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Evaluation of NAT1 gene expression and methylation in the blood of people with breast cancer

Behzad Ghasemi Jamal Abadi
M.SC, Department of Biology, Faculty of science, Branch, Islamic Azad University, East Tehran

Dr Kasra Esfahani
Assistant Professor, National Institute of Genetic Engineering and Biotechnology

Dr Hamidreza Kheiri Manjili
Assistant Professor, Department of Biology, Faculty of Science, Islamic Azad University, East Tehran Branch And Zanjan University, Faculty of Science, Department of Biology

***Abstract*---**Introduction: Breast cancer is one of the biggest health problems of women all over the world. Today, with the dramatic advancements made in this regard, we can see the increase in the life expectancy of women with this cancer. The current study aimed at investigating the NAT1 gene expression and methylation in the blood of people with breast cancer. This gene and its family, namely the group of NATs, especially NAT2 and NAT3, are one of the few genes that participate in the interaction of cancers such as breast by producing enzymes that carry factors such as oxygen and nitrogen, and their effect on carcinogens. Methods and Materials: In the current study, the rate of NAT1 gene expression is investigated by the use of the Real-time PCR technique. The methylation of NAT1 gene promoter is also evaluated by the use of MS PCR technique and blood samples of people with breast cancer and normal people among the Iranian population. Findings: The results obtained indicated that the NAT1 expression in the blood samples of patients with breast cancer is increased compared to normal blood samples and besides, there was a significant correlation between this gene's expression and age, family history, menopause, and also, the rate of the methylation promoter. The mean rate of NAT1 gene expression in non-methylated samples is 20% lower on average, and in methylated samples, it is 50% lower on average, thus, the mean rate of NAT1 gene expression in the methylated samples is higher than the non-methylated samples. Conclusion: Investigation of the increase in NAT1 gene expression and

methylation promoter can be used as a biomarker for the prognosis of breast cancer. On the other hand, it seems that more comprehensive research can be done on the proposal to prevent tissue from becoming cancerous by controlling the levels of enzymes derived from these genes, and people's lifestyles.

***Keywords*---**breast cancer, NAT1, real-time PCR, MS PCR.

Introduction

The investigations on breast cancer in 47 countries from 1987 to 2013 show that eh mortality rate due to breast cancer has been reduced in the last decades in 39 countries out of these countries due to advancements in diagnosis and treatment of this disease (Eid MM, et. al., 2021; Algarni SB, et. al., 2020). The researchers also found out that the mortality rate due to breast cancer has become lower among women under 50 years old of age than those above this age, worldwide (Cigudosa . JC., Parsa .N., Louie . DC., Filippa. DA., et al.,2003). Breast cancer is the most prevalent cancer among females. 7 to 11% of women during their lifetime, and annually, one million people all over the world are diagnosed with this disease, and despite the advancements in diagnostic methods for diagnosis of metastatic malignancies, there are still many restrictions in this regard. Meanwhile, the best diagnostic and study method is the investigation of the tumor markers in the blood. Among these markers are CEA and CAI5-3 which were investigated in breast tumors with metastasis to the underarm lymph nodes (Lee et al., 2013). Today, it has been approved that about a half of the breast cancers have estrogen and progesterone receptors on the tumor cells that lead to the growth of the tumor in the presence of these hormones (Allred. D., Bustamante. MA., Daniel. Co., et al., 1990).

The increase in age, obesity, decreased menopause age, improper diet, inadequate mobility, environmental pollution, anxiety, and stress are among the factors effective in the emergence of breast cancer (Travis . LB., Hill . DA., Dores . GM., et al .,2003). The relation between exposure to radioactive radiation and parasites and breast cancer is approved, especially in studies on the atomic bombings survivals in different areas of the world and the residents of the areas near high-voltage power posts (Presston . DL., Mattson . A., Holmberg . E., et al.,2002). The NAT1 (N-acetyltransferase) which is also referred to with other names such as the AAC1 and MNAT is located on chromosome 8 (8p22). The number of exons in this gene is 11 and in some sources, 9 (Krisnadi SR., et al.,1996). The coding zone which is located in exon 9 with 870 pb, has 2 promoters; the first promoter is located at the end of exon one and the second promoter is located upstream of Exon 4 (Yumi Endo1., et al;2014). The NAT1 activity has been seen in many non-hepatic tissues. The NAT1 and NAT2 as well as a pseudogene named NATP are located in the 8p22 region.

In a study, a report was provided for epigenetic control of NAT1 gene which was examined in 5'-untranslated region methylation conditions near the start codon for both normal and cancer tissues of the breast. In addition, they showed that mRNA expression of NAT1 in the breast malignant tissue is almost 1000 times

more than the normal tissues of the breast. The 5'-untranslated region of the NAT1 gene examined in this study (Butcher ., et al ., 2012) is located on an intron with a distance of 11 kilobases lower than the structural promoter for NAT1 (NATb). Thus, methylation in this distant region may be closely correlated with the methylation of CpG islands in the promoter region of the gene.

In a similar study by Kim et al. (2008), they showed that NAT1 gene methylation is significantly resistant to tamoxifen which indicates that NAT1 gene methylation might be stored as a marker for tamoxifen resistance. In the following, these studies indicated that the NAT1 gene has been partially silenced by DNA methylation (Kim et al., 2010). Wakefield et al. provided a report about methylation specific to the CpG tissue of the NAT2 gene of the mouse (Wakefield et al., 2010). They evaluated the methylation conditions of several CpG islands located between or near the central promoter and concluded that in this region, the gene is often non-methylated in all tissues studied. Therefore, based on the methylation values, it seems that it would change the special state of the tissue. Histone deacetylase (HDAC) inhibitors such as Sodium butyrate and richostatin A (TSA) can increase the Nat1 activity in human cancer cells (Paterson et al., 2011).

