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ORIGINAL ARTICLE

Altered expression patterns of circular RNAs Hsa-circ-0089172 as a Predicting and Promising Biomarker of Unexplained Infertility in Female Patients Suffering from Hashimoto's Thyroiditis

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ABSTRACT

Background
Unexplained infertility (UEI) is a complex pathological condition that involves environmental and genetic factors. Hashimoto's thyroiditis (HT) is the most common autoimmune disease in reproductive-age women. Circular RNAs (circRNAs) play a biological role in autoimmunity by regulating gene transcription. A previous study found upregulation of hsa_circ_0089172 in HT. We aimed in the current research to investigate the relative expression level of hsa_circ_0089172 in patients with HT and to assess its correlation with UEI among Egyptian women.
Methods: Among 60 women with HT (35 fertile and 25 women with UEI) and 60 healthy subjects as the control group. all subjects were investigated in clinical, laboratory, and radiological. Hsa- circ- 0089172 was examined by RT-PCR.
Results: There were significantly higher values of hsa_circ_0089172 in UEI group (3.4±0.26) compared to fertile group (2.34±0.33) and control group (0.91±0.216), P <0. 001. Interestingly we observed that anti-TPO and anti TG values were the independent factors associated with hsa_circ_0089172 in the prediction of UEI. hsa_circ_0089172 exposed a significant predictive value of HT, with an AUC of 0.990 (C. I=0.973-1.000), sensitivity of 91.7%, and specificity of 87.1%. Regarding the prediction of UEI among women with HT, the AUC of hsa_circ_0089172 was 0.994(C.I=0.981-1.000), a sensitivity of 94.9%, and a specificity of 89. 3%, P <0 .001
Conclusions: hsa_circ_0089172 level was upregulated in HT patients, particularly patients with UEI. Moreover, it was positively correlated with TSH, anti-TPO, and anti-TG
Key Words: Hashimoto's thyroiditis; unexplained infertility; RT-PCR; hsa_circ_0089172; anti-TPO.

INTRODUCTION

Hashimoto's thyroiditis (HT) is the highest prevalent autoimmune disease in reproductive age, it is estimated that approximately 5%-20% of females have HT. It should be noted that the existence of thyroid peroxidase antibodies (TPOAbs) and thyroglobulin antibodies (TgAbs) is mandatory for the diagnosis of HT [1]. Interestingly previous research detected that the influence of HT on female fertility variables includes miscarriage and decreased fertility [2].

Emerging evidence links unexplained infertility (UEI) to infertility regardless of normal routine infertility investigations [3]. The pathogenesis of UEI is multifactorial, involving various mechanisms such as a positive family history of UEI[4], HT as thyroid hormone has a worthy regulatory impact on female fertility at the levels of higher center for example hypothalamic-pituitary-ovarian axis [5]. Additionally, it has been proposed that epigenetic regulation such as circular (circRNAs), has key roles in the pathogenesis of infertility, and it is also proposed that they could be used as biomarkers of infertility [6].

CircRNAs are one of the non-coding RNAs (ncRNAs), that have been stated to be implicated in many pathological and physiological processes [7]. Previous studies detected upregulation of hsa_circ_0089172 in HT, also the level of this epigenetic was associated clearly with TPOAbs. [8]. the governing role of circRNAs in autoimmune diseases such as HT and UEI has not been defined. Importantly, it was noted that epigenetic regulations are vital for elucidating the molecular origin of HT in UEI. Consequently, there is a rising requirement for diagnostic markers of female UEI. To the best of our knowledge, this is the first Egyptian study that explored the altered expression patterns of circulatory levels of hsa_circ_0089172 in female Egyptian patients with HT in correlation with the risk of development of UEI.

METHODS

This case-control study was conducted on 60 healthy women as a control group and 60 patients with HT. Among patients with HT, 35 patients were fertile, and 25 patients had unexplained infertility. The medical history, physical examination, and laboratory tests were documented for each woman. The flowchart of the study is described in Figure 1. Routine diagnostic analyses were carried out according to Zagazig University Hospital,

***Ethics approval and consent to participate*:** Written notified consent was obtained from all studied women, and the research was accepted by the research ethical committee of the Faculty of Medicine, Zagazig University, (IRB≠, 54/11- **Feb**-2024).

