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Estimation of miRNA 208 Effects in Inducing Epithelial-Mesenchymal Transition by Targeting *CDH2* in Breast Cancer Patients of Iraqi Population

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Abstract:

Breast cancer is the most frequently diagnosed disease in women and remains a leading cause of cancer-related deaths worldwide. The small non-coding RNA molecule, MicroRNA-208 (miR-208), plays a crucial role in the development and advancement of numerous malignancies, including breast cancer. This study aimed to investigate miR-208 potential as an oncogene in breast cancer by examining its impact on cell motility and epithelial-mesenchymal transition (EMT). Tissue samples from 25 Iraqi women with malignant breast cancer and 25 with benign breast cancer were analysed. Total RNA was extracted from the samples and real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using SYBR Green technology to measure miR-208 expression levels. Tissue samples from both benign and malignant tumours were analysed to determine *U6* gene expression. The analysis showed that the typical Ct values for benign and malignant samples were 29.83 and 29.67 respectively. No significant difference in U6 expression levels was detected between benign and malignant tumours. However, upon comparing the initial malignant and the benign tumours samples, miRNA-208 ΔΔCT values were found to be substantially lower (-7.105 and -0.781 respectively) with a *p*-value of less than 0.05. The study found that malignant tumours had a significantly higher miRNA-208 folding value (2.896±) compared to benign tumours which had substantially lower miRNA-208 folding values (1.591 ±0.37). The study also found that the differences were statistically significant (*p*-value = 1.292*). In conclusion, the study found evidence linking miR-208 to the *CDH2* gene, indicating its role in the development and susceptibility to breast cancer in certain Iraqi women.

Keywords: Breast cancer, miR-208, *CDH2*, Epithelial-Mesenchymal Transition (EMT), N-cadherin, Gene expression, RT-PCR.

تقدير تأثير 208 miRNA في تحفيز الانتقال الظهاري – اللحمي المتوسط من خلال استهداف *CDH2* في مرضى سرطان الثدي في المجتمع العراقي

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الخلاصة

يعتبر سرطان الثدي من أكثر الأمراض التي يتم تشخيصها بشكل متكرر في النساء وتظل سببًا رئيسيًا للوفيات المرتبطة بالسرطان في جميع أنحاء العالم. تلعب جزيئة الرنا الصغيرة غير المشفرة ، miR−208208(miRNA) دورًا حاسمًا في تطور وتقدم العديد من الأورام الخبيثة ، بما في ذلك سرطان الثدي. هدفت هذه الدراسة إلى التحقيق في إمكانية استخدام مير − 208 كجين سرطاني في سرطان الثدي من خلال دراسة تأثيره على حركة الخلية والانتقال الظهاري − الجنيني (EMT) ،تم تحليل عينات الأنسجة من 25 امرأة عراقية عانين من سرطان الثدي الخبيث و 25 امرأة مصابة بسرطان الثدي الحميد. تم استخراج الرنا النوعي الكلي من العينات وأجريت تقنية الرنا النوعي العكسي بوليميراز السلسلة المتبدلة − سلسلة التفاعل (RT−PCR)باستخدام تقنية السايبر الخضراء لقياس مستويات تعبير مير − 208. تم تحليل عينات الأنسجة من الأورام الحميدة والخبيثة لتحديد تعبير جين .U6 أظهر التحليل أن القيم النمطية لـ Ct في العينات الحميدة والخبيثة كانت 29.83 و 29.67 على التوالي. لم يكن هناك فرق معنوي في مستويات تعبير U6 بين الأورام الحميدة والخبيثة. ومع ذلك ، عند مقارنة العينات الأولية بالأورام الخبيثة ، كانت قيم ΔΔCT miRNA208أقل بكثير (−7.105 و −0.781 على التوالي) مع قيمة p أقل من 0.05. أظهرت الدراسة أن للأورام الخبيثة قيمة طي miRNA 208 أعلى بشكل ملحوظ (2.896 ± 2.896) مقارنةً بالأورام الحميدة التي كانت لديها قيم طي miRNA 208 أقل بشكل كبير (1.591 ± 0.37). الدراسة وجدت أيضًا أن الاختلافات كانت ذات دلالة إحصائية) قيمة .(* 1.292 = p في الختام، وجدت الدراسة أدلة تربط بين miR−208وجين*CDH2* ، مما يشير إلى دوره في تطور وقابلية الاصابة بسرطان الثدي لدى بعض النساء العراقيات.

