Iraqi Journal of Science, 2026, Vol. 67, No. 1, pp: 19-29
DOI: 10.24996/ijs.2026.67.1.2

ISSN: 0067-2904

No association between *AQP3* SNP (rs16919255) and the Intracellular Level of the Promoter Markers HIF-1α, p53 and E2 in Breast Tumor Tissues of Adult Females in Thi-Qar Province, Iraq.

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Received: 24/ 5/2024 Accepted:16/ 1/2025 Published: 30/1/2026

Abstract

The upregulation of aquaporin 3 (AQP3) channels in the cell membrane promotes malignant cell migration, proliferation, and invasive capabilities. The translation inhibition of AQP3 mRNA in the 3'-UTR of *AQP3* crucial for reducing the synthesis of AQP3 channels and preventing or lowering breast cell migrations and proliferations. Promoter markers such as hypoxia inducible factor one alpha (HIF-1α), tumor suppressor protein (p53), and estradiol (E2) are associated with the progression of breast mass disease and also induce the AQP3 expression. The aim of this study is to investigation the possible association of SNP (rs16919255) in 3'-UTR of AQP3 gene with the intracellular levels of HIF-1α, p53, and E2. Sixty–five breast tumor tissues (0.7-1g) were collected from adult female patients aged between 18-80 years admitted at Al-Hussein Teaching hospital and AL-Haboubi Teaching Hospital. These samples were classified in two groups according to the tumor histopathological examination results, 21(32%) were malignant tissue with intensive ductal carcinoma not otherwise specified (IDC: NOS) and the other 44 (68%) were benign diagnosed as fibroadenoma. Tissue samples were washed with phosphate buffer saline for intracellular detection of HIF-1α, p53 and E2 levels using the ELISA technique, and were prepared for molecular analysis of SNP rs1691955 in the 3'-UTR AQP3 by PCR. Intracellular levels of HIF-1α, p53 and E2 levels were elevated in patients with IDC as compared to those with fibroadenoma. The molecular analysis of AQP3 SNP (rs16919255) revealed a 33% high occurrence of rs1691955 SNP in malignant case of IDC: NOS, whereas only one case (2%) in the fibroadenoma group exhibited this variant. Additionally, there was non-significant difference in these parameters between groups having rs16919255 variant and those without this SNP. The occurrence of rs1691955 SNP in 3'-UTR AQP3 gene didn't associated with the levels of intracellular promoters HIF-1α, p53 and E2 hormone.

Keywords: Aquaporin-3 gene single nucleotide polymorphism rs1691255, Breast tumor, Estradiol, Hypoxia inducible factor alpha, Tumor suppressor protein p53.

انعدام الترابط بين التغاير الجيني (rs1691955) لجين AQP3 مع تراكيز المحفزات HIF-1α و p53 و E2 داخل الخلية في النسيج الورمي للثدي للنساء البالغات في محافظة ذي قار \ العراق

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الخلاصة:

ان زيادة تخليق قنوات AQP3 في غشاء الخلية يساعد على هجرة وغزو الخلايا السرطانية. ان خطوة تثبيط وايقاف عملية ترجمة AQP3 mRNA في منطقة UTR– 3' لجين AQP3 مهمة جداً في تقليل تخليق قنوات AQP3 . العوامل المحفزة HIF–1α, p53, E2 ترتبط مع تقدم مراحل اورام الثدي وكذلك تحفز التعبير الجيني وتخليق قنوات AQP3 .تهدف هذه الدراسة للتحقق من مدى الترابط بين التغاير الجيني(rs16919255) AQP3 SNP في منطقة UTR– 3' مع مستويات المحفزات HIF–1α و p53 و E2 داخل الخلايا الثدي المصابة بالاورام. تم تجميع 65 عينه (1g– 0.7) لنسيج ورمي من الثدي لنساء بالغات تتراوح اعمارهن بين (82–18) سنه الراقدات في مستشفى الحسين التعليمي و مستشفى الحبوبي التعليمي. وفقا للتشخيص النسيجي فأن 21(32 %) عينة سرطانية خبيثة واخرى 44 (68 %) اورام ليفية حميدة . النماذج تم غسلها بمحلول بفرالفوسفات– سلاين PBS ثم قسمت لتقدير المستويات الخلوية للمحفزات HIF–1α و,p53 و E2 باستخدام تقنية ELISA وايضا دراسة التغاير الجيني وتحليل 3' UTR – AQP3 (rs1691955) SNP باستخدام تقنية PCR. أظهرت النتائج ان التغاير الجيني rs1691955 *AQP3*كان بنسبة 33% لدى عينات السرطان الثدي نوع IDC:NOS وبنسبة 2% لدى عينات الورم الحميد ، وايضا وجد ان ارتفاع معنوي ملحوظ في مستوى المحفزات HIF–1α و p53 وE2 داخل خلايا السرطانية مقارنة بالأورام الليفية الحميدة، بينما لم توجد فروقات معنوية في مستوى المحفزات داخل الخلية بين المجاميع الحاملة للتغاير الجيني rs1691955 والعينات التي لم يظهر لها التغاير الجيني. نستنتج من ذلك أنه لا يوجد ترابط بين ظهور التغاير الجيني rs1691955 في منطقةUTR–3' لجين AQP3 مع ارتفاع تراكيز العوامل المحفزة HIF–1α, p53 وهرمون E2 لتطور خلايا السرطان.

