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The Potential Regulatory Role of miR-378 in the Expression of Bone Morphogenetic Protein-15 in Infertile Women with Hyperprolactinemia (HPL)

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Abstract

The aim of this study is to investigate the relationship between microRNA 378 and BMP15 gene expression levels in blood samples collected from 50 healthy fertile females as controls and 50 hyperprolactinemia infertile females by quantitative real-time polymerase chain reaction (RT-qPCR). Specific primers were designed for this purpose based on the sequences of microRNA 378 and BMP15 retrieved from NCBI and designed by primer 3 software. The result assessing the expression level of BMP15 in hyperprolactinemia (HPL) was 0.220, while the control group's fold change value was 1.000. The HPL group showed downregulation in the expression of the BMP15 gene. While the fold expression values of the miRNA378 gene in the hyperprolactinemia (HPL) group were 2.227 and the control group's mean value was 1.000, the HPL group showed up-regulation in the expression of the miRNA378 gene. The results of the study revealed that miR-378 has directly regulated BMP15 gene expression; based on this, BMP15 gene expression was obviously decreased. Therefore, the expression level of BMP15 was negatively correlated with miR-378 expression.

Keywords: BMP15 gene, gene expression, miRNA-378 , HPL.

الدور التنظيمي المحتمل للموروث miR-378 في التعبير عن البروتينات المورفينية للعظام -15 في النساء المصابات بالعقم نتيجة فرط إنتاج البرولاكتين الدم (HPL)

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الخلاصة:

تهدف الدراسة الحالية إلى التحقق من العلاقة بين مستوى التعبير الجيني للموروث microRNA 378 و الموروث BMP15 في عينات الدم التي تم جمعها من 50 أنثى خصبة صحية كمجموعة سيطرة و 50 أنثى عقيمة نتيجة فرط إنتاج هرمون البرولاكتين HPL، حيث تم الكشف الكمي النسبي للتعبير باستعمال تقنية RT-PCR. من خلال استعمال بوائى متخصصة مصممة لهذا الغرض ، تم استرجاع تسلسل BMP15 و miRNA-378 من NCBI وتصميم البوائى بواسطة برنامج primer 3 . حيث أظهرت النتائج ، أن مستوى التعبير الجيني BMP15 لعينه من النساء العقيمات نتيجة فرط إنتاج برولاكتين الدم (HPL)

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بينما كان مستوى التعبير الجيني لمجموعة التحكم (1.000) ، أظهرت مجموعة HPL انخفاضاً ملحوظاً في التعبير الجيني للموروث BMP15. بينما كان مستوى التعبير الجيني للموروث miRNA378 في مجموعة النساء العقيمات نتيجة فرط إنتاج بروتينتين الدم (2.227) ، ومستوى التعبير الجيني لمجموعة التحكم (1.000) ، أظهرت مجموعة HPL ارتفاعاً في التعبير الجيني للموروث miRNA378. أثبتت نتائج الدراسة أن الانخفاض في التعبير الجيني للموروث miR-378 قد نظم بشكل مباشر تعبير البروتين BMP15 ؛ على أساس التعبير الجيني للموروث BMP15 حيث انخفض بشكل واضح. لذلك كان مستوى التعبير الجيني للموروث BMP15 مرتبطاً سلباً بالتعبير الجيني للموروث miR-378.

1. Introduction

Recent World Health Organization (WHO) research estimates that 8–10% of couples experience infertility issues [1]. Female infertility is a multifactorial syndrome that includes a wide range of disorders. In more than 50% of infertile women, the cause of their infertility is unknown (i.e., idiopathic), and it can be congenital or acquired. However, several factors are involved, including genetic, immunological, and environmental factors, as well as hormonal imbalance, but the pathway of interaction has not been precisely defined and is open to speculation [3, 4]. The bone morphogenetic proteins 15, which control female fertility [5, 6], have a big effect on follicular development and the rate of ovulation in mammals that only have one ovulation, like humans. The bone morphogenetic protein-15 was recognized as a candidate compound to stimulate oocyte development and open up fresh avenues for infertility treatment [7].

