Relationship of interferon regulator factor 5 and interferon-γ with missed abortion

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Abstract. The aim of the present study was to reveal the association of missed abortion, a process integrated with the immune system, with interferon regulatory factor 5 (IRF5) and interferon-γ (IFN-γ), and to demonstrate the function of these molecules by examining their levels in decidual tissue. This prospective cohort study included 13 patients with no additional systemic disease, between 6 and 10 weeks of gestation with negative fetal heartbeat, and 11 patients between 6 and 10 weeks of gestation with positive heartbeat who presented for voluntary termination of pregnancy. In the fresh decidual tissue materials recovered after therapeutic curettage, IFN-γ and IRF5 protein levels were determined by ELISA method and IFN-γ and IRF5 gene expression levels by qPCR method. The mean IFN-γ (86.5 vs. 27.3 pg/mg protein; P<0.001) and IRF5 (2.0 vs. 1.5 ng/mg protein; P<0.001) levels were significantly higher in pregnant women who had missed abortion compared to the voluntary abortion group. The increases in the mean IFN-γ/GAPDH (3.5 vs. 1.5-fold increase; P<0.001) and IRF5/GAPDH (3.9 vs. 1.4-fold increase; P<0.001) gene expression levels were significantly higher in the tissues of pregnant women with missed abortion than in the voluntary abortion group. A threshold value of 45.2 pg/mg protein for IFN-γ had a sensitivity of 100% and specificity of 100% in determination of missed abortion. The findings of present study revealed, to the best of our knowledge for the first time in the literature, that IFN-γ and IRF5 may be associated with missed abortion, and that IFN-γ and IRF5 protein levels and gene expression levels were significantly increased in the case of missed abortion. According to our findings, IFN-γ and IRF5 play an important role in placental invasion and pregnancy and can be used as markers for endometrial implantation.

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Introduction

Missed abortion (dead fetus) or early pregnancy loss seen in the first three months of gestation refers to the situation in which the abortion event has not been observed yet because the embryo that has lost its vitality has not been expelled from the uterus (1,2). Missed abortion can occur with a high frequency that can reach 50-70%, and is the most common complication of pregnancy (1-3).

Similar to spontaneous abortion, missed abortion may be caused by congenital anomalies, chromosomal anomalies, placental anomalies or embryonal factors such as exposure to toxic substances, maternal factors such as infection, endocrine disorders, renal diseases, trauma and immunological causes, and some paternal factors. However, the etiology of abortion is still not fully understood (1-4). In recent studies, it was considered that a significant proportion of all miscarriages was the result of problems with endocrine and immunological mechanisms. The causes of recurrent early pregnancy loss include immunological factors (20-50%), endocrine factors (17-20%), anatomical factors (12-16%), genetic factors (3.5-5%), infectious factors (0.5-5%), and other factors such as thrombotic factors, environmental factors and male factors (10%) (1,5,6). The presence of immune system elements such as antiphospholipid antibodies (i.e., lupus anticoagulant and anticardiolipin antibodies), that are autoimmune antibodies produced by plasma cells differentiated from B lymphocytes, in the decidua and their interaction may not only enable the structuring and continuation of the pregnancy, but may also pave the way for the events leading to the termination of pregnancy. Antiphospholipid antibodies react against the proteins that bind phospholipids on plasma membranes in an autoimmune disease, such as antiphospholipid syndrome, which provokes thrombosis in the vessels possibly leading to miscarriages (5-8). Missed abortion may lead to some important maternal complications such as septic abortion, septic shock or abnormal bleeding in some cases where it is not noticed for a long time. Therefore, early diagnosis of missed abortion is important. It has been stated that in addition to ultrasound imaging, some molecules can be used as biomarkers in the diagnosis of missed abortion (1-4,9). Interferon-γ (IFN-γ) was first identified in lymphocytes. It has been reported that IFN-γ is present as a Type I cytokine in the tissue mostly in the first trimester of pregnancy, but it cannot be detected in the third

trimester. It is considered that IFN-γ may be detrimental to normal fetal development. However, the specific mechanism remains unclear (10,11). In addition, IFN-γ-induced abortion was revealed in mice by suppressing regulatory T cells and Th17 cell polarization (12). In addition, to the best of our knowledge, the association between IFN-γ and uterine and placental biology have not been directly investigated, to date.