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1. Introduction:

Cancer diseases are indeed one of the most imperative diseases in the present and in future [1]. Affording to the WHO, by 2030, malignant tumors will cause 80% of death worldwide [2]. Cancer development and complication depend upon complex molecular processes in the structure of the genome, as in the change in the oncogene's activity [3].Cancer development depends strongly on complex molecular processes connected to the genome, such as changes in oncogenes activity and influenced by both exo- and endogenous factors [4]. Cell membrane aquaporin channels (AQP) are important in cell cancer migration; these channels are found in thirteen types (0-12) that are classified according to the type of materials that they transport. These membrane pores were initially identified as channels for water. AQP3 regulated the fluxes of water, glycerol, and hydrogen peroxide; these channels are expressed by AQP3 gene, which is located in chromosome 9 in p arm (p13.3). Its size is 6421 base pairs (bp), appeared to consist of a six exons distributed over 7 kilo bases, the sizes of the exons are 171, 127, 138, 119, 218, and 1035 bp, and those of introns are about 3530, 300, 350, 330, and 90 bp, as shown in Figures 1 and 2 [5,6].

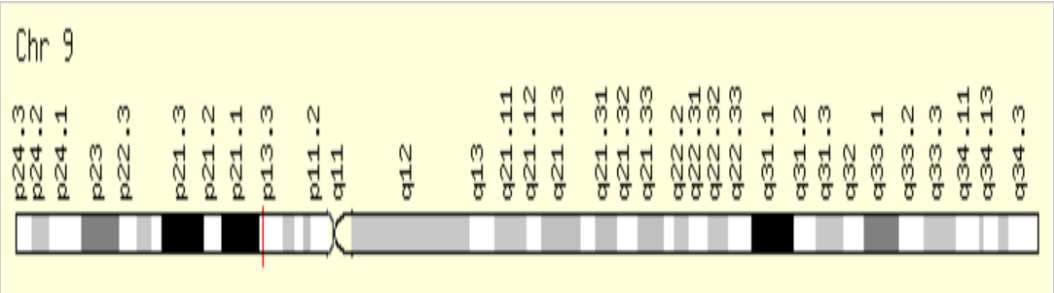

Figure 1: AQP3 gene in genomic location in chromosome 9 [5].

