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Haplotype-dependent binding of nuclear proteins to the promoter of the neural tube defects-associated platelet-derived growth factor alpha-receptor gene

Mascha Toepoel, Bart Ackerschott, Everardus J.J. van Zoelen*

Department of Cell Biology FNWI, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

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Abstract

We have previously shown that polymorphisms in the promoter of the human platelet-derived growth factor α-receptor (PDGFRA) gene can be grouped into five distinct haplotypes, designated H1, H2α, H2β, H2γ and H2δ, and that specific combinations of these promoter haplotypes predispose to neural tube defects (NTDs). These promoter haplotypes differ strongly in their ability to drive reporter gene expression in various human cell lines, with highest activity for H2α and H2β. Here, we show that the haplotype-linked *PDGFRA* promoter region extends to 3.6 kb upstream from the transcription start site, and contains a total of ten polymorphic sites. For two of these polymorphic sites, i.e. −909C/A and +68GAins/del, we observed differential binding of nuclear proteins from human osteosarcoma (HOS) cells. The protein complex binding specifically to −909C, which is present in all haplotypes except the low activity haplotype H2γ, contained members of the upstream stimulatory factor (USF) family of transcription factors. Furthermore, we identified a protein complex of 125 kDa which bound specifically to the low activity haplotype H1 at position +68GAdel and may represent an H1-specific *PDGFRA* transcriptional repressor. The current identification of cis-acting elements in the *PDGFRA* promoter and the transcription factors that bind them, provides a new strategy for the identification of genes that are potentially involved in neural tube defects.
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Keywords: PDGFRA; Promoter haplotype; Neural tube defect; EMSA; USF; Osteosarcoma

1. Introduction

With the recent completion of the overall sequence of the human genome, current genomic research now focuses on the estimated 0.1% difference in DNA sequences between individuals. Emphasis is put particularly on the role of single nucleotide polymorphisms (SNPs) and small insertion/deletions (ins/dels) within regulatory DNA sequences, since such polymorphisms may directly affect the expression levels of the nearby genes. Regulatory polymorphisms in multiple genes may not only determine the specific genetic characteristics of an individual, but also his/her predisposition for complex diseases.

In a previous study, we have identified seven SNPs and one dinucleotide ins/del in the 1.6 kb promoter region of the gene encoding the human platelet-derived growth factor α-receptor (PDGFRA). These polymorphisms appeared to be linked and gave rise to five distinct haplotypes, designated as H1, H2α, H2β, H2γ and H2δ [1]. H1 and H2α are the two most common haplotypes (approximately 22% and 65% of the Western European population [1–3]) and differ from each other on six positions in this promoter region, while H2β, H2γ and H2δ differ each on only a single nucleotide position from H2α. Upon transient transfection of these *PDGFRA* promoter haplotypes into the human osteosarcoma cell lines U2OS, HOS and SAOS, we observed an up to six-fold higher promoter activity for H2α and H2β when compared to H1, H2γ and H2δ [1]. This suggests that the corresponding polymorphisms may also influence gene

* Corresponding author. Tel.: +31 24 365 2707; fax: +31 24 365 2999.
E-mail address: vzoelen@science.ru.nl (E.J.J. van Zoelen).

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expression in vivo and, as a consequence, may contribute to the susceptibility to PDGFRA-related diseases.

Deregulated expression of *PDGFRA* has been linked to neural tube defects (NTDs) in mice [4,5] as well as in humans [1,6]. NTDs, including anencephaly and spina bifida, are common congenital malformations that involve defective development of the brain and/or spinal cord as a result of incomplete closure of the neural tube. NTDs occur with an average frequency of one per thousand births in the Western world and are considered to be multifactorial in origin, implying that both environmental and genetic factors contribute to their etiology [7].

In mice, *Pdgfra* deficiency leads to prenatal lethality and various developmental defects including severe spina bifida along the entire spinal column [4,5]. Moreover, some *PDGFRA* upstream regulators have been associated with NTDs, such as Pax1 [8–10], Pax3 [11–13] and PRX1/PRX2 [14,15], both in mice and men. This suggests that aberrant *PDGFRA* expression may predispose to NTDs in humans. Interestingly, we [1] and others [6] have shown that the H1 promoter haplotype of *PDGFRA* is associated with an increased risk of NTDs. In order to understand the role of *PDGFRA* haplotypes in NTDs at the molecular level, it will be important to identify transcription factors that are able to bind the *PDGFRA* promoter in a SNP-dependent manner. Recently, De Bustos et al. [3] have shown that the C to A substitution specific for the H2δ haplotype results in a loss of binding capacity of the zinc finger transcription factor ZNF148.

