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Research Article

Immunological insights into recurrent spontaneous abortions: The role of GATA3 and cytokine expression in maternal and placental tissues

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Abstract

Objectives: Recurrent spontaneous abortion (RSA) is the successive loss of pregnancy experienced by 1-2% of women with clinically recognized pregnancies. The role of cytokines and their regulators in RSA has gained significant attention in recent years. The GATA3 transcription factor has significant implications for maternal-foetal health outcome by modulating the immune T-cell population to T helper cell subsets that produce the proinflammatory (IFNγ) and anti-inflammatory cytokines (IL4) required for pregnancy maintenance.

Methods: The study involved 65 case-women with RSA and 70 control-women without a history of RSA. IL-4, IFNγ, and GATA3 transcription factor levels were analysed in maternal serum and placental tissues. Correlation analysis was performed for GATA3 expression and cytokine levels, and the results were tested for statistical significance.

Results: The mRNA expression of the GATA3 transcription factor was significantly reduced in both the maternal blood and placental tissues of the RSA group compared to the control group undergoing medical termination ($p \leq 0.05$*). Additionally, compared to the control group, the levels of the Th1 cytokine IFN-γ were significantly elevated (11034 pg/ml & 87.4735 pg/g), while the levels of the Th2 cytokine IL4 were significantly decreased (48.9832 pg/ml & 6320 pg/g) in RSA mother and their placenta samples respectively. Moreover, cytokine levels in the RSA group showed a significant correlation with GATA3 expression.

Conclusion: The study suggests that altered GATA3 levels and an increased IFN-γ/IL-4 ratio may increase the risk of recurrent spontaneous abortions in Telangana women.

Keywords: GATA3 transcription factor, IFNγ, IL4, maternal-foetal immunology, recurrent spontaneous abortion (RSA), TH1/TH2

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Recurrent spontaneous abortion has been redefined as the loss of two or more successive clinically recognised pregnancies before the 24th week of gestation, which poses a significant challenge in reproductive medicine [1]. The prevalence of recurrent spontaneous abortion is estimated to be approximately 1–2% of all couples attempting to conceive [2]. The etiology of RSA is multifaceted, with several contributing factors which include chromosomal abnormalities in either parent, uterine anomalies, hormonal imbalances, immunological factors, thrombophilia, or infections. Genetic factors, environmental influences, and lifestyle choices further contribute to the complexity of RSA etiology. Nevertheless, 50% of the exact cause behind the pathogenesis is unclear.

Cytokines are signalling molecules that orchestrate the immune responses crucial for implantation, placental development, and foetal growth. A delicate equilibrium between

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proinflammatory and anti-inflammatory cytokines is necessary to support maternal–foetal immune tolerance while ensuring protection against inflammation and rejection of semi allografts. During implantation, a controlled inflammatory response is vital for the invasion of trophoblast cells into the maternal endometrium followed by implantation. Proinflammatory cytokines, such as interferon gamma (IFNγ), tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), promote this early phase of pregnancy, and as pregnancy progresses, a shift towards an anti-inflammatory environment (IL10, IL4, and TGF beta) becomes essential to establish maternal–foetal tolerance. Piccinni and colleagues (2000) suggested that there was a decrease in Th1 cytokines and an increase in Th2 cytokines for the maintenance of a successful pregnancy [3]. Moreover, studies have shown that an excessive inflammatory response can lead to adverse pregnancy outcomes, such as recurrent loss of pregnancy and preterm birth [4,5]. GATA3 has emerged as an important transcriptional regulator of naïve T-cell differentiation into Th1 and Th2 effector cells that produce proinflammatory and anti-inflammatory cytokines, respectively, which are essential for maintaining successful and healthy gestation.

The placenta serves a vital role in immune modulation along with managing the hormonal, nutritional, and oxygen requirements of the foetus. The microenvironment established by the placenta plays a crucial role in the differentiation and function of immune cells infiltrating the implantation site. Helper T (Th) cells, pivotal in regulating immune responses, exhibit distinct contributions from different Th cell subsets during various stages of human pregnancy. Conversely, dysregulation of Th responses has been associated with numerous obstetric complications [6,7]. However, understanding the dynamics of foetal-maternal immune system throughout pregnancy remains incomplete.