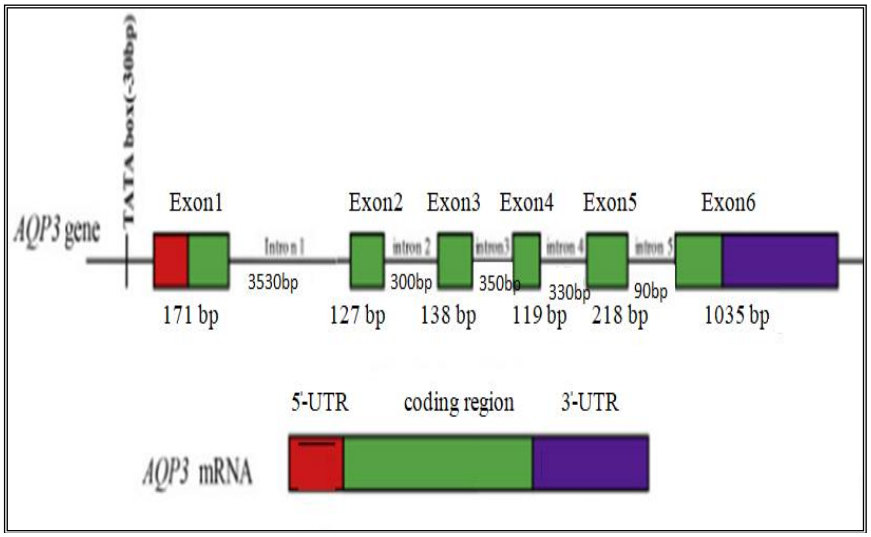

Figure 2: Human AQP3 and AQP3mRNA. The gene loci consist of six exons and five introns [5].

Tumor progression and metastasis need to increase the number of AQP3 channels in cell membrane to increase the yield of glycerol and hydrogen peroxide in the intracellular [7]. AQP 3 is involved in drug resistance in different malignant tumors [8, 9]. Gene expression processes of AQP3 can be regulated at the regulatory regions; the first is 5‘-UTR and the second is 3‘-UTR. The promoter and transcription factors bind to 5′-UTR to initiate AQP3 gene expression [8]. Promoter factors are associated with hypoxia condition, which induces hypoxia inducible factor one alpha (HIF-1α) that increase AQP3 gene expression [10]. Beyond HIF-1α, the activation of AQP3 gene expression in malignant cells involves additional regulatory factors, including the tumor suppressor protein P53 and the hormone estradiol (E2). There is significant elevation in the intra cellular levels of these markers and they are a useful prognostic marker indicating the proliferation, invasiveness, and metastases of breast tumors [11, 12, 13].

The 3‘ -UTR influences mRNA stability, translocation and translations inhibition by binding to Micro-RNA (miRNA), (Figure 3) [14]. The SNPs in the 3'-UTR affect and change the binding between the miRNA and the target AQP3 mRNA. It is critical because it determines AQP3mRNA stability and folding [15]. Conversely, allelic variations in the 3′-UTR have been associated with SNPs in miRNA-binding sites linked to hypertension, diabetes, Crohn disease and obesity in humans [16,17]. It is highly reasonable that such genetic variation in the 3'-UTR could also play a role in carcinogenesis [18,19].

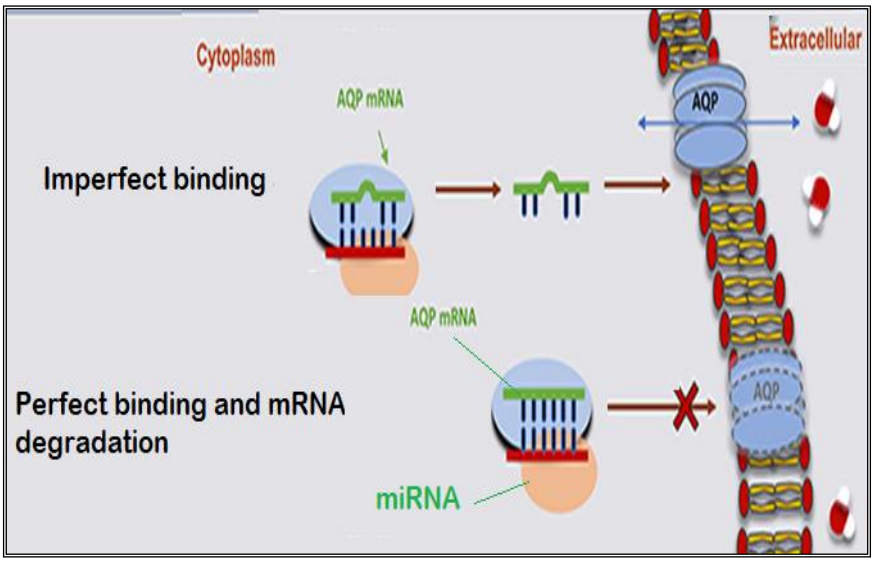

Figure 3: AQP mRNA translational inhibition or degradation through binding miRNA to 3'-UTR [14].

This study was designed to assess the occurrence of the SNP rs1691955 in the AQP3 gene in relation to the progression levels of *AQP3* promoters like HIF-α1, p53, and E2.