NAT1 has been seen in most microarray chips, thus, the laboratory research has generally revealed changes in values of mRNA of NAT1 concerning different types of cancer and secondary cancers. Probably, the best example of microarray data is the investigation of NAT1 in the main research by Perou et al. (Zhang .X., Perou., et al.,2008), who analyzed the gene expression in 29 samples of breast cancer and 3 control samples. Although this study did not report the values of mRNA of NAT1, it indicated the alternative analysis of the similar data for NAT1 expression relevant to the estrogen receptor expression. A positive correlation between NAT1 and estrogen receptor was strongly approved (Butcher. NJ., Minchin .RF., et al., 2012). It was shown in terms of Immunohistochemistry (IHC) that NAT protein in the cancerous tissues containing estrogen-receptor-positive is higher than those containing estrogen-receptor-negative. It asserts that NAT1 might be useful as an additional biomarker for the categorization of secondary types of breast cancer (Wakefield et al., 2008). The breast cancer microarrays analyses revealed that NAT expression luminal cancer has been higher than the quasi-basal cancers which are basically malignant and show weaker improvement (Alimonti et al., 2010). One of the most important findings of the scientists is the presence of mRNA of NAT1 and NAT2 in the human breast cells (Sadrieh. N., Davis. C. D., Snyderwine. E. G., 1996). Wakefield et al. reported the NAT1 gene expression in various types of cell lines in breast cancer (Wakefield et al., 2011).

In two studies on the relationship between red meat and NAT, no relationship between them and the risk of breast cancer was reported. In a study, a relationship between the NAT2 rapid and intermediate acetylator and the risk of breast cancer among women who constantly consume well-cooked meat was revealed (Stone et al., 1998). In two other studies, it has been indicated that people who smoke are at greater risk if they have a slow acetylator phenotype (Bouchardy, C., Mitrunen, K., Wikman.,1998). And in a study on smoking people, there were no differences between the rapid and slow acetylators in relation to the emergence of breast cancer. In another interesting study, it has been reported that in people with NAT1*11 allele who smoke and constantly consume well-

cooked red meat, the increase in the risk of breast cancer is obvious and significant (Williams. JA., Stone. EM., Millar. BC.,1998). The current study is the first of its kind in Iran. So far, no experiment has been done on the investigation of the NAT1 gene methylation and basically, no documentary research on the rate of NAT1 gene expression and its modifications due to methylation and its subsequent effects on the emergence or prognosis of breast cancer has been observed in the reports by previous researchers. The current study aimed to investigate the rate of NAT1 gene expression and methylation in blood samples of people with breast cancer.

Methods and Materials

In the current study, the real-time PCR technique was used to evaluate the rate of NAT1gene expression in 40 blood samples of people with breast cancer and 10 normal blood samples. Also, to investigate the methylation status of the NAT1 gene promoter, the MS PCR technique was used. Meanwhile, parameters such as kinship, pregnancy, lactation, hormone use, and alcohol consumption were the cases that were not in the scope of our study. A questionnaire about the age, family history of breast cancer, whether the patient has reached menopause or not, and a history of having other types of cancer was formulated.

The Stages of Evaluation of NAT Gene Expression

First, preparation of the blood samples of patients with breast cancer and the non-cancerous samples as the healthy samples was considered. Then, the RNA of the samples is extracted, and the optical absorption of samples was investigated with a spectrophotometer. In the following, the cDNA synthesis from the extracted RNAs, optimization of the Real-time PCR test of NAT and GAPDH genes were performed. Finally, the optimized test was performed on the samples.

Stages of Evaluation of Methylation Status of NAT1 Gene

It is the valuation of the probability of a relationship between the NAT1 gene expression status in breast cancer and the age, gender, and methylation status. The blood samples of people with breast cancer and the non-cancerous samples were prepared. In the following, DNA extraction of samples, evaluation of the quantity and quality of DNA by electrophoresis in agarose gel and nanodrop device, treatment of template DNA using bisulfite DNA, then optimization of MS PCR test of NAT1 gene and finally optimized test on samples were done.

Internal Control Gene

To measure the decrease or increase in target gene expression, it is compared to the internal control gene expression. In the current study, we have used glycerol-3-phosphate dehydrogenase (GPD2) as the internal control.

Real-time PCR and Quantitative Polymerase Chain Reactions

The SYBR Green method has been used in the current study for the investigation of genes expression. The calculation of THE efficacy of amplification of primers in

Real-Time PCR reaction was done with the LinRegPCR. To evaluate the modifications in genes expression by the use of the relative evaluation method, the efficacy of the genes under study and the reference genes should be as much equal as possible. Therefore, the efficacy of amplification of target and reference genes was calculated after each Real-time PCR reaction.

Data Analysis by the Use of Evaluation Method

Analysis of the data obtained from Real-time PCR based on the Cycle Threshold (CT) obtained for the target and reference genes. The difference of mean CT of the reference gene from the mean CT of the target gene was calculated as ΔCt index for both test and control groups (Equations 1 and 2). Also, the difference of CTs of test and control groups has been used to calculate the ΔΔCt index (Equation 3).

1- Control ΔCt= (mCt of the intended gene (control)-mCt of reference gel (control))
2- Control ΔCt= (mCt of the intended gene (test)-mCt of reference gel (test))
3- ΔΔCt= ΔCt (control)- ΔCt(test)
4- (Ratio of expression between the two samples of test and control)= $2^{-\Delta\Delta Ct}$

Real_Time PCR Gene-specific Primers for NAT1 and GAPDH

The sequence of the GAPDH gene which was used as the internal control and the sequence of NAT1 gene were obtained from the Gene Bank, and by the use of Primer Express Software, the specific primers were designed (Table 1). After designing the sequence of the primers in Runner Gene, they were Blast in NCBI, and then, they were evaluated by the Annealing Software so that it would not interfere with other organisms. The designed primer has 100% agreement with the sequences and was used in Iran for the first time. The GAPDH was used as the internal control which is from the housekeeping genes series and has a constant expression in all cells.

