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Association between HIC1 and RASSF1A Promoter Hypermethylation with MTHFD1 G1958A Polymorphism and Clinicopathological Features of Breast Cancer in Iranian Patients

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ABSRACT

Background: Ras-associated domain family 1 (*RASSF1A*) and hypermethylated in cancer (*HIC1)* genes are methylated more frequently in breast cancer. Genetic factors that alter the DNA methylation levels in normal and tumor tissues could therefore influence the susceptibility to this tumor phenotype. **Objective:** We determined the frequency of aberrant methylation of *HIC1* and *RASSF1A* gene promoters and their association with methylene tetrahydrofolate dehydrogenase (*MTHFD1*) G1958A polymorphism and major clinical and pathological features of breast cancer in Iranian women. **Methods:** DNA was extracted from 81 primary breast tumors and 100 control blood samples. Gene promoter methylation was analyzed by methylation-specific polymerase chain reaction. **Results:** Eighty four percent of the breast cancer samples showed total methylation in at least one of two tested loci. We detected *HIC1* hypermethylation in 79% of invasive and metastasis tumors and *RASSF1A* gene hypermethylation in 51% of them. We found no association between *HIC1* and *RASSF1A* gene hypermethylation and *MTHFD1* G1958A polymorphism, but a significant correlation between methylation of *HIC1* and *RASSF1A* promoters was indicated (r = 0.24, *P* = 0.02). There was a combination between hypermethylation of *HIC1* locus and nodal involvement in the studied population (p=0.03). We found a significant association between total methylation and nodal involvement (*P* = 0.01) as well as tumor size more than 2 cm in all cases (*P* = 0.02). **Conclusion:** Methylation of *HIC1* and *RASSF1A* promoters can be used as epigenetic markers to detect the malignant progression of breast carcinoma in Iranian women patients. *Iran. Biomed. J. 13 (4): 199-206, 2009*

Keywords: Breast cancer, Hypermethylated in cancer (*HIC1),* Methylation, Methylene tetrahydrofolate dehydrogenase (*MTHFD1*), Ras-associated domain family 1 (*RASSF1A*)

INTRODUCTION

Breast cancer is the most common malignancy in Iranian women [1]. Numerous studies have demonstrated hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is an important mechanism for gene inactivation in breast cancer [2, 3]. Expression of many genes was found to be lost in breast cancer because of aberrant promoter methylation, such as *RASSF1A* (Ras-associated domain family 1) and *HIC1* (hypermethylated in cancer) genes [2, 4].

RASSF1A encodes a 39-kDa predicted peptide that contains an N-terminal diacylglycerol binding domain, a ras-associated domain and a putative substrate for ataxia-telangiectasia-mutated (*ATM*) phosphorylation [5,6]. It has also been shown to suppress the growth of tumor cell lines as a major 3p21.3 tumor suppressor gene [5].

HIC1 encodes a transcriptional repressor with five Kruppel-like Cys_2-His_2 zinc fingers in the C terminus and a protein-protein interaction domain called the BTB/POZ domain at the N terminus [7]. It is a candidate tumor suppressor gene since it dramatically reduces tumorigenicity *in vivo* [8]. *HIC1* is located at 17p13.3, a region which is frequently hypermethylated or undergoes allelic loss in breast cancer [9].

*Corresponding Author; Tel. & Fax: (+98-711) 230 3029; E-mail: rasti31@yahoo.com. **Abbreviations:** Ras-associated domain family 1 (RASSF1A), hypermethylated in cancer (HIC1), methylene tetrahydrofolate dehydrogenase (MTHFD1), infiltrating ductal carcinoma (IDC), infiltrating lobular carcinoma (ILC), methylation-specific polymerase chain reaction (MSP), in vitro-methylated DNA (IVD), single nucleotide polymorphism (SNP), odds ratios (OR), confidence intervals (CI)

