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Original Article

The Impact of Long-term Exposure to Low Levels of Inorganic Arsenic on the Hypomethylation of SEPT9 Promoter in Epithelial-Mesenchymal Transformed Colorectal Cancer Cell Lines

Gholamreza Rafiei[1], Reza Shirkoohi[2], Mojtaba Saffari[1], Pouya Salehipour[1], Mohammad Hossein Modarressi[1*]

1. Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
2. Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran.

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Inorganic arsenicals are worldwide environmental contaminants that affect molecular characteristics in biological systems and lead to genomic and epigenomic instability as well as epithelial mesenchymal transition (EMT). In this study, we aimed to investigate whether low levels of sodium arsenite (iAsIII) can influence EMT and genomic instability through microsatellite analysis. We have also determined epigenomic instability by investigating the methylation status of *SEPT9* tumor marker in colorectal cancer (CRC) cell lines, Caco2 and HCT116, which were treated with iAsIII to assess IC50s. Short-term and long-term exposure to low concentrations (1 µM and 0.1 µM) of iAsIII in two separate experiments was implemented to analyze EMT, microsatellite status and the methylation pattern of *SEPT9* promoter. As expected, after 20 days of exposure to iAsIII, the expression of *CDH1* was significantly decreased while the expression of *CDH2*, *FIB1* and *VIM* was increased in Caco2 and HCT116, a finding that confirmed EMT induction. However, there was no detectable alteration in the size of microsatellites. As for the methylation pattern, *SEPT9* promoter was hypomethylated as a result of long-term exposure to 0.1 µM iAsIII in Caco2. Long-term exposure of HCT116 to both concentrations could induce hypomethylation of *SEPT9* promoter. Our findings indicate no linkage between EMT induction and microsatellite status in iAsIII-treated CRC cell lines. For the first time, the current study has shown that the induction of EMT by iAsIII is linked with *SEPT9* promoter hypomethylation in Caco2 and HCT116 in a concentration- and time-dependent pattern.

Key words: Arsenic; colorectal cancer; eEpithelial- mMesenchymal transition; microsatellite instability; *SEPT9*; methylation

Inorganic arsenic is known as one of the environmental factors involved in carcino-genesis. Arsenic- induced damage to genomic integrity is exerted through production of reactive oxygen species (ROS) (1). Moreover, inorganic arsenic triggers genomic instability through

*Corresponding author: Mohammad-Hossein Modarressi, Medical Genetics Department, Tehran University of Medical Sciences, Tehran, Iran. E-mail: modaresi@tums.ac.ir

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formation of single and double strand breaks, contributing to chromosomal instability (CIN) (2) and microsatellite instability (MSI) (3).

Arsenic can also modify the epigenomic landscape as it promotes the methylation in the promoter region of mismatch repair genes, hence, lead to epigenomic instability. This effect will further induce MSI as an outcome of exposure to arsenic as well (4-6). EMT is another incident to take place in cancerous and normal cells as a result of exposure to inorganic arsenic (7,8).

Effective treatment of cancers including colorectal cancer (CRC) is one of the main goals of various experiments in molecular biology and medical sciences. Many studies have focused on CRC classification based on molecular characteristics, therapeutic outcome, and prognosis (9-11). In terms of molecular characterization, MSI status is one feature of choice for classification of CRCs. This phenomenon can affect cancer treatment strategies as well as prognosis (12,13). Identification of tumors regarding MSI and EMT is beneficial to efficient management approaches of diseases (14,15), especially for CRC (16,17). Several studies have been conducted to detect specific tumor markers in blood or stool for early detection or screening of CRC. In this regard, methylation of septin 9 (*SEPT9*) gene promoter region has been introduced as a specific tumor marker which could be evaluated using cell free DNA obtained from the blood stream in symptomatic or asymptomatic patients with CRC (16,18). Therefore, efficient methods have been developed for investigation of promoter methylation pattern in *SEPT9* gene as a screening biomarker for CRCs in peripheral blood (17,18). On the other hand, MSI could be induced in transfected cell lines and animal models by short-term treatment with arsenic (3), and chronic exposure to arsenic can induce epigenetic alteration of mismatch repair-related proteins (19).

In the present study, we aimed to evaluate EMT and MSI as molecular characteristics of Caco2 and HCT116 cell lines in the presence of low concentrations of sodium arsenite (iAsIII) for short and long period of time. Moreover, for the first time we determined whether the treatment of the cell lines with iAsIII can alter the methylation status of *SEPT9* promoter region as the most important recognized CRC tumor marker.