Methodology

This study included sixty-five female patients diagnosed with breast mass diseases admitted to AL-Hussein Teaching Hospital, AL-Haboobi Teaching Hospital, and the Medicine College in Thi-Qar province, Nasiriyah city. Tumor specimens, weighing between 0.7 and 1 gram, were obtained from female patients immediately following surgical excision of the breast tumor mass (as shown in Figure 4). These tissue samples were classified into two groups according to the histopathological examination results; 21(38%) sample were diagnosed with malignant tissue (Intensive Ductal carcinoma (IDC)) with age range 32–80-year (57± 14.4 year) and 44 (62%) samples were diagnosed as benign tumor (fibroadenoma) with aged 18-42 year (32.55 ± 6.40 year). The samples were processed and transported to the laboratory in sterile tube (20ml) containing ice-cold phosphate buffer saline (PBS) (0.01 M, pH 7.4) in the ice box. The excess blood was removed from tumor tissue by washing with ice-cold. Then, it was divided into two tubes (1.5 ml) and classified according to the specific protocol for intracellular biochemical parameters analysis and DNA extraction for genetic study [15,16].

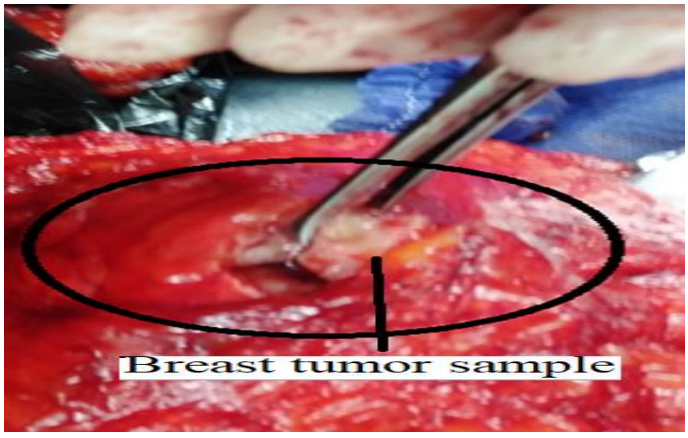

Figure 4: Removing the tumor mass in operating room from breast tissue of adult female.

Detection of intracellular biochemical parameters (HIF-1 α, p53, and E2):

A weight of 0.25g of fresh tumor tissue was homogenized using a tissue crusher (Heidoph /Germany) with cooled PBS (1g: 9ml) supplemented with protease inhibitors (0.25ml: 25ml) as in Figure 5. The intracellular solution was separated at 14,000 rpm /10min/4ºC using cooling centrifuge (Sigma /USA) and transferred to a new tube and kept at −20˚C (Deep freeze-ESCO /USA) for further biochemical investigation [20]. Analysis of biochemical parameters were made by Enzyme linked immune sorbent assay (ELISA) (HIF-1 α (Elabscience, USA), p53 (Elabscience, USA), and E2 (Monobind Inc, USA)) using BIOTIXS ELX800/ USA.

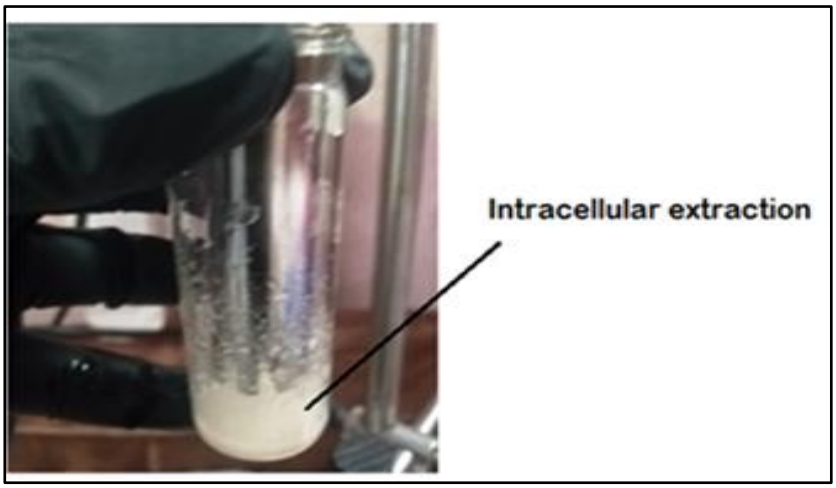

Figure 5: Preparation of intracellular fresh breast tumor tissue by silent crusher