Analysis of gene methylation patterns in breast tissue could be of profound significant in the early detection of cancer. However, we still know little of the mechanisms of aberrant methylation and whether germ-line factors can influence the level of genes hypermethylation. A germ-line variant in methylene tetrahydrofolate dehydrogenase (*MTHFD1*) gene that is involved in methyl group metabolism has been reported for susceptibility to DNA methylation in breast cancer [10]. *MTHFD1* is a trifunctional cytoplasmic enzyme, which catalyzes the conversion of tetrahydrofolate to the corresponding 10-formyl, 5,10-methenyl, and 5,10-methylene derivatives [11]. Ten-formyltetrahydrofolate and 5,10-methyl-enetetrahydrofolate are the donor cofactors for DNA biosynthesis. It has been previously shown that a G1958A polymorphism within the coding region of *MTHFD1* is associated with a higher frequency of tumor CpG island hypermethylation in the promoter regions of the *RARβ2*, *CDH1*, *ER*, *BRCA1*, *CCND2*, *p16* and *TWIST* genes in sporadic breast cancer [10].

In the present study, we determined whether *MTHFD1* polymorphism was associated with promoter hypermethylation of *RASSF1A* and *HIC1* genes. In addition, we investigated associations between this polymorphism, *RASSF1A* and *HIC1* genes hypermethylation, and the major clinicopathological features of breast cancer among Iranian patients. Results of this study show that total methylation of studied genes are associated with certain phenotype features of breast cancer. However, *MTHFD1* polymorphism does not appear to effect on *RASSF1A* and *HIC1* genes hypermethylation.

MATERIALS AND METHODS

Tumor and blood samples. A total of 81 invasive primary breast cancer tumors (68 infiltrating ductal carcinoma (IDC), 8 infiltrating lobular carcinoma (ILC), and 4 metaplast carcinoma) were obtained from the Department of Pathology at Shiraz University of Medical Sciences and from the Department of Pathology at Dena Hospital in Shiraz. All samples belonged to the patients who lived in Fars province. DNA was isolated from the frozen tissues by a standard phenol/chloroform procedure. The clinical and histopathological information was obtained by retrospective review of medical records. The histopathological diagnoses of breast tumors were confirmed and EDTA-anticoagulated blood samples of 100 healthy donors were obtained from Shiraz University Hospital (Iran). Patients were compared with an age matched control group. DNA was extracted from 1 ml of whole blood by DNA extraction kit (Cinnagen, Iran) and frozen until analysis at -20°C.

Treatment with sodium bisulfate and methylation-specific polymerase chain reaction (MSP). Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA [12]. MSP is the most widely used technique for studying the methylation of CpG islands. The sequences of PCR primers that was used to distinguish methylated and unmethylated *RASSF1A* and *HIC1* genes, annealing

Table 1. PCR primer sequences used for gene promoter methylation and gene polymorphism.

Name	Primers pair sequences (5' → 3')	Size (bp)	Anneal T (°C)
HIC1-M [13]	TCGGTTTTCGCGTTTTGTTCGT AACCGAAAACTATCAACCCTCG	95	60
HIC1-U [13]	TTGGGTTTGGTTTTTGTGTTTTG CACCCTAACACCACCCTAAC	118	60
RASSF1A-M [5]	GGGTTTTGCGAGAGCGCG GCTAACAAACGCGAACCG	169	64
RASSF1A-U [5]	GGTTTTGTGAGAGTGTGTTTAG CACTAACAAACACAAACCAAAC	169	64
MTHFD1[10]	CACTCCAGTGTTTGTCCATG GCATCTTGAGAGCCCTGAC	330	62

M, methylated-specific primers; U, unmethylated-specific primers.

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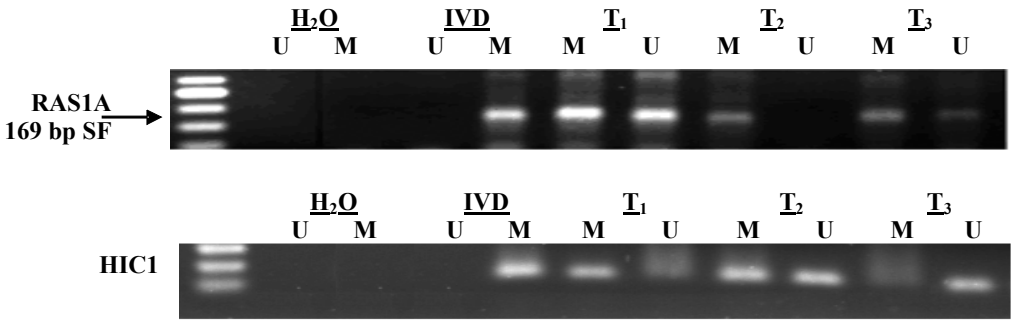

