Research Article / Araştırma Makalesi

Homeobox A1 Expression in Diabetic Pregnancy: Immunohistochemical and Computational Perspectives

Diyabetik Gebelikte Homeobox A1 Ekspresyonu: İmmünohistokimyasal ve Kompütasyonel Perspektifler

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Abstract

Background: This study aimed to evaluate the expression of Homeobox A Cluster 1 (HOXA1) protein in placentas from patients with gestational diabetes mellitus (GDM) using immunohistochemical techniques and to investigate the role of HOXA1 in GDM-associated biological mechanisms through computational analyses.
Materials and Methods: A total of 80 participants, including 40 healthy pregnant women and 40 women diagnosed with GDM, were enrolled. Placental tissues were examined histologically using hematoxylin-eosin and HOXA1 immunostaining. Additionally, HOXA1 and GDM-related proteins were retrieved from the STRING database and analyzed using Cytoscape software to identify shared interaction networks, determine node centrality (degree, closeness, betweenness), and perform Gene Ontology (GO) molecular function analyses.
Results: Histopathological analysis revealed significant increases in villous degeneration, fibrin deposition, hemorrhage, syncytial knot formation, and leukocyte infiltration in the GDM group. Immunohistochemical analysis demonstrated a marked upregulation of HOXA1 protein expression in the GDM group compared to controls. Computational analyses identified 15 shared proteins within the HOXA1 and GDM networks, with H3C12, H3-3B, H3C13, and ESR1 emerging as central regulatory proteins. GO analysis indicated that these proteins are primarily involved in chromatin organization, DNA binding, epigenetic regulation, and protein–protein interactions.
Conclusions: GDM induces significant histopathological and molecular changes in placental tissues, associated with increased HOXA1 protein expression. HOXA1 may play a critical role in the molecular mechanisms underlying GDM-related placental dysfunction and could serve as a potential biomarker and therapeutic target.

Keywords: HOXA1, gestational diabetes mellitus, placenta, topological analysis, bioinformatical analysis

Öz

Amaç: Bu çalışmada, gestasyonel diyabet mellitus (GDM) hastalarına ait plasentalarda Homeobox A Cluster 1 (HOXA1) proteininin ekspresyonu immünohistokimyasal yöntemlerle değerlendirilmiş ve HOXA1'in GDM ile ilişkili biyolojik mekanizmalardaki rolü hesaplamalı analizlerle araştırılmıştır.
Materyal ve Metod: Çalışmaya 40 sağlıklı gebe ve 40 GDM tanılı gebe olmak üzere toplam 80 kadın dahil edilmiştir. Plasentalar, hematoksilen-eozin ve HOXA1 immün boyama yöntemleriyle histolojik olarak incelenmiştir. Ayrıca, HOXA1 ve GDM ile ilişkili proteinler STRING veri tabanından elde edilerek Cytoscape programı kullanılarak ortak etkileşim ağları analiz edilmiş, düğüm merkeziliği (degree, closeness, betweenness) belirlenmiş ve gen ontolojisi (GO) moleküler fonksiyon analizleri yapılmıştır.
Bulgular: Histopatolojik analizde GDM grubunda villöz dejenerasyon, fibrin birikimi, hemoraji, sinsityal düğüm oluşumu ve lökosit infiltrasyonunda anlamlı artış saptanmıştır. İmmünohistokimyasal analizde, HOXA1 ekspresyonu GDM grubunda kontrol grubuna kıyasla belirgin şekilde artmıştır. Hesaplamalı analizlerde, HOXA1 ve GDM ağlarının kesişiminde 15 ortak protein belirlenmiş, H3C12, H3-3B, H3C13 ve ESR1 proteinleri ağın en merkezi düzenleyici proteinleri olarak tanımlanmıştır. GO analizi, bu proteinlerin kromatin organizasyonu, DNA bağlanması, epigenetik düzenleme ve protein-protein etkileşimleri gibi temel moleküler süreçlerle ilişkili olduğunu göstermiştir.
Sonuç: GDM, plasental dokularda önemli histopatolojik ve moleküler değişikliklere yol açmakta, HOXA1 proteininin ekspresyonunda artış ile ilişkilendirilmektedir. HOXA1, GDM'ye bağlı plasental disfonksiyonun moleküler mekanizmalarında rol oynayabilecek potansiyel bir biyobelirteç ve hedef molekül olarak öne çıkmaktadır.