Table 1: Gene-specific primers of Real-time PCR for NAT1 and GAPDH genes

Primer	Sequence	Tm °C	Aplicon size(bp)
NAT1(F)	GGATCCAGACGTGTACAGAAGGG	58.90	150 bp
NAT1(R)	TGCTGCTTCTTCCTGGCTTGA	59.11	150 bp
GAPDH(F)	ATGGAGAAGGCTGGGGCT	62.05	124bp
GAPDH(R)	ATCTTGAGGCTGTTGTCATACTTCTC	61.62	124bp

Optimization of Basic Factors of Real-Time PCR Technique for NAT1 Gene

After optimization of qPCR, different amounts of reaction materials of qPCR test in different temperature cycles were suggested for the genes under study, according to Table (3). To perform the qPCR test, a mixture of the reaction materials in Table (2) was prepared for each sample with a final volume of 20 µl.

Table 2: Materials in Real-Time PCR Reaction

Gene	qPCR Master mix(µl)	Forward Primer (µl)	Reverse Primer (µl)	DDW (µl)	Template (µl)
NAT1	10	0.5	0.5	8	1
GAPDH	10	0.25	0.25	7.5	2

The reaction mixture was loaded into each well of a 48-well plate or 8-well strips. The plate surface was covered with a clear cover or in case of the use of strip, they were covered with special lids. The plate or strip was placed inside the device and the temperature and time schedule was performed according to Table (3).

Table 3: The temperature and time schedule used in the quantitative polymerase chain reaction

Gene	Primitive Denaturation (°C)	Denaturation (°C)	Annealing Temperature (°C)
NAT1	95 1 cycle/10 min	95 40cycle/15 s	58 40cycle,1min
GAPDH	95 1 cycle/10 min	95 45cycle/15 s	59 45cycle,1min

Performing real-Time PCR Tests on the Patients Blood Samples

To investigate the methylated regions and their effects on cancer, MS PCR was used. To do so, 2 pairs of different primers were designed by which we can separate the methylated cytosine from the non-methylated cytosine and distinguish them from each other by PCR. First, the pattern DNA is treated by the use of sodium bisulfate, and when it is influenced by the sodium bisulfate, it becomes deaminated, i.e. methylated cytosine remains the same and does not change, but non-methylated cytosine is converted to uracil by loss of amine. In the next stage, two pairs of primers are designed; one for the methylated DNA and one for non-methylated DNA. After performing MS PCR, the methylated DNAs get an answer by the use of methylated primers (starting with G), and if they are non-methylated, they get an answer by the use of non-methylated primers (starting with A). to investigate the intended gene promoter methylation status, first, the DNA should be extracted and then it should be methylated.

Evaluation of the Quantity and Quality of the Extracted DNA

To perform an appropriate PCR, DNA with a suitable concentration and purity should be available. After extraction, the accuracy and quality of the extracted DNA need to be measured. The quality of the extracted DNA is investigated by the use of electrophoresis of it in the agarose gel. Also, the quantity of the extracted DNA is investigated by the use of the NanoDrop device.

Execution of Electrophoresis

To observe the results of DNA extraction, 35cc of 1.5% agarose gel was prepared. To prepared this gel, 0.52g of agarose was weighed and dissolved in 35cc of TBE

buffer and then, it was heated by the microwave until all agarose particles were dissolved. The agarose won't be dissolved in TBE solvent without heating. Every 30 seconds, we checked the solution in the microwave and whenever we found out it is boiling, we removed it from the microwave and checked its clarity. It is suitable if the solution was clear. then, we placed it in a stationary place to cool slightly. When the gel has cooled a little bit in the DARKROOM, 2 microliters of SYBR Green color is added to it and it is loaded into the previously-combed gel molds, and we wait until it cools and the gel becomes solid. After it is hardened after 20 to 30 minutes, the comb is removed and the gel is placed in the electrophoresis tank that contains TBE buffer in a way that the solution completely covers the gel surface. Hen, 5 µL of LADDER is loaded in the first well and 5 µL of each sample is mixed with 2 µL color and loaded into the wells. Then, the power generator is connected to the electrophoresis tank and during this operation, it should be noted that the flow direction is from negative pole to positive pole. Then, the device is powered on and adjusted on a voltage of 70 to 81 volts. After 30 minutes, when the samples pass through 2/3 of the gel, and the device notifies, the gel is removed from the tank and is placed inside the UV Illuminator device to observe the created bands.

DNA Modification Stages

To methylate the DNA, the (EPIJET BISULFITE CONVERSION K1461) kit was used. This kit has to be prepared after purchasing. To make it ready, the following stages are done: To prepare the modification reagent solution, first, 0.9ml of double distilled water is mixed with 200 µL of modification solution I and 60 µL of modification solution II and leave it for 10 minutes until it is fully dissolved. To prepare the wash buffer, 25ml of 96-100% ethanol is added to 9 ml of wash buffer. Then, to prepare the desulfination buffer, 10ml of 96-100% ethanol is added to 3.5ml of desulfonation solution.