Bone morphogenetic protein 15 (BMP15) is strongly associated with reproduction and women's reproductive diseases. As a multifunctional oocyte-specific secretory factor, BMP15 controls female fertility and follicular development [8, 9]. Oogenesis is regulated by almost 1,361 different gene transcripts expressed in human oocytes, genetic modification of these genes, as well as their regulatory molecules, including miRNAs [10, 11]. MicroRNAs are non-coding RNAs that bind target mRNA and inhibit protein synthesis. In order to suppress the production of its target mRNA, the miRNA acts as a guide by partnering with the mRNA's base sequence [12, 13]. They play important functions in ovarian follicle formation, oocyte maturation, embryo development, embryo-maternal communication, and pregnancy establishment [14]. Aberrant expression of miRNA is connected with a wide range of human health problems, including cancer, viruses, cardiovascular disease, and infertility [15]. The present study has shown the relationship of gene expression between the BMP15 and miRNA-378 genes as well as the regulatory role of their gene expression in predicting female infertility.

2. Material and methods

2.1: Patients and controls: The purpose of this research was to predict potential outcomes. During their visits to the Al-Elwiya Educational and Kadhimiya Teaching Hospital in Baghdad, Iraq, blood samples from a hundred fertile and infertile patients with an average age of 24–45 years were collected. This hundred women, which were divided into two groups, the first group consisting of 50 infertile women with hyperprolactinemia (HPL), and the second group consisting of 50 healthy fertile women, were selected according to standardized diagnostic criteria [16, 17].

2.2: Gene expression of BMP15: The expression of the BMP15 gene is determined via total RNA extraction followed by the reverse transcription quantitative polymerase chain reaction (RTqPCR) technique. The relative mRNA levels were determined by reverse transcription-quantitative PCR (RT-qPCR), as previously described [18]. Total RNA was extracted using

the TRIzol reagent (TransZol Up Plus, TransGen, biotech.) (Trizo, Trans; India), and the cDNA was synthesized with a real-time (RT) reagent SuperMix Kit and quantified using TransStart® Top Green qPCR Super Mix (TransGen, biotech. AQ131-01) by quantitative PCR (RT-qPCR) with specific primer pairs (Table 1) according to the previously described protocol [18]. The forward and reverse primers (oligonucleotide) of the BMP15 gene were designed. In addition, both forward and reverse primers for the housekeeping gene GAPDH are included (reference gene: glyceraldehyde-3-phosphate dehydrogenase) [19].

Table 1: All primer sequences utilized in BMP15 gene expression

Primer	Sequence (5'→3' direction)	primer size bp	Product size bp	Tm °C
<i>BMP15 (Gene Expression)</i>				
(Forward)	CTAGAAGAATCCCCTGGCGA	20	125	62
(Reverse)	ATGGTGCGGTTCTCTCTAGG			
<i>GAPDH- Glyceraldehyde 3-phosphate dehydrogenase</i>				
Forward	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reverse	GAGCCCCAGCCTTCTCCATG	20		

According to the manufacturer's instructions, the final volume of the reaction mixture was 20 µl, as follows: 10 µl of TransStart® Top Green qPCR Super Mix (2X) for 2-Step RT-qPCR, 1 µl of each primer (10 mM), 2 µl cDNA, and 6µl nuclease-free water. The mix was transferred to a real-time thermocycler (MIC-4 Real-time PCR System, MX 3000P™ / StratageneL/ USA), which was set to optimal cycles, including an initial denaturation of 30 seconds at 94 °C, followed by 5 seconds of denaturation at 94 °C, 15 seconds of annealing at 60 °C, 20 seconds of extension at 72 °C, and finally one cycle of the melt curve at 55 to 95 °C. For each gene, the fold change value was calculated using the 2- $\Delta\Delta C_t$ since the calibrator is the target gene in the control individuals, and the findings were presented as a fold change in the expression level of the target gene adjusted to an endogenous control (housekeeping gene) [20, 21].

2.3: Gene expression of miRNA378: Total miRNA was extracted from all samples using the EasyPure® miRNA Kit Reagent according to the manufacturer's instructions. The expression levels of miRNA 378 and U6 genes were assessed by measuring the threshold cycle (Ct) using the TransStart® Top Green qPCR Super Mix kit components. As recommended by the manufacturer, the method was carried out in a 20 µl reaction volume. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was carried out using TransStart® Top Green qPCR Super Mix (TransGen, biotech. AQ131-01) and cDNA as a template. As shown in Table 2, the forward and reverse primers (oligonucleotides) of the miRNA378 gene were designed. Also given the forward and reverse primers of the housekeeping gene U6.