Fig. 1. DNA methylation analysis of the *RASSF1A* and *HIC1* CpG islands by MSP. U and M represent amplified unmethylated and methylated bands, respectively. *In vitro*-methylated DNA (*IVD*) was used as a positive methylated control. H_2O is a negative control reaction without DNA. T indicates tumor sample.

temperatures, and the expected sizes of PCR products are summarized in Table 1. The *RASSF1A* [5] and *HIC1* [13] specific primers were ordered from Cinnagen, Iran. The PCR reaction mixture contained l× PCR buffer, dNTPs (each at 0.3 mM) and $MgCl_2$ (3 mM); all provided by enzyme supplier (Fermentas, Canada), primers (0.5 mM each per reaction), and bisulfite-modified DNA (100-150 ng) in a final volume of 25 µl. Reactions were hot-started at 95°C for 5 minutes before the addition of 1.25 units of *Taq* DNA polymerase (Fermentas, Canada). Amplification of two genes were performed under following condition: 40 cycles of 95°C for 40 s, at specific annealing temperature as listed in Table 1 for 40 s, and 72°C for 45 s; and a final extension of 7 min at 72°C. Human normal sample DNA was used as the control for unmethylated alleles. A reaction without DNA was used as negative control reaction for each set of PCR reactions. In each case *in vitro*-methylated DNA (*IVD*) was used as positive control. The amplified products were loaded onto a 2% agarose gel and visualized under UV illumination. The PCR for those samples demonstrating methylation was repeated to confirm the reproducibility of results.

Genotype analyses. Genotyping for the *MTHFD1* G1958A single nucleotide polymorphism (SNP) was carried out using PCR-Restriction fragment length polymorphism [10]. The sequence of PCR primers [10] used for *MTHFD1* G1958A (SNP), annealing temperature, and the expected size of PCR product are summarized in Table 1. The PCR product of 330 bp was digested with the restriction endonuclease *MSPI* (Fermentas, Canada) cutting the wild type (G) allele into two fragments. Restriction digests were analysed on 2% agarose gels.

Statistical analysis. The Chi-Square test was used to determine possible associations between genetic variants in *MTHFD1*, methylation of *RASSF1A* and *HIC1* genes, and various phenotypic features of breast cancer. Also, this test was used to evaluate *MTHFD1* genotypes association with gene methylation frequencies. Association between methylation frequencies of two loci was compared using the Pearson test. Logistic regression analysis was performed to estimate odds ratios (OR) and 95% confidence intervals (CI), and adjusted for age at diagnosis for breast cancer. Values of $P<0.05$ were considered to be statistical significant. All analyses were performed using the SPSS 12.0 statistical software (Chicago, IL, USA).

RESULTS

Analysis of methylation distribution in invasive tumors. We searched for aberrant promoter methylation at *RASSF1A* and *HIC1* gene loci in 81 primary breast cancers (Fig.1). Of 81 malignant tumors, 68 tumors (84%) were total methylated in at least one of two loci, 41 tumors (51%) displayed promoter methylation at *RASSF1A* gene and 63 tumors (79%) showed methylation of *HIC1* promoter (Table 2). Forty-four percent of breast tumor samples were methylated at two tested loci. The analysis of methylation distribution demonstrated a statistically significant association between methylation of *RASSF1A* promoter, and methylation at *HIC1* ($P = 0.02$, $r = 0.24$).

MTHFD1 (G1958A) polymorphism in test and control groups, and its association with promoter hypermethylation of RASSF1A and HIC1 genes. In the present study, the genotype of 71 invasive

Table 2. Association between *HIC1* and *RASSF1A* genes methylation and clinicopathological features of breast cancer.