MS PCR Reaction Optimization

After methylation of DNA, it is intended to investigate whether a specific region intended by the research (promoter of NAT1 gene) exists in CPG islands or not. To evaluate this status, the PCR test is used. The best method to use the PCR test is the differential method. To perform the MS PCR test, two pairs of primers are designed among which one is methylated and the one is non-methylated. The designed primers for the MS PCR are shown in the following table:

Table 4: The primers designed for methylated and non-methylated regions in the MS PCR reaction

Primer	Sequence	Tm °C	Amplicon Size(bp)
NAT1 MET(F)	TTATCGTGTTAGTTAGGATGGTTTC	57.84	134 bp
NAT1 MET(R)	AACTTATATAAAAATAAAAATATAACGTT	51.71	bp 134
NAT1 UNMET(F)	ATTGTGTTAGTTAGGATGGTTTTGA	58.15	bp 134

NAT1 UNMET(R)	TTTAACTTATATAAAAATAAAAATATAACA	50.94	bp 134

MS PCR Stages for Detection of Non-methylated Regions

First, the MASTER MIX with DW, 2X MASTER MIX, non-methylated forward primer, and non-methylated reverse primer are prepared for the 52 blood samples inside 1.5 ml microtubes. Then, they were vortexed and span and aliquoted in 50 0.2ml microtubes (23 µL of MASTER MIX is added to each microtube). Then, the relevant methylated DNA is loaded into microtubes with MASTER MIX to be placed in the PCR device according to Table (5). To observe the PCR results, 1.5% agarose gel was used (based on the instructions for gel preparation). When loading the samples, they are not mixed with the loading dye, since the MASTER MIX used is colored. The samples whose relevant bands are seen in this PCR should not show bands in the PCR with methylated primers.

Table 5: The optimized temperature schedule by the use of non-methylated primers for NAT1 gene

	Temperature℃	Time
Primitive denaturation	93℃	3min
Denaturation	93℃/40cycle	30s
Annealing	58℃/40cycle	30s
Extension	72℃/40 cycle	40s
Final extension	72℃	5min

MS PCR Stages for Detection of Methylated Regions

First, the MASTER MIX with DW, 2X MASTER MIX, methylated forward primer, and the methylated reverse primer is prepared for the 52 blood samples inside 1.5 ml microtubes. Then, they were vortexed and span and aliquoted in 50 0.2ml microtubes (23 µL of MASTER MIX is added to each microtube). Then, the relevant methylated DNA is loaded into microtubes with MASTER MIX to be placed in the PCR device according to Table (6).

Table 6: Schedule used for detection of methylated regions

	Temperature℃	Time
Primitive denaturation	93C	3min
Denaturation	93℃/40 cycle	30S
Annealing	60℃/40 cycle	30S
Extension	72℃/40 cycle	30S
Final extension	72C	5min

Findings

Results of Investigation of Light Absorption of RNA Available in the Samples by the Spectrophotometer

The results of light absorption of RNAs extracted to be used in cDNA synthesis were read by the Eppendorf spectrophotometry device and in this stage, samples with wavelengths of 260/280 nM with the concentration of 1.7 to 1.9 were approved and used for cDNA synthesis.

Results of Real-Time PCR Test for NAT1 and GAPDH Genes

The qPCR test was performed according to the protocol obtained from the optimization of this test for both genes under study on the cancerous and normal blood samples. The normal samples were used as a reference to compare the modifications in the expression of each gene with the cancerous samples. The diagram of the amplification of NAT1 and GAPDH genes of the samples was drawn by the device with the measurement of changes in fluorescence amount (Figures 1 and 2).