Introduction

Cancer metastasis stands as a significant contributor to cancer-related deaths. The process involves cancer cells within primary tumours exhibiting localized invasion into the surrounding microenvironment. Subsequently, these cells gain access to the vasculature, allowing them to disseminate to distant locations that offer a permissive environment for their growth and survival [1]. Breast cancer is a complicated disease that has various molecular subtypes and classifications. According to the International Agency for Research on Cancer's (IARC) 2020 World Cancer Report, breast cancer is not only most prevalent among women worldwide but is also the leading cause of cancer-related deaths in females [2]. Most malignancies in the ducts and lobules of the mammary glands are ductal cancers, predominantly hereditary disorders. Biomarkers are essential for providing insightful data for cancer diagnosis and prognosis [3][4]. It is a major cause of cancer-related female mortality in Iraq and ranks 1 among the population [5]. Eliminating breast cancer is a difficult task due to the dangerous side effects of conventional therapies like radiation and chemotherapy [6]. CT scan, an essential step in detecting breast cancer, involves using a specialized device to examine the breast for any tumours. A biopsy is then taken from any suspicious area and reviewed to determine if it is benign or malignant. This process helps determine the severity of the condition [7]. The process by which cancer cells go through genetic reprogramming and transform from a non-motile, epithelial phenotype to a migratory, mesenchymal-like phenotype is known as epithelial-to-mesenchymal transition (EMT) which has been observed in many epithelial malignancies. The promotion of metastasis in many types of cancers depends heavily on transition [8]. The downregulation of epithelial cadherin (E-cadherin) expression, together with the concomitant overexpression or de novo expression of neural cadherin (N-cadherin), is a common feature of the epithelial-to-mesenchymal transition (EMT). This process, also known as the "cadherin switch," has been connected to increased cell migration and invasion during EMT [9]. The notable effects of down-regulating E-

cadherin were the loss of stable epithelial cell-cell adhesive junctions, apicobasal cell polarity, and overall epithelial tissue structure. Disruption makes it easier for cancer cells to break free from the original tumour site which increases their capacity for movement and metastasis [10]. The classical cadherin family, a subset of the calcium-dependent adhesion molecule family, includes N-cadherin. Both homotypic (same cell type) and heterotypic (different cell types) cell-cell attachment is made possible by this molecule. N-cadherin is a traditional type I cadherin structurally with five extracellular domains linked to an active intracellular domain. When N-cadherin is involved in cell adhesion, certain methods are used. When N-cadherin monomers from different cells come into contact, they interact reciprocally by inserting a tryptophan residue side chain from one molecule's first extracellular domain (EC1) into the hydrophobic pocket of the other molecule's N-cadherin EC1. Trans-adhesion is the term used to describe this kind of contact. Furthermore, to develop in a stable manner, adjacent N-cadherin monomers on the same cell surface must group for N-cadherin-mediated adhesion. The His-Ala-Val (HAV) motif on N-cadherin's EC1 and a recognition sequence on the second extracellular domain (EC2) of the lateral N-cadherin monomer are both involved in this clustering process. "Cis adhesion" is the name given to this interaction type. The overall cell-cell adhesion mediated by N-cadherin is influenced by these trans and cis adhesion mechanisms working together [11]. The localization of N-cadherin on the cell membrane and its lateral clustering relies on the presence of p120 catenin. This essential protein plays a crucial role in directing N-cadherin to cholesterol-rich micro-domains within the cells [12]. Initial ligation of the extracellular domains of N-cadherin triggers events that activate Rac, a member of the Rho GTPase family. A variety of actions are subsequently started by this activated Rac, including the localized formation of actin filaments and the emergence of membrane protrusions at the locations of cell-cell contact [13]. Rac is initially activated by N-cadherin extracellular domain ligation, and then RhoA, another member of the Rho GTPase family, is afterwards. Rac's function is switched from activation to RhoA which is essential for the development of N-cadherin-based cell-cell junctions. In particular, RhoA causes catenin to be sequestered to the cadherin intracellular domain, strengthening and stabilizing the cell-cell junctions [14]. An in-depth understanding of each tumour's unique characteristics and behaviours can be gained by studying individual tumours. Small non-coding RNAs called microRNAs (miRNAs) control post-transcriptional gene regulation [15]. Specific and distinct geographical, temporal and chamber-specific expression patterns are displayed by miR-208 [16]. It has 2 subfamilies, miR-208a and miR-208b [17]. While miR-208 has been commonly linked to hypertrophy in earlier research, there is a growing body of investigations exploring its involvement in apoptosis. Several of these studies have suggested that miR-208a plays a role in reducing cell apoptosis [18]. Other research demonstrated miR-208's function in the invasion and metastasis of breast and pancreatic cancer cells [19].