RNA Isolation and qRT-PCR

Total RNA was separated from the PBMCs with TRIzol reagent (Invitrogen, California, USA) matching with the manufacturer's instructions. The primer sequences are shown in. β-Actin was used as a reference gene to quantitatively analyze the genes of interest in the study. The primers were as follows.

Gene	Forward primer (5'-3')	Reverse primer (5'-3'
Hsa_circ_0089172	5'-CCGATAGCACAATGCTTGCC-3'	5'-ATGGCTGTAGAAGGGGTTGC-3'
β-actin	5'-CACGAAACTACCTTCAACTCC-3'	5'-CATACTCCTGCTTGCTGATC-3'

STATISTICAL ANALYSIS

Statistical analysis was executed with SPSS software (version 22.0; IBM Corp.). The normality of variables was confirmed with the Kolmogorov-Smirnov test. An independent sample t-test and Mann-Whitney-U-tests were done. Also, we applied the following tests to analyze the results, Pearson correlation, linear regression test and the receiver operating characteristic (ROC) curve analysis. p-value < 0.05 was considered significant.

RESULTS

We enrolled 60 patients with HT and according to ASRM practice, we selected 25 patients with UEI in addition to 60 fertile women. To avoid any influence examined age on gene expression we match both case and control groups as regards age. Table 1 presents the anthropometric and laboratory variables of the studied subjects. Concerning age, BMI, estradiol, progesterone, or total testosterone. prolactin, AFC, FSH or, LH levels were non-significant differences, P>0.05. However, the HT group demonstrated significantly higher TSH, anti-TPO, and anti-TG levels than the control group. Additionally, free T4 levels were low in the HT group, P<0.001*.

To analyze the demographic, clinical, and laboratory features of the UEI group, we compared the fertile group and the UEI group in Table 2. We detected that among the HT group, the UEI had high TSH, anti-TPO, and anti-TG values, P<0.001*. Nevertheless, free T4 levels were significantly lower in the UEI group compared to the fertile group, P<0.001*. the studied hormonal profile detected no significant differences in estradiol, progesterone, prolactin, FSH, and LH, P>0.05.

Regards infertility demographic features in the UEI group. As shown in figure 2, 19 women had primary infertility,6 women had secondary infertility, the mean duration of infertility (8.4±1.9 years), 9 women had a history of previous miscarriage or abortion, and 4 women had a family history of UEI.

To assess the relative expression of hsa_circ_0089172 in the studied groups, we used the ANOVA test and we detected significantly higher values in the UEI group (3.4±0.26) compared

to the fertile group (2.34±0.33) and control group (0.91±0.216). Figure 3, P <0 .001.

We have demonstrated that by applying Pearson correlation, TSH, anti-TPO, and anti-TG values were significantly positively correlated with hsa_circ_0089172 values among the studied clinical and laboratory features of UEI women as described in Table 3, P <0 .001.

The results of the linear regression test to investigate the independent factors that were associated with hsa_circ_0089172 in the prediction of UEI was that only anti-TPO and anti-TG were the independent factors that were associated with hsa_circ_0089172, table 4, P <0 .001.

To examine the efficiency of hsa_circ_0089172 relative expression level in predicting HT among studied women. We applied ROC curve analysis and the results revealed that AUC of hsa_circ_0089172 =0.990 (C. I=0.973-1.000), a sensitivity of 91.7%, and specificity of 87.1% at a cutoff of 3.5 (Fig. 4), P <0 .001.

To analyze the effectiveness of hsa_circ_0089172 level in expecting UEI among women with HT, ROC curve outcomes showing that hsa_circ_0089172 demonstrated AUC of 0 .994(C. I=0.981-1.000), sensitivity of 94.9% and specificity of 89. 3% at a cutoff of 1.95 (Fig. 5), P <0 .001.