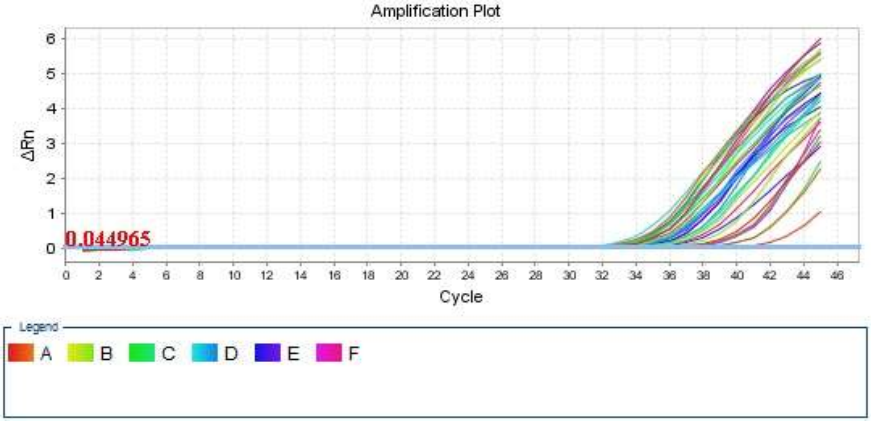

Figure 1: Diagram of NAT gene amplification in cancerous and healthy samples

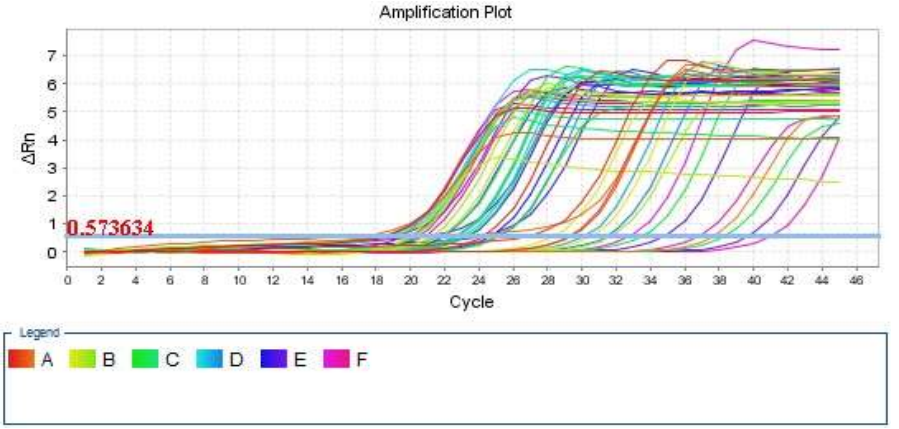

Figure 2: Diagram of GAPDH gene in the cancerous and healthy samples

Melt Curve Diagram

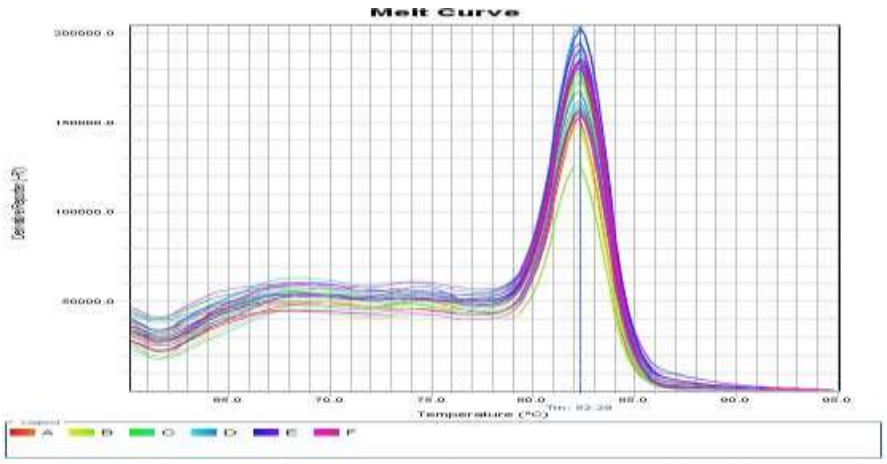

Figure 3: Melt curve of NAT1 gene in cancerous and healthy samples

To evaluate the specificity of primers and fluorescence color SYBR GREEN, assuring the amplification of specific portions and investigation of lack of non-specific portions and primer dimer in the PCR product, the melt curve for NAT1 and GAPDH genes (Figures 3 and 4) are separately drawn by the Step One Real-Time PCR Systems – Applied Biosystems device which is a confirmation of correct connection of the primers and their specificity to the intended genes. As a result, the PCR product obtained is exactly relevant to the intended gene.

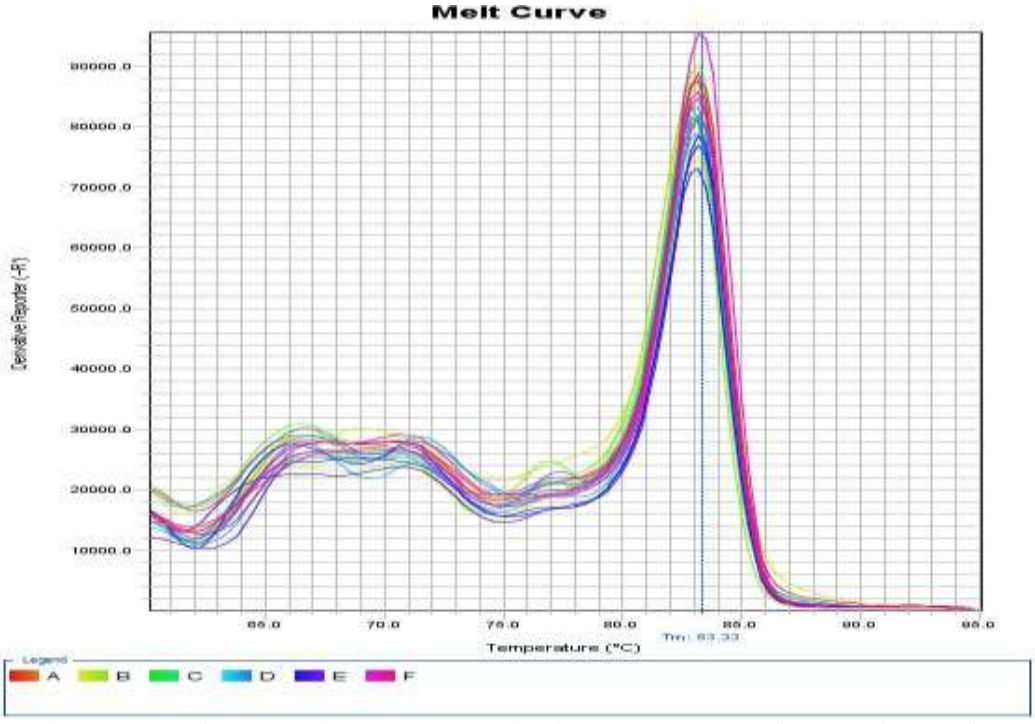

Figure 4: Melt curve of GAPDH gene in the cancerous and normal samples

Results of Rate of NAT1 Gene Expression

The Ct of the samples was calculated by the device after the amplification reaction and was converted to the RQ (Relative Quantification) or the expression rate. Then, the gene expression rate measurement was done by the ΔΔCt method. The cancerous samples' expression rate was compared to the normal samples. To do this comparison, the results obtained were compared to the expression rate of the same gene in the blood samples of formal people. The RQ of the samples was calculated by the device and the graph of the obtained results was drawn by the Graph pad software (P-Value<0.0001).

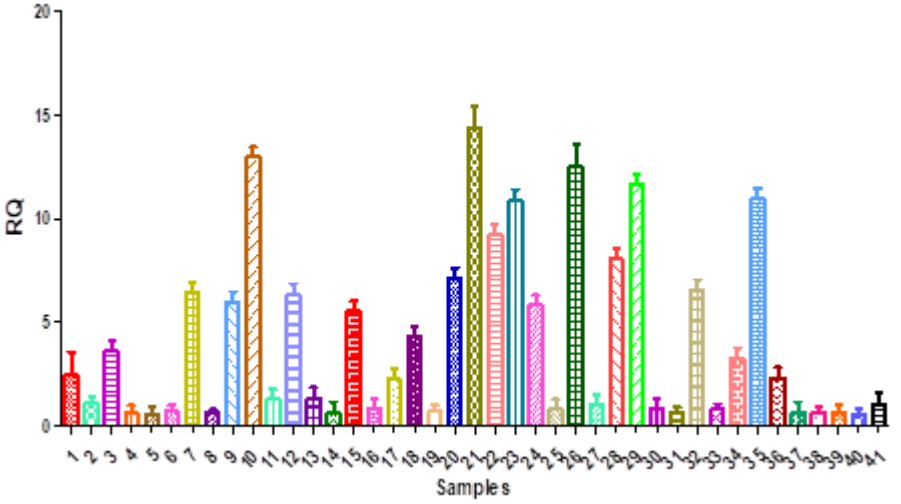

Figure 5: Analysis of investigation of the NAT1 gene expression in cancerous samples compared to the control samples (P-Value<0.0001)