Table 2: All primer sequences utilized in miRNA-378 gene expression

Primer	Sequence (5'→3' direction)	primer size bp	Product size bp	Tm °C
<i>miRNA</i>				
miRNA 378a-3p	TGGGGACTGGACTTGGAGTC	20	70	58
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT	22	73	
miRNA-	GCGAGCACAGAATTAATACGAC	22		

According to the manufacturer's instructions, the final volume of the reaction mixture was 20 μ l, and it consisted of 10 μ l of TransStart® Top Green qPCR Super Mix (2X), 1 μ l of each primer (10 mM), 2 μ l of cDNA, and 6 μ l of nuclease-free water. The mix was transferred to a real-time thermocycler (MIC-4 Real-time PCR System, MX 3000P™ / StratageneL/ USA). According to the thermal profile, the cycling protocol was optimized, including: initial denaturation (30 seconds at 94 °C), denaturation (5 seconds at 94 °C) (40 cycles), annealing (15 seconds at 60 °C) (40 cycles), extension (20 seconds at 72 °C) (40 cycles), and finally a melt curve (55 °C to 95 °C) (1 cycle). The $2^{-\Delta\Delta C_t}$ was calculated to determine the relative fold change in gene expression for each gene. Since the calibrator is the target gene in the control individuals, the findings were presented as the fold change value of the target gene adjusted to an endogenous control (housekeeping gene) [21, 22].

2.4: Literature findings: Google Scholar (<https://scholar.google.com/>) was used to study miRNA's role in HPL disease. The search terms used were “miRNA-378 down-regulates BMP15 in HPL” and “miRNA role in HPL infertility in women caused by gene silencing.” Google Scholar is a search engine that facilitates the user's access to books, journals, and articles on various topics, including sciences (medical fields) at most and social sciences at second [23].

2.4: MicroRNA and mRNA alignment

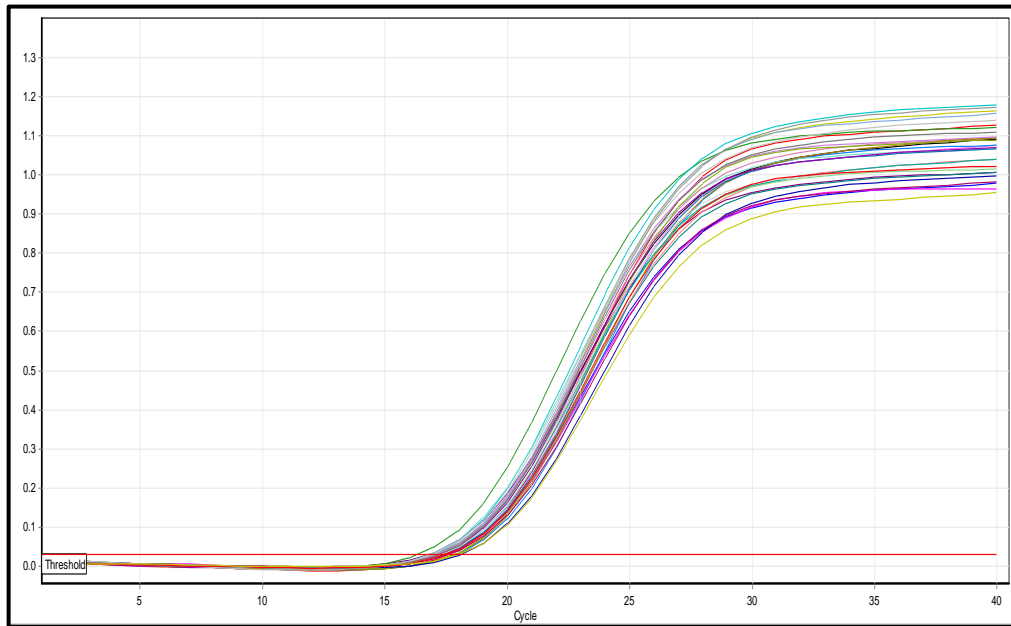
The alignment of microRNA sequences and BMP15 mRNA sequences was performed through the RNA22 tool (<https://cm.jefferson.edu/rna22/Interactive/>) [24]. The RNA22 tool takes sequences in Fasta format for microRNAs up to 50 and the mRNA sequence of one gene as an input. The alignment was performed using default parameters, but parameters were modified for the alignment of microRNA miR-378 and mRNA BMP15.

2.6: Statistical analysis: Data were statistically analyzed, and the IBM SPSS Statistics 26 program was used to detect the effect of different factors on study parameters. Statistically, significant comparisons between means were made using a one-way analysis of variance and the *T-test*. The results were shown as a mean + SD. The Chi-square test was developed to statistically compare the percentages of two categorical variables (0.05 and 0.01 probability). The Chi-square test was used to compare patient and control group frequencies of genotypes and allelic variants [25, 26].