Table 1: - Anthropometric and biochemical characteristics in HT patients

Parameter	Controls group, (*n*=60)	HT group, (*n*=60)	*P* value
Age (years)	25.1±9.6	27.6±9.3	0.134
BMI (kg/m^2)	22.9±1.8	23.2±1.6	0.336
FT4(ng/dl)	1.68±0.14	1.13±0.51	<0.001*
TSH (µIU/ml)	2.87±0.14	8.16±1.3	<0.001*
Anti TPO(IU/ml)	49.01±2.4	272.8±68.6	<0.001*
Anti TG (IU/ml)	0.58±0.14	4.16±1.19	<0.001*
Estradiol (pg/ml)	54.82±6.43	52.88±8.4	0.145
Progesterone (ng/ml)	11.2±3.1	11.86±2.8	0.268
Total testosterone (ng/mL)	0.64±0.1	0.63±0.09	0.744
Prolactin (ng/mL)	12.6±3.7	13.76±1.8	0.672
AFC	13.1±1.9	14.2±4.5	0.083
FSH (mIU/mL)	7.8±1.3	7.06±1.4	0.654
LH (mIU/ml)	5.5±1.2	4.4±3.5	0.656
AMH (ng/mL)	2.75±0.91	2.5±0.72	0.097

*** P < 0.05 .**

Table2: clinical and demographic characteristics of women with HT.

Parameter	Fertile group (*n*=35)	UEI group (*n*=25)	P value
age	26.3±5.6	27.3±4.4	0.460
Age of menarche	12.2±2.6	13.2±1.9	0.107
Marital status (duration in years)	8.3±2.6	7.9 ±3.1	0.589
BMI (kg/m^2)	23.7±2.7	25.7±2.5	0.421
FT4(ng/dl)	1.42±0.21	0.52±0.25	<0.001*
TSH (µIU/ml)	7.48±2.82	9.57±3.01	<0.001*
Anti TPO(IU/ml)	235.2±38.2	342.4±56.4	<0.001*
Anti TG (IU/ml)	3.2±0.6	5.63±0.28	<0.001*
Estradiol (pg/ml)	52.6±6.7	51.23±5.1	0.851
Progesterone (ng/ml)	11.9±1.5	11.4±0.6	0.12
Prolactin (ng/mL)	9.7±1.33	9.95±1.33	0.475
AFC	13.6±1.9	14.2±1.1	0.162

Parameter	Fertile group (*n*=35)	UEI group (*n*=25)	P value
FSH (mIU/mL)	7.5±1.33	7.9±1.43	0.270
LH (mIU/ml)	5.3±1.6	5.7±1.1	0.599
AMH (ng/mL)	2.49±0.42	2.51±0.31	0.833

*** P < 0.05.**

Table 3: Pearson correlation between Relative expression of hsa_circ_0089172 with clinical and laboratory characteristics in UEI group.

Variable	hsa_circ_008917	
	r	p
Age of menarche	0.068	0.607
FT4(ng/dl)	-0.047	0.726
TSH (µIU/ml)	0.385	<0.05*
Anti TPO(IU/ml)	0.612	<0.001*
Anti TG (IU/ml)	0.333	<0.001*
AFC	0.038	0.778
FSH (mIU/mL)	0.077	0.557
LH (mIU/ml)	0.038	0.778
AMH (ng/mL)	0.016	0.903

*** P < 0.05**

Table 4: linear regression analyses in UEI patients to test the influence of the main independent variables against hsa_circ_008917

Model	Unstandardized Coefficients		Standardized Coefficients	t	p	95% C. I	
	B	SE	Beta			Lower Bound	Upper Bound
(Constant)	4.660	0.405		11.502	<0.001*	3.848	5.472
AMH	-1.175	1.460	-0.129	-0.805	0.424	-4.102	1.752
Anti TPO	-0.871	0.307	-0.457	-2.840	<0.001*	-1.486	-0.256
Anti TG	-0.002	0.001	-0.278	-2.689	<0.001*	-0.004	-0.001
TSH	0.015	0.031	0.048	0.473	0.638	-0.048	0.078

