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Molecular Predictors of Effective Implantation and Live Birth in IVF Programs

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Abstract

The aim of the study was to improve the possibilities of predicting blastocyst implantation and live birth of ART programs in women of late reproductive age with tubal-peritoneal infertility based on immunohistochemical markers of the endometrium

***Methods and Results*:** The results of IVF and IVF/ICSI programs were analyzed in 68 patients of late reproductive age (36-44 years of age) with tubal-peritoneal factor of infertility. Morphological examination of the endometrium was performed on Day 7 after confirmed ovulation in the cycle preceding ART**.** The expression of vitamin D receptors (VDR) and HOXA11 in endometrial stromal cells was assessed by immunohistochemical method. The effectiveness of using the endometrial markers VDR and HOXA11 as potential predictors of ART programs efficiency was confirmed by prognostic models. The levels of the stromal expression of VDR<8.7% and HOXA11<6.1% (probability >0.27) were determined to be favorable for successful blastocyst implantation. The expression levels of VDR<8.3% and HOXA11<6.1% in endometrial stromal cells are prognostically favorable for live birth (probability >0.19) in women of late reproductive age with tubal-peritoneal infertility who undergoing ART treatment with their own oocytes.(**International Journal of Biomedicine. 2021;11(4):532-537.**)

Key Words: assisted reproductive technology • vitamin D receptor • HOXA11 • implantation • live birth

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Abbreviations

ART, assisted reproductive technology; **AUC**, the area under the ROC curve; **IVF**, in vitro fertilization; **IHCM**, immunohistochemical markers; **ICSI**, intracytoplasmic sperm injection; **PCC**, percentage correctly classified; **RIF**, recurrent implantation failure; **VDR,** vitamin D receptor.

Introduction

A new tendency in reproductive medicine is to improve the percentage of healthy perinatal outcomes for women using assisted reproductive technology (ART).[1] Recurrent pregnancy losses, corresponding to the loss of three or more consecutive pregnancies up to 20 weeks, are noted in 1%–2% of couples, and their causes remain unexplained in more than half of the cases.[2] The use of ART has improved the rates of pregnancy and live births.[3] However, recurrent implantation failure (RIF), determined by the absence of fertilization after several IVF attempts, showed the need to study the effect of the endometrial pattern on the outcomes of ART programs.[4-7] It is reported that suboptimal endometrial susceptibility and altered embryo-endometrial dialogue are responsible for

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two-thirds of implantation failures.[8] An unknown reason for the functional inferiority of the endometrium in women with tubal-peritoneal infertility indicates the need to choose the most informative methods to predict the outcomes of ART programs. The female reproductive system is unique in relation to other tissues and systems due to predominantly postnatal differentiation and transformations during the menstrual cycle and pregnancy.

Homeobox genes (as known as *HOX* genes) are leading candidates for the regulation of endometrium differentiation in preparation for embryonic implantation. HOX genes encode proteins that act as transcription factors.[9,10] Hoxa11/HOXA11 expression is preserved in the adult endometrium of mice[11,12] and humans.[12] Some of the *Hox/HOX* genes participate in remodeling of the genitourinary tract, and regulation of endometrial proliferation and cell differentiation, with the formation of susceptibility of the uterine mucosa to implantation or apoptosis and tissue rejection during each menstrual cycle.[13-16]

Homeobox-deficient mice (*Hoxa11*) were found to be infertile due to stromal-specific endometrial defects that exclude decidual transformations.[17,18]

The prognostic potential of vitamin D to ensure female fertility is determined by its extensive receptor network in the organs of the reproductive system, including the endometrium.[19] Recent studies have confirmed the steroidogenic effect of the active form 1,25(OH)2D and participation in the modulation of cell proliferation, differentiation and apoptosis of cells of the reproductive organs.[20,21] The effect of vitamin D on the endometrium is carried out by the formation in the cell nucleus of a ligand-independent transcription factor for the regulation of target genes and communication with cytosolic and membrane receptors.[22,23]

VDR is a part of a group of transcriptional regulators providing a variety of biological effects of 1,25(OH)2D and related compounds.[24] The recognition of molecular and cellular endometrial breakdowns during the "implantation window" as one of the significant reasons for ineffective IVF attempts explains the interest in studying the mechanisms of local activity of VDR and *Hox/HOX* genes.

The aim of the study was to improve the possibilities of predicting blastocyst implantation and live birth of ART programs in women of late reproductive age with tubal-peritoneal infertility based on ICHM of the endometrium.

Materials and Methods

Our prospective cohort study included 68 women of late reproductive age (36-44 years of age) with tubal-peritoneal infertility who underwent ART programs at the Center of Obstetrics and Gynecology.

