Molecular Biology Reports (2022) 49:847–857
https://doi.org/10.1007/s11033-021-06546-z

ORIGINAL ARTICLE

Investigation of promoter methylation patterns association with genes expression profile of *ISL1*, *MGMT* and *DMNT3b* in tissue of breast cancer patients

Hadi Yari[1] · Samira Shabani[1] · Nahid Nafissi[2] · Tayebeh Majidzadeh[1] · Frouzandeh Mahjoubi[1]

Received: 2 January 2021 / Accepted: 2 July 2021 / Published online: 8 January 2022

Abstract

Background and objectives Cancer initiation and progression could influenced by both genetic and epigenetic events revealing of the overlap between epigenetic and genetic alteration can give important insights into cancer biology.

Methods and results In this experiment *ISL1, MGMT, DMNT3b* genes were candidate to investigate both methylation status and expression profile by using methylation-specific PCR and real time PCR in 40 breast cancer patients, respectively, also we have assessed relation of the promoter methylation status and expression variation of the target genes. The mean level of methylation of *ISL1* and *MGMT* in tumor tissues were significantly greater than normal tissues. In Contrast, *DMNT3b* gene was showed lower mean level of methylation in tumor tissue compared to normal tissues, however, this was not statistically significant. Relative expression analysis was displayed a significant reduction in expression level of *ISL1* and *MGMT* in tumor tissues. Furthermore, there was a meaningful association between down expression of *ISL1* with histological grade, Her2 and ER status. Moreover, *MGMT* down expression was significantly associated with tumor sizes. Any remarkable relation was not observed between *DMNT3b* expression level and clinic pathological features. At the end, significant relation between methylation status and expression level has been revealed.

Conclusions In this study all observed results were exactly in line with the results were obtained from articles which were based on the methylation research and illustrate that the real-time PCR and methylation methods are in correlated with each other, furthermore, selected genes are capable to use as a potential biomarkers, however, more research on extended cases are needed.

Keywords Breast cancer · Methylation patterns · Expression level · Tumor tissue · Adjacent normal tissue

Introduction

In Europe, as well as in the United States, 1 in 3 people is diagnosed with cancer during their lifetime [1]. Breast cancer is one of the most common cancer among women and affects one in eight women. Its rate prevalence has increased considerably by 0.3% per year. At present, the woman typical risk of developing breast cancer during her life is about 13% in the United States [2, 3]. In Iran similarly the prevalence of this cancer has increased, one out of every 10 to 15 women is at risk of developing breast cancer. Surprisingly, its prevalence is under 45 years old in Iran in comparison with Western countries which is under 56 years [4]. Since 2007, in cases younger than 50, mortality rate from breast cancer has been steady, but in older women have continued to reduce. The death decrease rate was by 1.3% per year from 2013 to 2017 (American cancer society). It is believed

✉ Frouzandeh Mahjoubi
Frouz2@yahoo.com; frouz@nigeb.ac.ir

Hadi Yari
yari.hadi@gmail.com; h_yari@nigeb.ac.ir

Samira Shabani
samira.shabani.ir@gmail.com

Nahid Nafissi
nahid.nafissi@gmail.com

Tayebeh Majidzadeh
majidi_t@yahoo.com

[1] Human Genetics Dept., Medical Biotechnology Department, National Institute of Genetics Engineering and Biotechnology (NIGEB), Pajouhesh Blv, Tehran Karaj High Way, Tehran, Iran

[2] Surgery Department of General Surgery, Iran University of Medical Science, Tehran, Iran

that these mortality reduction is due to diagnosis of breast cancer in earlier stages via screening, improved awareness and also well treatments [5–7]. This issue emphasizes the importance of breast cancer diagnosing in the early stages with high sensitivity and specificity methods. Different studies consider Breast cancer progress as a stepwise procedure which is associated with genetic and epigenetic changes [8] and also studies have shown the overlap between epigenetic and genetic alteration in sporadic breast tumors can provide important insights into cancer biology and may afford new strategies for breast cancer prevention and diagnosis [9]. A variation in the methylation rate is a common change in the process of cancers that occurs in the early stages of cancer spread and this epigenetic alteration causes transcription and phenotypic variation [10, 11]. Epigenetic modifications importance in tumorigenesis have described in several researches, most often it is obvious that promoter-associated CpG Island methylation alterations take place in different cancers. However, in most articles, only changes in gene methylation have been examined and not much studies assesse the DNA methylation modification effects on gene expression pattern.

According to the above explanation, by going through published data in literature to find previously reported genes which underwent methylation changes *ISL1*, *MGMT*, *DMNT3b* genes, with conflicting data, were selected to investigate the promoter-associated CpG Island methylation pattern of *ISL1*, *MGMT*, *DMNT3b* genes and the association of DNA methylation variation effects on gene expression variation [12–16].

The *ISL1* gene encodes a member of the homeodomain LIM family of transcription factors. The protein from this gene binds to the insulin-promoting region and actually plays an important role in regulating insulin expression. This gene is a tumor suppressor gene which is in fact a natural controller over stem cells [15], Shin et al. have reported that *ISL1* gene has different expression pattern based on the subtypes of tumors, they explained ISL1 expression would be elevated in TNBC(Triple negative breast cancer) and its expression was lower in other molecular types [17]. The *MGMT* or O6 methyl guanine DNA methyl transferase gene (also O6-alkylguanine DNA alkyl transferase) which has vital role for genome stability is a DNA repair protein and has a protective function against the toxicity and carcinogenesis of agents [18]. This protein transports methyl from O6 alkaline guanine and other methylated parts of DNA to its molecule, which eliminates toxicity and inhibit potential mistake and mismatches during DNA replication and transcription. Therefore, it seems lack of this protein increases the risk of carcinogenesis [19], Nairui et al. reported MGMT promoter hyper methylation and also declared this gene as early stage biomarker [20], Chen et al. have showed that MGMT promoter hyper methylation can elevate risk of breast and gynecologic cancer [21]. *DMNT3b* gene is also one of the three target genes, The *DMNT3b* gene or DNA cytosine-5-methyl transferase 3 beta, is methyl transferase and is supposed to be involved in de novo methylation rather than in keeping methylation, Devon et al. have assessed breast cancer cell lines with hyper methylated profile and they find that *DNMT3b* promoter were significantly unmethylated and this lower methylation pattern lead to hyper activation of this gene [22].

