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HOXA1 Expression in preeclampsia: immunohistochemical and bioinformatic analyses

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Abstract

Objective: This study aimed to investigate the expression of Homeobox A1 (HOXA1) protein in placentas from pregnant women with preeclampsia using immunohistochemical techniques and to evaluate the potential role and interactors of HOXA1 in preeclampsia prognosis in silico.

Methods: Placenta samples taken from 40 healthy normotensive (control) and 40 preeclamptic pregnant women were used in the study. Samples were fixed in zinc formalin and embedded in paraffin. Demographic data of the patients were recorded. Sections from paraffin blocks were examined with Hematoxylin-Eosin and HOXA1 immunostaining methods. The HOXA1 protein-protein interaction network was constructed using STRING and imported into Cytoscape. Common proteins in the networks were identified and their relationships were examined. Protein class analysis was performed with PANTHER and gene ontology (GO) analysis was performed with ShinyGO

Results: Compared to control group, placentas of pregnant women diagnosed with preeclampsia showed increased chorionic villus degeneration (0.35±0.49 vs 2.75±0.44), dilation of vessels (0.30±0.47 vs 2.10±0.72), number of syncytial knots (0.35±0.49 vs 1.85±0.59), fibrin accumulation (0.45±0.51 vs 2.75±0.44) and hemorrhagel. HOXA1 expression significantly decreased in the preeclampsia (%34.93) group compared to control group (%28.17) (p=0.0008). From network analysis, five common protein interactors (CTNNB1, MYC, ESR1, GATA3, and JUN) were identified to be involved in the co-regulation of HOXA1-related preeclampsia pathways. Many of these targets serve as gene-specific transcriptional regulators that significantly influence transcriptional processes, cellular differentiation, chromatin organization, and immune response.

Conclusion: The downregulation of HOXA1 in preeclamptic placentas, combined with bioinformatic analyses, suggests potential mechanisms through which HOXA1-related pathways may influence preeclampsia pathogenesis.

Keywords: HOXA1, preeclampsia, gene ontology, PANTHER

Introduction

Hypertensive disorders are common and serious complications during pregnancy, posing risks to both mothers and their babies. These disorders include chronic hypertension (elevated blood pressure before the 20th week), gestational hypertension (high blood pressure after the 20th week without proteinuria), preeclampsia-eclampsia (hypertension with proteinuria), and chronic hypertension with superimposed preeclampsia.[1,2]

One of the primary causes of preeclampsia is uteroplacental ischemia, resulting from the failure of spiral arteries to remodel adequately. This inadequate blood flow causes placental hypoxia, leading to the release of factors such as soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sENG). These factors contribute to endothelial dysfunction, oxidative stress, and immune activation in the maternal system.[3-5]

Homeobox (HOX) genes are transcription factors characterized by a conserved homeobox sequence that plays a crucial role in placental development. These genes are involved in trophoblast invasion, decidualization, and placental vascularization.[6-8] Given their importance in these processes, dysregulation of HOX genes may contribute to the pathogenesis of preeclampsia.

This study aims to investigate the immune expression of the HOXA1 gene in placentas of women diagnosed with preeclampsia and explore its potential association with the condition using in silico analysis.

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Methods

Ethical approval was received from Non-Interventional Clinical Research Ethics Committee at Faculty of Medicine, Dicle University (date: 14/02/2024, approval number: 76). 40 healthy normotensive (control) pregnant women and 40 pregnant women diagnosed with preeclampsia were included in the study. Preeclampsia was defined as a pregnancy disorder associated with new-onset hypertension, often accompanied by proteinuria.[9] The hypertension criterion was defined as systolic blood pressure being 140 mm Hg or more or diastolic blood pressure being 90 mm Hg or more on two occasions at least 4 hours apart after the 20th week of gestation in a woman with previously normal blood pressure. Proteinuria was defined as 300 mg or more in 24-hour urine and a protein/creatinine ratio of 0.3 mg/dL or more.[10] The demographic data, lab results, and pregnancy outcomes for each patient were documented. Blood samples were taken upon admission, prior to delivery, and were subsequently analyzed for lab parameters. All patients were informed about the study and provided their consent to participate.All patients were informed and agreed to participate in the study. All participants signed a written consent form. Pregnant women with out of age range (minimum 18 years, maximum 40 years), chronic systemic diseases, drug use, smoking and other pregnancy complications (gestational diabetes mellites, placenta accrete, premature rupture of membrane, etc.) were not included. The control group consisted of pregnant women who were followed up regularly, did not have any problems during pregnancy follow-up, and met the exclusion criteria.