*** P < 0.05**

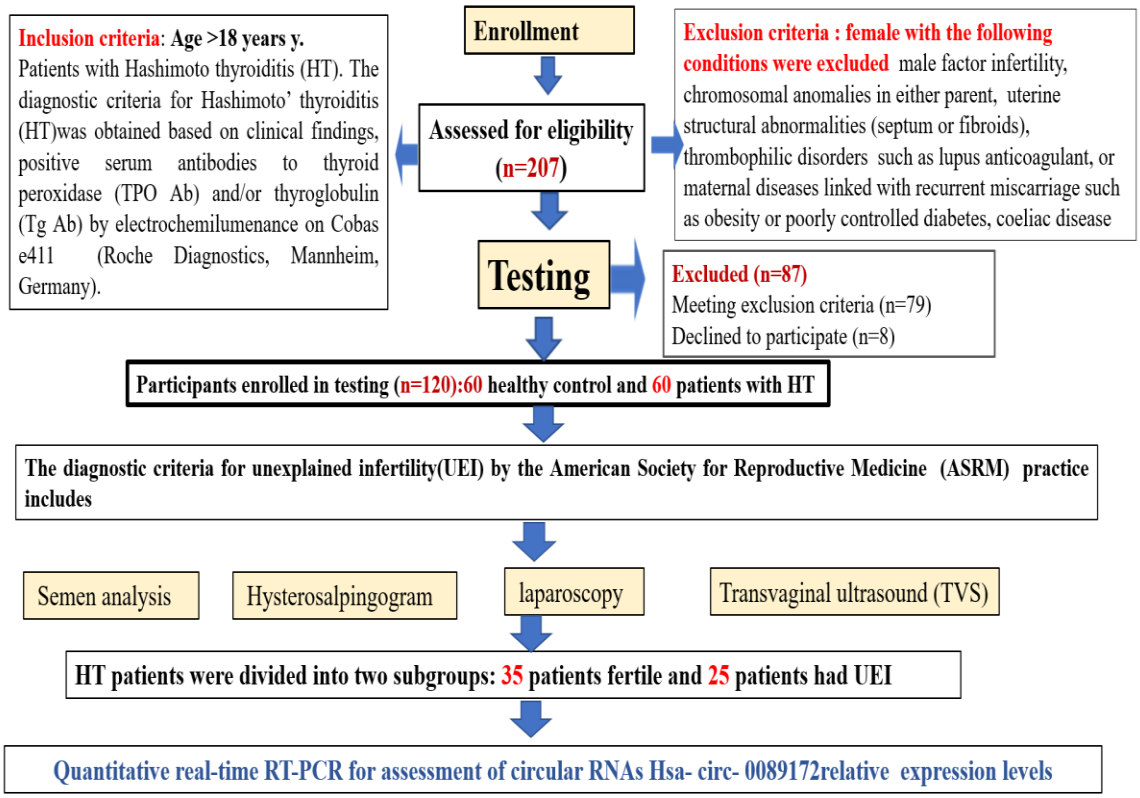

Figure 1: The flowchart of the study

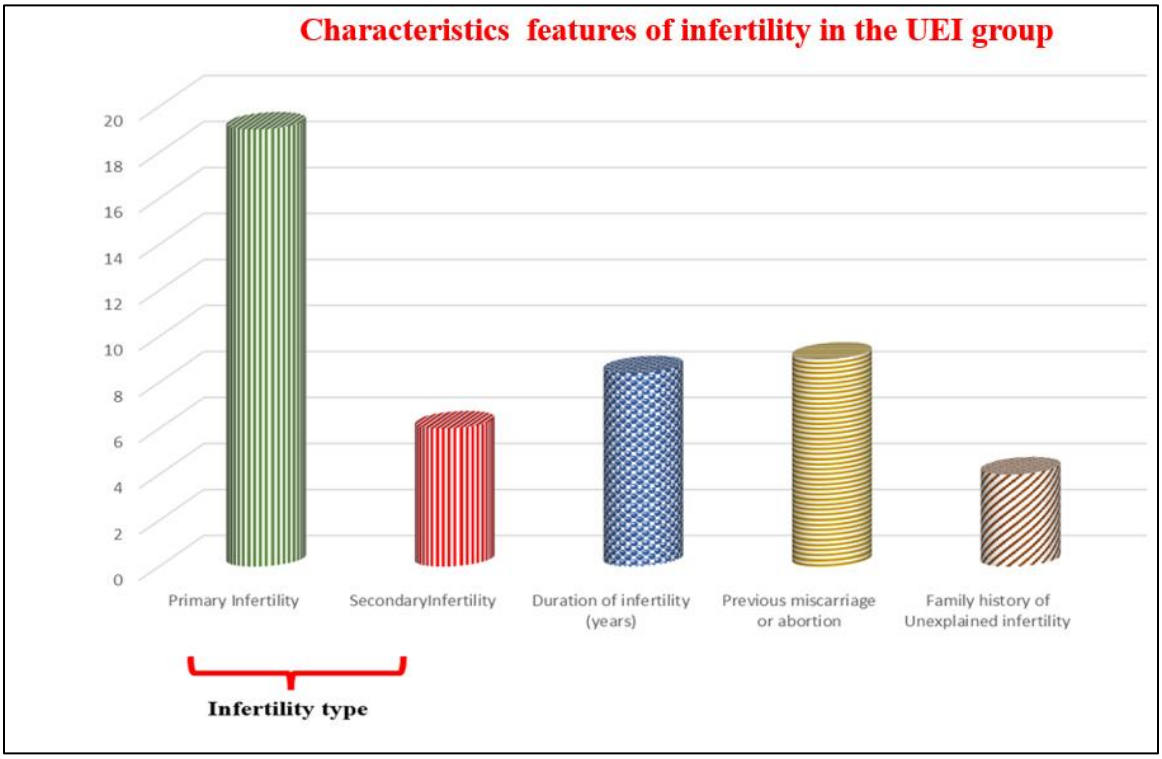

Figure 2: Characteristics features