As seen in the above graph, the NAT1 gene expression increase can be seen among the patients, however, it does not necessarily happen to all patients. On the other hand, the normal people did not show a significant expression. If the intended samples are compared, it would be seen that samples 1, ،3،7،9،10،12،15،17،18،20،21،22،23،24،26،28،29،32،34،35 and 36 have shown expression increase, but 19 remaining patients did not show a significant expression increase just like the normal people who are represented by number 41 and in black color. Since the NAT1 gene is considered as the gene responsible for the generation of enzymes activating the carcinogens, it was expected that we face an expression increase among the patients, which we did.

The results indicated that the mean NAT1 gene expression rate in patients was increased compared to the normal people by 0.53. There was no significant increase in expression among normal people. The expression increase can be variable based on age, gender, and methylation status (P-Value=0.0478). Also, the NAT1 gene expression rate in the patients, compared to the normal samples, is 45% on average among people under 50 years old, and about 55% among people above 50 years old. This classification was only done based on the age group and

not the gender (P-Value=0.0251). On the other hand, the rate of NAT1 gene expression among those who have reached menopause was higher than those who have not reached it yet, in a way that 63% of the women had reached menopause while 37% had not reached it. The patients with a family history of breast cancer showed a slightly higher increase in NAT1 gene expression.

The Results Obtained from DNA Extraction

The results of electrophoresis of the extracted DNA samples are shown in figures (6) and (7), all having sufficient quality. Based on the results obtained from the NanoDrop device, the concentration of the DNA extracted from the blood samples was estimated to be 20-150 ng/µl, which is a suitable concentration for molecular tests such as the PCR.

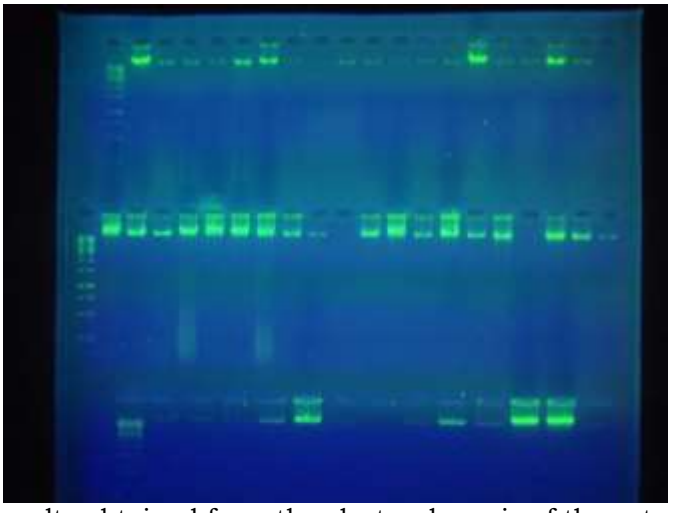

Figure 6: Results obtained from the electrophoresis of the extracted DNAs

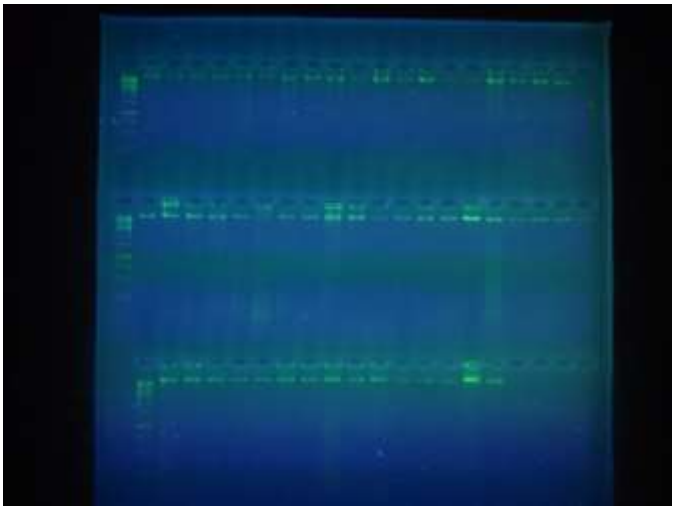

Figure 7: Results obtained from the electrophoresis of the extracted DNAs

Results Obtained from MS PCR by the Use of Methylated Primers

As seen in Figure (8), performing the MS PCR by the use of methylated primers on 50 intended samples to investigate the methylation status of NAT1 gene promoter, the band was observed in samples ‹30‹31 ‹27‹28 ‹7‹11‹12‹13‹14‹15‹16‹17‹18‹20 ‹2 49‹50 ‹38‹39‹40‹41‹42‹44‹45‹46 ‹36‹33‹34‹35, which is indicative of the methylation of the promoter in NAT1 gene in these samples. Since the NAT1 gene length is 134bp, the created bands should be in the scope of 134bp.