Anahtar Kelimeler: HOXA1, gestasyonel diyabetes mellitus, plasenta, topolojik analiz, biyoinformatik analiz.

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Introduction

Gestational diabetes mellitus (GDM) is a form of glucose intolerance that develops during pregnancy and typically resolves after childbirth. It arises due to hormonal alterations that impair pancreatic insulin production, leading to maternal hyperglycemia. Risk factors for GDM include obesity, advanced maternal age, family history of diabetes, and a previous history of GDM (1). The pathophysiology of GDM is multifactorial, encompassing β-cell dysfunction, chronic insulin resistance, oxidative stress, and neurohormonal imbalances(2, 3) . Elevated levels of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), produced by monocytes and macrophages, are known to impair insulin sensitivity, thereby promoting hyperglycemia and contributing to the development of GDM (4). If not properly managed, GDM can result in both maternal and fetal complications, including preeclampsia, macrosomia, neonatal hypoglycemia, and increased risk of type 2 diabetes in later life (5) .

The placenta is a key organ mediating nutrient and oxygen exchange between the mother and fetus, and its structural and functional integrity is critical for normal fetal development. In GDM pregnancies, placental histopathology frequently exhibits abnormalities such as villous edema, excessive fibrin deposition, hemorrhage, thickening of trophoblastic membranes, increased syncytial knot formation, and leukocyte infiltration(6-8). These structural alterations are thought to arise from impaired vascularization, abnormal angiogenesis, and inflammation induced by maternal hyperglycemia. Furthermore, studies have shown that GDM is associated with altered expression of genes and proteins involved in cell proliferation, apoptosis, immune signaling, and extracellular matrix remodeling(9, 10) . Despite these findings, the molecular mechanisms linking GDM to altered placental development remain incompletely defined.

HOX genes are a highly conserved family of transcription factors that regulate embryonic patterning, segmentation, and organogenesis. Originally characterized in Drosophila melanogaster, their mammalian counterparts play essential roles in cell fate determination, tissue differentiation, and spatial development during early embryogenesis (11, 12). Disruption of HOX gene expression has been implicated in various pathological conditions, including congenital malformations and cancers. Among these, HOXA1 is known to be a critical regulator of cell proliferation, differentiation, and migration. Recent studies have identified aberrant *HOXA1* expression in malignancies and suggested its involvement in abnormal tissue remodeling (13, 14). Although some HOX family members have been implicated in trophoblast differentiation and membrane formation, HOXA1 has not been comprehensively studied in the context of gestational diabetes.

Given its functional roles, dysregulated *HOXA1* expression may contribute to impaired placental development observed in GDM (7-9, 15, 16).

Therefore, the objective of this study was to investigate the expression and immunolocalization of HOXA1 protein in placental tissues from GDM and healthy pregnancies. In addition, we aimed to explore HOXA1-associated molecular mechanisms through computational protein–protein interaction and gene ontology analyses.

Materials and Methods

Patient selection and study design

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee for Non-Interventional Research of Dicle University Faculty of Medicine (Approval Date: 14/02/2024, No: 78). The study population included two groups: 40 pregnant women diagnosed with GDM and 40 healthy pregnant women as controls.

GDM was diagnosed based on the oral glucose tolerance test (OGTT) with 75 g of glucose performed between the 24th and 28th weeks of gestation. Inclusion criteria for the GDM group were fasting plasma glucose ≥92 mg/dL (5.1 mmol/L), ≥180 mg/dL (10.0 mmol/L) at 1 hour, and ≥153 mg/dL (8.5 mmol/L) at 2 hours post-glucose load, in accordance with established guidelines (10) .