Material and methods

Patients and samples

This research was approved by the National Institute of Genetic Engineering and Biotechnology (NIGEB). Written Consent form were taken from all 40 breast cancer patients admitted to Khatam Hospital (a referral governmental hospital) in Tehran those who whom underwent surgery. Tumor and adjacent normal tissues were obtained during surgery. The tissue specimen were stored at − 70 °C for RNA extraction. All patient's pathologic information was gained from Pathology Department. Breast issues staging was carried out as stated by the International Union against Cancer (UICC) which is based on (AJCC-TNM) classification [23, 24].

DNA extraction and bisulfite modification

DNeasy Blood and Tissue Kit (Qiagen, Germany)were used for Genomic DNA isolation from tumor and adjacent normal tissues according to the manufacturer's guidelines and subsequently Bisulfite Treatment was performed by EpiTect Bisulfite kit (Qiagen, Germany) that previously described [25]. In all cases, bisulfite conversion of DNA was confirmed by MS-PCR (primer sequences provided in Table 1) involved two separate PCR reactions using methylated/unmethylated-specific amplifiers flanking the CpG-rich *ISL1, MGMT* and *DMNT3b* promoter regions.

MS-PCR primer design

EPD (Eukaryotic Promoter database) and Promoter 2.0 Prediction Server online software were used to determine where the genes promoter are located and the CpG island for primer design was targeted by MehtPrimer 2.0 online software. All primers specificity was examined by Primer Design and search tools (bisearch.enzim.hu) online software. Primers were made by Metabion Co, Germany, and function of primers were investigated by control methylated DNA (Qiagen Co,). Information of primers are presented in Table 1.

Table 1 Primers sequences which is used for MS-PCR

Gene	sequencing primer		Amplicon size	Genomic coordinates
DMNT3b Methylated	5′-ATAAGGGGAGTCGGTATCGT-3′	Forward	121 bp	chr20:32,762,356 + 32,762,476
	5′-CGCTCGAAACGTCCACG-3′	Reverse		
DMNT3b Un methylated	5′-GGGTTATAAGGGGGAGTTGGT-3′	Forward	152 bp	chr20:32,762,356 + 32,762,501
	5′-AAAAACCAATCCCATCCATCAAA-3′	Reverse		
ISL1 Methylated	5′-GGAGAACGGTTTGAGTTTCG-3′	Forward	199 bp	chr5:51,383,188 + 51,383,386
	5′-CTCCATCGCCATTAATCTAACG-3′	Reverse		
ISL1 Un methylated	5′-GGGGAGAATGGTTTGAGTTTTG-3′	Forward	204 bp	chr5:51,383,188 + 51,383,391
	5′-CAACTCCATCACCATTAATCTAACA-3′	Reverse		
MGMT Methylated	5′-GTTAGGCGTATAGGGTAGCG-3′	Forward	309 bp	chr10:129,466,924 + 129,467,232
	5′-ACGAACTATCCCAACATATCCG-3′	Reverse		
MGMT Unmethylated	5′-TGGGTTAGGTGTATAGGGTAGTG-3′	Forward	314 bp	chr10:129,466,924 + 129,467,237
	5′-ACACAAACTATCCCAACATATCCA-3′	Reverse		

Genome build, Dec. 2013 (GRCh38/hg38)

Methylated specific PCR reaction and Sanger sequencing

After bisulfite conversion of all DNA samples, by using PCR thermal cycler (Bio-rad, T100™ Thermal Cycler) PCR reaction was carried out in all samples. The reaction was done in 25 µL of solution, containing 1 µM of each primer, 2 µL of DNA (as template), 12.5 µL of Taq DNA Polymerase Mix Red-Mgcl2/2 mM (Ampliqon, Denmark), and 8.5 µL water in 0.2 vials. The thermal cycle was set as follows: 1 cycle at 95 °C for 10 min as an initial denaturation step, 45 cycles at 95 °C for 30 s, 66 °C for 35 s, 72 °C for 30sand 1 cycle at70 °C for 10 min as final elongation Each sample was amplified using methylated and un-methylated primers. After performing all reactions, in order to the results confirmation, several methylated samples were subjected to Sanger sequencing of all 3 genes.

RNA purification and cDNA synthesis

TriPure Isolation Reagent and RevertAid First Strand cDNA Synthesis Kit were used for RNA purification (Roche applied sciences) and cDNA Synthesis (Thermo Fisher Scientific, Germany), respectively.

Real-time RT-PCR

Corbett Rotor-Gene 6000 real-time PCR thermal cycler and Real-Time RT PCR using SYBR-Green master (Roche Applied Sciences) were utilized to determined mRNA level expression. The reaction was done in 10 µL of solution, containing 0.5 µM of each primers, 1 µL of cDNA (as template), 5 µL of SYBR-Green Master, 3 µL water in 0.1 vials. The thermal cycle was set as follows: 95 °C for 5 min for initial denaturation step, an amplification program (95 °C for 20, 60 °C for 15 and 72 °C for 20 s respectively) repeated for 40 cycles. Primers were designed by oligo7 software. The specificity of primers were theoretically controlled by BLAST database. The primers were made by Metabion Co, Germany. Information of primers are showed in Table 2, the relative expression levels were normalized to the level of *B* actin as a housekeeping gene.

Table 2 Primers sequences which is used in real time RT-PCR

Primer	Sequence	Length	Genomic Coordinates
ISL1 F-Primer	5′-GTACATGCTTTGTTAGGGATGG-3′	122 bp	ENST00000511384.1 ISL1:167 + 288
ISL1 R-Primer	5′-ACGGGCACGCATCACGAAG-3′		
MGMT F-Primer	5′-TTCCAGCAAGAGTCGTTCACC -3′	159 bp	ENST00000306010 MGMT:384 + 542
MGMT R-Primer	5′- GATGGGGACAGGATTGCCTC -3′		
DMNT3b F-Primer	5′-GCCCATTCGAGTCCTGTC -3′	176 bp	ENST0000034 8286.6*DMN T3B*:1941 + 2116
DMNT3b R-Primer	5′-TGATGTTCCTCACGTCGTTC -3′		
B actin F-Primer	5′-GAGACCTTCAACACCCCAGCC-3′	161 bp	ENST00000493945 ACTB:528 + 688
B actin R-Primer	5′- AGACGCAGGATGGCATGGG-3′		