Material and Method

Bioinformatics Method

Online miRNA target prediction techniques described below were employed in this study to assess putative miR-208 target genes: Mir-TV Database (https://mirtv.ibms.sinica.edu.tw/), miRWalk (http://mirwalk.umm.uni- heidelberg.de/), and CoMeta Database (https://cometa.tigem.it/).

Samples Collection

The study included fifty participants with breast tumours of various ages, who were recruited from the Teaching Oncology Hospital in Baghdad governorate between March and May 2022. Tissue samples were collected from different classifications and stages of breast tumours using a Microtome equipment to obtain thin sections. Specimens can be categorized

according to their histopathology which involves the examination of a biopsy or surgical sample by a pathologist. This examination occurs after the specimen has undergone processing and histological sections have been mounted on glass slides for observation. Pathological grading systems are employed to assess the microscopic appearance of cells including abnormalities and deviations in their growth rate. The primary objective is to predict development at the tissue level based on these observations. This study divided the FFPET samples into two groups based on their histology and cytological characteristics. The first group comprised 25 samples classified as benign, while the second group comprised 25 as malignant. Excess paraffin from the sample blocks (Formalin-Fixed Paraffin-Embedded) was carefully trimmed and the resulting tissue sections were stored in Eppendorf tubes. Next 5–20 portions were taken out of each Eppendorf tube for further processing. Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections were deparaffinised using a solution that included different reagents such heptane, xylene, limonene, or CitriSolv.

Ethical Approval

The project was approved by the Genetic Engineering and Biotechnology Institute's Ethics Committee (**Date:** 15/5/2022**, Ref:** EC/A /1302).

miRNA Quantitation

miRNA was quantitated using the Qubit 4.0 platform that exhibits outstanding selectivity for miRNA in comparison to other RNA types.. The assay yields good accuracy results for initial sample concentrations ranging from 10 pg/L to 100 ng/L. The assay, when carried out at room temperature, may sustain steady signals for up to three hours. Furthermore, the performance of the Qubit 4.0 test can withstand typical impurities such as salts, free nucleotides, solvents, detergents or proteins without being adversely affected.

Quantitative PCR (qPCR)

After extracting total RNA samples, high-capacity cDNA Kit (ProtoScript® II First Strand cDNA Synthesis Kit, New England Biolabs) was used to reverse transcribe the RNA.

RNA Reverse Transcription

Reverse transcription technique with the ProtoScript® II First Strand cDNA Synthesis kit was used to evaluate the expression of PCR target genes. Using oligo-dT primers, all RNA species were converted into cDNA during this procedure which involved the conversion of RNA to cDNA. The ubiquitous tag sequence on the 5' end of these oligo-dT primers allowed for the subsequent amplification of mature miRNA during the real-time PCR step. Total RNA including miRNA, was the starting material for the reverse transcription procedure. The reverse transcription master mix contained all the elements required for cDNA first-strand synthesis, except RNA template. It was then carefully combined and added to PCR tubes. The template RNA was put into each tube, and then, following quick centrifugation, the lines were put into a thermal cycler. The reverse transcriptase enzyme was inactivated by incubating for 5 minutes at 95°C after 60 minutes at 37°C.

Primer Preparation and Optimization

miRNA genes amplicaion was carried out using specialized primers (Table 1). These primers were provided by MacroGen® in a lyophilized form in picomoles concentration. To create a stock of RNA solution, the lyophilized primers were dissolved in distilled water, resulting in a final concentration of 100 pmol. Subsequently, a working solution of the primers at 10pmol/µl concentration was prepared by combining 10µl of the primer stock solution with 90µl of deionized distilled water.