Only singleton pregnancies resulting in delivery between 37 and 41 gestational weeks were included. Placental samples were collected immediately after delivery for histological analysis. The control group consisted of women without metabolic disorders who also delivered at term and had healthy placentas.

Exclusion criteria included pregestational diabetes, chronic hypertension, history of preeclampsia, autoimmune or chronic inflammatory disease, fetal anomalies, intrauterine growth restriction (IUGR), multiple pregnancies, placental insufficiency, or placental abruption. Participants with a history of alcohol consumption were also excluded. Additionally, samples with inadequate or excessive fixation compromising immunohistochemical analysis were not included.

Histological Tissue Processing

Placental samples were collected from the Department of Gynecology and Obstetrics and immediately fixed in zinc-formalin solution. After 24 hours of fixation, tissues were rinsed in tap water and dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, 96%, and absolute alcohol). Following xylene clearing, the tissues were embedded in paraffin blocks.

Hematoxylin and Eosin (H&E) Staining

Paraffin-embedded sections were incubated at 37°C, and residual paraffin was removed by placing the slides in an oven at 58–62°C for six hours. After mounting on slides, sections were rehydrated through descending ethanol concentrations and rinsed in distilled water. H&E staining was performed using standard protocols. After staining, sections were dehydrated, cover-slipped with mounting medium, and examined under a Zeiss Imager A2 photomicroscope. Representative images were captured for analysis.

Immunohistochemical Staining

Sections mounted on poly-lysine-coated slides were incubated at 58–62°C for six hours to remove residual paraffin. Following deparaffinization in xylene and rehydration through decreasing ethanol concentrations, the sections were rinsed in distilled water. Endogenous peroxidase activity was blocked using hydrogen peroxide for 20 minutes. After PBS washing, Ultra V Block solution was applied for seven minutes to minimize non-specific binding.

Tissue sections were then incubated overnight at 4°C with primary antibody against HOXA1 (sc-293257, Santa Cruz, USA). The following day, sections were treated with a biotinylated secondary antibody and then exposed to streptavidin–peroxidase. The immune reaction was visualized using diaminobenzidine (DAB), and counterstaining was performed with hematoxylin. Slides were cover-slipped and examined using a Zeiss Imager A2 photomicroscope. Image analysis and quantification were performed using ImageJ software.

Semi-Quantitative Histological Scoring

Staining intensity for HOXA1 was evaluated using ImageJ (version 1.53, http://imagej.nih.gov/ij) according to the protocol described by Crowe et al. (11) . Five randomly selected fields per sample were analyzed. Brown staining indicated positive immunoreactivity, while blue staining indicated negative areas. For each field, the ratio of HOXA1-positive area to total tissue area was calculated. The mean value across five fields was recorded as the semi-quantitative immunohistochemical score for each sample.

Network Topology and Gene Ontology (GO) Analysis

To explore the potential mechanisms associated with increased HOXA1 protein expression in GDM placentas, in silico analyses were conducted. Interactors of HOXA1 were retrieved from the STRING database, expanded to include 500 additional proteins (confidence score ≥0.4), and visualized in Cytoscape (v3.10.2). A similar procedure was applied to extract 500 GDM-related interactors. These two proteins–protein interaction (PPI) networks were intersected to identify shared proteins. Topological analysis of intersected nodes was performed using the CentiScaPe 2.2 plugin in Cytoscape to calculate degree, closeness, and betweenness centrality values. Degree centrality reflects the number of direct connections to other proteins, closeness indicates a protein's overall proximity to all others in the network, and betweenness measures its bridging potential between nodes (12). GO Molecular Function (MF) annotation was performed for the shared interactors using the DAVID database (13). Annotations with a p-value <0.05 were considered statistically significant. A polar bar plot graph of enriched GO MF terms was generated using SRplot based on –log (p-value) scores (14) .