The *PDGFRA* haplotype-linked promoter SNPs identified thus far are located in the promoter region between +118 and −1589 relative to the transcription start site according to Afink et al. [16], which is the most conserved part of the promoter between human, mouse and rat. Studies on transgenic mice containing *PDGFRA* promoter constructs have shown, however, that the promoter region between −1589 and −3600 may contain additional elements necessary for proper *PDGFRA* transcription [17,18]. Analyses by Herrmann et al. [2] have indicated that this region contains at least a five base pairs ins/del at position −1631, which according to Zhu et al. [6] is haplotype coupled. In the present study, we show that this ins/del and an additional T/C SNP at position −2795 are linked to the H1 and H2 haplotypes, thereby extending the length of the various promoter haplotypes to at least 3600 bp upstream of the transcription initiation site. In order to identify haplotype-specific binding of transcription regulating proteins, we subsequently analyzed all eight haplotype-coupled SNPs and the two ins/dels present in the −3600/+118 *PDGFRA* promoter region for allele-specific binding of nuclear proteins from human osteosarcoma (HOS) cells. Here, we show by electrophoretic mobility shift assays (EMSAs) that the −909C/A SNP and the +68GAins/del show haplotype specific protein binding. Using supershift assays the protein binding to the −909C oligonucleotide could be identified as a member of the upstream stimulatory factor (USF) family of transcription factors, while the +68del oligonucleotide was shown to bind specifically a thus far unidentified protein complex with a molecular weight of 125 kDa. These data offer a possible mechanistic explanation for the different promoter activities of the various *PDGFRA* promoter haplotypes and are discussed in terms of the relation between promoter haplotypes and susceptibility to PDGFRA-related diseases such as NTDs.

2. Methods

2.1. Automated DNA sequencing

For analysis of the −3600 to −1535 region of the *PDGFRA* promoter, two overlapping fragments were amplified by PCR using the following primer pairs: forward primer F1 5′-AGGGAACTTTCACCTCAAGC-3′ with reverse primer R1 5′-CAGGCCACCATATGTACCC-3′ and forward primer F2 5′-CCCACATTCTTAACCCC-TTG-3′ with reverse primer R2 5′-GGAATGCTAACAG-GATACCG-3′. PCR mixtures contained 1× PCR buffer, 1 mM $MgCl_2$, 1 unit of Biotherm DNA polymerase (GeneCraft), 0.4 mM of each dNTP (Fermentas), 100 ng of each primer and 15 ng of genomic DNA in a total volume of 25 μl. Cycling parameters were 5 min at 95 °C, followed by 50 cycles of 30 s at 95 °C, 30 s at 60 °C and 2 min at 72 °C, with a subsequent extension of 10 min at 72 °C. PCR products were purified using the PCR purification kit (QIAgen). Automated sequencing was performed on the ABI310 genetic analyzer using the Big Dye terminator sequencing kit (Applied Biosystems).

2.2. Cell culture

Human osteosarcoma cells (MNNG/HOS TE85 Clone F-5, ATCC CRL1547) were maintained in bicarbonate-buffered Dulbecco's MEM/nutrient mix F12 (1:1) medium supplemented with 10% fetal calf serum (FCS, Life Technologies) in a 7.5% CO_2 atmosphere at 37 °C.

2.3. Preparation of nuclear extracts

HOS cells were grown to confluency on 56.7 cm^2 culture plates, washed once with ice-cold phosphate-buffered saline (PBS) and harvested on ice by gentle scraping in 1 ml PBS. Cells were centrifuged for 15 s at maximum speed in an Eppendorf centrifuge and the pellet containing the cells was resuspended in 400 μl buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). The lysate was subsequently incubated for 15 min on ice after which 25 μl of 10% NP40 were added. After vortexing for 15 s and centrifugation for another 30 s at maximum speed in an

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