As sample collection and tissue preparation, placentas were obtained from Department of Gynecology and Obstetrics, Medical Genetics and Pathology, Faculty of Medicine, Dicle University. For histological analysis, placental tissue samples were excised to small parts and fixed in zinc-formalin. After fixation, the tissues were passed through tap water, increasing ethanol 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. The placental tissue samples were first placed in a water bath at 37°C. Following this, they were transferred to an oven set between 58-62°C for 6 hours to remove excess paraffin. The sections were then deparaffinized with xylene and gradually dehydrated through a series of ethanol solutions with increasing concentrations. Hematoxylin-Eosin staining was then applied. After staining, the sections were quickly transitioned through progressively higher ethanol concentrations and then soaked in absolute alcohol for 2 minutes. Finally, they were placed in xylene and covered with a coverslip using a mounting medium. All prepared slides were examined with a Zeiss Imager A2 photomicroscope.

Placental sections from paraffin blocks were first heated in a double boiler at 37°C and then mounted onto polylysine slides. To remove excess paraffin, the slides were placed in an oven at 58-62°C for 6 hours. The sections were then deparaffinized using xylene and gradually rehydrated through a series of decreasing alcohol concentrations. After a 5-minute rinse in distilled water, the sections were incubated with hydrogen peroxide for 20 minutes. After the sections were washed with PBS, they were kept in Ultra V Block solution for 7 minutes. It was incubated with HOXA1 (sc-293257, Santa Cruz) primary antibody overnight at +4°C. After primary antibody application, the sections were washed with PBS and incubated with biotinylated secondary antibody for 14 minutes. Next, streptavidin-peroxidase was applied to the sections and left to incubate for 15 minutes. Diaminobenzidine (DAB) was then added to the sections, and the reaction was observed under a light microscope. Following this, the sections were washed three times with PBS, each wash lasting 15 minutes. After counterstaining with Harris hematoxylin, the sections were covered with mounting medium and covered with a coverslip. All preparations were imaged via Zeiss Imager A2 photomicroscope and processed using ImageJ software.

As semiquantitative analysis of HOXA1 expression, 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.[11] 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 semiquantitative immunohistochemistry scoring.[12]

In silico analysis for the common interactors of preeclampsia and HOXA1, a protein-protein interaction (PPI) network was constructed using the STRING database, with a medium confidence level (0.4) and a maximum of 500 interactors in the first and second shell. Subsequently, this network was transferred to the Cytoscape software (version 3.10.2). Additionally, a separate Preeclampsia

PPI network was generated using Cytoscape at a medium confidence level (0.4). The common proteins in these networks were obtained through a merge tool, and protein-protein associations were examined in the STRING database. Protein class analysis was performed using the PANTHER knowledgebase to elucidate the functional characteristics and roles of the shared proteins.[13] Furthermore, to highlight the biological significance of these common proteins, gene ontology (GO) analyses for biological processes (BP), cellular components (CC), and molecular functions (MF) were conducted using ShinyGO (version 0.77).[14] False discovery rate (FDR) values less than 0.05 were considered significant. The top ten significant GO terms obtained from this analysis were visualized through bar plots using SRPlot[15] based on fold enrichment values.