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (version 9.2.0; GraphPad Software, San Diego, CA). Data normality was assessed using the Shapiro–Wilk test. Non-normally distributed data were expressed as median (interquartile range). Comparisons between groups were made using the Mann–Whitney U test. Statistical significance was indicated as follows: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Results

Properties of patients

The demographic and laboratory characteristics of the study groups are presented in Table 1. There were no significant differences between the GDM and control groups in terms of age, gestational age, or HOMA-IR scores. However, body mass index (BMI) was significantly higher in the GDM group (P = 0.016). As expected, glucose values obtained from the OGTT were significantly elevated in the GDM group at all three time points (0 min, 1 hour, and 2 hours) compared to controls (P = 0.001 for all comparisons).

Histopathological findings

Figure 1 illustrates the histological features of placental sections stained with hematoxylin and eosin. In the control group (Figure 1A), placentas displayed well-organized floating chorionic villi with intact trophoblastic layers, villous stroma, and clearly defined chorionic capillaries. Fibrin accumulation was minimal, and no significant pathological alterations were observed. In contrast, placentas from the GDM group (Figure 1B) exhibited marked histopathological changes. These included widespread hemorrhage, leukocyte infiltration between villi, villous degeneration, and loss of structural integrity. Additionally, increased fibrin deposition, dilated and congested chorionic capillaries, and thickened syncytial knots were frequently observed. Histopathological scoring (Figure 1C) revealed that the GDM group had significantly elevated scores for villous degeneration, fibrin accumulation, hemorrhage, syncytial knot formation, and leukocyte infiltration compared to controls (P < 0.05 for all parameters). These findings indicate that GDM induces prominent structural disruptions in placental architecture.

HOXA1 Immunoexpression

Immunohistochemical analysis of HOXA1 protein expression is shown in Figure 2.In the control group (Figure 2A), HOXA1 immunoreactivity was observed in cytotrophoblasts, syncytiotrophoblasts, syncytial knots, and villous stromal cells. The staining was generally weak to moderate and distributed uniformly. In the GDM group (Figure 2B), HOXA1 protein expression was markedly increased. Strong and diffuse positive staining was noted in cytotrophoblasts, syncytiotrophoblasts, syncytial knots, villous stromal cells, and in various cells within the intervillous space. Semi-quantitative scoring of immunoreactivity (Figure 2C) confirmed a statistically significant increase in HOXA1 protein expression in placentas from the GDM group compared to controls (P < 0.001). These findings suggest that GDM is associated with upregulated HOXA1 protein expression in multiple placental compartments.