Table 3 Patient characteristics

Characteristics		No.of patients (N %)
Age (mean)	47.4	(31–72)
Cancer grade	Grade I	6(15)
	Grade II	20(40)
	Grade III	14(35)
Tumor grade	Stage I	7(18)
	Stage II	25(62)
	Stage III	8(20)
Lymph node status	Positive	31(77)
	Negative	9(17)
Tumor size	≤5	22(55)
	≥5	18(45)
HER 2 IHC	Positive	22(55)
	Negative	18(45)
ER	Positive	18(45)
	Negative	22(55)
KI67	0–6	4(10)
	6–10	8(20)
	≥10	38(70)
PR	Positive	24(60)
	Negative	16(40)

Statistical data analysis

The Real time RT-PCR raw data for each gene was evaluated by Linreg software. Subsequently, value and statistical significance of expression ratio results (tumor group difference to adjacent tissue group) analyzed by REST 2009 software as well as SPSS software V22.0 (SPSS, Inc., Chicago, IL). The normality assumption was checked by Kolmogorov–Smirnov test. The variances of the groups were determined by the analysis of variance (ANOVA) and independent sample T tests. Difference between pattern of methylation in normal and adjacent tumor tissues was evaluated by chi square. Comparison of the fold changes of the different methylation status groups was done by using One way Anova, also using LSD algorithm, and different methylation groups fold change one by one were compared.

Results

Patients' clinical and pathological data

In total, 40 patients with breast cancer were involved in this study. Tumor grade I, grade II, and grade III were identified by pathology check in 15%, 40% and 35% of the cases, respectively. Eighteen percent, 62% and 20% were at the stage I, stage II, and stage III, respectively. Size of tumor in 55% of patient was smaller than 5 cm and in 18% was bigger than 5 cm. Forty-five percent, and 60% of patients expressed ER and PR, respectively. Additionally, in 55% of patients HER2 were positive. You can find patient clinical and pathological data in supplementary data file.

ISL1, MGMT, DMNT3b genes promoter methylation status in breast cancer patients

The methylation status of *ISL1, MGMT and DMNT3b* genes was determined by methylated specific PCR in tumor and

Table 4 Methylation status in tumor and adjacent normal tissues in breast cancer patients

	Group		The chi-squer test	P value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Tumor	Adjacent normal						
ISL1								
Methylated	17	9	32.2	0.000				
Un methylated	5	29			87	72	76	85
Both	18	2						
MGMT								
Methylated	21	13	18.67	0.000				
Un methylated	8	25			87	62.5	76	86
Both	11	2						
DMNT3b								
Methylated	15	24	4.058	0.131				
Un methylated	19	12			87	60	76	86
Both	6	4						

P value less than ≤0.05 was considered significant. Both: mixed methylated and un-methylated alleles

PPV: positive predictive value, *NPV*: negative predictive value

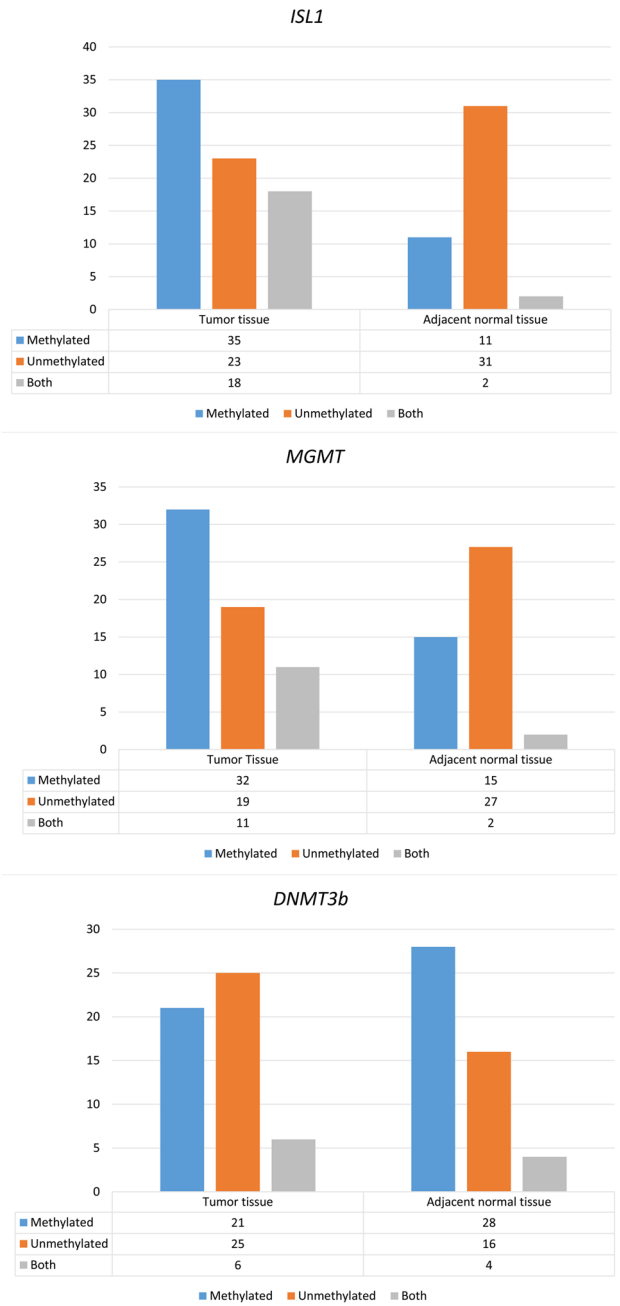

Fig. 1 Mean methylation levels of *ISL1, MGMT, DMNT3b* genes in tumor versus normal tissues

adjacent normal tissues of breast cancer and confirmed by Sanger sequencing of bisulfite converted DNA. Methylation frequency of promoter-associated CpG islands of both *ISL1, MGMT* genes were greater in tumor tissues of breast cancer compared to adjacent normal tissues. Conversely, *DMNT3b* methylation frequency in adjacent normal tissues was greater than tumor tissues. Also, sensitivities and specificities for each genes were investigated (Table 3). Also, mean levels of methylation of *ISL1, MGMT* and *DMNT3b* genes were examined in tumor and normal tissues for investigation of significant level methylation. The mean level of *ISL1, MGMT* in tumor tissues were significantly greater in comparison to normal tissues ($P \leq 0.05$). However, in tumor tissues, the mean level of methylation of *DMNT3b* gene was lower than normal tissues but that was not significant P (H) = (0.131) (Table 3).