Molecular analysis of SNP rs1691955 in AQP3:
DNA was extracted from fresh breast tumor tissue by using G-SPIN ™ Total DNA Extraction Mini KIT. Quantity, quality and integrity of DNA were checked using Nano-drop and agarose gel (0.8 % w/v) electrophoresis techniques [21].
Primers were designed using the Primer 3 version 4.0 program (Table 1). The specification of the primers was detected using the link below: http://WWW.NCBI.NIH. NIH.GOV/BLAST. The PCR master mix was provided by iNtRON Biolabotecnology, (Korea).

Table 1: Nucleotide sequence of forward and reverse primer of the 3-UTR AQP3 in exon 6.

Primer Direction	Nucleotide sequence	Started	Exon	The PCR product size
Forward (F)	5'-AGACAGCCCCTTCAGGATTT-3'	5691	6	226 bp
Reverse (R)	5'-TCCCTTGCCCTGAATATCTG-3'	5916		

The reaction mixture for PCR was prepared according to the addition order shown in Table 2.

Table 2: Addition steps for preparation PCR mixture.

Addition No	Materials added	Amount (µl)
1	Master mix PCR	5
2	Nuclease free water	12
3	Primer F (10pmol/µl)	1.5
4	Primer R (10pmol/µl)	1.5
5	DNA sample	5
Total reaction mixture		25

The protocol of PCR was developed using optimize Protocol Writer™, as detailed in Table 3. Following this, agarose gel electrophoresis (2%) was employed to identify the PCR results.

Table 3: PCR protocol for SNP 3rs1691955 SNP in *AQP3*.

step	Stage	Temperature(˚C)	Time	Cycle number
1	Initial denaturation	95	2 min.	1
2	Denaturation	95	30 sec.	29
3	Annealing	57.2	30 sec.	
4	Elongation	72	30 sec.	
5	Final extension	72	5 min	1

The fragment (226bp) in 3`-UTR in exon 6 of AQP3 was excreted according to the instruction of Wizard (R) SV Gel kit (Promega. USA.) and PCR Clean-up System (cat no# A9281) [22]. The fragments size 226 bp of 3'-UTR AQP3 gene from gel electrophoresis were analyzed for both directions forward and reverse according to the instruction of company Macrogen, Inc Geumchen, Seoul, South Korea. Sanger sequencing method was used for sequence analysis [23]. The results of sequencing tested for the alignment similarity was made using the software BioEdit Sequence Alignment version 7.1 (DNASTAR, Madison, WI, USA). The novelty of variant was checked by the use of data base of Single Nucleotide Polymorphism: dbSNP https:// www. ncbi.nlm.nih.gov/snp/?cmd=search).

Statistical analysis:

The obtained results were analyzed using SPSS program version 19. Firstly, Shapiro-willk normality test was used to check up the normality of data, t-test was used for analyzing the comparison of data between patients' groups used as mean ±SD. A probability (p) value ≤ 0.01 was considered statistically significant, while P ≥ 0.01 was considered non- significant differences [24].

Results:

The intracellular levels of the promoter in fresh breast tumor tissue are shown in Table 4 which revealed a significant difference between fibroadenoma and the IDC group. The levels of these parameters significantly increased (p≤ 0.01) in patients with malignant (IDC) compared to those in the benign fibroadenoma group.

Table 4: The concentration of intracellular parameters HIF-α1, p53, and E2 in tumor tissue of the 65 female patients with breast mass disease (21(38%) sample were diagnosed with malignant tissue (Intensive Ductal carcinoma (IDC)) and 44 (62%) samples were diagnosed as benign tumor (fibroadenoma)).

Parameters (pg/mg) means ± SD	Study groups		P value *
	Fibroadenoma (n=44)	IDC (n=21)	
HIF1α	65.34±2.01	742.49± 19.97	0.00
p53	1009.94±5.27	1755.46±24.61	0.00
E2	220.69± 1.50	339.14± 15.51	0.00

*The value of probability ≤ 0.01 refers to significant degree between all groups.
Figure 6 displays prominent bands, indicating the high quality and integrity of the whole extracted DNA from breast tumor tissues were checked using agarose gel (0.8 % w/v) electrophoresis techniques [21].