Interferon regulatory factors (IRFs) are intracellular communication factors. They are known to play central roles in cell differentiation and development, cell proliferation, DNA damage response and tumor suppression. IRFs are usually localized in the cytoplasm of an unstimulated cell in which they are located. IRF5 leads to the production of numerous inflammatory signals such as interleukin (IL)-6, TNF-α, IL-12, IL-23, which are involved in the pathogenesis of autoimmune disease (13-15). As aforementioned, autoimmune diseases may be associated with pregnancy loss with formation of thrombosis. In addition, polymorphism of the IRF5 gene, which has an important role in the regulation of genes related to immunity and apoptosis, and its association with recurrent pregnancy loss was demonstrated (16). It has been revealed that altering IRF5 levels impairs T helper (Th)-1/Th-2 balance causing abnormal release of cytokines (16). It has also been reported that cytokines such as IL-6 are crucial for the protection of pregnancy (17). In addition, it has been revealed that a high expression level of TNF-α increased the risk of recurrent pregnancy loss (18). In addition, to the best of our knowledge, the association between IRF5 and uterine and placental biology has not been directly investigated, to date.

The aim of the present study was to identify the association among missed abortion, a process integrated with the immune system, and IRF5 and IFN-γ, and to demonstrate the function of these molecules by examining their levels in decidual tissue.

Materials and methods

Ethical approval. The present study was approved (approval date and number: 12.08.21/28) by the Ethics Committee of Yeni Yüzyil University (Istanbul, Turkey). All of the participants enrolled in the study provided signed written informed consent. The present study was designed as a prospective cohort study.

Patients

Inclusion criteria. The study was performed with patients admitted to the Department of Obstetrics and Gynecology of Gaziosmanpasa Hospital (Yeni Yuzyıl University). This prospective cohort study included 13 patients with no additional systemic disease, between 6 and 10 weeks of gestation with negative fetal heartbeat, who presented to the Obstetrics and Gynecology department of our hospital and 11 patients between 6 and 10 weeks of gestation with positive heartbeat who presented for voluntary termination of pregnancy between September 2021 and December 2021.

Spontaneous pregnancy loss was diagnosed through transvaginal ultrasound and clinical assessment.

Exclusion criteria. Patients with uterine anomalies, patients with concomitant diabetes and blood pressure disease, and pregnant women who were not between the ages of 18-40 were excluded from the study. Women with a history of recurrent abortion (two or more abortions) were not included in the present study. All the patients were questioned about their history concerning inherited diseases, and it was confirmed that none of them had inherited diseases such as trombophilia, or additional diseases such as systemic lupus erythematosus.

Tests. Fresh decidual tissue materials recovered after therapeutic curettage were stored at -80˚C. The materials were sent to the Research Laboratory of Biochemistry of the Faculty of Medicine of Adnan Menderes University (Aydin, Turkey) for IRF-γ and IRF5 analysis. IFN-γ and IRF5 protein levels in the materials were determined by ELISA method, and IFN-γ and IRF5 gene expression levels by quantitative polymerase chain reaction (qPCR) method.