The GATA family member GATA3-binding protein is a zinc finger transcription factor that recognises G-A-T-A in the promoter of target genes. GATA3 is involved in the control of CD4+ effector T-cell differentiation into Th2 subtypes that produce the anti-inflammatory cytokines IL4 and TGF-beta by simultaneously inhibiting the production of Th1 cell lineages that promote proinflammatory cytokines. GATA3 facilitates the conversion of the Th2 gene locus into an open conformation and increases accessibility to transcription activators along with GATA3, which initiates the transcription of Th2 genes, such as interleukin 4 (IL4), interleukin 5 (IL5), and interleukin 13 (IL13), and simultaneously suppresses the transcription of the potent proinflammatory Th1 cytokine interferon gamma (IFNγ), thereby skewing naïve T cells to Th2 effector cells, which are crucial for foetal implantation and maintenance [8].

Interleukin-4 is a pleiotropic anti-inflammatory cytokine of 20 kDa that contributes to the maintenance of pregnancy by coordinating vascularisation, placental development, and immune response adaptations at the maternal–foetal interface. IL-4 binds to its receptor, IL-4Rα, and activates the signal transducer STAT 6 signalling pathway, which helps polarize antigen-stimulated naïve Th cells into Th2 effector cells and promote Th2 responses. By suppressing the production of IFNγ, STAT6 may directly inhibit the growth of Th1 cells by inducing the zinc-finger transcription factor GATA3 (GATA-binding protein 3). The interleukins IL4, IL10 and IL13 are reported to have homeostatic functions during pregnancy [9]. These anti-inflammatory cytokines promote spiral artery remodelling by inducing the expression of leptin receptors for the ligand leptin, which plays a significant role in lipid metabolism, angiogenesis, vascular function, placental development and intrauterine foetal development [10,11]. IL-4 enhances the expression of VCAM-1 and induces changes in the morphology of human umbilical vascular ECs during the implantation phase of pregnancy [12,13].

IFNγ is a potent cytokine that is essential for the differentiation of naïve T cells into Th1 cells. IFNγ, which helps in the propagation of the immune response, belongs to the type II interferon subfamily. It has been proposed that during pregnancy, IFNγ is essential for the initiation of endometrial vasculature remodelling and angiogenesis during implantation. Previous findings have shown that the rejection of allografts is supported by elevated levels of IL-2 and IFNγ [14,15]. Conversely, it was observed that there was an increased production of IFNγ by natural killer cells during the first week of pregnancy, and these levels dampened during the latter phase for maintenance of the semi-allogenic foetus, thereby establishing a reduced Th1 profile and increased Th2 cytokine profile. An imbalance in IFNγ levels has been demonstrated to lead to severe pregnancy outcomes, such as spontaneous abortion, preterm birth, preeclampsia, and gestational diabetes [4,16–18]. IFNγ, the major Th1 cell differentiation cytokine, is regulated by various transcription activators, such as RUNX3, Tbet and STAT. Studies have shown that GATA3 not only promotes Th2 cell differentiation but also inhibits Th1 cell differentiation [19] through its interaction with activators and repression of their action. Ectopic GATA3 expression in developing Th1 cells inhibits IFN-γ production by repressing the expression of STAT4, which is normally highly expressed in Th1 cells compared with Th2 cells [20], and by suppressing T-cell differentiation into Th1 cytokine-producing cells.

However, to date, no studies have been investigated whether the differential protein levels of IL4 and IFNγ are associated with altered levels of GATA3 factors in the etiology of RSA. Therefore, taking into account the immunomodulatory properties of GATA3 and the importance of IL4 and IFNγ during pregnancy, the present study aimed to evaluate the correlation between altered GATA3 expression and cytokine levels (IL4 and IFNγ) in RSA pathogenesis.

Materials and Methods

Study cohorts

A total of 135 women who attended the Department of Gynaecology and Obstetrics, Government Maternity Hospital, Petlaburz, Hyderabad, Telangana were enrolled in the study.

Table 1. Quantitative real-time PCR primer sequences

Gene	Primer sequence	Product size
GATA 3	Forward: GGCGCCGTCTTGATACTT Reverse: CCGTCTCTCTCTCTTCTTCTC	100 bp
Beta Actin	Forward: GGATCAGCAAGCAGGAGTATG Reverse: AGAAAGGGTGTAACGCAACTAA	96 bp

PCR: Polymerase chain reaction.

