

HOXA1 expression in placentas of woman with fetal growth restriction

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Abstract

Objective: In this study, we examined the HOXA1 expression in the placentas of women diagnosed with fetal growth restriction (IUGR) by immunoeexpression and in silico analysis.

Methods: Placenta samples from 40 control (healthy) and 40 pregnant women diagnosed with IUGR were included in the study. The samples were fixed in zinc-formal and embedded in paraffin. Demographic information of the patients was recorded. Sections taken from paraffin blocks were analyzed by Hematoxylin-Eosin and HOXA1 immunostaining. The protein-protein interaction network of HOXA1 was constructed using the STRING database and analyzed with Cytoscape. The route description was made with the DAVID web tool.

Results: In histopathological examination, intense fibrin accumulation, structural degeneration of placental components, congestion, dilatation and increased syncytial nodes were observed in the IUGR group compared to the control group. HOXA1 gene expression was significantly increased in the IUGR group. The HOXA1 PPI network contained 201 nodes and 3876 edges. MCODE analysis identified 8 modules, the highest scoring module was related to the “Systemic lupus erythematosus”, “Alcoholism” and “Neutrophil extracellular trap formation” pathways.

Conclusion: With immunoeexpression and in silico analysis, we showed HOXA1 is a player of immune pathways, tissue development, and placental regulation, suggesting potential research avenues in understanding IUGR mechanisms.

Keywords: Homeobox genes, HOXA1, fetal growth restriction, placenta

Introduction

In the intrauterine period, the evaluation of fetal health. Intrauterine growth restriction (IUGR), is a condition in which a fetus falls below its normal expected growth potential.^[1] Common risk factors for IUGR development encompass maternal issues such as hypertension, diabetes, heart and lung diseases, anemia, poor nutrition, smoking, and drug use; fetal issues like genetic disorders including aneuploidy, birth defects, fetal infections, and multiple pregnancies; and placental issues such as placental insufficiency, infarction, and mosaicism.^[2] In high-income countries, IUGR affects 3% to 9% of pregnancies, while in developing countries, this figure can be as high as 25%.^[2] Maternal health, nutrition, smoking, and substance use, along with fetal and placental factors,

all play roles in fetal growth. Placental insufficiency is a common underlying cause of IUGR in otherwise healthy fetuses, where the placenta's dysfunction limits the fetus from achieving its natural growth potential.^[3] The most common placental conditions are changes in uteroplacental and fetal-placental circulation. In the majority of IUGR cases, there is a decrease in maternal uteroplacental blood flow due to insufficient or incomplete invasion of the spiral arteries in the placental bed by trophoblasts.^[4] Several genetic and immunological factors influence placental development, crucial for proper angiogenesis.^[5] Vascular Endothelial Growth Factor (VEGF), Endothelial Nitric Oxide Synthase 3 (NOS3) and Hypoxia-Inducible Factor 1 Alpha (HIF1A) are known to play a role in angiogenesis and vasculogenesis. Placental infar-

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ction, widespread and irregular villous inflammations are the most common placental pathologies associated with IUGR in the literature.^[6-8]

HOX genes constitute a group of transcription factors containing a conserved homeodomain, initially discovered in the fruit fly *Drosophila*.^[9] These genes are pivotal during embryonic development, influencing the organization of body structures along the head-to-tail axis and participating in early morphogenetic processes.^[10] High expression of HOXA7 gene were detected in the placentas of women with preeclamptic patients.^[11] In another similar study, placental HOXA13 hypermethylation was shown in preeclamptic placental tissues and the relationship between HOXA 13 and preeclampsia was noted.^[12]

HOXA genes are essential for proper development of the fetal vascular system in the placenta.^[13] There is little information these genes might contribute to the development of IUGR associated with placental inadequacy. This study aimed to investigate the expression levels of HOXA1 gene family members within the placenta and its potential distribution across the placental decidual layer.