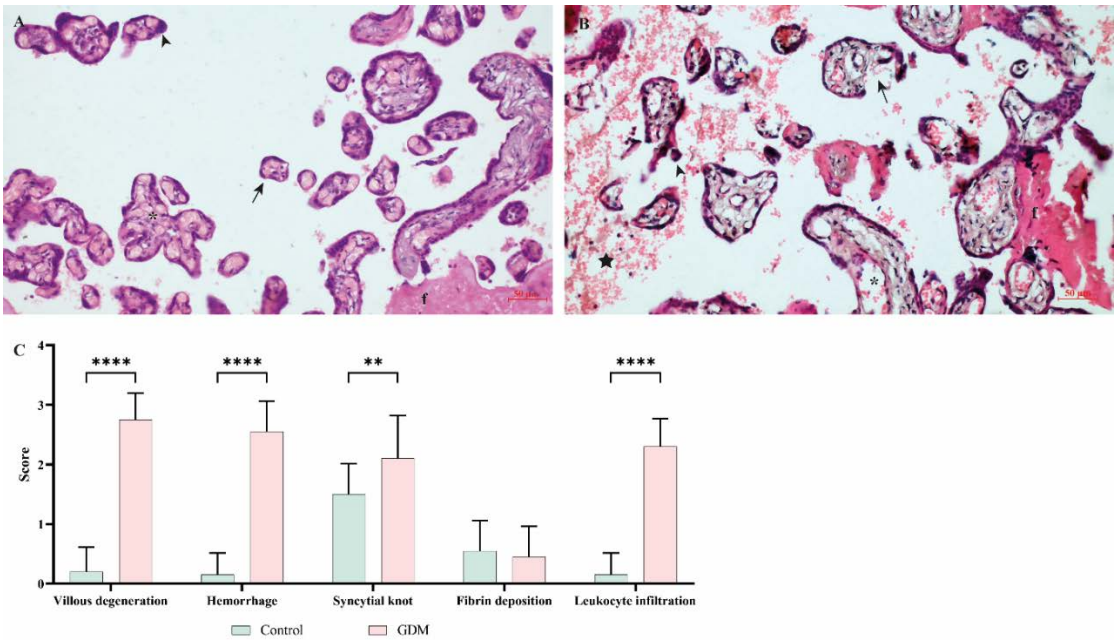

Figure 1. Cross sections of placental samples from pregnant women and histological scores. A) Control group; B) GDM group, C) Histopathological scoring, arrow: chorionic villus, arrowhead: syncytial knots, f: fibrin deposition, asterisk: villous stroma, star: hemorrhage, Bar: 50 µm, Magnification: 20X

Table 1. Demographic and laboratory parameters of patients.

Parameters	Control (n: 40)	GDM (n: 40)	P value
Age (year)	28.0 ± 5.8	29.4 ± 4.8	0.703
Gestation (week)	26.9 ± 1.8	26.1 ± 1.7	0.390
BMI (kg/m^2)	28.5 ± 5.7	32.4 ± 4.9	0.016
HOMA-IR score	1.57 ± 1.18	1.94 ± 1.04	0.061
OGTT (75 gr) 0. scale glucose (mg/dl)	80.2 ± 6.6	94.0 ± 8.1	0.001
OGTT (75 gr) 1. scale glucose (mg/dl)	152.5 ± 11.2	210.2 ± 21.0	0.001
OGTT (75 gr) 2. scale glucose (mg/dl)	139.0 ± 10.3	173.5 ± 20.3	0.001

BMI: Body mass index, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance, OGTT: Oral Glucose Tolerance Test

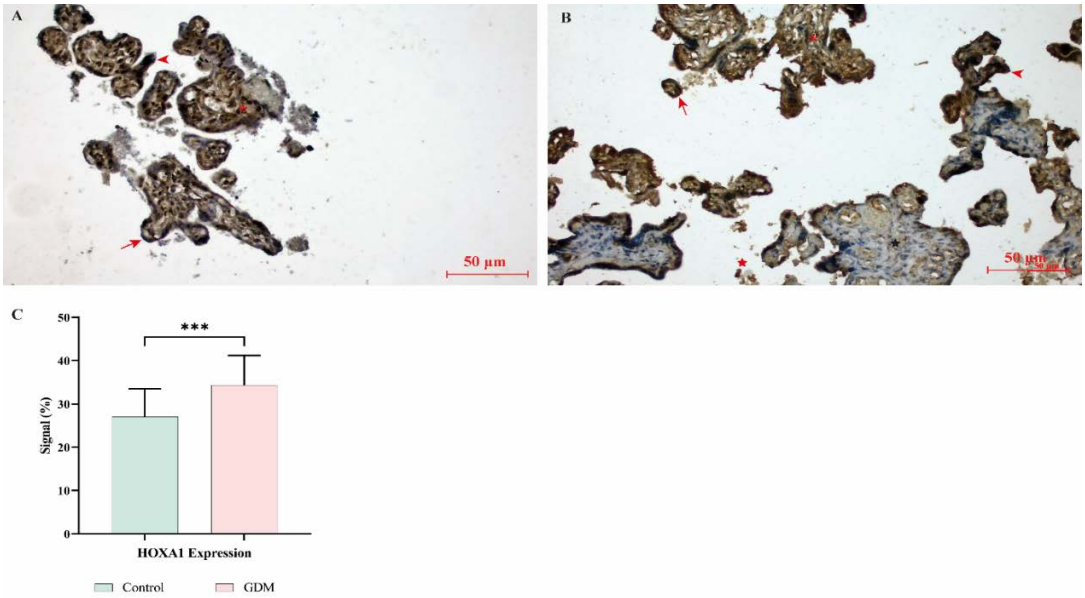

Figure 2. HOXA1 immune staining and semiquantitative scoring of placental sections per group. A) Control group; B) GDM group, C) Semi-quantitative scoring, arrow: chorionic villus, arrowhead: syncytial knots, f: fibrin deposition, asterisk: villous stroma, star: hemorrhage, Bar: 50 µm, Magnification: 20X