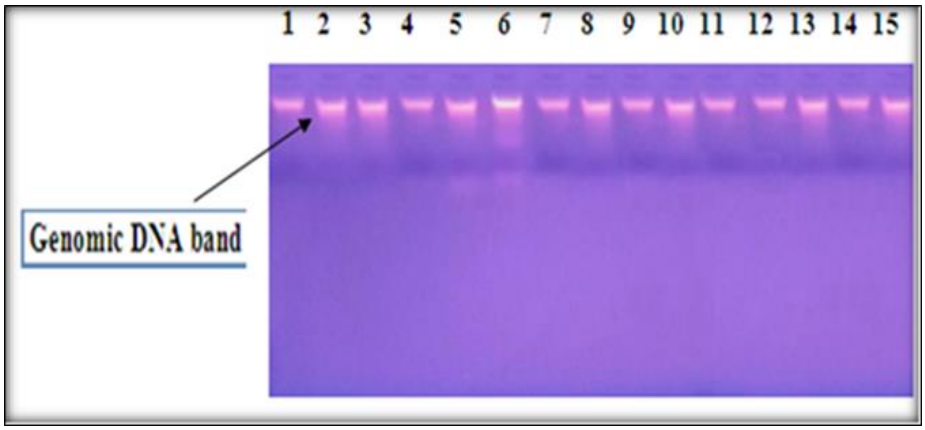

Figure 6: Agarose gel electrophoresis of the whole extracted DNA from breast tumor tissues loaded with using agarose 0.8% w/v for 30 min at 100v. (1-7 fibroadenoma cases and 8-15 malignant cases) by agarose gel electrophoresis).

The results of PCR products were examined using gel electrophoresis and detected under the UV light, show the presence of bands of 226 bps was indicative of 3'-UTR AQP3 gene. Numbers 1-7 referred to females with benign breast tumor and 8-14 referred to females with breast cancer (Figure 7).

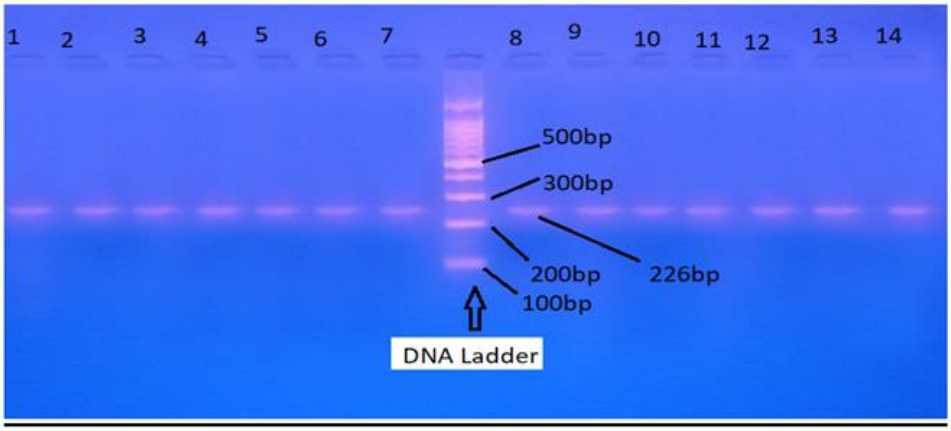

Figure 7: Gel electrophoresis of PCR products were examined under the UV light. The presence of bands of 226 bps was indicative of the AQP3. Numbers 1-7 referred to females with benign breast tumor and 8-14 referred to females with breast cancer.

The chromatogram of this SNP was detected in only two polymorphic patterns, GG, and GA. The homozygous G/G status was observed in the majority of samples, while the heterozygous G/A pattern was observed in S2, S5, S6, S11, S17, S18, S20 of IDC cases. In the analysis of rs16919255 SNP within invasive ductal carcinoma (IDC), 7 samples (33%) exhibited the variant, while 14 samples (67%) did not possess this genetic variation in their sequences.

In Table 5, the biochemical parameters HIF-1α, p53, and E2 in the study groups were analyzed according to AQP3 SNP (rs16919255) distribution. No significant difference in HIF-1α, p53, and E2 levels could be observed between cases with and without rs16919255 SNP.

Table 5: The association of HIF-1α, p53, and E2 level with the occurrence of AQP3 SNP 16919255 in malignant group.