Methods

Study design

Ethical approval was received from Dicle University Faculty of Medicine Non-Interventional Studies Ethics Committee (Date: 14/02/2024, Issue: 77). The placentas of 40 control (healthy) women and 40 women diagnosed with IUGR were included in the study. IUGR was defined as fetuses with an estimated weight below the 10th percentile for gestational age.^[14] Demographic characteristics, ultrasonographic examinations and pregnancy outcomes of each patient were recorded. Blood samples were obtained prior to delivery and sent for laboratory analysis for various parameters. All participants were fully informed about the study and provided their consent to participate, documented by signing a written consent form. Multiple pregnancies, pregnant women under 18 years of age and over 40 years of age, BMI>35, those with chronic, mental and physical diseases, those with serious hepatic, renal, gastrointestinal chronic/acute inflammatory disease, hyperthyroidism, hypothyroidism, hypertension, Diabetes Mellitus, those with a history of malignancy, pregnant women with macrosomia fetuses were not included in the study. IUGR group; The single IUGR group consisted of pregnant women who were not diagnosed with anything other than IUGR during their pregnancy follow-ups, had an estimated fetal weight (EFW) below the 3rd percentile^[15,16], and met the exclusion criteria who were followed up regularly. Control group; No problems in pregnancy follow-up, regular follow-up, EFW 10-90. it created sing-

leton pregnant women who were within the percentiles and met the exclusion criteria.

Histological tissue preparation Placentas were obtained from Dicle University Faculty of Medicine, Department of Gynecology and Obstetrics. For histological analysis, placental tissue samples were excised to an appropriate size and fixed in zinc-formalin. After fixation, the tissues were passed through tap water, increasing alcohol series and xylene stages and were embedded in paraffin blocks. 5 µm sections were cut from paraffin blocks. Sections were stained with Hematoxylin-Eosin and immunostaining of HOXA1.

Immunohistochemical Staining Placental sections retrieved from paraffin blocks were transferred onto poly-lysine-coated slides using a double boiler set at 37°C. Excess paraffin on the slides was melted off by incubating them in an oven maintained at 58-62°C for 6 hours. The sections underwent deparaffinization in xylene and were gradually rehydrated through a series of decreasing alcohol concentrations, followed by a rinse in distilled water for 5 minutes. Hydrogen peroxide was applied to the sections and allowed to react for 20 minutes, after which they were washed with PBS and treated with Ultra V Block solution for 7 minutes. Subsequently, the sections were incubated overnight at +4°C with the primary antibody against HOXA1 (sc-293257, Santa Cruz, USA). Following overnight incubation, the sections were washed with PBS and exposed to a biotin-conjugated secondary antibody for 14 minutes. This was followed by a 15-minute incubation with streptavidin-peroxidase. Diaminobenzidine (DAB) was then applied to visualize the antibody-antigen reaction, and the sections were observed under a light microscope. After rinsing with PBS three times for 15 minutes each, the sections were counterstained with Harris hematoxylin. Finally, coverslips were mounted, and the slides were examined using a Zeiss Imager A2 photomicroscope. Image processing and measurements were conducted using ImageJ software.

ImageJ analysis The staining intensity of HOXA1 expression was measured with Image J software (version 1.53, <http://imagej.nih.gov/ij>). The measurement was calculated according to the method of Crowe et al..^[17] Ten fields from each sample per group were analyzed and quantification was recorded. In the samples, the brown color represents positive expression of the antibody of interest, while the blue color represents negative expression of the antibody of interest. Signal intensity (expression) from an area was calculated by dividing the intensity of the antibody of interest by the entire area of the sample. The staining area/whole area value was calculated for each

sample from ten fields. A mean value for the groups was measured and analyzed for semi-quantitative immunohistochemistry scoring.^[18]

Module and Pathway Analysis of HOXA1

After examining the expression of HOXA1 in the placentas of IUGR, we aimed to elucidate the potential mechanisms by which HOXA1 may exert its effects. To achieve this, we first constructed a protein-protein interaction (PPI) network for HOXA1 using the STRING database (additional interactor number: 200). This network was then imported into Cytoscape software (version 3.10.2). Using the Molecular Complex Detection (MCODE) plug-in, we performed module detection within the PPI network. The MCODE parameters used for the analysis were; degree cutoff: 2, node score cutoff: 0.2, K-core: 2, and maximum depth: 100. MCODE applies specific scoring and parameter thresholds to identify densely connected regions in the PPI network.^[19] This method of module detection aids in identifying significant HOXA1 subnetworks that could represent functional units or protein complexes relevant to IUGR. Next, pathway annotation for each module was conducted using the DAVID web-based tool which provides gene visualization, annotation, and integrated discovery, facilitating a thorough understanding of the biological roles of individual genes.^[20] Annotation was performed on the category with the highest percentage of involved genes, and the top five pathways with significant associations were listed. Pathways with a p-value less than 0.05 were evaluated as significant.

