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Placental Expression of ANGPT1, FGF2, PDGFB Genes in Unexplained Recurrent Pregnancy Loss: A Case- Control Study

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ABSTRACT

Background: Recurrent pregnancy loss (RPL) is defined as three or more successive pregnancy loss before the completion of 20th week of gestation. The imbalance between pro angiogenic and anti angiogenic factors can lead to impaired placentation, and result in RPL. Angiopoietins 1 (ANGPT1), Fibroblast Growth Factor (FGF2), and Platelet Derived Growth Factor B (PDGFB) are potent angiogenic factors involved in different developmental processes including embryonic development.

Methods: Gene expression analysis of targeted ANGPT1, FGF2, PDGFB genes and a house keeping gene GAPDH was carried out in the placental tissue collected from 35 women with unexplained recurrent pregnancy loss and compared with the placental tissue obtained from 20 women with medically terminated pregnancy. Placental tissue was collected in RNA later followed by RNA isolation, quantification, cDNA synthesis and real time qPCR using allele specific primers and SYBR green.

Results: The mRNA expression of ANGPT1 gene was significantly downregulated in the placenta of recurrent pregnancy loss cases (1.96-fold, $p<0.0001$) in comparison to the placenta of medically terminated pregnancies., No significant difference was observed in the placental expression of FGF2 and PDGFB genes in recurrent pregnancy loss cases and the control subjects.

Conclusion: In conclusion the results of the present study suggest that altered expression of ANGPT1 gene in placenta may disturb placental angiogenesis and contribute to recurrent pregnancy loss. However, no significant difference was observed in the placental expression of FGF2 and PDGFB genes between RPL and control subjects.

Keywords: Recurrent Pregnancy Loss; Angiopoietin 1 (ANGPT1); Fibroblast Growth Factor (FGF2); Platelet Derived Growth Factor B (PDGFB)

Abbreviations: RPL: Recurrent Pregnancy Loss; PE: Preeclampsia; FGF2: Fibroblast Growth Factor 2; PlGF: Placental Growth Factor; VEGF: Vascular Endothelial Growth Factor; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; IDT: Integrated DNA Technologies; BLAST: Basic Local Alignment Search Tool; ERK: Extracellular Signal-Regulated Kinase

Introduction

Recurrent pregnancy loss (RPL) is defined as three or more successive pregnancy loss before the completion of 20th week of gestation, occurs in 1–2% of child-bearing women [1]. The synchronized regulation of immunological, metabolic, vascular, and endocrine processes is required for maintenance of human pregnancy. Abnormal regulation of these processes may lead to recurrent pregnancy loss (RPL). Angiogenesis, the formation of new blood vessels from the pre-existing blood vessels, is a fundamental process occurring during embryonic development and reproductive cycle [2]. Effective placentation requires the establishment of a competent vascular network formed by vasculogenesis, which involves the de novo formation of vessels from endothelial progenitor cells and branching and the non branching angiogenesis, which is the remodelling of the pre-existing vessels [3]. The imbalance between pro angiogenic and anti angiogenic factors can lead to impaired placentation, leading to major pregnancy complications, such as preeclampsia (PE) and intrauterine growth restriction (IUGR), which may lead to poor obstetric consequences [4,5]. Angiopoietins are mainly produced by the placenta during pregnancy and seem to play an important role in endothelial cell survival and the remodelling of vessels, and acts complementary to the VEGF system, and contribute to the later stages of angiogenesis [6]. Fibroblast growth factor 2 (FGF2) which is also known as basic heparin-binding growth factor that occurs in several isoforms.