Assessment of the predictive value of 2 or 3 genes promoter methylation variation combination

Regarding our description on previous section, none of the genes has reasonable predictive value or sensitivity and specificity. Thus, their promoter methylation variation were combined two by two and all three genes as one. As mentioned in Table 4, just *MGMT* and *ISL1* combination has convincing sensitivity and negative predictive value, 91% and 90%, respectively.

Expression pattern of ISL1, MGMT and DMNT3b genes in breast cancer patients

Relative expression analysis of *ISL1* showed a significant reduction in expression level of *ISL1* in tumor tissues by 0.273 fold change P (H) = (0.0012). (Fig. 1A).

Investigation of association between clinico-pathological features and *ISL1* expression level demonstrated a significant association between *ISL1* expression and histological grade ($P \leq 0.007$). Furthermore, there was a significant association between down expression of *ISL1* and ER status ($P \leq 0.045$). In addition, there was a significant relationship between decreased expression level of *ISL1* and Her2 status ($P \leq 0.043$). No association between *ISL1* down expression

Table 5 Predictive value of 2 or 3 genes promoter methylation variation combination

Combination	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MGMT + *ISL1*	31	9	27	3	91	75	77	90
MGMT + *DMNT3b*	23	11	22	5	82	66	67	81
ISL1 + *DMNT3b*	21	13	21	3	87	61	61	87
ISL1 + *DMNT3b* + *MGMT*	19	9	21	4	82	70	67	96

TP true positive, *FP* false positive, *TN* true negative, *FN*: false negative, *PPV* positive predictive value, *NPV* negative predictive value

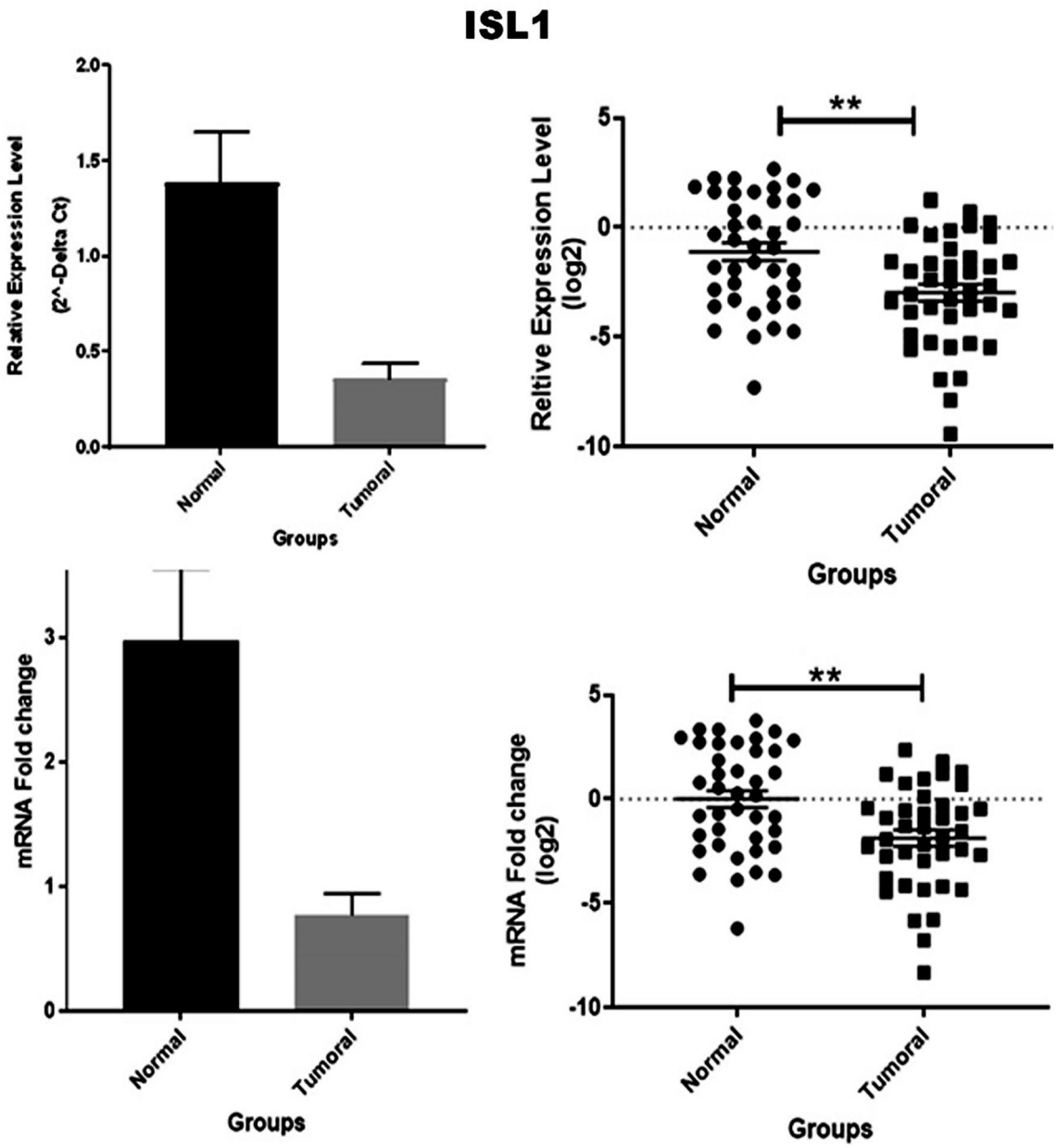

Fig. 2 Expression pattern of *ISL1* in breast cancer tumors using the $2^{-\Delta\Delta Ct}$ method P (H) = (0.0012)

age, PR status, Ki67 status, tumor stage, lymph node involvement and tumor size were detected.

Relative expression analysis of *MGMT* displayed a noticeable change between tumor groups in comparison to control group in a way that *MGMT* was decreased in tumor groups in contrast to normal tissues by 0.308 fold change P (H) = (0.003). (Fig. 1B).

In addition, a significant relationship between reduced expression level of *MGMT* and tumor size were confirmed by analysis of correlation between clinico-pathological features and *MGMT* down expression ($P \leq 0.05$). No correlation was found between reduced expression levels of *MGMT* and age, ER status, PR status, Ki67 status, Her2 status, tumor stage, lymph node involvement and histological grade.

