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ACVR2A promoter polymorphism rs1424954 in the Activin-A signaling pathway in trophoblasts

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ABSTRACT

Introduction: Pre-eclampsia is a pregnancy-specific disorder and characterized by reduced trophoblast invasion and reduced spiral artery remodeling in the first trimester placenta. A polymorphism located in the promoter region of *ACVR2A* (rs1424954 (A > G)) has previously been shown to be significantly associated with pre-eclampsia.
Methods: The effects of this variant on *ACVR2A* expression and its function in the Activin-A signaling pathway were studied by transfections in SGHPL-5 extravillous trophoblasts followed by qRT-PCR.
Results: Here we show that the *ACVR2A* promoter susceptibility variant causes a downregulation of *ACVR2A* expression. We also provide evidence for transcription of a so-called PROMPT (PROMoter-uPstream-Transcript) in the opposite direction of *ACVR2A*, containing the polymorphism, and downregulated when the susceptibility allele is carried, which either shares the same promoter as *ACVR2A* or is a non-coding RNA that is able to enhance *ACVR2A* transcription. Furthermore, when the effect of the susceptibility variant is mimicked by knockdown of *ACVR2A*, physiologic concentrations of Activin-A cause a reduction in *NODAL* mRNA expression in the SGHPL-5 trophoblasts, indicative of a protective effect as reduction in *NODAL* expression is associated with an increase in trophoblast invasion. However, at pathologic levels of Activin-A, as found in pre-eclampsia, this effect is no longer seen, and we show this is potentially caused by a lack of downregulation of *ACVR2B*.
Discussion: The combined data suggest a double hit phenomenon in which the first hit, the promoter variant, together with the second hit, pathological levels of Activin-A, lead to high levels of *NODAL*, associated with reduced trophoblast invasion and observed in pre-eclamptic placentas.

1. Introduction

Pre-eclampsia (PE) is a potentially life threatening disease of human pregnancy affecting 2–8% of all pregnancies which remits after delivery [1], thus making the placenta central in PE. During PE, migration properties of the placental trophoblast cells are thought to be impaired, leading to insufficient invasion of the trophoblasts into the maternal decidua causing improper remodeling of spiral arteries.

In two large positional candidate gene-based association studies involving the Norwegian and Australian populations, *ACVR2A* was identified on chromosome 2q22 as a candidate for a common susceptibility gene for pre-eclampsia [2–4]. By measuring the squared value of the pairwise correlation among intragenic genotypes (r^2) with the observed patterns of LD referred to as iso-correlated redundant variant (IRV) sets, three very strong ($r^2 > 0.7$) IRV sets were seen in the Norwegian population-based study (HUNT study). One of them, IRV1 defined by seven SNPs (rs1424954, rs1014064, rs2161983, rs2288190, rs10497025, rs3768687 and rs3764955) exhibited an OR > 1, indicating that pre-eclamptic women are more likely than control women to carry the major allele at these loci [2,3].

We decided to functionally test the allele-dependency of one of these, i.e. rs1424954 within IRV1 of the *ACVR2A* gene for several reasons. i. This SNP showed the strongest association in the independent Australian study as well [2]. ii. The association of pre-eclampsia with rs1424954 has been replicated in the Brazilian population [5]. iii. rs1424954 (chr2:148600794) is an upstream

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gene variant within or near the promoter region of *ACVR2A* located at 177 bp upstream of the transcription start site (chr2:148602564). This makes a functional effect likely. iv. The expression patterns of the receptor, *ACVR2A*, and its ligand, Activin-A in normal and abnormal (pre-eclamptic) placentation [6–9], the upregulation of Activin-A in serum of pre-eclamptic mothers [10–13], along with experimental evidence in cell culture model systems (SGHPL5, HTR8/SVneo) [14–18] indicate the existence of a functional pathway in the early placenta with interaction between maternal decidua and fetal trophoblast. This pathway involves at least one receptor (*ACVR2A*) and two ligands (Activin-A and Nodal) as we recently showed that Nodal knockdown in the maternal decidua upregulates *NODAL* mRNA expression in fetal extravillous trophoblast cells, potentially via upregulation of Activin-A in the maternal decidua [14]. Furthermore, invasion potential of trophoblast cells is inhibited by Nodal with placentas of pre-eclamptic patients showing upregulated Nodal expression [19]. Finally, high doses of Activin-A in human trophoblasts cells (HTR8/SVneo) have been shown to upregulate Nodal expression [17], while knockdown of *ACVR2A* in HTR8/SVneo cells showed an invasion promoting effect with ACVR2A protein significantly upregulated in term PE placentas [18].

We therefore tested the functional effect and allele-dependency of rs1424954 in this Activin A- *ACVR2A*-Nodal pathway to see if this could explain the association between the susceptibility allele of rs1424954 and pre-eclampsia.

2. Material and methods

2.1. Construction of plasmids containing the ACVR2A promoter region and exon 1

A 2434 bp DNA fragment (chr2: 148600343-148602776) containing the *ACVR2A* promoter region (HG_KWN:35322) (MPromDB) and *ACVR2A* exon 1 was cloned into pF12A-RM-flexi vector (Promega) according to the manufacturer's instructions, using the following primers (Forward: 5′-TCT TCC ATT CCA GAA CCG TTA-3′ and Reverse: 5′-CTG AAG AAC AGG AGA TAA GAA AGA C-3′). The rs1424954 variant was introduced by using the Quickchange XL site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. Integrity and orientation were verified by sequencing.