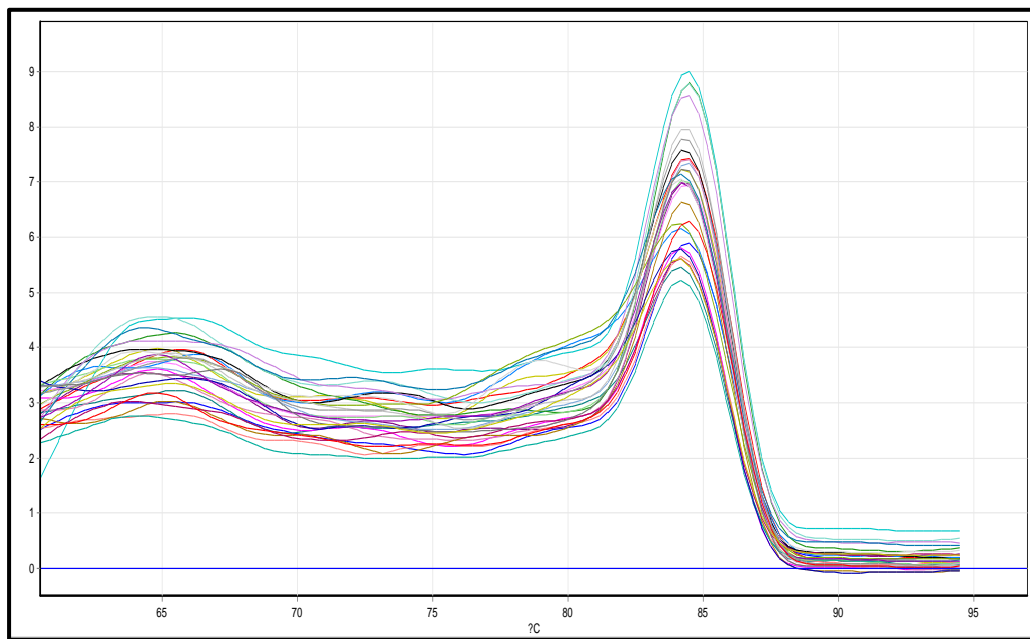
3. Result and discussion

3.1: BMP15 and miRNA378 genes Expression: The expression levels of BMP15 and miRNA378 genes were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and the gene expressions were normalized against those of housekeeping genes GAPDH and RNAU6 and quantified by the ΔC_t value and folding ($2^{-\Delta\Delta C_t}$) method. As shown in Table 3, the level of relative gene expression of BMP15 in the infertile group was significantly higher in those with HPL (7.533 ± 1.05) compared with the fertile control group (6.725 ± 0.18).

Also, the expression level of the BMP15 gene in infertile females was lower in those in the HPL group (0.220) than in the control group (1.000). The HPL group showed down-regulation in the expression of the BMP15 gene. These findings imply that the fold of gene expression differed significantly among these groups ($p = 0.001^{**}$).



(A)



(B)

Figure 1: The normalized fluorescence and the melting curve of BMP15 (A) The BMP15

Table 3: The gene expression level of the BMP15 gene among the study groups

Study Groups	Mean ± SD of					The gene expression Depending on $2^{-\Delta Ct}$
	BMP15 Ct value	ΔCt	ΔCTC	$\Delta \Delta Ct$	$2^{-\Delta \Delta Ct}$	
Group 1: Control	23.651± 1.03	6.725± 0.18	5.041± 0.83	1.683± 0.77	0.36±0.19	1.000
Group 2: HPL	25.182± 0.96	7.533± 1.05	6.532±	1.001± 1.32	0.71±0.69	0.220

	0.89					
P-value	0.0001**	0.0001**	0.0001**	0.005**	0.002**	0.0001**

These results show that the BMP15 gene is less active in the HPL group. This suggests that the BMP15 gene could be used as a biomarker to spot infertility early on. The amplification plots and dissociation curves for the BMP15 gene are shown in Figure 1. gene amplification was plotted using qPCR samples that covered all research groups. The CT values varied between 17 and 20. (B) MP15 gene dissociation curves using qPCR samples that covered all research groups Melting temperatures varied from 78 °C to 81 °C. The images were captured using the Qiagen Rotor-Gene Q qPCR apparatus

While Table 4 observed that, the ΔCt mean \pm SD showed a significant ($p = 0.0001^{**}$) difference in the hyperprolactinemia (HPL) (2.862 ± 1.40) group when compared to the corresponding fertile control group (4.268 ± 0.90).