Inclusion criteria for the study were tubal factor of infertility, normal or reduced ovarian reserve with a preserved regular ovulatory menstrual cycle, normozoospermia or minor pathozoospermia of the husband (donor), use of the patient's own oocytes, embryos of good and excellent quality.

Exclusion criteria were infertility due to the absence of ovulation; endometriosis, uterine fibroids 4 cm or more, uterine factor of infertility, chronic active endometritis, HIV infection, hepatitis B and C, severe pathozoospermia, systemic diseases; somatic diseases in the stage of exacerbation or decompensation.

Depending on the outcomes of ART programs, 4 groups were formed, two of them according to the incidence of clinical pregnancy: Group 1 (n=18) – with pregnancy, Group 2 (n=50) – with a negative result; and two groups according to the indicator of live birth (frequency of deliveries with a live fetus(es) [take-home baby]): Group 3 (n=14) – with a favorable result, Group 4 (n=54) –with a negative result.

Endometrial sampling by aspiration biopsy using a Pipelle catheter during the period of the proposed "implantation window" was performed in 68 women in the cycle preceding the ART programs (on Days 17-25 of the menstrual cycle, depending on the results of ultrasound monitoring and on Day 7 after the peak of luteinizing hormone.

For histological examination, the standard method of fixation in 10% neutral formalin was used, followed by dehydration, degreasing and embedding in paraffin in a histological machine, according to the generally accepted technique. From paraffin blocks, sections with a thickness of 5μm were prepared, several (5-10) on 10-15 glass slides. After dewaxing, the sections were stained with hematoxylin and eosin; the avidin-biotin immunoperoxidase method was used for the immunohistochemical study of endometrial biopsies.

The polyclonal antibodies (PCAB) used in the study were designed to work with paraffin sections. Treatment options for dewaxed sections were selected depending on the manufacturer's instructions (Table 1). A Histophine detection system (Nichirei Corp., Japan) was used to visualize primary antibodies.

Table 1.

Panel of antibodies for immunohistochemical study.

Antibodies	Clone	Working dilution	Manufacturer
VDR	polyclonal	1:100	GeneTex, USA
HOXA11	polyclonal	1:250	GeneTex, USA

The stained samples were automatically classified by the program into 10 color channels, depending on the color and the intensity of staining. After classification, pseudo-color masks were applied to the structures of interest. In this case, the relative density (%) of the studied structures was calculated in relation to the total area of the studied frame.

The results of VDR and HOXA11 expression were calculated by counting and finding the percentage of the relative density of stained endometrial stromal cells. Morphofunctional assessment of the endometrium was performed using licensed software (Morphology 5.2., Russia). The preparations were examined and photographed using a Primo Star microscope(Carl Zeiss, Germany), with the help of a Pixera Pro 150ES digital camera (Pixera, Japan) at an operating magnification of x400.

Embryo transfer was carried out in "fresh" cycles and cycles with the transfer of thawed embryos. For the pregnancy rate indicator, only clinical pregnancy (sonographic presence

of fetal egg/eggs) was taken into account, and for live births – the number of ART programs that ended with live births/ fetuses.

All patients were informed about the purpose and design of the work and gave their consent to participate in the study and publish its results in the open press. The study was approved by the SUSMU Ethics Committee.

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The normality of distribution of continuous variables was tested by the Kolmogorov-Smirnov test with the Lilliefors correction and Shapiro-Wilk test. Continuous variables with normal distribution were presented as mean (standard deviation [SD]); non-normal variables were reported as median (Me) and interquartile range (Q1-Q3). Means of 2 continuous normally distributed variables were compared by independent samples Student's t test. Differences of continuous variables departing from the normal distribution, even after transformation, were tested by the Mann-Whitney U-test. Threshold values (cut-off point) were calculated by the ROC analysis method. To construct a prognostic model and estimate OR (odds ratio), we used the method of multiple logistic regression; 95% CI (confidence interval) was calculated. A value of $P<0.05$ was considered significant.

Results

The onset of clinical pregnancy was detected in 18(26.5%) patients with their own oocytes in ART programs. In Group 1 and Group 2, VDR expression in the endometrial stroma was 7.35(4.9;8.1)% and 8.56.3;9.8)% (P=0.016), respectively, and HOXA11 expression – 5.1(4.3;6.1)% and 7.4(5.4; 8.7)% (P=0.001), respectively.

The AUC was determined for implantation markers: VDR of the stroma – 0.739±0.063 (P=0.003) and HOXA11 – 0.767±0.064 (P=0.000).