Statistical analysis was carried out using GraphPad Prism 7 (GraphPad Prism, San Diego, CA, USA) and SAS v9.3 (SAS Institute Inc., Cary, NC, USA) software. Data were expressed as mean ± standard deviation. The Shapiro-Wilk test was used to assess the normality of the data distribution. Histopathological and signal (%) score were evaluted by between control and preeclampsia group by Mann-Whitney U test. A p-value of less than 0.05 was considered statistically significant. Sample size for each group was determined using G Power analysis (version 3.1), and Cohen's criteria were based on the study by Alviggi et al.[16]

Results

Demographic parameters and laboratory results of control and pregnant women diagnosed with preeclampsia are listed in Table 1.In pregnant women with preeclampsia, systolic and diastolic blood pressure and 24-hour urine protein were significantly higher and platelet count was lower compared to control group.

Hematoxylin Eosin staining of placentas taken from patients in the groups was shown in Figure 1. No pathology was found in the placental components in the control group sections. In the chorionic villi, syncytiotrophoblast and cytotrophoblast cells were observed in the outermost parts, and chorionic capillary and stromal connective tissue were observed in the inner parts. No dilatation or congestion was observed in the vascular structures (Figure 1A). Increased degeneration and fibrin deposition in chorionic villi were observed in the preeclampsia group compared to the control group. Vascular dilation and congestion were observed in the chorionic capillaries. There was a notable rise in the number of syncytial knots (Figure 1B).In semi-quantitative histopathological scoring (control vs preeclampsia), a significant increase in chorionic villus degeneration (0.35±0.49 vs 2.75±0.44), dilation of vessels (0.30±0.47 vs 2.10±0.72), number of syncytial knots (0.35±0.49 vs 1.85±0.59), fibrin accumulation (0.45±0.51 vs 2.75±0.44) were observed (Figure 1C).

Table 1. Demographic parameters and laboratory results of the patients

Parameters	Control (n=40)	Preeclampsia (n=40)	Significance (p-value)
Maternal age, years	28.3 ± 4.5	31.3 ± 5.2	0.106
Gravida, n	2.6 ± 1.2	2.4 ± 1.1	0.067
Parity, n	1.3 ± 1.2	1.1 ± 0.9	0.057
Systolic blood pressure, mmHg	109.1 ± 8.6	160.2 ± 16.3	<0.001
Diastolic blood pressure, mmHg	62.3 ± 5.8	98.9 ± 9.67	<0.001
24-hour proteinuria output	165.4 ± 67.3	1263 ± 319.8	<0.001
Platelet count, 10^9/L	279.4 ± 69.8	171.2 ± 67.4	<0.01
LDH, U/L	297.8 ± 82.7	329.8 ± 97.8	0.412
AST, U/L	21.9 ± 8.8	37.6 ± 9.2	0.169
ALT, U/L	16.1 ± 4.3	32.6 ± 11.5	0.391
Gestational week at birth	38.3 ± 1.4	35.4 ± 2.7	0.032

LDH: Lactate dehydrogenase, AST: Aspartate transaminase, ALT: Alanine aminotransferase

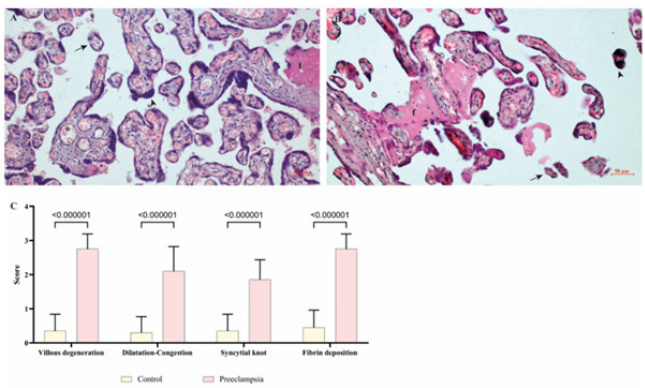

Fig 1. Hematoxylin Eosin staining and scoring of placental tissues. A) Control group; B) Preeclampsia group, C) Histopathological scoring, arrow: chorionic villus, arrowhead: syncytial knot, f: fibrin deposition, asterisk: chorionic capillary, Bar: 50 ✂m, Magnification: 20X