Table 1: PCR Primers

No.	Primer Name		Primer Sequence (` 5----3`)	Reference
1.	**MiRNA-208**	RT primer	CTCAACTGGTGTCGTGGAGTCGG CAATTCAGTTGAGGCTTTTTG	[20]
		Forward Primer	ACACTCCAGCTGGGATAAGACGA GCA	
		reverse primer	TGGTGTCGTGGAGTCG	
2.	**U6 Housekeeping gene (Reference gene)**	Forward Primer	CTCGCTTCGGCAGCACA	[21]
		reverse primer	AACGCTTCACGAATTTGCGT	

miRNA Detection by RT-qPCR

miRNA levels were measured by RT-qPCR using SYBR Green reagent. After 40 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds, the amplicaton conditions were set as following. The second section of the methodology involved choosing and processing cDNA samples from malignant and begin simultaneously. Every sample was run through two different PCR tubes, one for miRNA-208 and the other for U6 snRNA, the study's housekeeping gene. The fluorescent signal that SYBR Green emits was used to estimate the amount of the amplified products. Specific ingredients were carefully combined to create the reaction of all mixed components and their respectivequantities(Table2).

Table 2: Reaction components of miRNA expression.

No.	Component	20 ul Reaction
1.	Luna Universal qPCR Master Mix	10 ul
2.	Forward primer (10 µM)	1ul
3.	Reverse primer (10 µM)	1ul
4.	Template cDNA	5ul
5.	Nuclease-free Water	3ul

The PCR tubes were subjected to a rapid centrifugation step lasting 1 minute at 2000g to eliminate bubbles and gather the liquid. Real-Time PCR program was configured according to the specified thermocycling protocol (Table 3).

Table 3: Real-Time PCR program for amplification of miRNA 208 and U6 snRNA

No.	Cycle Step	Temperature	Time	Cycles
1.	Initial Denaturation	95°C	60 seconds	1
2.	Denaturation	95°C	15 seconds	40
	Extension	60°C	30 seconds (+plate read)	
3.	Melt Curve	60-95°C	40 minute	1

Statistical Analysis

The Statistical Analysis System [22] program was used to detect the effects of both malignant and benign groups in gene expression of miRNA-208/ fold change. T-test was used to compare significant difference between means.

Results and Discussion

In Silico Study

Multiple web servers were used to browse, search and query miRNA, and target gene data. This allowed for the combination of expression data from hundreds of tissues and cellular

conditions to infer miRNA targets and miRNA-regulated gene networks. These networks were then used for dynamic visualization of clinical data and possible predictions of miRNA-binding sites within the complete sequence of all known genes of three genomes (human, mouse and rat).

RNA Extraction

After RNA extraction, a wide range of RNA concentrations, from low to high concentrations (42.5-38.17ng/l) were obtained. Notably, the total RNA concentrations of the tumour samples did not differ significantly from one another. Furthermore, the RNA purity evaluation reported no appreciable variations within the same group.

miRNA 208 Expression

The miR208, and the reference gene U6, were amplified in the molecular experiment to determine each threshold cycle (Ct) value (Figures 1& 2).

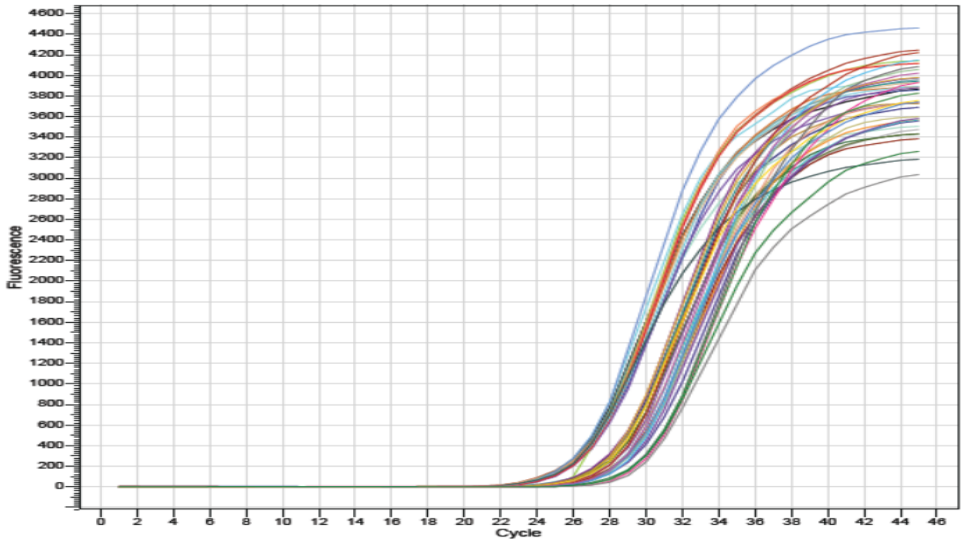

Figure 1: Amplification plots for miR208 expression obtained by Real-Time PCR.