Features (n)*		Methylated promoter region				Total methylation	*P*
		HIC1 gene	*P*	***RASSAF1A gene***	*P*		
Total	81 (100%)	63 (79%)		41 (51%)		68 (84%)	
Age **			NS		NS		NS
≤ 47 years	46 (58%)	37 (80.4%)		27 (58.7%)		41 (89.1%)	
> 47 years	34 (42%)	25 (73.5%)		13 (83.2%)		26 (76.5%)	
Side			NS		NS		NS
Right	36 (50%)	29 (80.6%)		21 (58.3%)		32 (88.9%)	
Left	36 (50%)	27 (75%)		16 (44.4%)		28 (77.8%)	
Nodal involvement			0.03		NS		0.01
Negative	36 (46%)	24 (66.7%)		17 (47.2%)		26 (72.2%)	
Positive	41 (54%)	36 (87.7%)		22 (53.7%)		39 (95.1%)	
Tumor size			0.06		0.3		0.02
≤ 2 cm	26 (32.5%)	17 (65.4%)		12 (46.2%)		18 (69.2%)	
> 2 cm	54 (67.5%)	45 (83.3%)		29 (53.7%)		49 (90.7%)	
Histological type			NS		NS		NS
IDC	68 (85%)	51 (75%)		34 (50%)		56 (82.4%)	
ILC	8 (10%)	7 (87.5%)		3 (37.5%)		7 (87.5%)	
Metastasis	4 (5%)	4 (100%)		4 (100%)		4 (100%)	
Histological grade			NS		NS		NS
I	10 (14%)	9 (90%)		5 (50%)		10 (100%)	
II	47 (70%)	34 (72.3%)		22 (46.8%)		38 (80.9%)	
III	11 (16%)	10 (90.9%)		8 (72.7%)		10 (90.9%)	
ER status			NS		NS		NS
Positive	52 (81%)	42 (80.8%)		23 (44.2%)		44 (84.6%)	
Negative	12 (19%)	11 (91.7%)		8 (66.7%)		12 (100%)	
PR status			NS		NS		NS
Positive	41 (64%)	35 (85.4%)		17 (41.5%)		36 (87.8%)	
Negative	23 (36%)	18 (78.3%)		14 (60.9%)		20 (87%)	

P, p value obtained from Chi-Square; NS, not significant (*P* value is >0.05); ER, estrogen receptor; PR, progesterone receptor. *Data missing for age and tumor size and type in one case, tumor side in 9 cases, nodal involvement in 4 cases, tumor grade in 13 cases and estrogen receptor and progesterone receptor status in 17 cases. ** Mean age is 47 years.

tumors and 100 control blood samples were analyzed for *MTHFD1* (G1958A) SNP (Table 3). A representative genotype analyses for *MTHFD1* is shown in Figure 2. Patient and control groups were age-matched, and there were no significant differences in their ages between two groups (*P* = 0.6). Also, we found no difference for this SNP between test and control population. However, they were in Hardy-Weinberg equilibrium. Moreover, the variant A allele frequencies were same for test (0.47) and control (0.47) population. In addition, *MTHFD1* (G1958A) polymorphism had no significant effect on the frequency of CpG island promoter methylation in *RASSF1A* and/or *HIC1* genes (Table 4).

Associations between MTHFD1 polymorphism, RASSF1A and HIC1 genes hypermethylation, and the major clinicopathological features of breast cancer. No significant association was observed between polymorphisms in *MTHFD1* gene and the clinicopathological features of patient age, nodal

Table 3. Genotype frequencies of the *MTHFD1* polymorphism G1958A in patient groups and control individuals.

Groups		MTHFD1 G1958A			*P* value
		GG	AG	AA	
Patients	71 (100%)	21(30%)	34 (48%)	16 (22.5%)	0.6
Controls	100 (100%)	24 (24%)	55 (55%)	21 (22%)	

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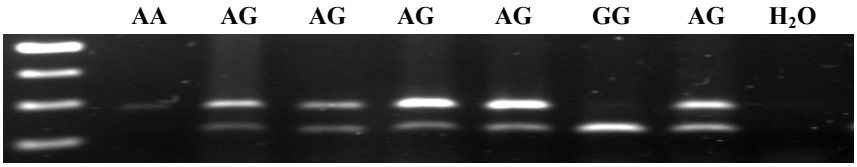

Fig. 2. Genotype analysis for the *MTHFD1* gene. The wild type (G) allele was digested with restriction endonuclease *MSP1*. H_2O is a negative control reaction without DNA.