The threshold value of VDR expression in endometrial stromal cells at the cut-off point was 8.7%. The sensitivity and specificity of the method were 100% and 40%, respectively (Fig.1). The threshold value of HOXA11 expression in endometrial stromal cells, calculated for successful implantation at the cut-off point, was 6.1%. The sensitivity and specificity of the method were 80% and 73%, respectively (Figure 2).

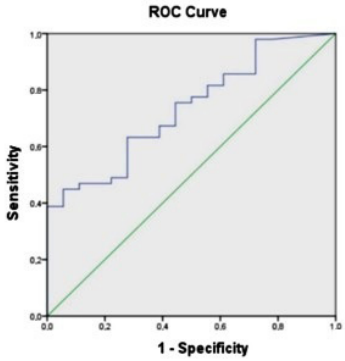

Fig.1. *ROC-curve of the relationship between successful implantation (the onset of clinical pregnancy) and the VDR expression in endometrial stromal cells*

AUC 0.739±0.063 (P=0.003); 95% CI: 0.616-0.862.

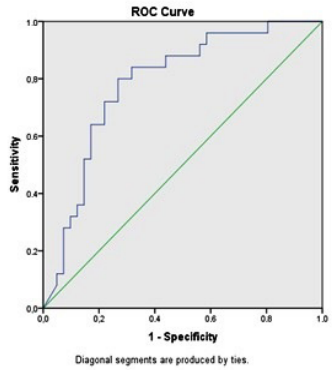

Fig.2. *ROC-curve of the relationship between successful implantation (the onset of clinical pregnancy) and the HOXA11 expression in endometrial stromal cells.*

AUC 0.767±0.064 (P=0.000); 95% CI: 0.642-0.892.

To create a mathematical model that allows predicting implantation, we used multiple regression analysis with forced inclusion of two variables: VDR, HOXA11 (Table 2).

Table 2.

Calculation of the blastocyst implantation prognosis

Parameter	B	Value	OR(Exp(в)	95% CI for OR
HOXA11	-0.487	0.003	0.615	[0.444;0.850]
VDR	-0.302	0.012	0.740	[0.585;0.935]
Constant	4.121	0.005	61.649	

The equation was as follows:

$$p = \frac{1}{1 + e^{-(4.121 - 0.487 \cdot x_1 - 0.302 \cdot x_2)}},$$

where x_1 is the value of HOXA11 expression in the endometrial stroma, x_2 is the value of VDR expression in endometrial stromal cells, p is the probability of implantation.

If the probability calculated by the model is >0.27, then the onset of implantation is predicted. The resulting model is statistically significant (P<0.001). Model sensitivity (83.3%), specificity (74.0%), and PCC (76.5%) indicate a high predictive ability.

The ROC curves for predicting blastocyst implantation, taking into account the expression of individual markers in the endometrium, are presented in Figure 3.

Live birth in ART programs occurred in 14(21.7%) women. The expression of IHCM in the endometrial stroma in Groups 1 and 2 was as follows: 7.1(4.9;8.0)% and 8.5(6.1;9.8)%, respectively, for VDR (P=0.240); 5.0(4.3;6.1)% and 7.1(5.3;8.7)%, respectively, for HOXA11 (P=0.006). In patients of Group 1, the decrease in HOXA11 expression turned out to be statistically significant (P=0.006).

The AUC of IHCM expression in women with their own oocytes was 0.728±0.067 (P=0.009) for the VDR in the stroma and 0.744±0.071 (P=0.005) for HOXA11.

The value of the threshold parameter of HOXA11 expression, prognostically favorable for live birth, was

6.1%. The sensitivity and specificity of the method were 80% and 63%, respectively. The threshold value of VDR expression in stromal cells was calculated and turned out to be prognostically favorable for live birth was 8.3%. The sensitivity and specificity of the method were 93% and 53%, respectively.

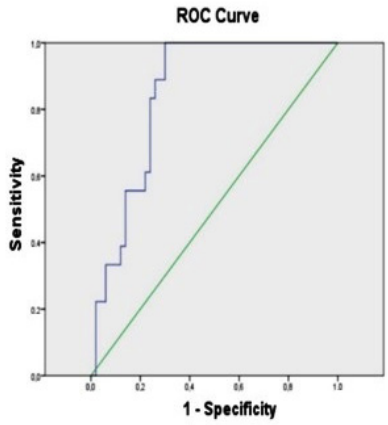

Fig.3. *AUC for the blastocyst implantation prognosis model.*

AUC= 0.846 (P=0.000); 95% CI: 0.756-0.935.