Relative expression analysis of *DMNT3b* demonestrated that *DMNT3b* expression increased significantly in tumor tissues in contrast to adjacent normal tissues by 2.001 fold change P (H) = (0.0098). (Fig. 1C).

Although increased expression was investigated in *DMNT3b* gene in tumor tissues, no reasonable relation was observed between *DMNT3b* expression level and patient's

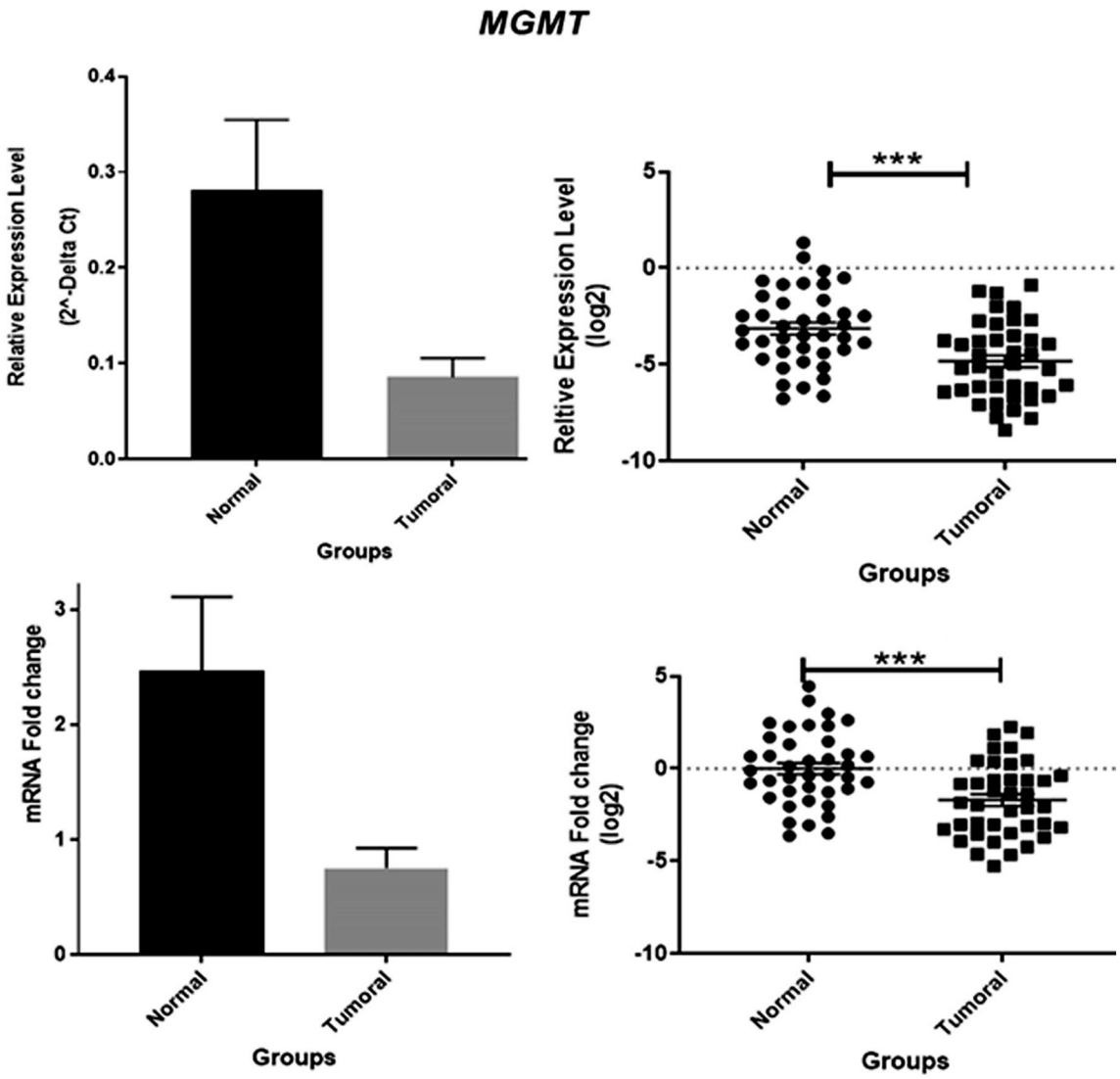

Fig. 3 Expression pattern of *MGMT* in in breast cancer tumors using the $2^{-\Delta\Delta Ct}$ method P (H) = (0.003)

Table 6 Comparison of promoter methylation status and fold change using one way annova

Gene	Tissue Type	P Value	df
ISL1	Adjacent tumor	0/011	2
	Tumor	0/00	2
DMNT3b	Adjacent tumor	0/00	2
	Tumor	0/01	2
MGMT	Adjacent tumor	0/013	2
	Tumor	0/00	2

P value less than ≤ 0.05 was considered significant

age, ER status, PR status, Ki67 status, Her2 status, tumor stage, lymph node involvement and histological grade.

Promoter methylation and gene expression correlation

The promoter methylation status and expression of all 3 genes showed significant change in tumor tissues and normal adjacent tumor tissues. Thus, correlation between methylation status of genes promoter and their changes in expression level in all samples were analyzed. In this step all samples were separated by their methylation status in 3 groups; methylated, un-methylated and both (being mix of methylated and

Table 7 Multiple comparisons of fold changes variation between different methylation status groups using one way annova by LSD algorithm

Comparison	*ISL1* (P Value)		*DMNT3b* (P Value)		*MGMT* (P Value)	
	Adjacent	Tumor	Adjacent	Tumor	Adjacent	Tumor
Methylated vs Un-methylated	0/04	0/00	0/00	0/00	0/020	0/00
Methylated vs Both	0/72	0/01	0/09	0/519	0/909	0/135
Un-methylated vs Both	0/23	0/00	0/05	0/04	0/320	0/00

*P value less than ≤ 0.05 was considered significant

Table 8 Promoter methylation correlation to ΔCt

Delta Ct	ISL1			DMNT3b			MGMT		
	Min	Average	Max	Min	Average	Max	Min	Average	Max
Methylated	3/37	4/99	9/42	3/14	4/4	6/44	3/5	5/6	8/32
Un-methylated	− 2/67	− 0/16	2/97	− 1/04	1/19	2/95	− 1/32	1/64	3/23
Both	0/14	1/76	3/28	1/42	2/6	3/24	2/63	4/4	6/16

un-methylated). Analysis have shown significant differences in all 3 genes and two types of samples,

Also, LSD algorithm was applied for multiple comparison of each two groups for precision elucidation. As shown in Table 5, all paired groups had significant differences except *ISL1* methylated and un-methylated versus both in normal adjacent tumor tissue, *DMNT3b* methylated versus both in tumor tissue and *MGMT* methylated versus both in all two types of tissues and un-methylated versus both in normal adjacent tumor tissue, This results may occurred due to the fact that one allele of these genes has normal promoter methylation status (Fig. 2).