Sixty-five women with RSA along with aborted tissue and seventy healthy women who underwent medical termination of pregnancy were considered cases and control subjects, respectively. Demographic details were recorded with the help of a standard proforma, and prior consent was obtained from all the subjects. The study was approved by the institutional ethics committee (Ref. No. 24/EC/NEW/INST/2023/4032. dtd. 22/12/2023) of the Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, Telangana. The study was designed in accordance with the Helsinki Declaration.

Inclusion criteria

Both the case and control subjects with a mean age of less than 40 years and who were gestational matched were selected. Women with two or more consecutive spontaneous abortions were considered cases, whereas participants with no previous history of spontaneous abortions and who had at least two live births were considered controls.

Exclusion criteria

The case-control study subjects who demonstrated physiological anomalies, chromosomal and hormonal abnormalities, diabetes, APA antibodies, hypothyroidism, blood pressure and who consumed contraceptive pills were excluded from the study.

Sample collection

A total of 4 ml of blood sample and 100 mg aborted placental tissues from women with RSA and who underwent medical termination of pregnancy (MTP) were collected for quantitative analysis of cytokines. The samples were collected in Hi media RNA later as per standard operating conditions and stored at -80°C for RNA isolation.

Cytokine quantification

The protein levels of IL-4 and IFN-γ in the case, control, and maternal–placental study groups were quantified via commercially available ELISA kits. The IL-4 GENLISA™ ELISA and IFN-γ GENLISA™ ELISA were procured from Kishgen Biosystems for the quantification of cytokines in serum and tissue samples.

Tissue homogenate processing

For the enzyme-linked immunosorbent assay of conceptus, the tissues weighing 100mg were initially washed with PBS at pH 7.4 to remove excess blood and homogenised with 5 ml volume of PBS by mere mixing with a glass homogeniser on ice as described by the Weel et al. [21]. This ensures a weight-based normalisation of cytokine levels. Finally, the supernatant for the analysis was collected by centrifuging the homogenised samples at 12,000 rpm for 15 minutes and was used to determine cytokine levels which expressed as pg/g of placental tissue. The intra-assay and inter-assay coefficients of variation for all measurements were <8% and 10%, respectively, and were recorded in pg/ml for serum samples.

RNA isolation and cDNA conversion

Total RNA was isolated from both case and control maternal blood as well as their respective placental tissues via the Macherey-Nagel NucleoSpin RNA isolation Kit according to the manufacturer's instructions, and any DNA contamination was removed via on-column DNase treatment (Qiagen, Inc.). The quality of the RNA was assessed by measuring the 260/280 absorbance. The amount of RNA per microliter was quantified via a NanoDrop, and each sample was normalised prior to cDNA conversion. One microgram of total RNA was used for first-strand cDNA synthesis in a 20 µL reaction mixture via an iScript cDNA synthesis kit.

Expression analysis of GATA3

Three replicates of real-time PCR experiments were performed for each sample in a 96-well plate via an ABI 7000 Sequence Detection System from Applied Biosystems (Applied Biosystems). The primers for the target genes GATA3 and beta-actin were designed with Primer Express software (Applied Biosystems), and the sequences used for PCR are presented in Table 1. A total volume of 20 µl was used with 10 µl of SYBR Select Master mix (Cat. no. 4472908; Thermo Fisher Scientific, Inc.), 1 µl of each primer (10 µM), and 4 µl of 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 was also performed to check that no primer dimers or false amplicons interfered with the result. The Ct value was extracted for both the reference gene and target gene with an auto baseline and manual threshold, and the fold change in expression was calculated via the ΔΔCt method.