status, tumor size, left or right sides, histological type or grade, estrogen and progesterone status. We next examined whether *RASSF1A* and *HIC1* gene hypermethylation were associated with the clinicopathological features of breast cancer (Table 2). The mean age of Iranian breast cancer patients at diagnosis was 47 years, ranging from 23 to 75 years. *RASSF1A* methylation gene was observed in 34 (50%) and *HIC1* methylation in 51 (75%) of 68 IDC samples. Therefore, methylation of the *RASSF1A* or *HIC1* CpG island (total methylation) was detected in 82.4% of these IDC lesions. In addition, aberrant promoter methylation for *HIC1* gene was found in 7 of the 8 (87.5%) ILC samples. However, three of these samples were methylated at both loci. All four metaplast carcinoma samples showed methylation of both CpG islands (Table 2). We found that *HIC1* methylation occurred in 36 of 41 (87.7%) malignant tumors with positive lymph nodes. These results showed a significant association between either *HIC1* (OR = 3.6, 95% CI, 1.1- 11.5, $P = 0.03$) or total (OR = 7.5, 95% CI, 1.5- 37, $P = 0.01$) methylation and the patients lymph nodes involvement. Total methylation also was significant for tumor size more than 2 cm (OR = 4.3, 95% CI, 1.2-15.1, $P = 0.02$). The same trend was observed for the association between *HIC1* methylation and the tumor size more than 2 cm ($P = 0.06$) (Table 2). However, no statistically significant differences in the frequencies of *RASSF1A* and *HIC1* genes promoter methylation were found between the patients at age's ≤ 47 and > 47 years old; left or right sides; histological grade or type, estrogen and progesterone status.

DISCUSSION

The rational of undertaking this study was based on the report that breast cancer patients who were homozygous for the *MTHFD1* (G1958A) genotype showed a higher frequency of methylation of multiple genes than that of wild-type homozygotes and it was suggested that gene hypermethylation may be affected by germ-line variant in this methyl group metabolism gene [10]. In this study, we first determined the frequency of *RASSF1A* and *HIC1* genes methylation in primary tumors because they are two most frequently hypermethylated markers for breast cancer [14, 15] and then investigated whether *MTHFD1* polymorphism was associated with promoter hypermethylation of these two genes. Aberrant methylation of the *RASSF1A* promoter region was commonly observed in 49% of American patients [5]. It was detected in 61% [16] and 95% [17] of primary breast tumors in China. Whereas, study in India has shown 72% of the cases were *RASSF1A* methylated [18]. In addition, *HIC1* gene was proposed to be commonly hypermethylated in 48% [19] and 64% [20] of breast carcinoma in Italy. It was hypermethylated in 67% of primary breast cancer in American people [9].

According to our results, promoter methylation of *HIC1* (79%) occurred more frequently than *RASSF1A* methylation (51%) in malignant tumors in Iranian population. Recently, it has been suggested that the reason of different gene promoter methylation frequencies in breast cancers in various

Table 4. Polymorphism in *MTHFD1 G1958A* genotype and the frequency of CpG island promoter methylation of *HIC1* and *RASSF1A* genes.

Gene methylation		MTHFD1 G1958A			*P* value
		GG	AG	AA	
H1C1	57 (80.3%)	17 (29.8%)	26 (45.6%)	14 (24.6%)	NS
RASSF1A	39 (54.9%)	14 (35.9%)	19 (48.7%)	6 (15.4%)	NS
Total	62 (87.3%)	20 (32.3%)	28 (45.2%)	14 (22.6%)	NS

NS, not significant (*P* value is >0.05)

reports might be because of different patients with ethnic and socioeconomic variables in each area [21]. Our study showed there was no association between *MTHFD1AA* carriers and promoter hypermethylation of *RASSF1A* and/or *HIC1* genes in Iranian population (Table 4). Also, this result could be supported by the finding that there was not any differences in the A allele frequencies between breast cancer (0.47) and control (0.47) population for *MTHFD1* (G1958A) genotype. Therefore, our findings did not support the previous report that *MTHFD1* (G1958A) polymorphism is the only polymorphism in methyl group and folate metabolism genes that is associated with the frequency of methylation in breast cancer [10]. However, it may be because of the low number of methylated genes or cases that we studied. Germ-line variants in methyl group and folate metabolism genes may play an important role in other kinds of cancers such as colorectal cancer [22-24].