Table 2. Node centrality values of GDM associated HOXA1 interactors

Gene	Description	Uniprot ID	Degree	Closeness	Betweenness
H3C12	H3 Clustered Histone 1	P68431	14.0	0.0714	5.873
H3-3B	H3 Histone, Family 3B	P84243	14.0	0.0714	5.873
H3C13	H3 Clustered Histone 13	Q71DI3	14.0	0.0714	5.873
ESR1	Estrogen Receptor 1	P03372	14.0	0.0714	5.873
CEBPA	CCAAT Enhancer Binding Protein Alpha	P49715	13.0	0.0666	4.858
H3-4	H3.4 Histone, Cluster Member	Q16695	13.0	0.0666	2.173
SIRT1	Sirtuin 1	Q96EB6	12.0	0.0625	0.363
DNMT1	DNA Methyltransferase 1	P26358	12.0	0.0625	0.363
JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	P05412	12.0	0.0625	1.658
H3-5	H3.5 Histone	Q6NXT2	12.0	0.0625	0.363
H3-2	H3.7 Histone (Putative)	Q5TEC6	12.0	0.0625	0.363
DNMT3B	DNA Methyltransferase 3 Beta	Q9UBC3	11.0	0.0588	0.181
DNMT3A	DNA Methyltransferase 3 Alpha	Q9Y6K1	11.0	0.0588	0.181
TCF7L2	Transcription Factor 7 Like 2	Q9NQB0	7.0	0.0476	0.0
RXRA	Retinoid X Receptor Alpha	P19793	5.0	0.0434	0.0