of infertility in the UEI group

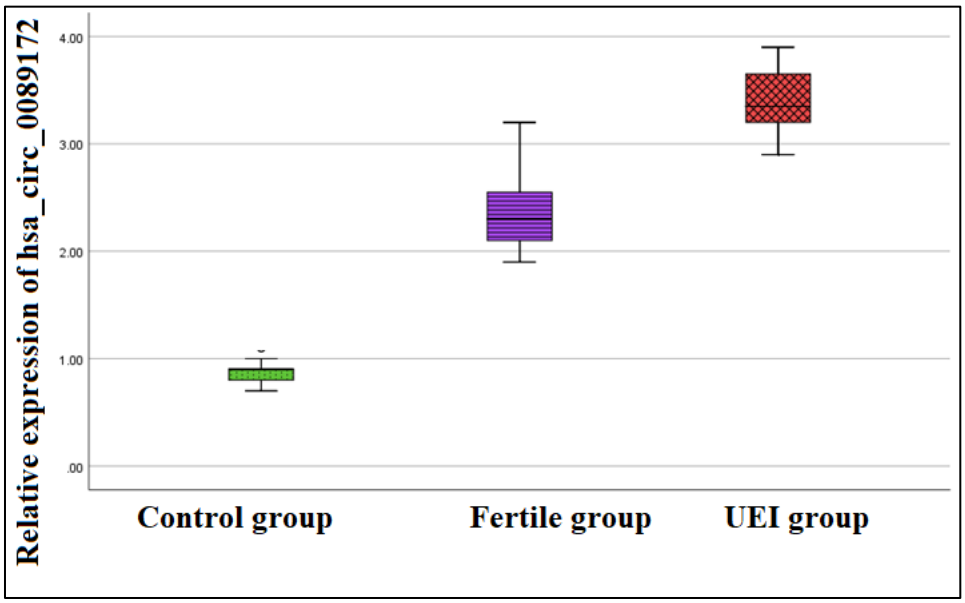

Figure 3: Comparison between studied groups regards the relative expression of hsa_circ_0089172