Gene expression of IFN-γ and IRF5 by reverse transcription (RT)-qPCR. From each fresh tissue total RNA was isolated with a commercial isolation kit (Intron Biotechnology, Inc.; cat. no. 17211). A total of 1 µg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosytems; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The primer sequences were as follows: IRF5 forward, 5'-TCCTGGAAGTGGATTTGG GC-3' and reverse, 5'-CTTTCCCTGCTCATGGCTGA-3'); IFN-γ forward, 5' CCAGAGCATCCAAAAGAGTGTG-3' and reverse, 5'-TCAGCTTTTCGAAGTCATCTCGTT-3'); and GAPDH forward, 5'-AGGGCTGCTTTTAACTCTGGT-3' and reverse, 5'-CCCCACTTGATTTTGGAGGGA-3'; Sigma Aldrich; Merck KGaA). A total of 100 ng of cDNA was amplified using SYBRGreen PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI StepOne Plus detection system, programmed at 95˚C for 10 min, then 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The results were analyzed using StepOne Software v2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.) using $2^{-\Delta\Delta Cq}$ method (19) and normalized to GAPDH mRNA. Data were expressed as fold induction relative to the control.

Protein levels of IFN-γ and IRF5 by ELISA. Tissue samples were homogenized using RIPA cell Lysis and Extraction Buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) including added protease inhibitor cocktails (product no. P2714; Sigma-Aldrich; Merck KGaA) on ice. Tissue samples were homogenized and kept on ice for 30 min. Samples were centrifuged at 10,000 x g for 20 min at 4˚C and the supernatant was used for measurement. Protein concentrations were measured with Bradford method (20). The levels IFN-γ and IRF5 were measured with the sandwich ELISA in accordance with the manufacturer's protocols (cat. nos. EH0164 and EH3278; Fine Test; Wuhan Fine Biotech Co., Ltd.) with inter-assay cv: <12%, and intra-assay cv: <10% respectively. IFN-γ and IRF5 values are presented as ng/µg protein. All ELISA measurements were performed using a microplate reader (BioTek Instruments, Inc.). Results were presented as ml/mg of protein.

Statistical analysis. The sample size of the study was calculated using G*Power software (ver. 3.1.9.7; Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany; gpower.hhu.de/). While calculating the sample size, the effect

Table I. Demographic characteristics of the study population (mean ± SD).

Characteristics	Missed abortion (n=13)	Voluntary abortion (n=11)	P-value
Age (years)	29.2±4.2	29.7±6.5	0.796
Gravida	1.8±0.7	1.9±1.0	0.703
Parity	0.5±0.7	0.8±0.9	0.382
Weight (kg)	61.3±11.7	66.2±9.9	0.287
Height (cm)	159.5±5.6	162.1±4.1	0.209
BMI (kg/m^2)	24.1±4.1	25.3±4.3	0.483
Gestational week	8.1±0.8	6.0±0.6	**<0.001**

Bold indicates a statistically significant difference.