Statistical analysis Statistical analysis was performed using IBM SPSS 25.0 software (IBM, Armonk, New York, USA). We performed a Shapiro-Wilk test to assess the normality of our data distributions. The results indicated that the data did not follow a normal distribution. Consequently, we opted for non-parametric tests, which do not require the assumption of normality, to analyze our data. The Mann-Whitney U test (a non-parametric test) was employed to compare two independent groups (control and IUGR) since our data did not meet the normality assumption. This test is suitable for continuous and ordinal data, which is consistent with the nature of our measurements (immunoexpression levels, demographic characteristics, and ultrasonographic measurements). To quantify the magnitude of the observed differences, we calculated the effect size using Cohen's criteria^[21] via G*power software, version 3.1. This approach allows us to determine the practical significance of our findings beyond mere statistical significance with medium Effect ($d = 0.57$), representing a moderate, visible difference. Significance level was considered for p values <0.05 .

Results

Demographic findings Demographic parameters, ultrasonographic measurements and pregnancy outcomes of control and pregnant women diagnosed with IUGR were listed in Table 1. Fetal measurements and birth weights are significantly lower in pregnant women with IUGR.

Table 1. Demographic parameters, ultrasonographic measurements and pregnancy outcomes of the patients. BPD: Biparietal diameter measurement, AC: Abdominal circumference, EFW: Estimated fetal weight, FL: Femur length

Parameters	Control Group (n=40)	IUGR group (n=40)	Significance (p-value)
Maternal age, years	29.27 ± 5.23	28.74 ± 6.20	0.684
Body mass index, kg/m ²	29.97±5.52	28.51±5.62	0.189
Gravida, n	3 ± 1	2 ± 1	0.084
Parity, n	1 ± 1	0 ± 1	0.015
Gestational week at delivery	39.2 ± 1.1	37.6 ± 0.66	<0.001
BPD, mm	90 ± 4.3	85.6 ± 4	<0.001
AC, mm	320.2 ± 20.8	287.2 ± 16.3	<0.001
EFW, gr	2846.1 ± 481.1	2210.6 ± 342.4	<0.001
FL, mm	69.3 ± 4.3	65.9 ± 4.6	0.002
Birth weight, g	3436.3 ± 428	2442.4 ± 206.6	<0.001
1- min APGAR score	8.8 ± 0.48	6.47 ± 1.41	0.001
5- min APGAR score	9.80 ± 0.48	8.23 ± 0.9	0.001

Histopathological findings Figure 1 shows Hematoxylin Eosin staining transverse sections of placentas taken from patients belonging to the groups. In the placentas of the control group, the chorionic villi generally maintained their structural integrity. Cytotrophoblast cells, syncytiotrophoblast cells and villous stromal regions were evident. No pathology was observed in the vessels. Fibrin deposition and the number of syncytial nodes were low (Figure 1A). An intense fibrin deposition was observed in the placentas of patients in the IUGR group compared to the control group. Structural integrity of the villi was impaired and most of them were degenerated. Increased congestion and dilatation in floating villi were present. The number of syncytial nodes was increased compared to the control (Figure 1B). In histological scoring between groups, it was observed that villous degeneration, dilatation/congestion, syncytial knot, and fibrin deposition parameters increased significantly in the IUGR group compared to the control group (Figure 1C). Our findings showed in IUGR placentas, there are notable disruptions in villous architecture and increased pathological changes such as fibrin deposition and syncytial knot formation. These alterations likely contribute to the impaired placental function seen in IUGR, which may adversely affect

fetal growth and development. The significant differences observed in histopathological parameters between the control and IUGR groups underscore the potential impact of these pathological changes on the clinical outcomes of pregnancies complicated by IUGR.