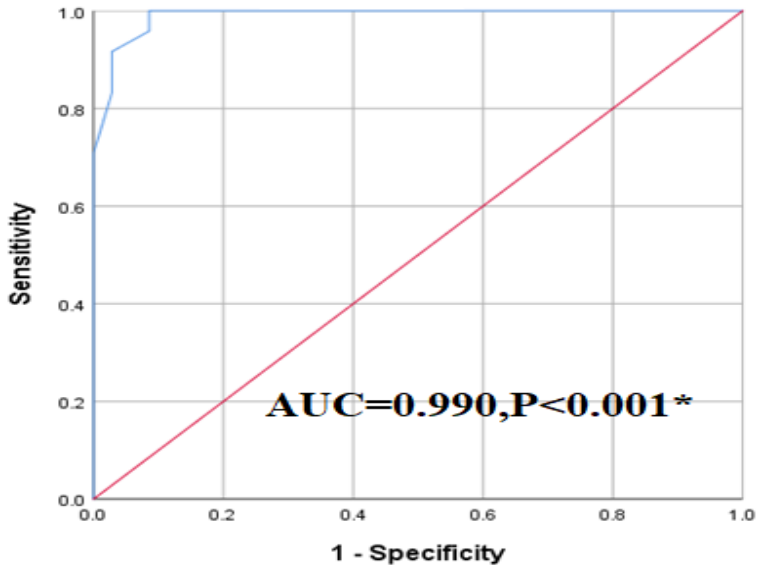

Figure 4: ROC analysis to investigate the efficiency of hsa_circ_0089172 relative expression level in predicting of HT among studied women.

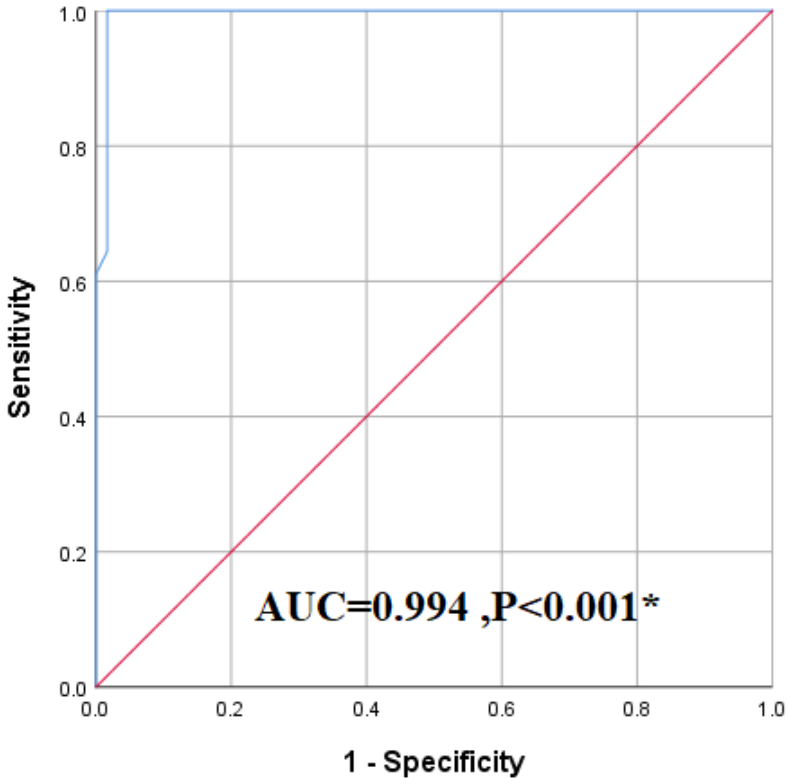

Figure 5: ROC analysis to investigate the efficiency of hsa_circ_0089172 relative expression level in predicting UEI among women with HT.

DISCUSSION

Accumulating evidence investigated the pathophysiological associations between TAI and infertility and they demonstrated that different mechanisms attributed to UEI including thyroid hormone and immunological action of HT on the female reproductive system [9].

Our findings demonstrated that among 60 women with HT,25(41.7%) women suffered from UEI. Out of 25 women with UEI,19 women had primary infertility and 6 women had secondary infertility. Similarly, the results of our previous study found that about 37.5 % of the studied females who suffered from HT had UEI [10].