FGF2 has pleiotropic roles in many cell types and tissues; it is a mitogenic, angiogenic and survival factor which is involved in cell migration, cell differentiation and in a variety of developmental processes [7]. PDGF-B function is crucial in physiological events such as embryonic development and wound healing. Several types of human cancers have been shown to increase in PDGF-B and/or PDGFR-b expression, affecting tumour growth and angiogenesis [8]. Though, there were emerging evidence on the role of angiopoietins in pregnancy related complications. Majority of the studies have been focused on the serum levels and placental expression of various angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and its receptors in normal and pathological pregnancies [9,10]. Only limited data is available on the role of the Angiopoietin/Tie signalling system in recurrent pregnancy loss, a second vascular endo-thelium specific receptor tyrosine kinase pathway apart from the VEGF system [11]. There is dearth of information on the role of FGF2 and PDGFB genes in recurrent pregnancy loss. In view of the above, the present study was taken up to understand the placental expression of ANGPT1, FGF2, PDGFB genes in unexplained recurrent pregnancy loss.

Materials and Methods

The present case-control study consists of gestational age matched 35 women with unexplained recurrent pregnancy loss and 20 women with medically terminated pregnancy, who were enrolled from Government Modern Maternity Hospital, Petlaburj, Hyderabad. The clinical history of the study group was collected using a standard questionnaire and prior informed consent was obtained from all the study subjects. The study was approved by the Institutional Ethics Committee. Placental tissue was obtained from both the case and control group to study the mRNA expression of ANGPT1, FGF2 and PDGFB genes using Real-Time qPCR.

Inclusion and Exclusion Criteria

Placental tissue from women with unexplained recurrent pregnancy loss were considered as cases. While, the pregnancy loss due to known causes such as chromosomal abnormalities, uterine anomalies, endocrine disturbances, antiphospholipid syndrome, inherited thrombophilia and infections were excluded from the study. Placental tissue from women with unwanted medically terminated pregnancies who has successfully given birth to at least two children and does not have any medical history of pregnancy loss were considered as control subjects.

Tissue Collection

Placental tissue was collected in RNA later solution (Invitrogen, Thermo scientific solutions) from both recurrent pregnancy loss cases and medically terminated pregnancies and stored at -800C till further use.

Total RNA Isolation and Purification

Total RNA from placental tissue was extracted using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Purity and concentration of RNA was estimated by Nanodrop (Eppendorf).

Primer Designing

The forward and reverse primers for the target genes ANGPT1, FGF2 and PDGFB and endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed by obtaining all mRNA transcripts sequences from NCBI. All the transcripts were compared using Clustal omega multi sequence alignment. Primer quest tool from Integrated DNA Technologies (IDT) was used to design the appropriate primers and the obtained primers were checked by Basic local alignment search tool (BLAST). The primers sequences were synthesised from Eurofins India Pvt Limited.

cDNA Synthesis and Expression Analysis by SYBR Green Real Time qPCR

cDNA was synthesised by using QuantiTect Reverse Transcription Kit (Qiagen). The reaction was incubated for 30 minutes at 420C and 3 minutes at 950C according to the manufacturer's instructions. Amplification of the target genes ANGPT1, FGF2, PDGFB and housekeeping gene GAPDH were done by quantitative real-time (qRT)-PCR using gene specific oligonucleotides (Table 1). Three

replicates of real-time PCR experiments were performed for each sample in 96-well plate using an ABI 7000 Sequence Detection System from Applied Biosystems (Applied Biosystems). A total volume of 20-µl reaction was performed with SYBR Select Master mix of 10 µl (cat. no. 4472908; Thermo Fisher Scientific, Inc.), 1 µl of each primer (10 µM), and 4 µl template cDNA. The amplification protocol consisted of an initial denaturation step at 95°C for 4 min, followed by two-step PCR for 40 cycles at 95°C for 30 sec and 60°C for 30 sec. A melting curve analysis were also performed to check no primer dimmers or false amplicons interfered with the result. The Ct value was extracted for both reference gene and target gene with auto baseline and manual threshold and fold change of expression was calculated by ΔΔCt method.

Table 1: Primers sequences used for qPCR.