The gene expression of the miRNA378 gene in hyperprolactinemia (HPL) was (2.227), while the control group value was (1.000). The HPL group showed up-regulation in the expression of the miRNA378 gene. These findings imply that the level of gene expression differed significantly among the two groups ($p = 0.001^{**}$).

Table 4: The gene expression level of miRNA 378 among the study groups

Study Groups	Mean \pm SD of					
	miRNA378 Ct value	ΔCt	ΔCTC	$\Delta \Delta Ct$	$2^{-\Delta \Delta Ct}$	Gene expression depending on $2^{-\Delta Ct}$
Group 1: Control	21.255 \pm 1.04	4.268 \pm 0.90	3.210 \pm 0.00	1.058 \pm 0.90	0.5595 \pm 0.24	1.000
Group 2: HPL	22.049 \pm 1.49	2.862 \pm 1.40	3.413 \pm 0.86	-.550 \pm 1.04	1.7152 \pm 0.73	2.227
P-value	0.0001**	0.0001**	0.003**	0.0001**	0.0001**	0.0001**

• Values are means \pm standard deviation of means.
• ** Indicate the highly significant ($p < 0.01$).

The amplification plots and dissociation curves for the miRNA378 gene are depicted in Figure 2.

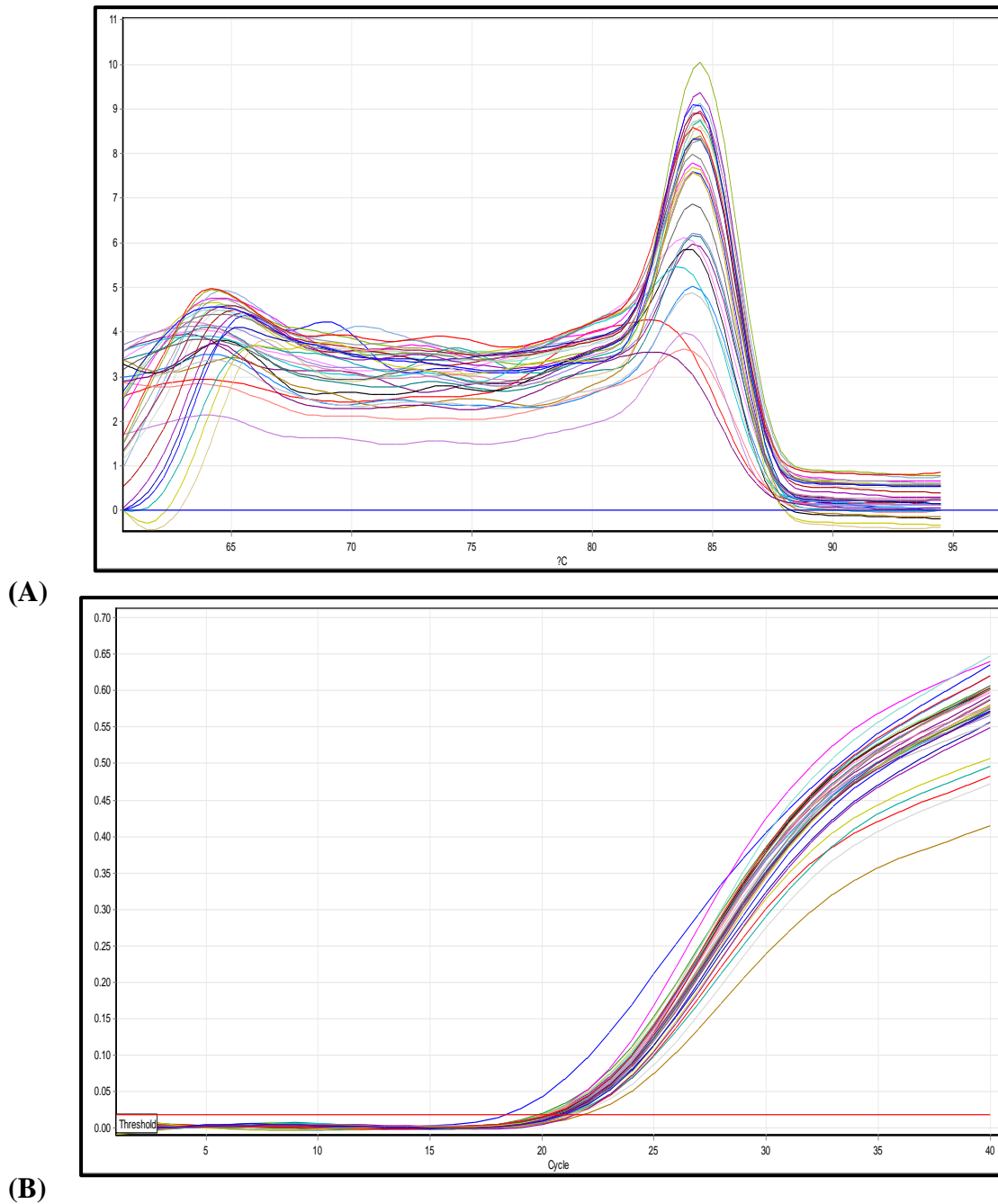


Figure 2: The normalized fluorescence

and the melting curve of miRNA 378 (A) The miRNA 378 gene amplification was plotted using qPCR samples that covered all research groups. The CT values varied between 19 and 21. (B) miRNA378 gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 78 °C to 81 °C. The images were captured using the Qiagen Rotor-Gene Q qPCR apparatus.

A number of miRNAs are overrepresented in the mammalian oocyte, as determined by expression profiling studies. This suggests that miRNAs have a role in many steps of the oogenesis process. One miRNA may control many RNAs, or a single gene might be regulated by numerous miRNAs, adding complexity to the interaction between genes and miRNAs [27]. MiRNAs play an essential function in the cell, and abnormalities in miRNA regulation have been linked to several illnesses, including sterility [28]. Recent studies suggest that miR-378

is a candidate miRNA that controls the expression of BMP15 [29]. This was confirmed by the fact that miR-378 was highly expressed in hyperprolactinemia patients. The results showed that miR-378 did, in fact, directly control the expression of the BMP15 protein since the expression of the BMP15 gene was clearly lower [30].

3.2: Identification of miRNA and associated genes through literature findings