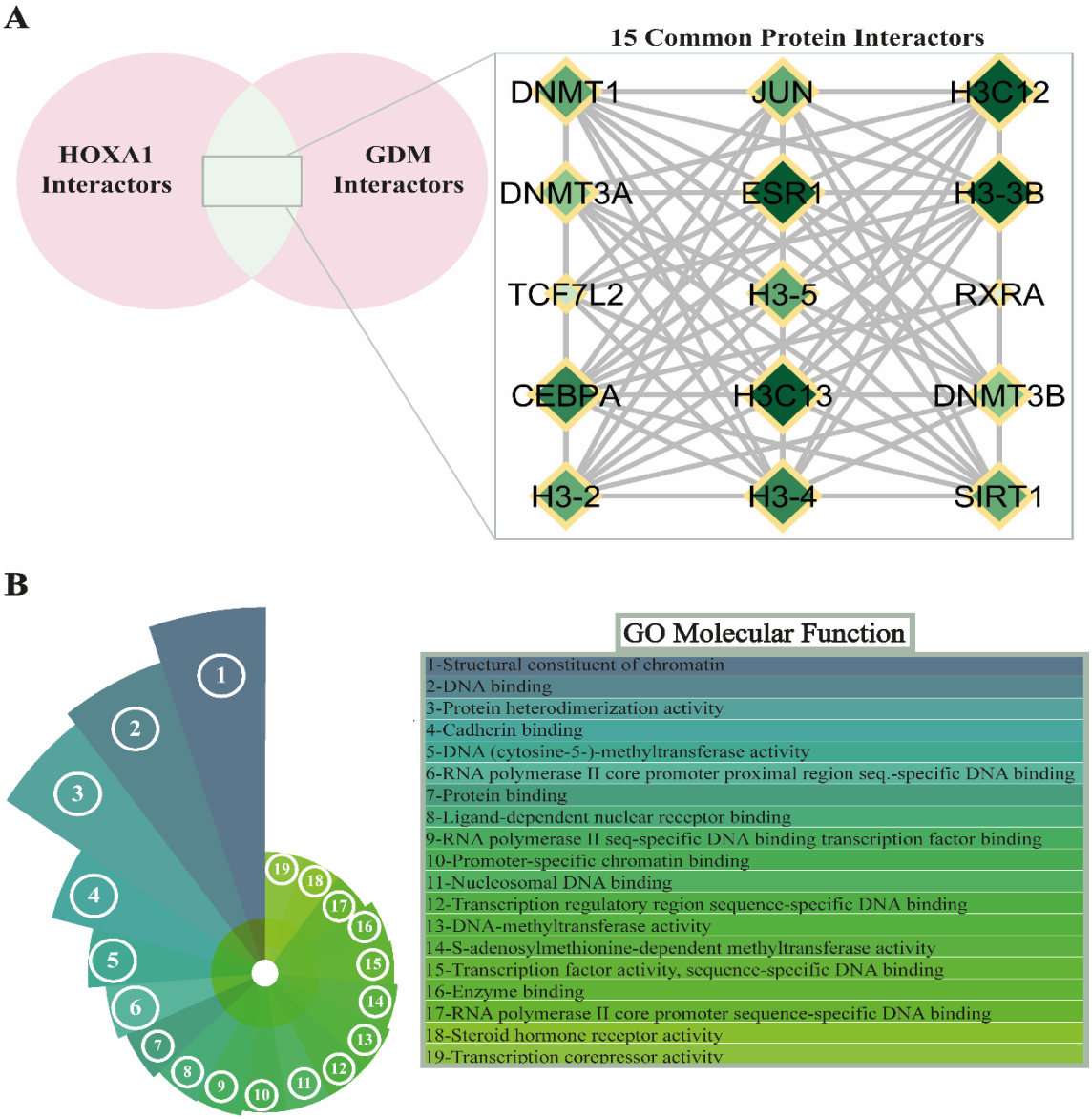

Figure 3. Node centrality and functional annotation analysis of common targets of HOXA1 and GDM. A. Node centrality analysis of 15 intersected proteins using the Centiscape. Larger nodes represent higher degree scores, while darker green color indicates higher closeness scores. B. Polar bar plot represents the significant GO Molecular Function terms of GDM-associated HOXA1 targets. The size of bars is proportional to "-log10(p-value)" values. Terms with a p-value less than 0.05 are considered statistically significant.

Node Centrality and GO Molecular Function Analysis of GDM associated HOXA1 interactors

The intersection of HOXA1 and GDM protein–protein interaction (PPI) networks yielded 15 common protein interactors, as listed in Table 2. The original HOXA1 and GDM networks contained 29,391 and 16,015 edges, respectively, prior to intersection. Node centrality analysis using degree, closeness, and betweenness metrics identified H3C12, H3-3B, H3C13, and ESR1 as the most central nodes within the intersected network (Figure 3A). These proteins exhibited the highest scores across all three centrality measures, indicating their potential regulatory importance within the HOXA1-GDM network. Gene Ontology Molecular Function (GO MF) enrichment analysis of the 15 shared proteins revealed significant associations with chromatin organization, DNA binding, epigenetic regulation, and protein–protein interactions (Figure 3B). These functions may underlie the mechanistic link between increased HOXA1 protein expression and placental pathology in GDM.

Discussion

Gestational diabetes mellitus (GDM) is a transient but significant metabolic disorder that can adversely impact both maternal and fetal health. While it is often limited to pregnancy, women with GDM are at elevated risk of developing type 2 diabetes mellitus (T2DM) later in life (17). Furthermore, GDM may unmask an underlying predisposition to metabolic dysregulation or autoimmune β-cell dysfunction, reinforcing the importance of early diagnosis and intervention.