S.No	Gene	Forward primer	Reverse primer
1.	ANGPT1	CCAAAGAGGCTGGAAGGAATA	GTACTGCCTCTGACTGGTAATG
2.	FBF2	GCTGGTGATGGGAGTTGTATTT	CTGCCGCCTAAAGCCATATT
3.	PDGFB	CTGTTGAGGTGGCTGTAGATG	GATGAAAGGAACCAGAGGAAGAG
4.	GAPDH	CTCTCTGCTCCTCCTGTTCG	CCATGGTGTCTGAGCGATGT

Statistical Analysis

The difference between the level of expression in ANGPT1, FGF2, PDGFB genes and housing keeping gene was determined by Livak method [12] in recurrent pregnancy loss and medically terminated pregnancies. Comparison of values between the two groups were performed by paired, two tailed, non-parametric t-test using Graph pad Prism version 9.3.1(471). Statistical significance was established at $p<0.05$.

Results

The clinical characteristics of the study group are represented in the (Table 2). The mean age of the cases was 25.23±2.51, while the mean age of controls was 29.21±3.54. The body mass index of the cases was 24.48±3.54, whereas in controls the body mass index was 23.55±3.02. The gestational age of the cases was 11.02±3.12 (weeks), while 12.14±3.72 (weeks) in controls. Relative quantification of mRNA expression of ANGPT1, FGF2 and PDGFB genes from placenta of recurrent pregnancy loss and medically terminated pregnancies was done in order to know whether the altered expression of these genes plays a role in the pathogenesis of unexplained recurrent pregnancy loss. Real time qPCR results for the relative mRNA expression of ANGPT1, FGF2 and PDGFB genes in both the case and control groups are represented in (Figure 1). Our results showed a significantly downregulated mRNA expression of ANGPT1 (1.96-fold, $p<0.0001$) gene in the placenta of recurrent pregnancy loss compared to medically terminated pregnancies (4.64-fold). However, no significant difference was observed in the placental expression of FGF2 and PDGFB genes in recurrent pregnancy loss cases and the control subjects (Table 3).

Table 2: Clinical characteristics of the study subjects.

Category	Sample size	Age (years)	BMI (kg/m^2)	Gestational age (weeks)
Cases	35	25.23±2.51	24.48±3.54	11.02±3.12
Controls	20	29.21±3.54	23.55±3.02	12.14±3.72

Table 3: Fold change expression and P value of the study group.

Gene		ΔCt	ΔΔCt	2^-ΔΔCt	SD	SE	P value
ANGPT1	Cases (35)	3.78	-0.84	1.96	0.90	0.16	<0.0001
	Controls (20)	2.47	-2.15	4.64	1.40	0.35	
FGF2	Cases (35)	3.58	-2.60	6.51	2.76	0.50	<0.5165
	Controls (20)	3.41	-2.77	7.00	1.73	0.43	
PDGFB	Cases (35)	2.98	-3.20	9.43	2.28	0.42	<0.9833
	Controls (20)	3.01	-3.17	9.41	2.68	0.67	

