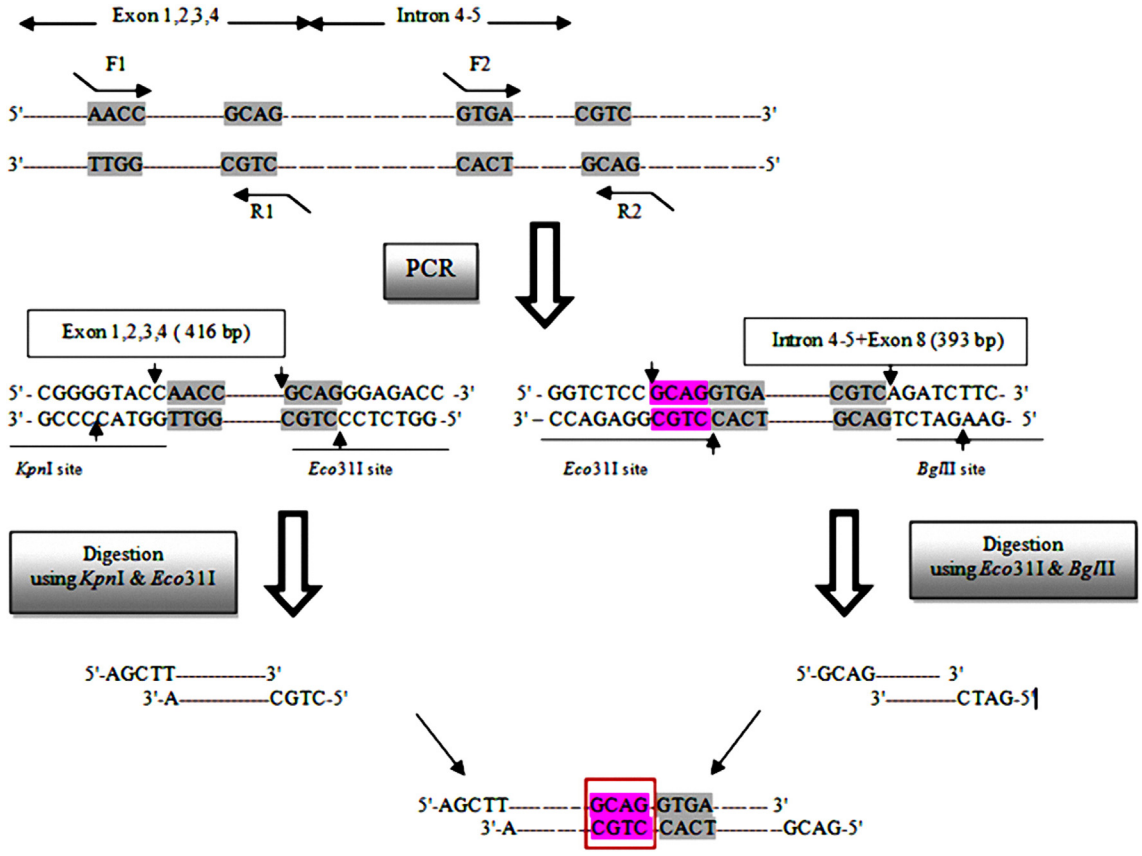

Fig. 1. Schematic diagram of the recombinant VEGF-111 cDNA synthesis. The underlined sequences indicate the restriction sites of enzymes which were introduced by the primers. The starting and ending bases of two fragments are shaded gray.

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