Statistical analysis

The data obtained were statistically evaluated using statistical package IBM© SPSS statistics 24.0 software. The sam-

Table 2. Demographic characteristics of the case-control group study subjects

Demographic characters	Case subjects n=65		Control subjects n=70		p	OR CI (95%)
	n	%	n	%		
Age (mean age±SD) (n)						
≤30 years	37	52.3	63	90.0	0.014*	2.704
>30 years	28	47.7	17	10.0	χ^2=7.983	(1.303–5.612)
No of miscarriages						
Women with 2 pregnancy loss	16	24.6	Nil		<0.0001***	–
Women with ≥3 pregnancy loss	49	75.4				
Consanguinity						
Yes	18	27.5	27	38.5	0.247	1.64
No	47	72.3	43	61.4	χ^2=1.795	(0.7935–3.388)
Time of previous abortion (gestation weeks)						
>12 week	50	80	41	58.6	0.037*	2.358
≤12 week	15	20	29	41.4		(1.116–4.98)
Socio economic status						
Rural	49	75.4	42	60	0.883	2.042
Urban	16	24.6	28	40	χ^2=2.131	(0.974–4.278)
Education						
Primary level	39	60	48	68.6	0.390	0.6875
Secondary level	26	40	22	31.4	χ^2=1.08	(0.3388–1.395)
Smoking						
No	44	67.7	56	80	0.194	1.909
Yes	21	32.3	14	20	χ^2=2.658	(0.872–4.177)

*: p≤0.05; **: p≤0.001; ***: p≤0.0001. OR: Odds ratio; CI: 95% Confidence interval; SD: Standard deviation; BMI: Body mass index.

ple size was calculated using a free open access software OpenEpi (http://www.OpenEpi.com/). All the quantitative data obtained were represented in mean±standard deviation. The strength of risk for the variables and recurrent spontaneous abortion was evaluated from Odds ratio at 95% of their confidence interval. Pearson test of correlation was performed to find relationship between the independent and dependent variables. Logistic regression was carried out to evaluate any confounding effect of independent variables on the disease condition. A p- value less than 0.05 was considered statistically significant.

Results

The mean age of the participants enrolled in the study was under 40 years, and the participants were grouped into those with a mean age below 30 years and those with a mean age above 30 years, as depicted in Table 2. The cut off maternal age as 30 years was selected as a woman's fertility begins to decline gradually around age 30, with a more rapid decrease after age 35 and increased the risk of miscarriage and other adverse pregnancy outcomes with advancement of maternal age [22]. The data indicated a notable difference in the mean age between the case and control groups, with χ^2=7.985, p≤0.05*. Specifically, the study demonstrated a twofold increased susceptibility to recurrent spontaneous abortion (RSA) among case subjects in the older group (≤30 years) compared with control subjects in the same age group (OR=2.7, p=0.01*). A comparison of the basal metabolic rates between the case and control groups revealed a significant difference (p=0.005*), with the case study group having a higher BMI (25.13±3.54) than the control group. Further classification on the basis of the number of pregnancy losses revealed a significantly greater proportion (75.4%) of cases with more than three pregnancy losses than those with 2 pregnancies lost (p≤0.0001**). The inclusion of women with consanguineous marriages was not associated with disease susceptibility. Additionally, the evaluation of the gestational period and susceptibility to RSA revealed statistical significance, with case subjects demonstrating a twofold increased risk during early gestation (OR=2.3, p=0.037*). Furthermore, socioeconomic status, education, and smoking habits were not significantly associated with disease susceptibility.

As depicted in Table 3, the levels of IFNγ and IL4 varied among the respective case and control groups, and the difference was statistically significant. Compared with their respective controls, women with RSA and their placental tissues presented lower levels of IL4 (63±20 pg/ml & 48.98±32 pg/g; p≤0.05*) and elevated levels of IFNγ (110±34 pg/ml & 87.47±35 pg/g; p≤0.05*, respectively). Furthermore, the

Table 3. Analysis of the levels of the circulating cytokines IL4 and IFN-γ in placental tissue and maternal blood

	Placental tissue		Maternal blood serum	
	IFN-γ (pg/g) Mean±SD	Il4 (pg/g) Mean±SD	IFN-γ (pg/ml) Mean±SD	Il4 (pg/ml) Mean±SD
Case	87.47±35	48.98±32	110±34	63±20
Control	71±28	66.47±21	95±30.1	79±31
p	0.003**	0.0002***	0.007**	0.001**

P<0.001 **: Significant; p<0.0001 ***: Significant. IL4: Interleukin 4; IFN-γ: Interferon gamma; SD: Standard deviation.