Note: SD=Standard deviation, SE= Standard error

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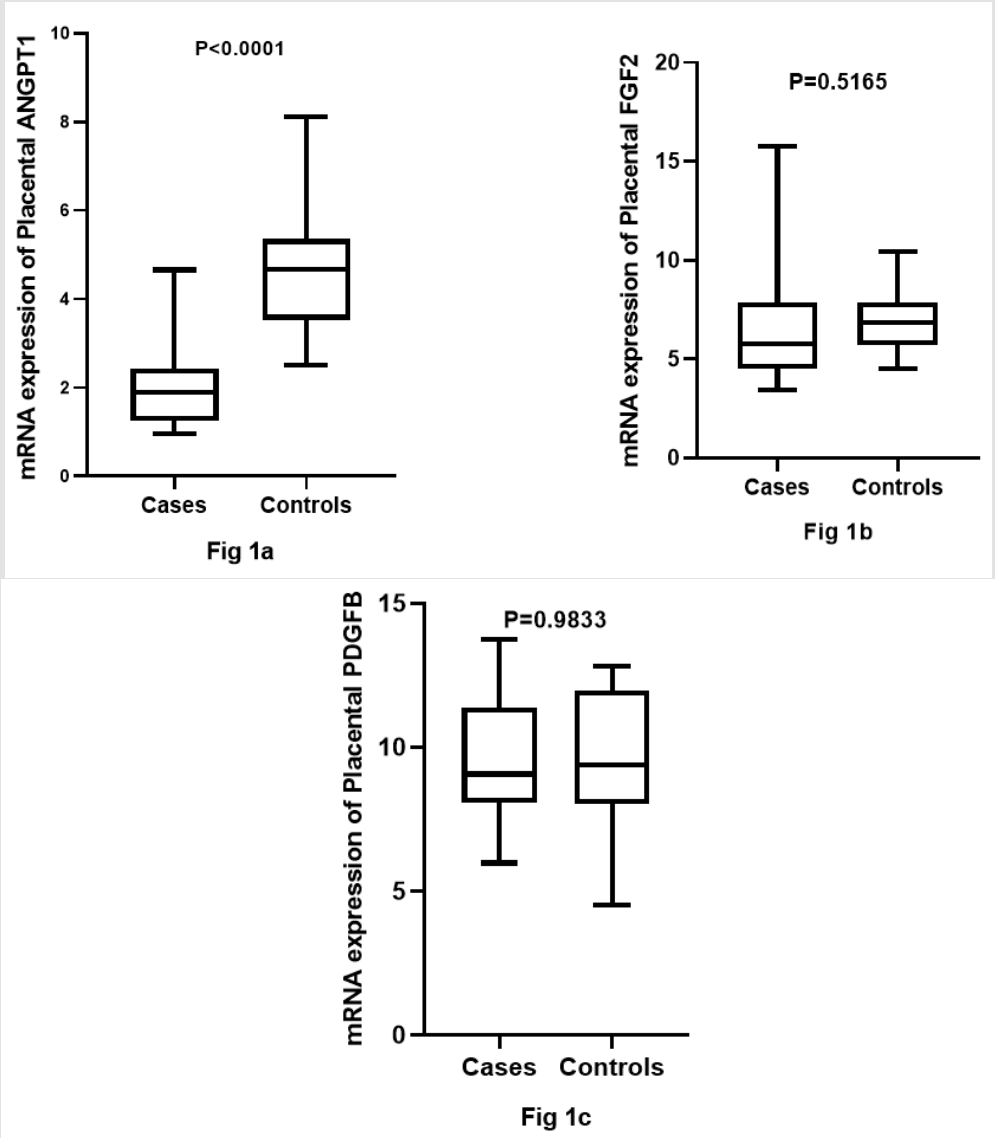

Figure 1:
a. mRNA expression of placental ANGPT1 in cases and controls.
b. Placental mRNA expression of FGF2 in cases and controls.
c. Placental mRNA expression of PDGFB in cases and controls.

Discussion

As angiogenesis is essential to normal placental, fetal growth and development, several growth factors allegedly including ANGPT1, FGF2 and PDGFB regulate angiogenesis, as they are widely expressed during embryonic development, and act by controlling neovascularization and in turn RPL pathogenesis. Exploring the significance of ANGPT1, FGF2 and PDGFB genes in the establishment of a competent feto maternal vascular system that is essential for proper placental function and fetal growth. The angiopoietin system includes four ligands (Ang 1, Ang 2, Ang 3 and Ang 4), among them the most well characterized are Ang 1 and Ang 2, and two corresponding tyrosine kinase receptors (Tie 1 and Tie 2) [6,13]. Ang 1 and Ang 2 both bind to Tie 2, an endothelial cell specific tyrosine kinase receptor with similar affinity [14,15]. Ang 1 acts as a paracrine agonist to Tie 2, results in receptor dimerization and induces its phosphorylation on several cytoplasmic residues to activate downstream signalling pathways, including the phosphoinositide 3 (PI3) kinase/Akt and extracellular signal-regulated kinase (ERK) pathways [16]. Angiopoietins are involved in the migration and