HOXA1 expression was decreased in preeclamptic placenta. Figure 2 displays the HOXA1 immunostaining results for the placental tissues across different groups. In the placental sections from the control group, there was a strong expression of HOXA1 observed in the various components of the placenta. HOXA1 immune reaction was observed more intensely, especially in the syncytial knots, villous stroma and fibrin deposition (Figure 2A).In the sections of the preeclampsia group, it was observed that

HOXA1 expression decreased compared to the control group. While moderate expression was observed in areas with fibrin accumulation and syncytial knots, generally negative HOXA1 expression was observed in the connective tissue areas of chorionic villi. Mild HOXA1 expression was also observed in some cells in the intervillous area (Figure 2B).In semi-quantitative HOXA1 expression analysis, it was observed that HOXA1 immune reactivity was significantly reduced in preeclampsia group (%28.17) compared to the control group (%34.93) (p=0.0008) (Figure 2C). According to our findings, HOXA1 expression was statistically downregulated in placental tissues in the preeclampsia group.

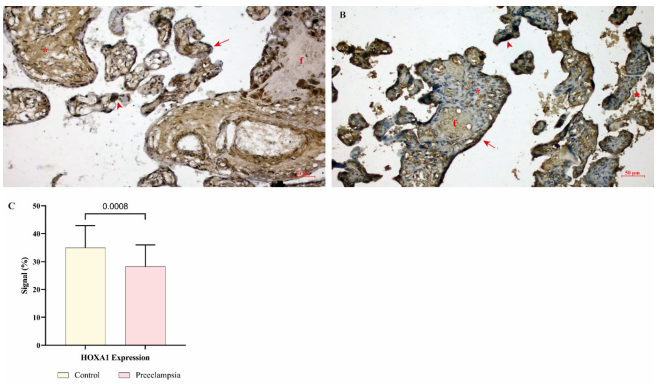

Fig 2. HOXA1 immunostaining and scoring of placental tissues. A) Control group; B) Preeclampsia group, C) Semi-quantitative scoring, arrow: trophoblastic layer, arrowhead: syncytial knot, f: fibrin deposition, asterisk: villous stroma, star: intervillous area, Bar: 50

As a result of network analysis, the characteristics of the protein-protein interaction (PPI) networks were determined: the PPI network obtained for HOXA1 from the STRING database consisted of 588 knots and 35,540 edges, while the preeclampsia PPI network contained 550 knots and 22,716 edges. The intersection of both networks identified 8 common protein interactors: MYC Proto-Oncogene (MYC), Integrin Subunit Alpha 2b (ITGA2B), Jun Proto-Oncogene (JUN), Kallikrein Related Peptidase 2 (KLK2), GATA Binding Protein 3 (GATA3), Histamine N-Methyltransferase (HNMT), Catenin Beta (CTNNB1), and Estrogen Receptor 1 (ESR1). Within the STRING protein network constructed with these proteins, the highest number of protein-protein associations (4 edges) occurred between CTNNB1-MYC, ESR1-MYC, GATA3-ESR1, and JUN-GATA3. However, ITGA2B and KLK2 proteins showed no interaction within the network (Figure 3A). The common 8 proteins were predominantly classified as gene-specific transcriptional regulators (57.1%), followed by protein modifying enzymes (14.3%) and cell adhesion molecules (14.3%) (Figure 3A). Gene ontology (GO) analysis revealed their involvement in various biological processes. In terms of biological processes (GO BP), these proteins were associated with regulating DNA binding and transcriptional processes, as well as myeloid cell differentiation and response to stimuli. Regarding cellular components (GO CC), they were associated with various protein complexes involved in transcriptional regulation and chromatin organization. Additionally, the molecular function analysis (GO MF) highlighted their roles in DNA binding, transcriptional regulation, and interaction with transcription factors and coregulators (Figure 3B).