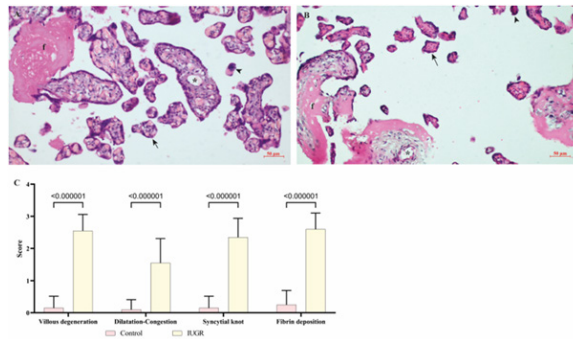


Fig 1. Hematoxylin Eosin staining and scoring of placental tissues. A) Control group; B) IUGR group, C) Histopathological scoring, arrow: chorionic villus, arrowhead: syncytial node, f: fibrin deposition, asterisk: chorionic capillary, Bar: 50 μ m, Magnification: 20X

Placental tissues in the groups were stained with HOXA1 antibody and the results were shown in Figure 2. Although HOXA1 immune reaction was generally negative in cytotrophoblasts, syncytiotrophoblasts and syncytial nodes, weak expression was observed in villous connective tissue (Figure 2A). In the IUGR group, HOXA1 expression significantly increased compared to the control group. Intense HOXA1 expression was observed in fibrin deposition, the trophoblastic layer and syncytial nodes. Intense HOXA1 expression was also observed in the inflammatory cells in the intervillous area. HOXA1 immune reactivity was negative in some villous connective tissue cells area (Figure 2B). Placental sections in the groups were analyzed by semi-quantitative measurement, and a significant increase in HOXA1 expression was noted in the IUGR group compared to the control group (Figure 2C). According to our findings, HOXA1 plays a significant role in the pathophysiology of IUGR. The increased expression of HOXA1 in the IUGR placentas, particularly in areas associated with pathological changes such as fibrin deposition and inflammatory infiltration, indicates that HOXA1 may contribute to the impaired placental function observed in IUGR. This differential expression of HOXA1 highlights its potential as a biomarker for placental dysfunction and provides insight into the molecular mechanisms underlying IUGR, which could inform future therapeutic strategies.

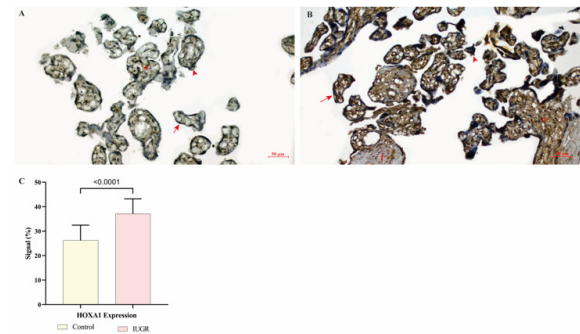


Fig 2. HOXA1 immunostaining and scoring of placental tissues. A) Control group; B) IUGR group, C) Semi-quantitative scoring, arrow: trophoblastic layer, arrowhead: syncytial node, f: fibrin deposition, asterisk: villous stroma, star: intervillous area, Bar: 50 μ m, Magnification: 20X

In silico findings

The STRING analysis of the HOXA1 PPI network resulted in 201 nodes and 3876 edges. Using the MCODE app in Cytoscape software, the module analysis of the HOXA1 PPI network identified 8 clustering modules, ranked by their computed score values. The scores for modules 1 through 8 were 60.688, 9, 7.133, 6.5, 4.75, 3.75, 3, and 2.8, respectively. The number of nodes in each module was 65, 9, 31, 9, 9, 9, 3, and 6, respectively (Figure 3). These findings provide a detailed view of the HOXA1 interaction landscape and highlight key clusters of interactions that may be relevant to IUGR. The high score of modules 1, which contains 65 nodes, suggests a highly interconnected network of proteins interacting with HOXA1, indicating a central role in various biological processes. This module's prominence may reflect crucial pathways and molecular mechanisms disrupted in IUGR. Modules with lower scores but still significant interactions, such as modules 2 and 3, point to additional pathways and processes potentially involved in the pathology of IUGR.