Consistent with the results, a previous study evaluated the prevalence of TAI in infertile women and found that approximately 26% of women had anovulation [11], while 30 % of women had idiopathic infertility and endometriosis [12]. Hamad et al observed that the prevalence of TAI in patients exhibiting IVF/ICSI is 25.3% which is higher than the fertile women [13].

On the other hand, other studies observed that the prevalence of TAI in infertile women was non-significant compared to the general population and ranged from 6.8 to 14.5% [13,14]. the discrepancy between studies' results could be due to many factors such as differences in sample size, age, and methods of autoantibody assay.

Our studies revealed that approximately 9 women had a history of previous miscarriage or abortion, and 4 women had a family history of UEI. Similar findings were observed in other studies [4,15].

Mirzaei et al found that circRNAs are plausible contributors to many diseases [16]. Nevertheless, the regulatory mechanism of circRNAs in HT is unknown [17]. To analyze the regulatory role of hsa_circ_0089172 levels in the studied groups, we used the ANOVA test and we found that there was a significant overexpression of hsa_circ_0089172 in UEI group in comparison to the fertile group and control group, P <0 .001. Similar to the current research findings, Xiong and his colleagues detected overexpression of hsa_circ_0089172 in HT compared to control [8].

To further explore the associations of hsa_circ_0089172 with other studied parameters we detected that TSH, anti-TPO, and anti-TG were

significantly positively correlated with hsa_circ_0089172 in women with UEI, P <0 .001. Additionally, we further confirmed that the independent factors that were associated with hsa_circ_0089172 in the prediction of UEI were only anti-TPO and anti-TG, P <0 .001.

To examine the efficiency of hsa_circ_0089172 relative expression level in predicting HT among studied women. We applied ROC curve analysis and the results revealed that hsa_circ_0089172 exhibited the most robust predictive value, with an area under the curve (AUC) of 0.990 (C. I=0.973-1.000), a sensitivity of 91.7%, and specificity of 87.1% at a cutoff of 3.5 (Fig. 4), P <0 .001.

Xiong and his colleagues observed that hsa_circ_0089172 had positive associations with TPOAb [8], even though they found a non-meaningful correlation between hsa_circ_0089172 and TGAb in HT [8].

An interesting study by Dang et al. observed that circRNAs are implicated in implantation [18]. Thus, alteration of these epigenetic markers could lead to infertility. Ultimately, we confirmed whether hsa_circ_0089172 could be used as a diagnostic marker of HT and UEI. Our finding regards the power of hsa_circ_0089172 in diagnosis HT and UEI depended on the ROC curve results and we detected that the effectiveness of the hsa_circ_0089172 level in predicting HT, ROC curve results exposed that hsa_circ_0089172 exhibited the strongest predictive value, with a sensitivity of 91.7% and specificity of 87.1% at, P <0.001. Concerning UEI, hsa_circ_0089172 had a strong diagnostic power of UEI among women with HT with a sensitivity of 94.9% and specificity of 89. 3%, P <0.001.According to this finding the level of hsa_circ_0089172 has a high significant value in HT diagnosis, in particularly females with UEL.Similarly, the ROC curve finding in Xiong et al study detected that hsa_circ_0089172 as one of the studied markers has significant power in the diagnosis of HT[8].

The study of Xu et al. found 11 aberrant expressed circRNA in endometriosis, in particular, circ_0004712 and circ_0002918 were upregulated in ovarian endometriosis and they suggested that they could be diagnostic markers of ovarian endometriosis [19]. Also, Liu et al. revealed altered expression of hsa_circ_103,716 and hsa_circ_070616 in repeated implantation failure [20].

LIMITATIONS OF THE STUDY

The results of this research have few limitations, a small sample size. And we did not investigate the tissue level of hsa_circ_070616. In the future, we will handle a large sample study and we will combined tissue and circulatory tests for better evaluation of the studied marker.

CONCLUSION

Our study results detected that the hsa_circ_0089172 level was upregulated in HT patients, in particularly patients with UEI. Also, it was associated with TSH, anti-TPO, and anti-TG. Thus, circulatory hsa_circ_0089172 could be used for diagnosis of EUI associated with HT.

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