Table 4. Binary logistic regression analysis for predictors associated with RSA

	Cytokines	Odds ratio	95% CI	p
Maternal serum	IFNγ	1.07	1.002–1.094	0.053
	IL4	0.87	0.631–0.979	0.61
Placental	IFNγ	1.73	1.806–3.041	0.027*
Tissue	IL4	0.66	0.422–0.837	0.001**

The variables in the binary logistic regression are adjusted for age, BMI, gestational period, consanguinity, socioeconomic status and smoking habits. *: p≤0.05; **: p≤0.001. RSA: Recurrent spontaneous abortion; CI: Confidence interval; IFN-γ: Interferon gamma; IL4: Interleukin 4.

above parameters were adjusted for the demographic variables studied to determine whether any confounding effect resulted in the disease condition. However, binary regression analysis revealed that placental tissue levels of IFNγ (OR=1.73, p value=0.027*) and IL-4 (OR= 0.66, p=0.001*) were significant single predictors of RSA, as shown in Table 4.

The relative expression of GATA3 was assessed in the case and control groups of women with recurrent spontaneous abortion and their respective placental tissues to determine whether altered levels of GATA3 contributed to the pathogenesis of RSA. Figure 1a, b depict the comparative case–control mRNA expression levels of GATA3 in placental tissues and maternal blood, respectively. The relative expression of the GATA3 transcription factor revealed that GATA3 mRNA levels were downregulated in women with RSA (p<0.043*) and in the respective placental tissues by one-fold (p<0.002**) compared with those in the control groups. Finally, the correlation between GATA3 expression and the levels of IL4 and IFNγ was assessed using a Pearson correlation test. This assessment was conducted in women from the RSA group and their corresponding tissues. The correlation was deemed statistically significant if the p value was below 0.05. The Pearson correlation test showed a strong positive correlation between IL4 levels and GATA3 expression in placental tissue (r=0.782, p=0.0001***) and in the corresponding maternal group (r=0.572, p= 0.003**), as depicted in Figure 2a, b. Additionally, a negative correlation was found between IFNγ levels and GATA3 expression in both placental tissues and the maternal group. However, the correlation was moderate in the maternal group (r=-0.6601, p=0.017*), whereas it was strong in the placental group (r=-0.7827, p=<0.00001**), indicating statistical significance, as illustrated in Figure 3a, b.

Discussion

The study aims to investigate the expression profile of the GATA3 transcription factor, which modulates T helper cell activity, along with their cytokines, particularly IL4 and IFNG, obtained from women with recurrent spontaneous abortion along with their respective placental tissues and those with no history of RSA who underwent medical termination of pregnancy and their placental tissues.

Previous studies have emphasized the importance of down-regulating TH1 cytokines and upregulating TH2 cytokines during pregnancy. TH1 immunity, characterized by immune-inflammatory responses, predominates during the pre-implantation period and shortly after placental implantation [4]. Subsequently, early inflammatory TH1 immunity transitions to TH2 anti-inflammatory responses. The prevailing TH2 immunity at the placental implantation site helps maintain a balance with TH1 immunity, thereby safeguarding foetal and placental development [23,24] .Various immunomodulatory gene products, including transcription factors such as GATA3 protein, play crucial roles in orchestrating the TH1/TH2 shift throughout pregnancy. GATA3 is a key regulator of CD4+ T cell differentiation into Th2 effector cells. It activates the expression of signature Th2 cytokines like IL4, IL5, and IL13, which are pivotal for trophoblast establishment, maintenance, and differentiation. GATA3 executes these diverse functions by directly activating target genes or binding to cofactors that regulate gene expression and epigenetic modifications. GATA3 directly binds to the promoters of IL5 and IL13, intragenic areas of IL4, and the CGRE region within the IL13 locus, thereby promoting Th2 differentiation [25]. Additionally, GATA3 inhib-