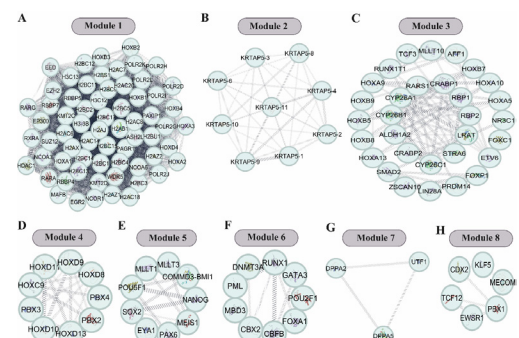


Fig 3. The eight most significant clustering modules identified in the HOXA1 PPI networks are shown. (A) Module 1; (B) Module 2; (C) Module 3; (D) Module 4; (E) Module 5

Pathway analysis for each module revealed that module 1 was predominantly related to “Systemic lupus erythematosus”, “Alcoholism”, “Neutrophil extracellular trap formation”; module 2 was associated with “Keratinization” and “Developmental Biology”; module 3 was linked to the “Signaling by Retinoic Acid (RA)” and RA biosynthesis pathway”; module 5 was connected to the “Signaling pathways regulating pluripotency of stem cells” and “Transcriptional misregulation in cancer”; and module 6 was related to the “Acute myeloid leukemia” pathway. No annotations were obtained for modules 4, 7, and 8 (Table 2). Our results suggest that HOXA1 may influence a wide range of biological processes and pathways in the pathophysiology of IUGR.

Table 2. Pathway Annotations for the modules of the HOXA1 PPI Network

Category	Module 1 Pathways	Count	p value
KEGG	Systemic lupus erythematosus	36	7,60E-49
KEGG	Alcoholism	37	2,20E-45
KEGG	Neutrophil extracellular trap formation	37	4,10E-45
KEGG	Viral carcinogenesis	21	2,80E-18
KEGG	ATP-dependent chromatin remodeling	16	1,50E-15
Category	Module 2 Pathways	Count	p value
REACTOME	Keratinization	9	1,80E-14
REACTOME	Developmental Biology	9	3,00E-08
Category	Module 3 Pathways	Count	p value
REACTOME	Signaling by Retinoic Acid	6	7,90E-09
REACTOME	RA biosynthesis pathway	5	4,50E-08
REACTOME	Transcriptional regulation of pluripotent stem cells	5	6,50E-08
REACTOME	Vitamins	3	4,20E-05
REACTOME	Signaling by Nuclear Receptors	6	1,20E-04
Category	Module 5 Pathways	Count	p value
KEGG	Signaling pathways regulating pluripotency of stem cells	5	2,40E-06
KEGG	Transcriptional misregulation in cancer	5	7,90E-06
Category	Module 6 Pathways	Count	p value
KEGG	Acute myeloid leukemia	2	4,60E-02

Discussion

In IUGR, the placenta is on average half the size of the normal placenta, and there are malformations in the villous tree and placental vascular network, as well as changes in the function and development of trophoblasts. In their study, Novac et al.^[5] observed placental infarction, increased syncytial nodes, and intervillous fibrinoid accumulation in the placentas of IUGR pregnancies. Sun et al.^[20] demonstrated reduced proliferation and increased apoptotic death of trophoblasts in IUGR placentas. Mifsud defined placental infarction due to maternal vascular insufficient perfusion as an area where the intervillous space is reduced and villous aggregation occurs. Degeneration of syncytiotrophoblast cells was demonstrated by pyknosis, karyorrhexis, and the presence of fibrin-like eosinophilic amorphous material. He also stated that in vessels with insufficient extravillous trophoblast invasion, the arterial walls are irregular and necrotic, and fibrin-like eosinophilic material and numerous lipid-laden macrophages are present.^[21] Our findings, consistent with these studies, show that placentas in the control group preserved their normal structural features, while placentas in the IUGR group exhibited significant structural deteriorations. Dense fibrin accumulation, villous degeneration, increased number of syncytial nodes and dilatation/congestion of vessels observed in the IUGR group indicate that the functional capacity of the placenta decreases and placental insufficiency increases. These changes may negatively affect the adverse effects of IUGR on placental functions and therefore the growth and development of the fetus.