Material and methods

Cell culture

Two human CRC cell lines including Caco2 (ATCC® Number: HTB-37) characterized as mismatch repair-sufficient (sMMR) and HCT116 (ATCC® Number: CCL-247) as mismatch repair-deficient (dMMR) were obtained from the Pasteur Institute of Iran, and cultured in RPMI 1640 with 10% (v/v) fetal bovine serum (Gibco, USA) at the temperature of 37 °C humidified air with a CO_2 pressure of 5%.

Chemo-sensitivity evaluation

Approximately 2×10^3 cells per well of each cell line separately seeded in triplicate in 96-well plates for 24 hours prior to treatment. Chemo-sensitivity of cell lines to iAsIII (Merck, Germany, Cat No: 106277) was evaluated by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay after 36 h exposure to serial dilutions of iAsIII (20). The percentage of inhibition at each concentration of iAsIII was calculated using the following formula with respect to the optical density (OD) of the blank well:1 – ($OD_{observed}$ / $OD_{control}$) × 100 = % inhibition

Treatment with iAsIII

According to MTT assay results, different concentrations of iAsIII (1 µM and 0.1 µM, considered as low concentrations) without any significant inhibitory effects on cellular viability were chosen for short-term (36 h) and long-term (20 days) treatment. These concentrations were respectively less and more than the safe concentration of inorganic arsenic recommended by

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World Health Organization (WHO) (21). After seeding of approximately 1×10^6 cells on 6 cm plates for short-term treatment and 5×10^5 cells on 10 cm plates for long-term treatment, each plate was treated with the aforementioned concentrations of arsenic. Negative controls comprised one plate of each cell line cultured under the same condition with no treatment. All experiments were performed in duplicate.

DNA and RNA extraction

For DNA and RNA extraction, each plate was treated with trypsin, washed with phosphate-buffered saline (PBS) twice, and detached cells were divided into two populations; the first population was incubated with 10 µl of proteinase K (20 mg/ml) (BioNeer, Korea), 500 µl of SE buffer (75 mM NaCl; 25 mM Na2 EDTA; pH 8.0) and 120 µl of 20% SDS for 2 h at 56 °C (NaCl, Na_2EDTA and SDS from Merck, Germany). After cell lysis, DNA was extracted using 500 µl of phenol/chloroform (1:1) (Merck, Germany), and was precipitated in absolute ethanol through centrifugation for 20 min at 11000 g, 4 °C. The pellet was then washed with 70% ethanol (Merck, Germany) and solved in 50 µl distilled water.

The second population of cells was treated with RNX plus™ solution (Cinnagen, Iran) according to the manufacturer's instruction for RNA extraction. Extracted RNA was kept in -80 °C for further assays.

Epithelial-Mesenchymal Ttransition analysis

EMT analysis was conducted by expressional analyzis of vimentin (*VIM*), E-cadherin (*CDH1*), N-cadherin (*CDH2*) and fibronectin (*FN1*) at RNA level. cDNA synthesis was carried out using BioFACT™ (Daejeon, Korea) according to the manufacturer's instruction. Real-time PCR was setup according to Niknami et al. (22). The levels of expressed mRNAs were assessed and normalized to *GAPDH* housekeeping gene, which is not affected by iAsIII treatment (23,24). Primer sequences used for EMT-related genes are listed in Table 1. Fold changes of gene expression was calculated according to $2^{-\Delta\Delta Ct}$. The entire procedure was performed in duplicate for each plate (i.e. four times for each concentration).

Microsatellite instability (MSI) assay

Five microsatellites were selected as potentially more sensitive to mismatch repair deficiency for analysis of MSI, upon Selective Targets in Human MSI-H Tumorigenesis Database (SelTarbase) and previously published literature (25-27). Primers were designed for multiplex PCR with respect to sequence homology and delta Gibbs free energy to

Table 1. Primers of EMT-related genes for gene expression.