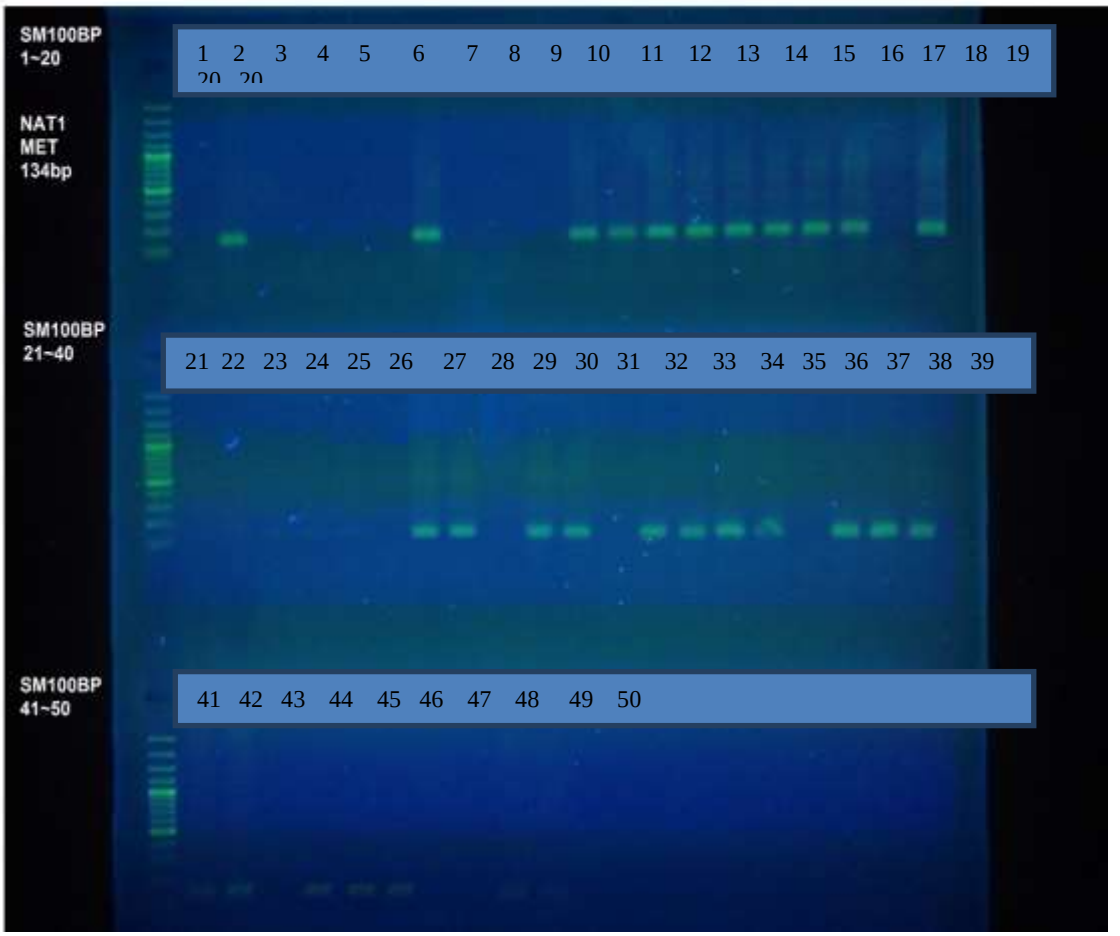

Figure 8: The results obtained from MS PCR by the use of methylated primers

Results Obtained from MS PCR by the Use of Non-Methylated Primers

As seen in Figure (9), performing the MS PCR by the use of non-methylated primers on 50 intended samples to investigate the methylation status of NAT1 gene promoter, the band was observed in samples ‹19 ‹8‹9‹10 ‹1‹3‹4‹5‹6 ‹26‹29‹32‹37‹43‹48 which is indicative of the non-methylation of the promoter in NAT1 gene in these samples. Since the NAT1 gene length is 134bp, the created bands should be in the scope of 134bp.

Figure 9: The results obtained from MS PCR by the use of non-methylated primers

Table 7: The NAT1 gene-specific PCR test results for evaluation of methylation

Sample number	Un Met	Met	Sample number	Un Met	Met	Sample number	Un Met	Met	Sample number	Un Met	Met	Sample number	Un Met	Met
1	+	-	11	-	+	21	-	+	31	-	+	41	-	+
2	-	+	12	-	+	22	-	+	32	+	-	42	-	+
3	+	-	13	-	+	23	-	+	33	-	+	43	+	-
4	+	-	14	-	+	24	-	+	34	-	+	44	-	+
5	+	-	15	-	+	25	-	+	35	-	+	45	-	+
6	+	-	16	-	+	26	+	-	36	-	+	46	-	+
7	-	+	17	-	+	27	-	+	37	+	-	47	-	+
8	+	-	18	-	+	28	-	+	38	-	+	48	+	-
9	+	-	19	+	-	29	+	-	39	-	+	49	-	+
10	+	-	20	-	+	30	-	+	40	-	+	50	-	+

Based on the results in Table (7), the results obtained from the MC PCR for the NAT1 gene showed that 33 DNAs of the patients are methylated (83%) as well as 2 normal samples which are methylated (20%). Thus, 70% of the samples are methylated for the NAT1 gene promoter.

Investigation of Mean Rate of NAT1 Gene Expression based on Gene Promoter Methylation

The results indicated that in 33 patients, the NAT1 gene is methylated while in 7 patients, the gene promoter is not methylated. The mean rate of NAT1 gene expression in non-methylated samples is lower by 20% on average, while it is close to 50% on average in methylated samples. Therefore, the mean rate of NAT1 gene expression in methylated samples is higher than the non-methylated ones (Figure 10).