The other aim of this study was to determine whether *RASSF1A* and *HIC1* genes methylation in breast cancer were associated with distinctive pathological characteristics of this cancer in Iranian patients. Epigenetic characterization of breast tumors may provide highly specific and sensitive molecular markers for cancer progression [25]. *RASSF1A* and *HIC1* are breast tumor-related genes that use as potential biomarkers for detection of cancer in serum [26-28] and epigenetic differences in breast tumors [15, 20, 29]. It has been demonstrated that methylation of *RASSF1A* correlated with nodal metastasis [18] and an advanced tumor stage as well as poor prognosis [25]. *HIC1* gene methylation was reported in 64% of invasive ductal carcinoma of the breast [20]. Our most significant finding was the association of either *HIC1* (OR = 3.6, 95% CI, 1.1- 11.5, $P = 0.03$) or total (OR = 7.5, 95% CI, 1.5- 37, $P = 0.01$) methylation with the patients lymph nodes metastasis. In addition, total methylation was significantly correlated with tumor size more than 2 cm (OR = 4.3, 95% CI, 1.2-15.1, $P = 0.02$). A slight association was also observed between *HIC1* methylation and tumor size more than 2 cm ($P = 0.06$). Our findings support pervious data in association of methylation of these genes with breast tumor malignancy and metastasis, because increased tumor size and nodal metastasis are two criteria for cancer progression. Also, these results support the notion that promoter methylation of either *HIC1* or with *RASSF1A* genes may provide highly specific and sensitive molecular markers for cancer progression. Therefore, we can use these epigenetic markers for tumor prognosis. Especially, *HIC1* hypermethylation was linked to tumor malignancy in Iranian breast cancer population.

Also, we showed it was methylated in 79% of invasive and metastatic breast tumors. It has been reported early detection of aberrant methylation of *RASSF1A* gene may serve as a diagnostic and prognostic of breast cancer [25, 26, 30-32]. In addition, there are controversial reports about the relationship between the promoter methylation of the *RASSF1A* gene and clinical parameters in different populations [16-18, 30, 33]. However, we could not find any correlation between *RASSF1A* gene methylation and clinicopathological features of breast cancer. *RASSF1A* gene methylation was shown to correlate with negative estrogen and progesterone receptor status [33], estrogen receptor positive tumor in early stage of tumor progression [29] and ages between 32 and 55 years old [30]. Nonetheless, there was a report that found no association between promoter methylation of *RASSF1A* gene and clinical parameters [16]. Our data are consistent with this pervious report. The *RASSF1A* and *HIC1* promoter hypermethylation occurred at a high frequency in breast cancer tumors and methylated [17] or hemi-methylated in normal breast tissues [9]. It has been suggested that *RASSF1A* and *HIC1* genes methylation are early events in breast cancer pathogenesis [9, 17, 34]. These are critical growth regulatory genes in breast cancer and their promoter methylation are more carcinogenic and are likely to establish breast cancer [18, 20, 29]. Interestingly, we found a significant association between methylation of the *RASSF1A* promoter, and methylation at *HIC1* promoter ($P = 0.02$, $r = 0.24$). Coincident methylation of some other tumor suppressor genes has also been reported previously [19, 29] in breast cancer. These associations represent the epigenetic inactivation of tumor suppressor genes does not occur randomly [19, 29] and suggest the existence of specific molecular associations between hypermethylation of *RASSF1A* and *HIC1* genes in breast tumors. It is possible that the promoters of these tumor suppressor genes are susceptible for CpG methylation that leads to transcriptional silencing of these genes and establishment of breast cancer.

In conclusion, we reported frequent promoter methylation of *RASSF1A* (51%) and *HIC1* (79%) genes in Iranian breast cancer population. We found a correlation between total methylation of these genes and certain pathological parameters of breast

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cancer; confirming the hypothesis that using these epigenetic markers are important for tumor progression in Iranian patients. However, *MTHFD1* polymorphism does not appear to effect on *RASSF1A* and *HIC1* genes hypermethylation. Nevertheless, further studies with large samples are demanded to confirm our finding.

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