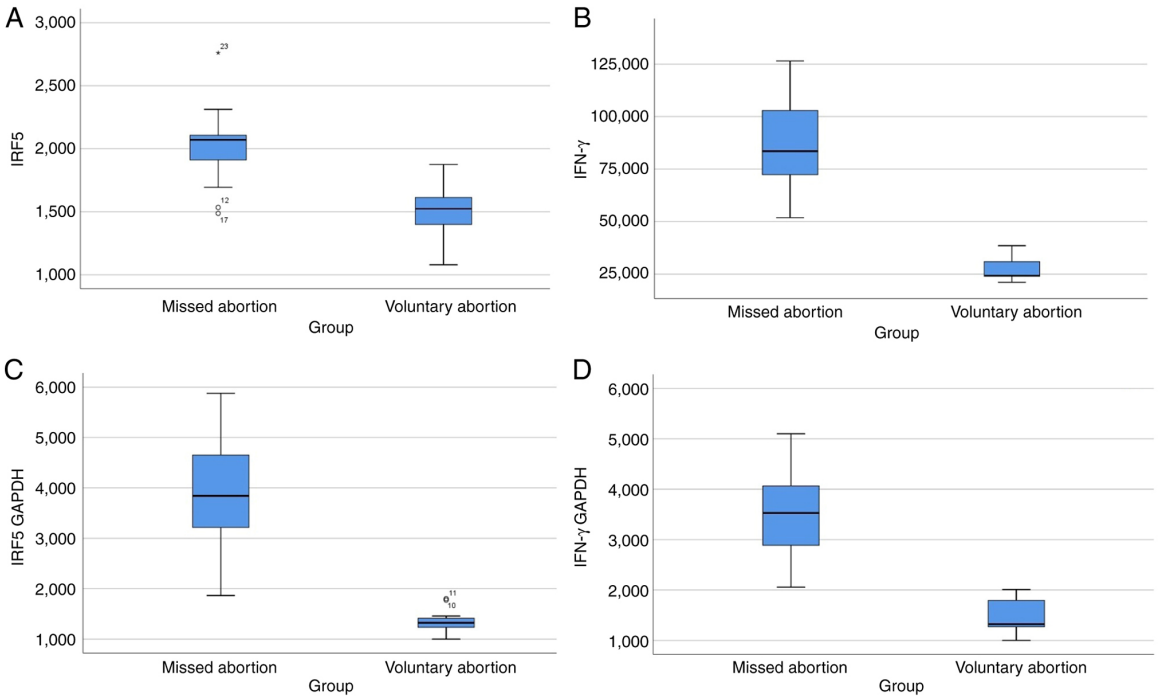

Figure 1. Protein and gene expression levels of IRF5 and IFN-γ in missed abortion and voluntary abortion groups. Boxplots showing the comparisons of the mean (A) IRF5 and (B) IFN-γ protein levels and (C) IRF5 and (D) IFN-γ gene expression levels in the missed abortion and voluntary abortion groups. IRF5, interferon regulatory factor 5; IFN-γ, interferon-γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

size was considered as 0.2, the type 1 error as 0.05, and the power as 0.8.

The statistical analysis was performed using SPSS 25.0 (IBM, Inc.) package software. Descriptive data were expressed as number and percentage. Normality of the continuous variables was confirmed with the Kolmogorov-Smirnov Test. Differences between groups in terms of continuous variables were studied with the Student's t-test. The differences between the groups in terms of median values of the non-normally distributed variables were analyzed with the Mann-Whitney U test. The correlation between continuous variables was evaluated with Spearman correlation analysis. The capacity of IFN-γ to predict missed abortion was analyzed using receiver operating characteristic (ROC) curve analysis. Logistic regression analysis was used to determine odds ratio for variables in terms of predicting missed abortion. The results were evaluated at 95% confidence interval and P<0.05 values were considered to indicate statistically significant differences. Box-and-whisker plots were used to present quartiles, minimum and maximum values, medians and outliers of the groups for comparison.

Results

The mean age of the pregnant women was 29.4±5.1 (age ranges, 19-39 for the voluntary abortion group; and 23-36 for the missed abortion group) (Table I). The mean IFN-γ (86.5 vs. 27.3 pg/mg protein; P<0.001) and IRF5 (2.0 vs. 1.5 ng/mg protein; P<0.001) levels were found to be significantly higher in pregnant women with missed abortion compared with the voluntary abortion group (Table II and Fig. 1).

The increases in the mean IFN-γ/GAPDH (3.5 vs. 1.5-fold increase; P<0.001) and IRF5/GAPDH (3.9 vs. 1.4-fold increase; P<0.001) gene expression levels were significantly higher in

Table II. Comparison of the mean protein and gene expression levels of IFN-γ and IRF5 in decidual tissues between the groups.

Groups	Missed abortion (n=13)	Voluntary abortion (n=11)	P-value
IFN-γ (pg/mg protein)	86.5±22.9	27.3±5.3	<0.001[a]
IRF5 (ng/mg protein)	2.0±0.3	1.5±0.2	<0.001[a]
IFN-γ/GAPDH gene expression (fold increase)	3.5±0.9	1.5±0.4	<0.001[a]
IRF5/GAPDH gene expression (fold increase)	3.9±1.2	1.4±0.3	<0.001[a]

[a]P<0.05. IFN-γ, interferon-γ; IRF5, interferon regulatory factor 5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table III. Correlation analysis.