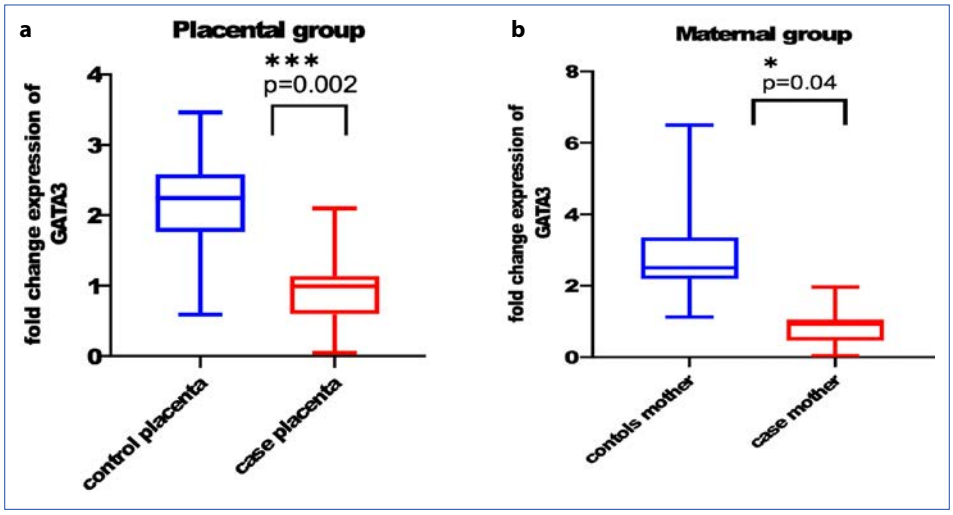

Figure 1. Graphical representation of the relative expression of GATA3 in case-control placental tissue and maternal blood.

GATA3: GATA binding protein 3.

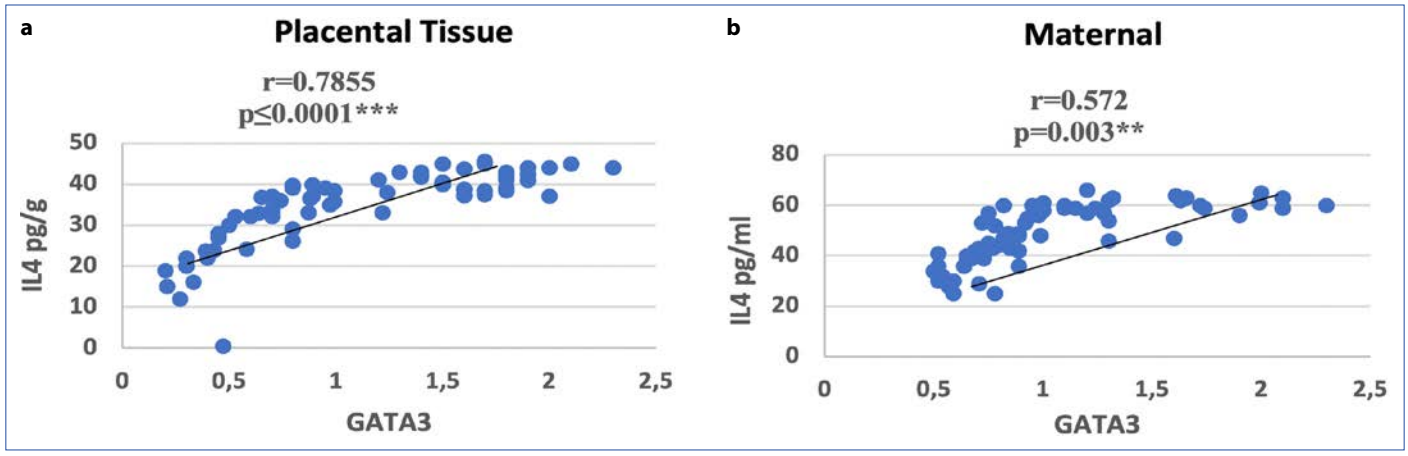

Figure 2. Correlation of GATA3 expression and IL4 levels, where r is the correlation coefficient and p is the statistical significance.

IL4: Interleukin 4.