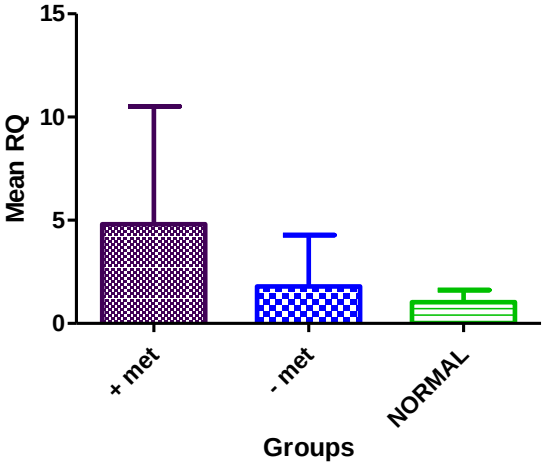

Figure 10: Mean RQ of NAT1 gene based on its promoter methylation among cancerous and normal people (P-value=0.264)

The P-Value obtained from the analysis of modification of NAT1 gene expression based on the age, menopause, and family history of breast cancer, as well as the methylation status, is 0.251, 0.010, 0.080, and 0.264, respectively, which are all significant. Therefore, there is a significant correlation between the increase in NAT gene expression and age, menopause, family history, and finally, the rate of promoter methylation.

Discussion

In the current study in which the NAT1 promoter methylation status was investigated in people with breast cancer by the use of the MSP technique, it was revealed that hypermethylation of the NAT1 promoter is associated with increased transcription. Full and partial methylations were observed in blood cell lines which are indicative of an increased expression. These findings are in line with those of David Barker et al. who had concluded that the increase in NAT1 gene

transcription in the breast cancer cells works as a gene generating transferase enzyme. These findings are also in line with those of Jaki Tiyang et al. who concluded that with the decrease in cell growth in the G2 phase and the subsequent decrease in NAT1 gene expression the growth of cancerous cells is also reduced (Jaki Tiyang et al., 2010).

Recent research has investigated the primary relationship between the acetylator phenotype and the prevalence of cancer. Detection and cloning of NAT1 and NAT2 has been considered by many researchers about 10 years ago. Most of the research has investigated the NAT2 genotype and prevalence of cancer, both alone and in combination with its phenotype. Recent research has focused on the NAT1 genotype. These investigations have been usually associated with NAT2 genotype and since the carcinogenic multiple metabolism enzymes are involved in the activation and inactivation of chemical carcinogens, most studies have evaluated the interactions between genes that metabolize multiple carcinogens (Bigler. J., Chen. C., Potter, J. D., 1997).

Several studies have investigated a relationship between NAT2 acetylator genotype or phenotype and breast cancer, however, the findings are very contradictory. NAT2 acetylator phenotype has been not relevant in three studies on breast cancer (Ladero. J. M.. et al ., 1987). On the other hand, in a primary report, the rapid/intermediate NAT2 genotypes have been related to the risk of breast cancer in women who have constantly consumed well-cooked meat (Artamonov. VV., 2004).

Recent research has found the relationship between the NAT1 acetylation polymorphism and the risk of contracting breast cancer. The NAT1 enzyme activity (not the NAT2 activity) has been detected in the human mammary epithelial cells. A study has reported the few effects of smoking on breast cancer based on the NAT1 genotype, except for the women with NAT1*10 allele after menopause. A very recent study has reported an increased risk of breast cancer in smokers who constantly consume well-cooked meat and have the NAT1*11 allele (Zheng. W., 1999).

Jaki Tiyang et al. (2010), using an inhibitor that reduces the cell growth in the G2 phase, concluded that the NAT1 expression rate is also reduced and subsequently, the growth of breast cancer cells is also reduced. Therefore, the NAT1 activity is related to breast cancer (Jaki Tiyang et al., 2010). David Barker et al. (2007) found an increase in mRNA transcription from breast cancer cells, related to the NAT1 gene. When the transcription is high and the activity of the NAT1 coding exons promoter is obvious, the cancer cells grow twice as fast (Barker et al., 2007). Williams (2010) found out that people with breast cancer who have ER+ respond better to the hormone and drug therapy (Albrecht. ED., Pepe .GJ.,2010). On the other hand, overexpression of the N-acetyltransferase enzyme of NAT1 leads to an increase in carcinogenicity, especially in people with ER+ for whom the high amount of this enzyme was problematic. Liton et al., (2011) found out that the effects of rapid and slow acetylation of the Acetyltransferase enzymes from the NAT1 and NAT2 genes on the aromatic compounds can be the reason behind 20% of the breast cancers among the

women who have reached menopause (Liton.B., Armenian. SH., Sun. CL., and et al., 2011).

Bell et al. (2014) investigating the Caucasian race and comparing it to the African race, concluded that the NAT1 gene has had slow acetylation in the Caucasians while in the Africans, it has been rapid (Olshan. AF., Weissler. MC., Bell DA.,2014).

Gushal et al. (2000) and again, Tang et al. (2006) detected the mutation in NAT1 gene at the site c-8 of nucleoside atoms inside the epithelial cells in the mammary glands of mice with breast cancer (Perera. FP., Tang .D., et al.,2006). Pefa et al. (2009) studied the DNA adducts associated with the NAT1 and NAT2 genes in human breast epithelial cells, thought to be the source of breast cancer (Vähäkangas .K., Myllynen .P.,2009). Pro et al. investigating the gene expression in 39 breast cancer samples and 3 control samples, conducted comprehensive research on the relationship between the rapid and slow acetylations of this gene (Chelouti. H., 2017). Adam et al. showed with their studies that there is a direct relationship between the NAT1 and ER+. However, they also approved that the ER is not accountable for the level of NAT1 expression in breast cancer (Adam et al., 2013).

Conclusion

Our data showed that generally, the amount of this gene is higher in the blood samples of the patients than the normal blood samples. Also, the results obtained from the investigation of the methylation status of NAT1 gene promoter indicated that 83% of the DNA in patients is methylated and only 2 of the normal blood samples were methylated. Besides, there was a significant correlation between the mean expression rate of the NAT1 gene and the age and the methylation status, however, there were no significant relationships between the mean expression rate of the NAT1 gene and the gender.

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