		Missed abortion			Voluntary abortion		
Groups		IRF5	IFN-γ	IRF5/GAPDH	IRF5	IFN-γ	IRF5/GAPDH
IFN-γ	r	0.286			0.555		
	P-value	0.344			0.077		
IRF5/GAPDH	r	0.324	0.489		-0.164	-0.527	
	P-value	0.280	0.090		0.631	0.096	
IFN-γ/GAPDH	r	0.489	0.440	0.478	0.273	0.027	0.518
	P-value	0.090	0.133	0.098	0.417	0.937	0.102

IFN-γ, interferon-γ; IRF5, interferon regulatory factor 5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table IV. Logistic regression analysis.

Parameters	S.E.	Wald	df	P-value	Exp(B)
Univariate					
IRF5	2.922	6.512	1	0.011	0.001
IFN-γ	349.663	0.000	1	0.994	0.088
Gestational week	7057.031	0.000	1	0.998	0.000
Multivariate					
IRF5	132916.620	0.000	1	>0.999	1183E+15
IFN-γ	1812.911	0.000	1	0.999	0.126
Gestational week	91895.750	0.000	1	>0.999	0.000
Constant	327262.188	0.000	1	>0.999	1306E+37

S.E., standard error; df, degree of freedom; Exp(B), odds ratio; IRF5, interferon regulatory factor 5; IFN-γ, interferon-γ.

the tissues of pregnant women with missed abortion than in the voluntary abortion group (Table II and Fig. 1).

No significant correlation was identified between IFN-γ and IRF5 levels in the correlation analyses performed separately in the missed abortion and control groups (for all P>0.05; Table III). Logistic regression analysis revealed a significant association between IRF5 and missed abortion, as revealed in Table IV (P=0.011), however, IFN-γ and gestational week were not found to be directly related with missed abortion (P>0.05 for both) (Table IV).

ROC analysis revealed that a threshold value of 45.2 pg/mg protein for IFN-γ had a sensitivity of 100% and specificity of 100% in determination of missed abortion [area under curve (AUC), 1.0; P<0.001; lower bound, 1.0; and upper bound, 1.0) (Fig. 2A). A threshold value of 1.7 ng/mg protein for IFN-γ had a sensitivity of 84.6% and specificity of 90.9% in determination of missed abortion (AUC, 0.909; P=0.001; lower bound, 0.787; and upper bound, 1.0) (Fig. 2B).

Discussion

Some physiological regulations, such as changes in cytokine production, occur in the immune response in order to prevent rejection of the fetal allograft during pregnancy. However,