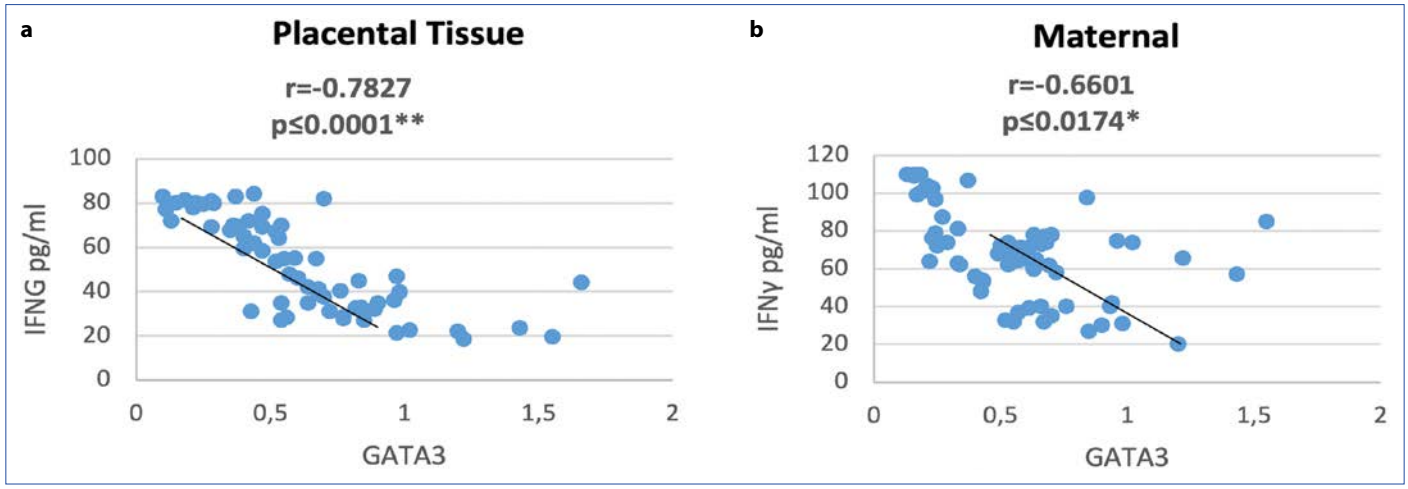

Figure 3. Correlation of GATA3 expression and IFNγ levels of placental and maternal blood, where r is the correlation coefficient and p is the statistical significance.

IFN-γ: Interferon gamma.

its Th1 differentiation by suppressing the production of STAT4 and IL12Rb2, both crucial for Th1 differentiation. Moreover, GATA3 interacts physically with Runt-related transcription factor 3 (Runx3), a regulator that stimulates Th1 differentiation, to suppress exomes expression and IFN-γ production.

Previous studies by Home et al. [26], and Saha et al. [27], demonstrated that the knockdown of GATA3 inhibited trophoectoderm maintenance, and also decreased expression of GATA3 in blastocysts of mouse models that further affected embryo hatching as well as the implantation respectively [26,27], underscoring the significance of GATA3 expression during pre-implantation. GATA3 expression promotes a TH2 response and modulates TH1 response, balancing TH2/TH1 levels crucial for maintaining successful pregnancy. Studies on tolerance induction to allografts have shown decreased levels of TH1 cytokines such as interleukin (IL)-2 and IFNγ, and increased levels of TH2 cytokines such as IL-4 and IL-10. Conversely, rejected allografts exhibited elevated levels of IL-2 and IFN [28].

GATA3 enhances IL4 production along with Stat5 by directly binding to the IL4 locus, promoting the opening of IL4 chromatin during the early stage of implantation while inhibiting the production of IFNγ, a potent cytokine in TH1 response and differentiation. Ectopic GATA3 expression in developing Th1 cells inhibits IFN-γ production through repressing the expression of STAT4, which is normally highly expressed in Th1 cells compared with Th2 cells and there by suppressing T cell differentiation to Th1 cytokine producing cells [8]. Further a study by Ribeiro et al. [29], reported that lower expression of GATA3 in preeclampsia women demonstrated altered levels of inflammatory cytokines, where there was increased levels of pro-inflammatory and lower anti-inflammatory cytokines. The present study results are in consistent with these findings, showing decreased levels of GATA3 protein expression in maternal blood and respective placental tissues from women with recurrent spontaneous abortions compared to those from healthy women who underwent medical termination of pregnancy. Furthermore, the study revealed differences in levels of potent Th1/Th2 cytokines IFNγ / IL4 crucial for pregnancy maintenance, indicating that altered levels of GATA3 and Th1/Th2 cytokines play a significant role in the etiology of recurrent spontaneous abortion.

Conclusion

The current study indicates a relationship between cytokine levels and GATA3 expression and suggests that immune pathway abnormalities may play a major role in the development of RSA. This highlights GATA3 as a potential target for reestablishing immunological homeostasis during pregnancy.

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Ethics Committee Approval: The study was approved by The Osmania University Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, Telangana Ethics Committee (No: 24/EC/NEW/INST/2023/4032, Date: 22/12/2023).

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