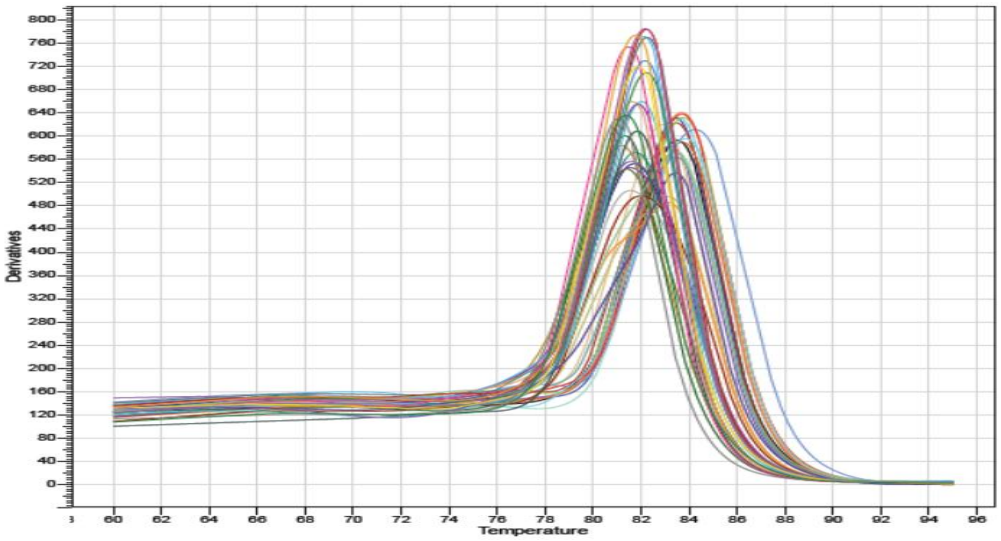

Figure 2: The miR208 expression melting curve.

The melting temperature curve was calculated using the CT values which were also used to quantify real-time RT-PCR data that was inversely related to the amount of beginning template. The expression fold of miRNA-208 is displayed in Table 4.

Table 4): miRNA-208 expression fold.

Group	Mean ± SE				
	miR-208 Ct	**U6 Ct**	**ΔCt**	**ΔΔCt**	**Fold Change**
Benign	31.61	29.83	1.767	-7.105	1.591 ±0.37
Malignant	30.66	29.67	0.986	-0.781	2.896±
***P*-value**	--	--	--	--	1.292 *
T-test	--	--	--	--	0.0478
(P≤0.05) Significant					

Using RT-PCR for miRNA-208, *in vitro* examination of gene expression produced interesting results. Based on the gene expression data of U6 in malignant and benign tumours, the average Ct values for malignant and benign tumours were 29.67 and 29.83 respectively. No significant differences were observed in U6 expression levels between benign and malignant tumours. However, upon comparing benign and malignant tumours, miRNA-208 ΔΔCT values were found to be considerably lower in the former (-7.105 and -0.781 respectively), indicating a significant difference (p<0.05). In particular, it showed that the miRNA-208 folding value in malignant tumours was noticeably greater (2.896±) than in benign tumours (1.591 ±0.37). It's notable that these differences were found to be statistically significant (p-value = 1.292*). Additionally, miRNA-208 folding value increased consistently and significantly as the disease's severity increased. Since *CDH2* has been identified by bio-informatic predictions as a target gene of miR208, N-cadherin expression also increased. This regulation mechanism suggests the potential that miRNA208 functions as an oncogene in breast cancer by promoting cell motility and invasion because it targets *CDH2*.