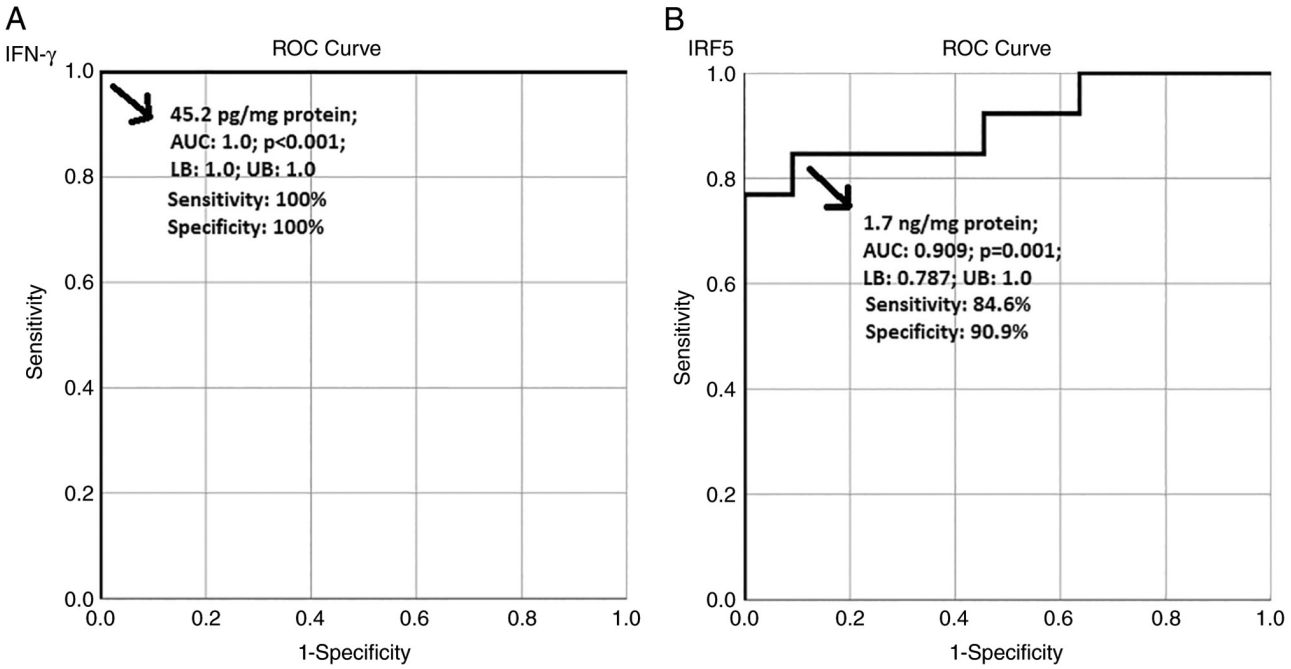

Figure 2. ROC analysis of IFN-γ and IRF5. (A) ROC analysis revealed that a threshold value of 45.2 pg/mg protein for IFN-γ had a sensitivity of 100% and a specificity of 100% in determination of missed abortion (AUC, 1.0; P<0.001; LB, 1.0; and UB, 1.0). (B) A threshold value of 1.7 ng/mg protein for IRF5 had a sensitivity of 84.6% and a specificity of 90.9% in determination of missed abortion (AUC, 0.909; P=0.001; LB, 0.787; and UB, 1.0). ROC, receiver operating characteristic; IFN-γ, interferon-γ; IRF5, interferon regulatory factor 5; AUC, area under curve; LB, lower bound; UB, upper bound.

complications such as early termination of pregnancy, including missed abortion, can be observed as a result of some deterioration in the immune response due to numerous factors including dysregulation of a cytokine (21). In the present study, it was demonstrated that IFN-γ and IRF5, which are among the molecules involved in the immune response, may be associated with missed abortion.

IFN-γ is an immune system molecule with a wide variety of functions, mostly associated with helper T cells. IFN-γ levels are increased in numerous infectious and autoimmune diseases and cancers. Some variants of IFN-γ have been reported to be associated with spontaneous preterm birth (10,11).