To create a mathematical model that allows predicting live birth, we used multiple regression analysis with the forced inclusion of two variables: VDR, HOXA11 (Table 3).

Table 3

Calculation of the live birth prognosis

Parameter	B	Value	OR(Exp(в)	95% CI for OR
HOXA11	-0.308	0.030	0.735	[0.556;0.971]
VDR	-0.329	0.006	0.720	[0.568;0.912]
Constant	2.825	0.033	16.860	

The equation was as follows:

$$P = \frac{1}{1 + e^{-(2.825 - 0.308 \cdot x_1 - 0.329 \cdot x_2)}},$$

where x_1 is the value of HOXA11 expression in the endometrial stroma, x_2 is the value of VDR expression in endometrial stromal cells, p is the probability of live birth.

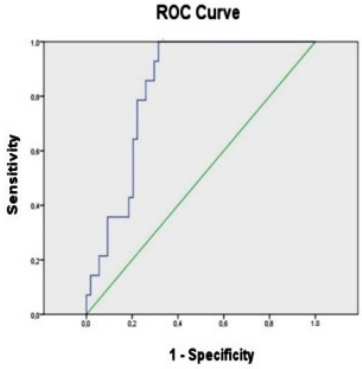

Fig. 4. *AUC for the live birth prediction model*

AUC= 0.831 (P=0.000); 95% CI: 0.736-0.925.

The probability calculated by the model of >0.19 means a favorable prognosis for live birth (*P*<0.001). Model sensitivity (86.0%), specificity (70.0%), and PCC (78.6%) indicate a high predictive ability. ROC curves for predicting live birth, taking into account the expression of individual markers in the endometrium, are shown in Figure 4.

Discussion

Predicting the outcomes of ART programs is possible if the "subtle" endometrial patterns of fertility regulation are taken into account. The results obtained showed that effective interaction between the endometrium and the blastocyst during the "implantation window" suggests a lower level of VDR expression in endometrial stromal cells.

These results explain the data of studies indicating a decrease in VDR expression in the middle stage of the secretion phase (during the "implantation window") in healthy women and a decrease in the antiproliferative effect at low levels of VDR expression.[25] This is probably due to the progesterone-like activity of vitamin D along with the limitation of local pro-inflammatory reactions necessary for blastocyst implantation. The susceptibility of the endometrium during the implantation period was due to a decrease in VDR activity, which probably affects not only the metabolic profile of cell subpopulations, but also the ratio of immune cells.

The data we obtained differ from the results of researchers who indicated the relationship between positive outcomes of IVF and IVF/ICSI programs and increased VDR expression. In the study by J. Guo et al.,[26] for example, the absence of significant statistical differences (*P*=0.083), the younger age of women with pregnancy (*P*=0.032), the small sample size (n=16), and the study of endometrial samples at different phases of the menstrual cycle in different women are factors that limit the significance of the results.

In earlier studies, we identified a decrease in the expression of the VDR protein in the stromal epithelium (*P*=0.016) of women with pregnancy, as opposed to the glandular layer.[27] It has been calculated that a 1% decrease in VDR expression in the stroma increases the chance of a favorable outcome by 1.35 times.[28]

The combination of the optimal expression level of VDR and HOXA11 has a beneficial effect on implantation and the outcomes of IVF and IVF/ICSI programs. Therefore, it is possible to assert that a lower expression of endometrial markers is crucial for the synchronized dialogue between the endometrium and the embryo, as well as for live birth.

Our research results differ from those given in the literature. Thus, Makker A. et al.[29] revealed no differences in protein expression in patients with infertility and uterine fibroids that do not deform the cavity, compared with healthy fertile women. According to other data,[30] the minimum level of HOXA11 in the endometrium was found only in infertility of unknown origin, which was significantly lower than in the control group, in contrast to women with other types of infertility (*P*=0.005). A decrease in HOXA11 expression in the middle of the secretory phase during implantation and in the early stages of pregnancy was determined only in the

glandular endometrium, in contrast to stromal cells, in which high expression persisted throughout the menstrual cycle.[31]

Conclusion

The effectiveness of using the endometrial markers VDR and HOXA11 as potential predictors of ART programs efficiency is confirmed by prognostic models. The levels of the stromal expression of VDR<8.7% and HOXA11<6.1% (probability >0.27) were determined to be favorable for successful blastocyst implantation. The expression levels of VDR<8.3% and HOXA11<6.1% in endometrial stromal cells are prognostically favorable for live birth (probability >0.19) in women of late reproductive age with tubal-peritoneal infertility who undergoing ART treatment with their own oocytes.

Competing Interests

The authors declare that they have no competing interests.

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