Promoter methylation correlation to ΔCt

Correlation between promoter methylation and gene expression were studied to see if we could predict methylation statue using the ΔCt (refer to Supplementary data file). Regarding *ISL1* gene, if the ΔCt was between $-2.67 < X < 3$, the sample could be predicted as un-methylated and if the ΔCt was $X > 3$ the sample would be methylated. The discrimination between the samples with both methylated and un-methylated (Hetero) status was not possible (Fig. 3).

Regarding *DMNT3b* gene if the ΔCt was between $-1.04 < X < 3$ it could be concluded that the sample was un-methylated and if the ΔCt was $X > 3$ it could be concluded that the sample was methylated. Once again, the discrimination between the samples with both methylated and un-methylated (Hetero) status was not possible (Table 6).

Regarding *MGMT* gene the sample with $-1.32 < X < 3.2$ is un-methylated and with ΔCt is $X > 3.2$ is methylated. Likewise, the discrimination between the samples with both methylated and un-methylated (Hetero) status is not possible.

Discussion

Breast cancer prevalence has increased considerably by 0.3% per year, and most important cause of cancer-related death among women is breast cancer [26],.At present, the woman typical risk of developing breast cancer during her life is about 13% in the United States [2, 27, 28]. In Iran similarly the prevalence of this cancer has increased, one out of every 10 to 15 women is at risk of developing breast cancer (Table 7). Surprisingly, its prevalence is under 45 years old in Iran in comparison with Western countries which is under 56 years [4, 29, 30]. Fortunately, although the incidence of this cancer has increased in recent years, its mortality rate has dropped significantly [31]. It is supposed that extensive screening, for this cancer, which leads to early detection of and also improved treatment methods caused reduction in mortality rate [32].

Cancer initiation and its progression is influenced by both genetic and epigenetic events [33]. Illustrating the overlap between epigenetic and genetic alteration in sporadic breast tumors can provide important insights into cancer biology and may afford new strategies for cancer prevention and diagnosis [5, 9]. Screening and diagnostic tests based on DNA methylation are one of the new methods that have been considered in recent decades [33].Furthermore, DNA methylation is one of the crucial factor for controlling gene expression and maintaining of genomic structure [34]. It has been long time considered that DNA methylation is a significant regulator of gene expression [11, 34, 35]. By going through published data in literature to find previously reported genes which underwent methylation changes, *ISL1, MGMT* and *DMNT3b* genes were candidate to better understand the role of DNA methylation variation effects on gene expression variation [15, 19, 22].

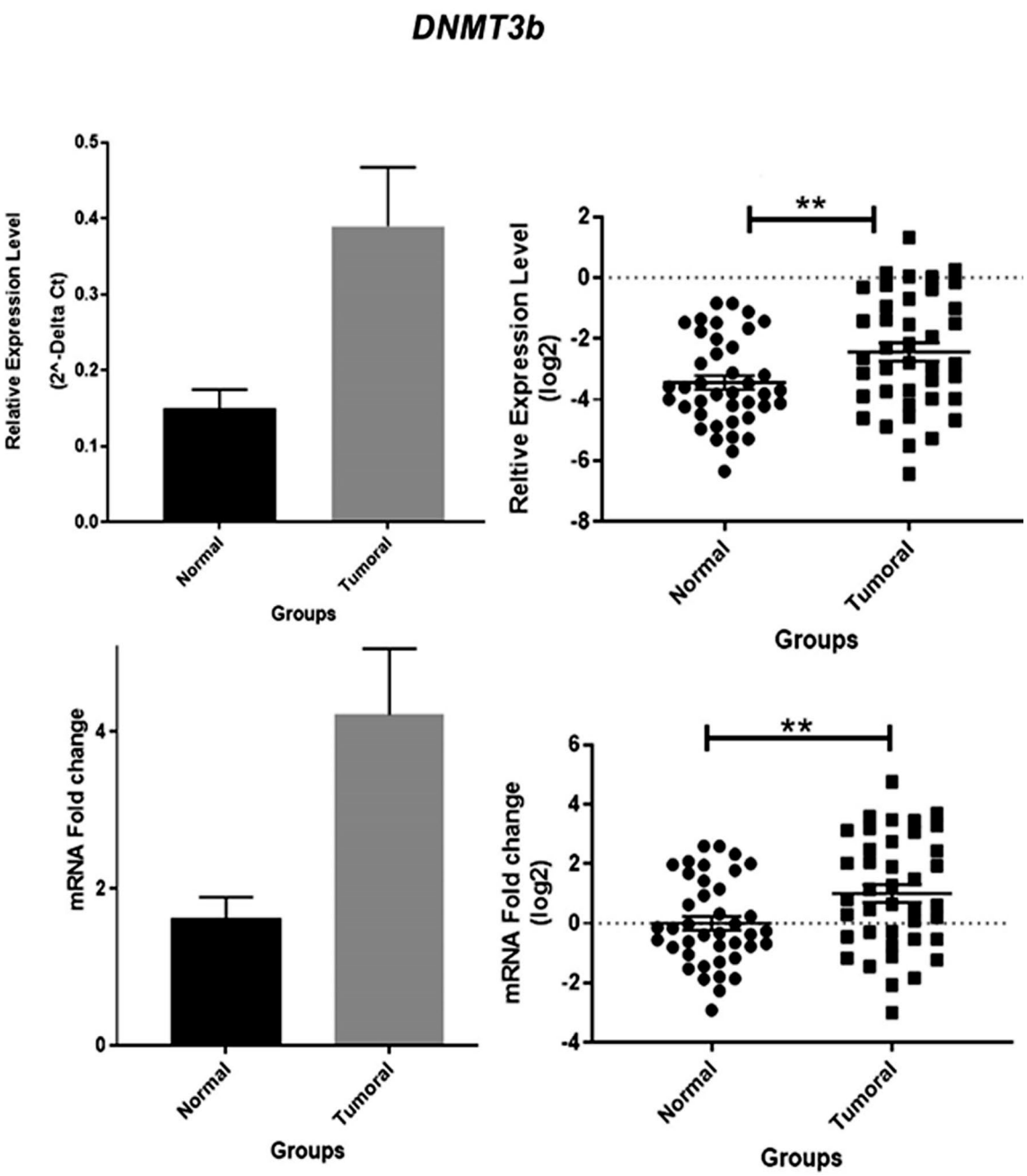

Fig. 4 Expression pattern of *ISL1* in in breast cancer tumors using the $2^{-\Delta\Delta Ct}$ method P (H) = (0.0098)