Some studies have shown that IFN-γ levels, collected from peripheral blood samples, are significantly higher in pregnant women with recurrent spontaneous abortion than in the control group (22,23). Xiao *et al* (22) revealed that IFN-γ levels collected from peripheral blood samples were significantly higher in the recurrent abortion group in comparison with the control group (27.8 ng/l vs. 18.4 ng/l). Peng *et al* (23) reported that the IFN-γ/IL-4 ratio collected from peripheral blood samples, was significantly higher in the unexplained recurrent abortion group than that of the control group. Gharesi-Fard *et al* (24) demonstrated that IFN-γ levels assessed in the culture supernatants were significantly higher in the recurrect spontaneous abortion group in comparison to the unsuccessful pregnancy group (412.8 pg/ml vs. 254.3 pg/ml). These studies support the association of IFN-γ with abortion, however these levels (as numerical values) cannot be compared with our results due to the difference of the samples and the difference of units (our results were reported as 86.5 vs. 27.3 pg/mg protein) among the studies. In an experimental study, it was reported that IFN-γ induced the production of some cytokines, such as CXCL12 or IL-6, that have been revealed to cause abortion (25). In another study, it was revealed that the level of IFN-γ was significantly increased in decidual cells in the first trimester and therefore its association with spontaneous abortion should be investigated (26). In an experimental study, IFN-γ released during placental development was identified to induce abortion (27). In another study, IFN-γ was shown to cause abortion by modulating some receptors, such as Ly-49A or Ly-49G2, in natural killer cells, and it was revealed that IFN-γ could be used as a prognostic marker for embryo abortion (28). In addition, in an experimental study, it was determined that high-dose IFN-γ induced abortion by suppressing regulatory T cells (12). Although these data indicated an association between IFN-γ and abortion, they could not clearly reveal whether there is an association between IFN-γ and missed abortion. In our study, the mean IFN-γ level was found to be significantly higher in pregnant women with missed abortion compared with the voluntary abortion group (86.5 vs. 27.3 pg/mg protein). In addition, in the present study, the mean increase in IFN-γ/GAPDH gene expression in the tissues of pregnant women with missed abortion was found to be significantly higher than in the voluntary abortion group (3.5 vs. 1.5 fold increase). In addition, for the first time in the literature, to the best of our knowledge, ROC analysis revealed a clear threshold value for IFN-γ in determination of missed abortion with sensitivity and specificity values both of which were 100%. These findings reveal that the protein level and gene expression level of IFN-γ in the tissue were significantly increased in cases of missed abortion, that this molecule may be directly related to missed abortion, that the protein level of IFN-γ and gene expression of IFN-γ play an important role in implantation and pregnancy, and that this molecule can be used as a marker for implantation.

IRF5 plays an important role in antiviral and inflammatory response processes and plays a key role in the regulation of dendritic cells, macrophages and B cells (14,15). It has been shown that polymorphism in IRF5 can lead to some autoimmune diseases and recurrent spontaneous abortions (13,16). However, it has been indicated that the mechanism of the functional role for IRF5 in pregnancy and abortion needs to be explored (16). In addition, there is no clear data revealing the association between IRF5 and missed abortion, to date. In the present study, the mean protein level of IRF5 in pregnant women with missed abortion was found to be significantly higher than in the voluntary abortion group (2.0 vs. 1.5 ng/mg protein). Furthermore, the mean IRF5 gene expession level in pregnant women with missed abortion was found to be significantly higher than in the voluntary abortion group (3.9 vs. 1.4 fold increase). In addition, ROC analysis revealed a very reliable threshold value for IRF5 in determination of missed abortion with sensitivity and specificity values over 80%. Moreover, logistic regression analysis showed a significant association between IRF5 and missed abortion. These findings demonstrated that the protein level of IRF5 and the gene expression level of IRF5 in the tissue, were significantly increased in the cases of missed abortion, that this molecule may be directly related to missed abortion and can be used as a predictive marker in the poor implantation process of the embryo.

In the correlation analyses, it was determined that the protein levels and increase in the gene expression levels of IFN-γ and IRF5 were not significantly correlated with each other. This may be due to the fact that the number of participants in the groups was not high.

As a limitation of the present study, since it was planned as a cohort study, pregnant women were not followed-up for a long period of time after abortion and long-term changes in the levels of these molecules were not examined. In addition, although the number of pregnant women included in the study was sufficient for statistical analysis, the small number of participants was another limitation of the study.

The findings obtained in the present study, to the best of our knowledge, for the first time in literature, reveal that IFN-γ and IRF5 may be associated with missed abortion, that the protein and gene expression levels of IFN-γ and IRF5 are significantly increased in the cases of missed abortion, and that IFN-γ and IRF5 play an important role in placental invasion and pregnancy and can be used as markers for endometrial implantation. Further large-scale studies are required to provide more insight into the roles of IFN-γ and IRF5 in missed abortion.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DH designed the study and wrote the manuscript. SG collected and analyzed data. OC analyzed the data and wrote and edited the manuscript. DH and OC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval date and number: 12.08.21/28) by the Ethics Committee of Yeni Yüzyil University (Istanbul, Turkey). All of the participants enrolled in the study provided signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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