Intracellular Parameters Mean ± SD (pg/mg)	Malignant cases		P-value*
	With SNP rs 1691255 N=7 (33%)	Without SNP rs 1691255 N=14 (64%)	
HIF-1α	744.02 ± 22.40	741.72 ± 19.49	0.45
p53	1756.73 ± 29.00	1754.83 ± 23.78	0.31
E2	339.57 ± 15.32	338.93 ± 16.17	0.89

* P-value >0.01: Non-significant.

Discussion:

The SNP (rs16919255) in *AQP3* didn't show relation to the abnormality degree of *AQP3* promoters' hypoxia marker, tumor suppression and estradiol hormone, as shown in Table (5). About the clinical significance of SNP (rs16919255) in *AQP3*, there was no report on the clinical variations in NCBI [25]. Several factors attributed to promote cell migration, invasion and proliferation, some of these are associated with extracellular complications such as abnormal biological processes, hormonal changes, microenvironment factors [26], low oxygen support "hypoxia" [27], and other intracellular factors which affect the structure and function of the cell such as mutations in tumor suppression proteins that controlling the cell cycle and DNA repair in the nucleus [28,29], also other changes in an intracellular proteins localization, and the expression level of some genes, which will promote migration like aquaporins. The severity and risk of malignant cells are related to their ability to grow rapidly and separate or migrate away from the primary site of solid tumor to invade the other healthy tissues and organs, in addition to their abnormal functions [30]

The HIF-1α, p53 dysfunction, and hormone (E2), increase AQP3 expression as potential valuable indicators for highest-risk patients. AQP3 localization is affected by the degree of hypoxia it's exclusively expressed in the cytoplasm, as well as enhanced AQP3 goes beyond their water, hydrogen peroxide, and glycerol permeability to cells casing increasing the large, volume and complexity of cells. In AQP3 gene promoter location or part; there are two identified hypoxia response elements (HREs). AQP3 facilitates formation of reactive oxygen species (ROS) and uptake by cells through pathway ROS-HIF-1α-AQP3-ROS loop. Also, *AQP3* overexpression increase the stability of HIF-1α and reduce the HIF degradation [31,32]. A report has revealed that the response element of p53 in AQP3 gene can advocate

members of the p35 family like p63 and p73 which can induce an activation of AQP3 gene expression [33]. Some studies tried to stabilize the structure of tumor suppressor protein p53 by de-phosphorylation through de-phosphorylation of Mouse double minute 2 (MDM2) in order to prevent tumor growth in breast cancer harboring Wild-type p53 [34]. Estrogen directly regulates AQP3 through binding estrogen response element (ERE) to the promoter side in *AQP3* in the promoter of the *AQP3* structure [35].

Unregulated sequences of the 3′-UTR in *AQP3* cause changes in the interaction with the miRNA which may be weakening binding or altered miRNA target that increases expression with 4-fold and modulating nuclear export of AQP3 mRNA in cytoplasm [36]. Breast cancer metastases are associated with the degree of down regulation of microRNA [37]. The up regulation of AQP3 may be associated with epithelial mesenchymal transition (EMT), as AQP3 reduces the expression of E-Cadherin through activation of the PI3K /AKT/SNAIL pathway. AQP3 channels often promote cell migration and invasion due to loss of cellular polarity and cell-cell junction, influencing the reorganization of actin [38]. The vital role of miRNA874 by inhibiting translation and formation AQP3 channels and lower rate of the EMT but in cancer cell had unregulated miRNA and also suppressed the activation of PI3K/AKT signaling pathway because phosphorylation of PI3K and AKT blocks the transmission of downstream signals to regulate tumor cell proliferation and decrease synthesis of E-cadherin [39].

Conclusion

In summary, the presence of SNP rs16919255 in the 3′-UTR *AQP3* was not associated with the elevation of the intracellular levels of promoter markers (HIF-1α, p53, and E2) of AQP3 gene expression in breast tumor tissue.

Acknowledgements:

Full thanks and acknowledgements to the staff of surgical unit, histopathological laboratory in Al-Hussein Teaching Hospital and Al-Haboobi Teaching Hospital for their contributions in samples collection and processing, also my deep gratitude to the patients for their cooperation and accepting to participate and in obtaining information and sampling to complete this work.

Conflict of Interest

The authors declare no conflict of interest

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