The expression of *ISL1* gene is normally inhibited in breast tissue during pregnancy, and abnormally, this gene is expressed during breast cancer [15]. For the first time, Kim et al. showed that hyper methylation of *ISL1* could be an independent predictor of cancer development and recurrence in bladder cancer [36]. In other research Kitchen et al. reported an increased methylated promoter-associated island of *ISL1* genes in progressive high-grade bladder tumour tissues [15].Furthermore, Convey et al. revealed hyper methylation of *ISL1* genes in 517 breast tumors by using microarray analysis [37].

MGMT gene is directly involved in the DNA repair system, thus, its unusual function can lead to cancer (Table 8). In several studies it has been shown that methylation of this gene increases in breast cancer and its hyper methylation is directly related to tumor survival [38], Chen et al. have declared that down regulation of *MGMT* along with *P16* promotes could have the anti-proliferative and pro-apoptotic

effects of 5-Aza-dC and radiation on cervical cancer cells [39].

The protein of *DMNT3b* gene is usually located in the nucleus and is well controlled during development. Devon Roll et al. demonstrated the association of overexpression *DMNT3b* on hyper methylation phenotype in cell lines breast cancer patients [22]. In 2013, Naghitorabi et al. measured the methylation level of *DMNT3B* gene in breast cancer patients and reported a hypo methylation of this gene in breast cancer patients compared to controls [40].

Up to date, methylation statues of *ISL1, MGMT* and *DMNT3b* has been studied in by numbers of researchers, but expression level of these genes have not been studied. In this regard in this paper, both methylation status and the expression level of *ISL1, MGMT* and *DMNT3b* were investigated (Fig. 4).

We found that the rate of methylation of *ISL1* and *MGMT* genes reduced in tumor samples compared to adjacent normal samples, and relative expression analysis displayed a significant reduction in expression level of *ISL1* and *MGMT* in breast cancer compared to adjacent normal tissues P (H) = (0.0012) and P (H) = (0.003), respectively. Furthermore, there was a significant association between down expression of *ISL1* and histological grade, Her2 status and ER status ($P \leq 0.007$), $P \leq 0.045$), ($P \leq 0.043$) respectively. Moreover, *MGMT* down expression was significantly associated with tumor sizes ($P \leq 0.05$).

Unlike *ISL1, MGMT* gene it was identified that *DMNT3b* expression increased significantly in tumor tissues in contrast to adjacent normal tissues P (H) = (0.0098) but remarkable relation was not observed between *DMNT3b* expression level and clinic-pathological features [41]. Previous studies have reported that *ISL1, MGMT* were hyper methylated in tumor tissues whereas *DMNT3b* gene was hypo methylated. The hypothesis of this study was that if a gene methylation statues varies during cancer initiation and progression this change must also be seen in the expression level of genes. The gained results of this study were exactly in line with the obtained results from articles which were based on the methylation research. It was revealed that the results of the real-time and methylation methods are consistent with each other.

Conclusion

In this research, we approached to results that support previous data which were based on the relationship between methylation status of *ISL1, MGMT* and *DMNT3b* genes and tumor behavior and characteristics.

Our finding also reported DNA methylation variation effects on gene expression variation. Finally, due to the noticeable change in promoter methylation and gene expression of *ISL1, MGMT* and *DMNT3b* genes more investigating is recommend, Since methylation of these genes could be detected in normal adjacent tumor, the potential methylation statue of these genes as diagnosis biomarkers is now questionable, however, by further investigation these genes maybe be introduced as prognosis /and prediction biomarkers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06546-z.

Author contributions HY conception and carried out the experiment, SS verifying statistical analysis and were involved in manuscript drafting, NN supervise the project and prepared samples, TM conception and design and manuscript drafting, FM supervised the project, grant and revised the draft. All authors read and approved the final manuscript.

Funding This project was partially supported by NIGEB including grant no. 738 and partially by Iran University of Medical Science.

Data availability The data that support the findings of this study are openly available from the corresponding author upon possible request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest and also competing interests**.**

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and ethic permission Number is, IR.NIEGEB.EC.1395.5.6.B.

Informed consent All patients who participated in this study were fully aware of the study process and their consent to participate in the study was received and we assured them about personal information protection.

References

1. Wittenberger T, Sleigh S, Reisel D, Zikan M, Wahl B, Alunni-Fabbroni M et al (2014) DNA methylation markers for early detection of women's cancer: promise and challenges. Epigenomics 6(3):311–327
2. Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, Harlow SP et al (2011) Effect of occult metastases on survival in node-negative breast cancer. N Engl J Med 364(5):412–421
3. Rosner D, Lane WW (1993) Predicting recurrence in axillary-node negative breast cancer patients. Breast Cancer Res Treat 25(2):127–139
4. daloeiy mrn, tabarestani s. review artile, molecular genetics, diagnosis and breast cancer theraphy(in persian)
5. Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B et al (2003) The case for early detection. Nat Rev Cancer 3(4):243–252
6. Horner M (2009) SEER cancer statistics review, 1975–2006. National Cancer Institute, Bethesda