2.2. Cell culture and transfection

For cell culture, $2*10^5$ SGHPL-5 cells, kindly provided by Dr Judith Cartwright, St George's University of London, UK, were plated in 12 well plates. Cells were cultured in IMDM medium containing fetal bovine serum (FBS) at 37 °C under 5% CO_2. Plasmids containing the *ACVR2A* promoter region and exon 1 with wildtype allele (A) or susceptibility allele (G) were transfected into cells using FuGENE HD Transfection Reagent according to manufacturer's instructions (Promega). Forty-eight hours after transfection, RNA was isolated. Four independent transfection experiments were performed in triplicate.

siRNA transfection experiments were performed by using lipofectamine RNAiMAX (Invitrogen) in combination with 15 pmol *ACVR2A* siRNAs (Qiagen flexitube gene solution) or AllStars Negative control siRNAs (Qiagen) in media supplemented with 0.1% FBS. After 8 h, recombinant Activin-A (Life technologies) was added at four different final concentrations (0, 5, 50 and 100 ng/ml). After 48 h, RNA was isolated. Three independent transfection experiments were performed in triplicate.

2.3. RNA isolation and quantitative RT-PCR

RNA isolation was performed using the RNeasy kit (Qiagen) including on-column DNase treatment. For plasmid transfections, RNA was reverse transcribed to cDNA (Superscript III First strand synthesis system, Invitrogen) using a primer specific for exon 1 of *ACVR2A*. −RT reactions were performed to monitor and, if necessary, correct for transfection efficiency. Subsequently quantitative real time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with ACVR2A forward (5′-CGA GAA CTT CCT CCG GAT T-3′) and reverse primer (5′-CAA ACG CCA ACT TTG CAG-3′). For quantification of PROMPT, PROMPT specific primers (forward: 5′-CCA AAA CTT TGT AGA GTA CAT TAA CAG-3′, reverse: 5′-TCC AGC ACC CAG AAG TAT CA-3′) and probe (5′-TGA CTA AGG GAT GAC TGC CTT TCT CT-3′) were designed and strand-specific RT-qPCR was performed. For siRNA transfections, quantitative RT-PCR was performed using Taqman RNA-to-Ct Kit (Applied Biosystems) in combination with gene expression assays (Applied Biosystems) for *ACVR2A* (for confirmation of knockdown, see Supplementary Fig. 1), *ACVR2B* and *NODAL*. For all quantitative RT-PCRs normalization was done by measuring *GAPDH*.

2.4. Data analysis

All data were analyzed by calculating the delta Ct values and corrected for transfection efficiency. Relative expression levels are expressed as mean ± SEM unless stated otherwise. Statistical analysis was done by Student's *t*-tests using Prism 5.0c (GraphPad Prism, San Diego, CA, USA). All graphs are based on the combined data of independent transfections.

3. Results

3.1. Effect of the rs1424954 promoter polymorphism on ACVR2A exon 1 expression

To investigate the effect of the SNP located in the promoter region of *ACVR2A*, we cloned the promoter region containing the wildtype (A) and susceptibility (G) allele together with *ACVR2A* exon 1 in a vector containing an inactive promoter. These constructs were transiently transfected in SGHPL-5 cells, representative of first trimester extravillous trophoblast cells, after which RNA was isolated and analyzed by quantitative RT-PCR measuring exon 1 expression of *ACVR2A* (NM_001616). This showed that the promoter containing the G allele gave significantly lower expression levels for exon 1 than the A allele (Fig. 1).

This reduced expression of *ACVR2A* with the susceptibility allele present in the promoter might be caused by reduced binding of a transcription factor to regulatory sequences controlling transcription initiation. Another possibility that could cause reduced expression is the fact that the SNP is located in a potential PROMPT (PROMoter uPstream Transcript) regulating *ACVR2A* gene transcription [20]. Ongoing high-coverage RNA-sequencing experiments have confirmed the presence and size of a PROMPT transcript. For this, paired-end sequencing (2 × 125 bp) (HiSeq2500, version 4 chemistry) was done of cDNA libraries generated from polyA + mRNA of SGHPL-5 cells. Mapping with transcriptome reconstruction was done with the Tuxedo pipeline under RABT assembly conditions. By this, a PROMPT transcript upstream of *ACVR2A* was identified and found to correlate exactly with the BE044444 and BM709416 expressed sequence tags (GRCh37/hg19 assembly) providing the 5'- and 3'-end boundaries, respectively, and forming a transcript of 1059 bases. To validate if indeed a PROMPT was expressed, we first applied strand-specific quantitative RT-PCR to determine its normal endogenous presence as tested in untransfected cells. This confirmed the presence of a transcript transcribed from the opposite strand as *ACVR2A* (Fig. 2A). After transfection of the constructs consisting of the promoter region, *ACVR2A* exon 1, but which also include the PROMPT region, PROMPT expression showed the same directional

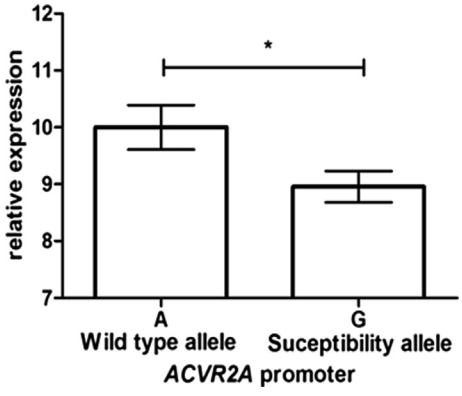

Fig. 1. *ACVR2A* exon 1 mRNA expression. RT-qPCR measuring *ACVR2A* exon 1 transcription in SGHPL-5 cells after transfection with plasmids containing the *ACVR2A* promoter and *ACVR2A* exon 1 with either the wildtype allele (A) or the susceptibility allele (G). Bars are mean ± SEM from four experiments, * indicates $P < 0.05$.