Consistent with previous studies, our findings revealed notable structural abnormalities in placentas from GDM-affected pregnancies. These included villous degeneration, syncytial knot proliferation, fibrin deposition, leukocyte infiltration, and hemorrhagic areas, all of which are indicative of placental dysfunction(18-20). These histopathological features likely reflect impaired vascular remodeling, increased oxidative stress, and inflammatory responses driven by maternal hyperglycemia. The structural disorganization may compromise placental efficiency, thereby increasing the risk of adverse perinatal outcomes.

At the molecular level, our immunohistochemical analysis showed significantly elevated HOXA1 protein expression in multiple placental compartments from the GDM group. Increased immunoreactivity was observed in cytotrophoblasts, syncytiotrophoblasts, stromal cells, and intervillous cells, suggesting a widespread upregulation of this transcription factor. As a member of the highly conserved HOX gene family, HOXA1 plays critical roles in cell proliferation, differentiation, and developmental patterning, but its role in placental development under hyperglycemic conditions has remained unexplored (21, 22).

Our in silico analysis provides additional insights into the molecular context of HOXA1 in GDM. The intersected HOXA1-GDM network identified 15 shared interactors, with H3C12, H3-3B, H3C13, and ESR1 emerging as central regulatory proteins. These proteins are involved in chromatin remodeling, DNA binding, and epigenetic regulation—processes that are essential for trophoblast invasion, differentiation, and maternal-fetal interface integrity. Dysregulation of these pathways may contribute to the altered placental phenotype observed in GDM. Prior studies have also reported epigenetic abnormalities in GDM placentas, including DNA methylation changes and miRNA dysregulation, which may influence gene expression relevant to fetal growth and metabolism (23-26).

Importantly, the increased expression of HOXA1 protein in GDM placentas, supported by both histological and bioinformatic data, raises the possibility that HOXA1 could serve as a biomarker of placental dysfunction in diabetic pregnancies. If further validated, HOXA1 expression levels could potentially aid in identifying pregnancies at higher risk for adverse outcomes. Moreover, given its role in regulating transcriptional programs involved in cell fate and vascular remodeling, HOXA1 may represent a therapeutic target for modulating placental function in GDM.

Limitations

This study has several limitations that should be acknowledged. First, it was observational in nature and based on tissue-level analysis, limiting the ability to infer causality. Second, although immunohistochemistry provides spatial information, it does not capture the functional consequences of altered HOXA1 protein expression. The lack of in vitro or in vivo mechanistic studies limits our understanding of how HOXA1 modulates placental cell behavior under diabetic conditions. Future studies incorporating gene knockdown or overexpression models will be necessary to clarify these pathways.

Conclusion

In conclusion, this study demonstrates that GDM induces significant histopathological and molecular alterations in placental tissues. Increased HOXA1 protein expression was observed in multiple placental cell types in the GDM group, suggesting that this transcription factor may be involved in the pathophysiology of diabetic pregnancies. Our in silico analysis further revealed that HOXA1 is functionally connected to key proteins involved in chromatin organization, DNA binding, and epigenetic regulation—processes that are critical for placental development and fetal health. Among the identified interactors, H3C12, H3-3B, H3C13, and ESR1 emerged as central regulatory nodes in the GDM-associated HOXA1 network. Taken together, these findings suggest that HOXA1 may play an important role in the molecular mechanisms underlying placental dysfunction in GDM. HOXA1 also holds potential as a biomarker for placental pathology and may serve as a target for therapeutic modulation in future interventions aimed at mitigating GDM-related complications.

Further research—including functional studies—is warranted to validate the mechanistic roles of HOXA1 and to explore its potential utility in clinical applications.

Ethical Approval: *This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee for Non-Interventional Research of Dicle University Faculty of Medicine (Approval Date: 14/02/2024, No: 78).*

Author Contributions:
Concept: A.S.A
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Design : A.S.A, F.A.
Data acquisition: A.S.A, E.A, A.A.
Analysis and interpretation: T.K., F.A.
Writing manuscript: A.S.A., O.A., A.A., G.E.A.A.
Critical revision of manuscript: Z.T., F.A.
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