proliferation of trophoblasts and the regulation of nitric oxide release during placentation [17]. Ang 1 is expressed in the placenta from the very early stages of pregnancy and mediate a number of endothelial and non endothelial effects that are thought to be pivotal for proper placental development [17-19]. Throughout gestation, the serum levels and placental expression of Ang 1 normally increases, [18,20].

Low ANGPT-1 levels lead to vessel destabilization and a decrease in the angiogenic sprouting promoting vessel leakage [21]. A case–control study was conducted by (Daponte, et al. [22]) to evaluate whether a single serum measurement of angiopoietin-1 (ANG-1) and angiopoietin-2 (ANG-2) at 6–8 weeks gestation can differentiate failed pregnancies, such as ectopic pregnancies (EP) or missed abortions (MA), from healthy intrauterine pregnancies (IUP). Serum and tissue mRNA determination of ANG-1 and ANG-2 levels were done by ELISA and RTPCR, and found that ANG-1 and ANG-2 concentrations and their ratio were lower in EP and MA cases compared to IUP [22]. Earlier studies revealed no marked changes in the placental expression of Ang 1, Ang 2 and Tie 2 from preeclamptic pregnancies [18,23,24]. Whereas, altered placental expression of the angiopoietin/Tie 2 system has been observed in pregnancies complicated by placenta accreta and in women with recurrent abortion [25,26]. Our study showed a significantly downregulated mRNA expression of ANGPT1 gene in the placenta of recurrent pregnancy loss compared to medically terminated pregnancies. The protein encoded by FGF2 gene binds to heparin and possess broad mitogenic and angiogenic activities, which have been implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth. Deprivation of endogenous FGF2 might result in dysregulation of the activities of other survival and angiogenesis-related genes [27].

Only few studies have been carried out on role of FGF2 gene in pregnancy related complications. (Choi, et al. [28]) compared the gene expression level for angiogenesis genes in chorionic villi from RPL patients and those from normal controls, by Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The results revealed that 7 angiogenesis related genes including basic fibroblast growth factor (bFGF), were less expressed in chorionic villi of RPL patients [28]. Perhaps, the present study could not show any significant difference between the placental expression of FGF2 gene in recurrent pregnancy loss and medically terminated pregnancies. The PDGF family of proteins are important in physiological and pathological events, because of their direct effects on the state of the vasculature. Glioma angiogenesis and growth is enhanced by PDGF-B through stimulating VEGF expression in the tumor endothelia, increasing endothelial cell migration and mitogenesis, and by promoting recruitment of pericytes into growing vessels, thus facilitating vessel assembly [8]. Genetic studies demonstrated that lack of PDGF-B and PDGFR-b expression disrupts normal vascular development during embryogenesis, leading to premature death [29]. There is lack of human studies showing the importance of PDGFB gene in pathophysiology of pregnancy related complications. However, the present study didn't show any significant difference between the placental expression of PDGFB gene in recurrent pregnancy loss and medically terminated pregnancies.

Conclusion

Out study supports the notion that ANGPT1 is essential for the proper reorganization of the placental vascular system even from the very first stages of pregnancy. There is a need of studies on angiopoietins in the field of RPL to not only highlight the important role of angiopoietins in pregnancy, also to elucidate the aetiopathogenesis and the underlying mechanisms. Yet, available information on the function of FGF2 and PDGFB during pregnancy is still limited. The pathophysiological significance of our results should further be studied in a larger population to implicate them as factors in the prognostic and therapeutic implications.

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Conflict of Interest

None to declare.

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