Literature was studied to verify MicroRNA-378 and its affected gene, BMP15. Through a literature review, it was inferred that due to the up-regulation of miRNA-378, the BMP15 gene was down-regulated along with other genes such as GDF9, ZP3, and CX37 in females with hyperprolactinemia (HPL) [31]. Another study showed that the BMP15 gene was down-regulated due to the up-regulation of miRNA-21 in non-fertile women [32]. The BMP15 gene, along with GDF9, CX37, and ZP3, was found to be down-regulated due to overexpression of microRNA-224 in infertile females with polycystic ovary disease. However, gene silencing is not the only factor responsible for infertility in women; up-regulation of genes can also enhance hyperprolactinemia (HPL). According to a study, down-regulation of miR-320 was found to be the cause of the up-regulation of gene ET-1 in certain HPL patients [33]. As most of the studies depict, gene silencing is the evident cause of HPL in females. Hence, the up-regulated microRNA, miRNA-378, was considered for further analysis in order to unravel the potential biomarker in HPL.

3.3: Alignment analysis of BMP15 and miR-378

The RNA22 tool showed alignment results for all of the 4 microRNAs, such as hsa-miR-378a-5p, hsa-miR-378b, hsa-miR-378e, and hsa-miR-378i, against gene BMP15. The alignments were filtered based on a P-value < 0.05. Resultantly, 3 of the alignments, such as hsa-miR-378b-BMP15, hsa-miR-378e-BMP15, and hsa-miR-378i-BMP15, showed P-values less than 0.05, that is, 0.03 [34, 35]. The filtered miR-378-BMP15 alignments were found to be statistically significant due to their lowest P-value (Table 5).

Table 5: The P-values of the alignments of hsa-miR-378b-BMP15, hsa-miR-378e-BMP15, and hsa-miR-378i-BMP15

miRNA Name	binding site	folding energy	Heteroduplex	p value
hsa_miR_378i	603	-8.1	ATATCACACA ACTTGTTCAGC :	0.0336
hsa_miR_378b	605	-6.8	GGAAGACTGAGGATCAGGTCA ATCACACA ACTTGTTCAGC :	0.0336
hsa_miR_378e	606	-8	AAGACGGAGGTTTCAGGTCA TC-ACACA ACTTGTTCAGC :	0.0336
			AGGACTGAGGTTTCAGGTCA	

- (p > 0.05) mean non-significant difference
- (p < 0.05) mean significant difference

Conclusion

BMP15 expression is inversely linked with miR-378 expression. So, this study found that the Iraqi women who complain about HPL have a lower level of BMP15 gene expression because their miRNA-378 expression is higher. This is also the cause of female infertility.

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