7. Radpour R, Barekati Z, Kohler C, Holzgreve W, Zhong XY (2009) New trends in molecular biomarker discovery for breast cancer. Genet Test Mol Biomark 13(5):565–571
8. Adegboyega PA, Mifflin RC, DiMari JF, Saada JI, Powell DW (2002) Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps, and adenomatous colorectal polyps. Arch Pathol Lab Med 126(7):829–836
9. Romagnolo DF, Daniels KD, Grunwald JT, Ramos SA, Propper CR, Selmin OI (2016) Epigenetics of breast cancer: modifying role of environmental and bioactive food compounds. Mol Nutr Food Res 60(6):1310–1329
10. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome—biological and translational implications. Nat Rev Cancer 11(10):726–734
11. Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, Degner JF et al (2011) DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol 12(1):R10
12. Meiers I, Shanks JH, Bostwick DG (2007) Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. Pathology 39(3):299–304
13. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S (2001) Hypermethylation of 14-3-3 σ (stratifin) is an early event in breast cancer. Oncogene 20(26):3348–3353
14. Montavon C, Gloss BS, Warton K, Barton CA, Statham AL, Scurry JP et al (2012) Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer. Gynecol Oncol 124(3):582–588
15. Kitchen MO, Bryan RT, Haworth KE, Emes RD, Luscombe C, Gommersall L et al (2015) Methylation of HOXA9 and ISL1 predicts patient outcome in high-grade non-invasive bladder cancer. PLoS ONE 10(9):e0137003
16. Subramaniam D, Thombre R, Dhar A, Anant S (2014) DNA methyltransferases: a novel target for prevention and therapy. Front Oncol 4:80
17. Shin E, Lee Y, Koo JS (2016) Differential expression of the epigenetic methylation-related protein DNMT1 by breast cancer molecular subtype and stromal histology. J Transl Med 14(1):1–11
18. Tano K, Shiota S, Collier J, Foote RS, Mitra S (1990) Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O6-alkylguanine. Proc Natl Acad Sci 87(2):686–690
19. Spitzwieser M, Holzweber E, Pfeiler G, Hacker S, Cichna-Markl M (2015) Applicability of HIN-1, MGMT and RASSF1A promoter methylation as biomarkers for detecting field cancerization in breast cancer. Breast Cancer Res 17(1):1–13
20. An N, Shi Y, Ye P, Pan Z, Long X (2017) Association between MGMT promoter methylation and breast cancer: a meta-analysis. Cell Physiol Biochem 42(6):2430–2440
21. Chen R, Zheng Y, Zhuo L, Wang S (2017) Association between MGMT promoter methylation and risk of breast and gynecologic cancers: a systematic review and meta-analysis. Sci Rep 7(1):1–8
22. Roll JD, Rivenbark AG, Jones WD, Coleman WB (2008) DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer 7(1):15
23. Koudehi AT, Mahjoubi B, Mirzaei R, Shabani S, Mahjoubi F (2018) AKAP4, SPAG9 and NY-ESO-1 in Iranian colorectal Cancer patients as probable diagnostic and prognostic biomarkers. Asian Pacific J Cancer Prev 19(2):463
24. Motalebzadeh J, Shabani S, Rezayati S, Shakournia N, Mirzaei R, Mahjoubi B et al (2018) Prognostic value of FBXO39 and ETS-1 but not BMI-1 in Iranian colorectal cancer patients. Asian Pacific J Cancer Prev 19(5):1357
25. Jamebozorgi I, Majidizadeh T, Pouryaghoub G, Mahjoubi F (2018) Aberrant DNA methylation of two tumor suppressor genes, p14ARF and p15INK4b, after chronic occupational exposure to low level of benzene. Int J Occup Environ Med 9(3):145
26. Mendoza G, Portillo A, Olmos-Soto J (2013) Accurate breast cancer diagnosis through real-time PCR her-2 gene quantification using immunohistochemically-identified biopsies. Oncol Lett 5(1):295–298
27. DeWeese TL, Laiho M (2011) Molecular determinants of radiation response. Springer Science & Business Media, New York
28. Hallajian Z, Mahjoubi F, Nafissi N (2017) Simultaneous ATM/BRCA1/RAD51 expression variations associated with prognostic factors in Iranian sporadic breast cancer patients. Breast Cancer 24(4):624–634
29. Waks AG, Winer EP (2019) Breast cancer treatment: a review. JAMA 321(3):288–300
30. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A et al (2019) Breast cancer statistics, 2019. CA: Cancer J Clin 69(6):438–451
31. Parkin DM, Fernández LM (2006) Use of statistics to assess the global burden of breast cancer. Breast J 12:S70–S80
32. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128(4):683–692
33. Pan Y, Liu G, Zhou F, Su B, Li Y (2018) DNA methylation profiles in cancer diagnosis and therapeutics. Clin Exp Med 18(1):1–14
34. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B et al (2014) Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res 24(6):766–769
35. Silva JM, Gonzalez R, Dominguez G, Garcia JM, España P, Bonilla F (1999) TP53 gene mutations in plasma DNA of cancer patients. Genes Chromosomes Cancer 24(2):160–161
36. Kim YJ, Yoon HY, Kim JS, Kang HW, Min BD, Kim SK et al (2013) HOXA9, ISL1 and ALDH1A3 methylation patterns as prognostic markers for nonmuscle invasive bladder cancer: array-based DNA methylation and expression profiling. Int J Cancer 133(5):1135–1142
37. Conway K, Edmiston SN, May R, Kuan PF, Chu H, Bryant C et al (2014) DNA methylation profiling in the Carolina Breast Cancer Study defines cancer subclasses differing in clinicopathologic characteristics and survival. Breast Cancer Res 16(5):450
38. Natarajan A, Vermeulen S, Darroudi F, Valentine MB, Brent TP, Mitra S et al (1992) Chromosomal localization of human O6-methylguanine-DNA methyltransferase (MGMT) gene by in situ hybridization. Mutagenesis 7(1):83–85
39. Chen Gd, Qian Dy, Li Zg, Fan Gy, You Kl, Wu Yl (2017) Down-regulation of p16 and MGMT promotes the anti-proliferative and pro-apoptotic effects of 5-A za-d C and radiation on cervical cancer cells. Cell Biochem Funct 35(8):488–496
40. Naghitorabi M, Asl JM, Sadeghi HMM, Rabbani M, Jafarian-Dehkordi A, Javanmard HS (2013) Quantitative evaluation of DNMT3B promoter methylation in breast cancer patients using differential high resolution melting analysis. Res Pharm Sci 8(3):167
41. Amara K, Ziadi S, Hachana M, Soltani N, Korbi S, Trimeche M (2010) DNA methyltransferase DNMT3b protein overexpression as a prognostic factor in patients with diffuse large B-cell lymphomas. Cancer Sci 101(7):1722–1730