Breast cancer is one of the malignant tumours that affects women both in developed and the developing countries. Breast cancer cells exhibit a high degree of metastatic tendency, similar to other tumour forms. In addition, breast tumour cells can become resistant to certain medications. The present research focused on highlighting the role of EMT in breast tumour malignancy and its resistance to medications. Since it is an associated mechanism to cancer propagation, the process of Epithelial-Mesenchymal Transition (EMT) enhances the migratory ability of breast carcinoma cells and the process of breast cancer invasion induced by EMT is characterized by increased N-cadherin and vimentin levels, as well as decreased E-cadherin levels [23].

Studies have indicated a significant correlation between miR-208 expression and the onset and progression of liver cancer. As a result, detecting miR-208 expression holds considerable importance in exploring the development of various diseases. The invasive nature of tumour cells and their ability to form metastases play a crucial role in cancer progression. An integral aspect of invasiveness involves the process of epithelial-mesenchymal transition (EMT) [24].

Epithelial-mesenchymal transition (EMT) is a complex process with a primordial role in cellular transformation, whereby an epithelial cell transforms and acquires a mesenchymal phenotype. This transformation plays a pivotal role in tumour progression and self-renewal, and exacerbates resistance to apoptosis and chemotherapy. EMT can be initiated and promoted by deregulated oncogenic signalling pathways, hypoxia and cells in the tumour microenvironment, resulting in a loss-of-epithelial cell polarity, cell–cell adhesion and enhanced invasive/migratory properties [25].

The repressors of the E-cadherin (*CDH1* gene), ZEB1 (zinc finger E-box-binding homeobox 1), and SIP1 (Smad-interacting protein 1, ZEB2, and SMADIP1) operate as EMT-activators and suppress a variety of cancer forms, including pancreatic cancer [26]. It has been demonstrated that proteins ZEB1 and SIP1 and members of the miR-200 family (miR-200a, b, c, miR-141, mir208, and miR-429) reciprocally negatively regulate one another in a feedback loop process that governs the EMT [27].

Overexpression of miR-208 led to a decrease in E-cadherin expression, implying that it could assist in promoting EMT in pancreatic cancer cells [28]. In human oesophageal squamous cell carcinoma, miR-208 has been shown to encourage cell proliferation [29] and colorectal cancer carcinogenesis [30] to promote cell proliferation and invasion in hepatocellular carcinoma and non-small cell lung cancer, or to reduce cell apoptosis in gastric cancer [31]. It was discovered in 2015 that TGFß1 transcriptionally controls miR-208-3p which specifically targets ARID2 [32].

A significant association was found between the high expression of miR-208a, lymph node metastasis and tumour-node-metastasis stage. The suppression of miR-208a reduced NSCLC cells' ability to proliferate and invade. It was found that miR-208a in NSCLC cells could directly target the gene *SRC* kinase signalling inhibitor 1 (SRCIN 1). Additionally, knocking down SRCIN 1 salvaged the effects of miR-208a on NSCLC cells [33].

A study found that miR-208a overexpression is linked to the growth, proliferation, migration and invasion of HCC via blocking the epithelial-mesenchymal transition (EMT) [34][35].

Numerous signalling pathways and variables are involved in the regulatory processes that govern the interaction between *CDH2* and miRNA-208. However, targeting downstream effectors or upstream regulators of *CDH2* and miRNA-208 may obstruct their interaction. Blocking the connection between *CDH2* and miRNA-208 can be achieved by inhibiting signalling pathways such as the Wnt/β-catenin or the TGF-β that regulate *CDH2* and miRNA-208 expression or activity. These pathways initiate EMT programs in tumour cells by inducing a core set of EMT transcription factors consisting of three distinct protein families: the zinc-finger E-box-binding homeobox factors ZEB1 and ZEB2, the basic helix-loop-helix factors TWIST1 and TWIST2, and the SNAIL family of zinc-finger factors SNAI1 (also known as SNAIL) and SNAI2 (also known as SLUG) [36].

Transcription factors work in different combinations to stimulate the production of mesenchymal-associated genes such as vimentin and *CDH2* (N-cadherin). At the same time, they restrain genes expressions linked to epithelial cells like *CDH1* (E-cadherin). Additionally, the Rho GTPase family members, including RhoA, RAC1 and CDC42, regulate cytoskeletal alterations and cellular motility [37][38].

Conclusion

Finally, based on the bioinformatics prediction results, the current study demonstrated a substantial relationship between miRNA-208 and the *CDH2* gene, poining to a possible connection between breast cancer susceptibility and progression in some Iraqi women.

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