HOX genes are effective in various physiological and pathological processes. It plays a crucial role in the correct formation of the uterus in embryonic development and is essential for processes such as implantation, decidualization, and immune modulation. As a result, alterations or mutations in HOXA expression can lead to irregular uterine morphology, impairments in implantation, and issues with fertility.^[22] Alfaidy and colleagues have shown that homeobox genes regulated by growth factors such as EG-VEGF control angiogenesis during pregnancy by regulating extravillous trophoblasts, which are particularly involved in extravillous angiogenesis.^[23] Murti showed that trophoblast functions are affected as a result of the homeobox genes being affected in the IUGR placenta.^[24] In their research, Novakovic et al. demonstrated that HOXA10 RNA levels showed a notable decrease alongside increased DNA methylation in second and third trimester placentas compared to the first trimester. They linked these findings to the regulation of HOXA10 gene activity during pregnancy, suggesting its critical role in placental development. Additionally, they observed a

reduction in the staining intensity of HOXA10 protein in term placental tissues. This suggests that diminished HOXA10 expression, localized primarily in the syncytiotrophoblast, could potentially impact syncytiotrophoblast function or trophoblast differentiation.^[25]

According to immunohistochemical findings in our study, placentas in the control group exhibit mild HOXA1 expression, while placentas in the IUGR group show a significant increase in HOXA1. This increase suggests that HOXA1 may play an important role in placental pathology in IUGR. Increased expression of HOXA1 may lead to changes in processes such as cell proliferation, differentiation, and apoptosis in the placenta, which may affect the functional capacity and structural integrity of the placenta. To further explore the role of HOXA1 in IUGR, we performed in silico analysis, identifying eight distinct modules within the HOXA1 PPI network that could be relevant to IUGR. Module 1 exhibited the highest score among all identified modules, indicating a greater degree of connectivity among the proteins within this module. This suggests that these proteins are more likely to participate in shared biological processes and functional interactions.^[26] Therefore, Module 1 provides valuable annotation data for elucidating the potential mechanisms underlying the association between HOXA1 and IUGR. Module 1 was predominantly associated with pathways including systemic lupus erythematosus, alcoholism, and neutrophil extracellular trap formation. These associations suggest a strong link between immune response pathways and IUGR, indicating that regulation of the immune system might play a crucial role in the development of this condition. Previous research has highlighted the importance of maintaining a suppressed pro-inflammatory Th1/Th17 immunity for the successful progression of pregnancy. However, in instances of IUGR, there appears to be an amplified inflammatory reaction, yet the mechanisms orchestrating this abnormality remain elusive. One of the studies has indicated the potential role of FasL+ exosomes in modulating NF- κ B p65 within T-cells during pregnancy. It proposed that decreased exosome production might play a part in the disruption of p65 and the ensuing inflammation implicated in the development of IUGR.^[27] Consequently, it is conceivable that immune mechanisms correlated with HOXA1 may have an influence on IUGR. Beyond module 1, pathway annotation data obtained from other modules indicate that HOXA1 may play a role in tissue development, RA-dependent pathways, and cell growth and differentiation pathways. The literature highlights the importance of homeobox genes in placental development^[24], the regulatory role of RA in the placenta^[28], and the critical regulatory mechanisms involved in placental

vascular proliferation, invasion, and differentiation.^[29] Therefore, these findings are consistent with the functional annotation data obtained for these modules and may potentially contribute to the development of IUGR.

Conclusion

In conclusion, upregulation of HOXA1 expression in placental tissues in the IUGR group indicates that this molecule may be an important biomarker in the pathophysiology of IUGR and can be considered as a potential therapeutic target to be targeted in the management of IUGR. In silico analysis supported the significant role of HOXA1 in IUGR, particularly through its involvement in immune response pathways. These insights provide a comprehensive perspective of HOXA1's potential mechanisms in IUGR and highlight the importance of further research to elucidate these pathways and the role of HOXA1 in the diagnosis and treatment of IUGR.

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