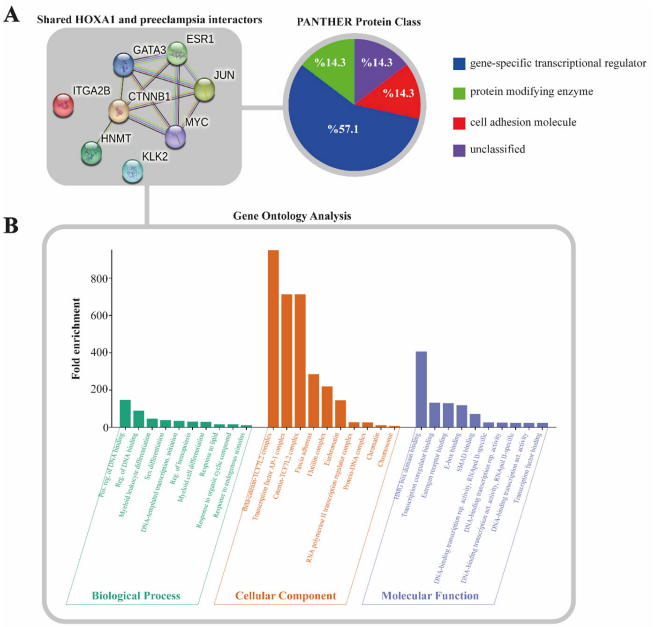

Fig 3. STRING protein network and gene ontology analysis of shared HOXA1 and preeclampsia protein interactors. A. The STRING PPI network illustrates the relationships between proteins, with nodes representing proteins and edges depicting interactions (light blue and pink: known interactions, light green: text-mining, black: co-expression). Additionally, the Panther pie chart displays the distribution of protein classes among the shared proteins identified in the HOXA1 and preeclampsia interactors. B. The bar chart illustrates the GO biological process, cellular component, and molecular function analyses of the common proteins. Act: activator, pol:polymerase,pos: positive, reg: regulation, rep: repressor.

Discussion

Preeclampsia is a condition affecting multiple organ systems that develops after the 20th week of pregnancy, characterized by high blood pressure and the presence of protein in the urine.[17] Preeclampsia affects between 1.5%

and 16.7% of pregnancies globally and is responsible for around 60,000 maternal deaths and over 500,000 premature births annually. It ranks as the second leading cause of maternal mortality worldwide.[2]

Histological alterations in placental villi and vasculature are observed in preeclampsia.[18,19] Pietro et al. showed that placental infarction, hypoplasia and agglutination in the villi were observed in preeclampsiadue to hypertension in the vascular bed of the intervillous space.[20] Ojha et al. found that in placentas from preeclamptic patients, there was an increase in the number of syncytial knots, areas of fibrinoid necrosis, hyalinization, and calcification.[21] In another study, in placenta sections with preeclampsia, increased fibrinoid tissue was observed in the root villi, and hyalinized and necrotic areas were observed in the chorionic villi. Severe inflammation and bleeding foci have been detected in the intervillous region.[22,23] Our findings show that, consistent with these studies, preeclampsia causes significant histopathological changes in placental tissues. While the placentas in the control group maintain their normal histological features, serious structural deteriorations are observed in the preeclampsia group (degeneration and increased fibrin accumulation in chorionic villi, vascular dilatation and congestion in chorionic capillaries). This may lead to insufficient blood flow to the fetus and therefore problems in the growth and development of the fetus. These findings highlight the importance of monitoring placental health and taking necessary precautions in the management of preeclampsia.

HOX genes, a subgroup of the homeobox superfamily, have important roles in apoptosis, receptor signaling, differentiation, motility, and angiogenesis.[24] Homeobox genes have been shown to be expressed in trophoblastic tissue, mature placentas, and the developing embryo.[25] Zang and colleagues showed that HOXA11 is strongly expressed in cytotrophoblasts and is subsequently downregulated as these cells differentiate into syncytiotrophoblasts.[26] Asir and colleagues determined that the HOXA7 protein level increased in placentas that experienced premature birth, that this gene is important for placental development and fetal membrane integrity, and that transcription control may give clues about placentation disorders.[27] HOXA10, a homeobox transcription factor regulated during development, is strongly expressed in stromal cells undergoing decidualization. It is believed that the increase in stromal cell proliferation serves as the trigger for the decidualization process.[28] Jia and colleagues showed that the HOXB3 gene, belonging to the homeobox gene family, transcriptionally specifies Notch1 to activate the Wnt/β-catenin pathway, resulting in accelerated proliferation, migration, and invasion of the trophoblast. They also revealed that the HOXB3 gene alleviates preeclampsia and improves fetal health.[29]

Our HOXA1 immunohistochemical findings indicate that preeclampsia markedly reduces the expression of HOXA1 protein in placental tissues. While placentas in the control group exhibited intense HOXA1 expression, this expression was observed to decrease in the preeclampsia group. It suggests that preeclampsia downregulates HOXA1 expression in the placenta, which may have negative effects on placental function and structure. Reduction of HOXA1 may affect processes such as cell proliferation, differentiation and apoptosis, which may play an important role in the pathophysiology of preeclampsia.

To investigate potential functional annotations associated with HOXA1, which is downregulated in preeclamptic placentas, pathway, classification, and GO analyses were conducted on the intersected interactors of preeclampsia and HOXA1. The characteristics observed in the PPI networks, particularly the higher number of edges among specific proteins, suggest their potential centrality and functional significance within the networks.[30] Specifically, the presence of four edges between CTNNB1, MYC, ESR1, GATA3, and JUN indicates strong interactions among these proteins, potentially highlighting important biological processes or signaling pathways they may collectively regulate. These involvement of these in preeclampsia has been supported by various studies. CTNNB1 demonstrates decreased mRNA and protein expression in preeclamptic placental tissues [31], while epigenetic regulation within the c-myc promoter region has been implicated in preeclampsia development.[32] Disruption of ESR1 has been associated with severe preeclampsia [33] and GATA3 has been implicated in placentally mediated imprinting in preeclampsia.[34] Furthermore, experimental studies support the potential involvement of JUN modulation in preeclampsia.[35] Conversely, the absence of interaction of ITGA2B and KLK2 proteins within the network suggests their limited involvement or distinct roles compared to the other identified proteins. The predominance of gene-specific transcriptional regulators among the common proteins suggests their potential involvement in regulating gene expression. These findings were supported by GO analysis, revealing critical roles of these proteins in regulating DNA binding and transcriptional processes, fundamental aspects of cellular function. Moreover, their association with myeloid cell differentiation and response to stimuli suggests potential implications in immune response and cellular differentiation pathways, often dysregulated in diseases like preeclampsia.[36,37] In terms of

cellular components, their involvement in various protein complexes related to transcriptional regulation and chromatin organization underscores their role in modulating gene expression at the chromosomal level. Additionally, highlighted molecular functions, such as DNA binding, transcriptional regulation, and interaction with transcription factors and coregulators, further emphasize their involvement in transcriptional control mechanisms. These findings offer valuable insights into the functional roles of shared targets and provide potential molecular mechanisms underlying the HOXA1 associated preeclampsia pathogenesis.

Conclusion

HOXA1 expression was significantly reduced in placental tissues from the preeclampsia group, suggesting that this molecule could play a crucial role in the development of preeclampsia. It may serve as a valuable biomarker and a potential target for diagnosing and treating the condition. The experimentally observed downregulation of HOXA1 in preeclamptic placentas is further elucidated by bioinformatic analyses, which highlight critical interactions of preeclampsia-associated HOXA1 interactors such as CTNNB1, MYC, ESR1, GATA3, and JUN.These proteins might play central roles in transcriptional regulation and immune response, processes that may be disrupted in preeclampsia. Therefore, HOXA1 downregulation might contribute to preeclampsia pathogenesis through these pivotal molecular pathways.

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