Gene of interest	Forward primer (5′→3′)	Reverse primer (5′→3′)
VIM	GGCTCAGATTCAGGAACAGC	AGCCTCAGAGAGGTCAGCAA
CDH1	GAATGACAACAAGCCCGAAT	TTTGTCAGGGAGCTCAGGAT
CDH2	AACAGCAATTGATGCTGACG	GGATTGCCTTCCATGTCTGT
FN1	CCAGGCAGTACAATGTGGGT	TGGAATAGAGCTCCCAGGCT
GAPDH	GGGCCATCCACAGTCTTC	GAAGGCTGGGGCTCATTTG

Table 2. Microsatellite characteristics.

Marker pseudo name	GeneBank number	Gene/ Position	Repeat size/ sequence	PCR product size (bp)	Primer dye
NR-21	XM_033393	SLC7A8/5′UTR	21/A	~110	FAM
NR-27	AF070674	BIRC3/5′UTR	27/A	~83	TAMRA
BAT-25	X69313	c-kit/intron 16	25/A	~127	JOE
BAT-26	AY601851	hMSH2/intron 5	26/A	~105	JOE
MONO-27	AC007684	MAP4K3/intron 13	27/A	~157	TAMRA

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Table 3. Primers, probes and blocker sequences of *SEPT9* and *ACTB* for methylation evaluation.

Gene	Type	Sequence (5′→3′)	Concentration (µM)
SEPT9	Forward	AAATAATCCCATCCAACTA	1.5
	Reverse	GATT-dSp[1]-GTTGTTTATTAGTTATTATGT	1.5
	Blocker	GTTATTATGTTGGATTTTGTGGTTAATGTGTAG-SpC3[2]	1.0
	Probe	FAM-TTAACCGCGAAATCCGAC-BHQ_1	0.075
ACTB	Forward	GTGATGGAGGAGGTTTAGTAAGTT	0.2
	Reverse	CCAATAAAACCTACTCCTCCCTTAA	0.2
	Probe	TEXAS RED-ACCACCACCCAACACACAATAACAAACACA-BHQ_2	0.075

1.a basic d-spacer base, 2.C3 spacer to prevent amplification of non-methylated site

avoid undesired interactions. Amplification program was performed according to Buhard et al. with some modifications (28). PCR products were loaded on capillary electrophoresis (Applied Biosystems® 3500, USA), and the results were analyzed using GeneMarker® software for detection of MSI. The highest peak (in relative fluorescence unit) for each marker was assumed as an allele. Before and after treatment, markers are considered as MSI if the size of alleles are different from each other by more than two nucleotides. The characteristics of the microsatellites are listed in Table 2.

***SEPT9* promoter methylation assay**

HeavyMethyl duplex PCR was applied for analysis of *SEPT9* promoter methylation status. For bisulfite conversion, EpiTect® Fast DNA Bisulfite kit (Qiagen, Germany) was used; each treatment reaction was performed on 1 µg of extracted DNA based on the manufacturer's instruction. Amplification of *SEPT9* as the gene of interest and beta-actin (*ACTB*) as the internal control was accomplished at the same tube with 1 µl of converted DNA, using Amplicon RealQ Plus 2x Master Mix (Amplicon, Denmark) for probe without ROX. Probes, primers and blocker sequences and their concentrations are shown in Table 3. Real-time PCR was used on Rotor-Gene Q (Qiagen, Germany) as mentioned before with some modifications (18). After prior heating at 95 °C for 15 min, the amplification program was implemented as the following; 50 cycles of 95 °C for 10 s, 56 °C for 30 s, and one cycle of 40 °C for 30 s. The percentage of *SEPT9* methylation (PMR) was calculated according to the following formula: PMR% = ((SEPT9/ACTB) treated cell line /(SEPT9/ACTB) non treated cell line) × 100

The entire procedure was performed in triplicate for each concentration.

Statistical analysis

Statistical analysis of obtained data was performed using the SPSS software package, version 19.0 (SPSS, Chicago, IL). Student's t-test and one-way ANOVA were performed to compare the means of gene expression levels between treated and non-treated cell lines. Differences with a p-value of <0.05 were considered statistically significant. GraphPad Prism software was applied to draw graphs.

Results

The inhibitory effect of iAsIII on CRC cell lines

Treatment of Caco2 and HCT116 cells with iAsIII for 36 h exerted an inhibitory effect on cell proliferation (Fig.1). Evaluated IC50s for iAsIII-treated Caco2 and HCT116 were 25 µM and 100 µM, respectively. In both cell lines, iAsIII concentrations of less than 12.5 µM had no significant inhibitory effect on cell proliferation.

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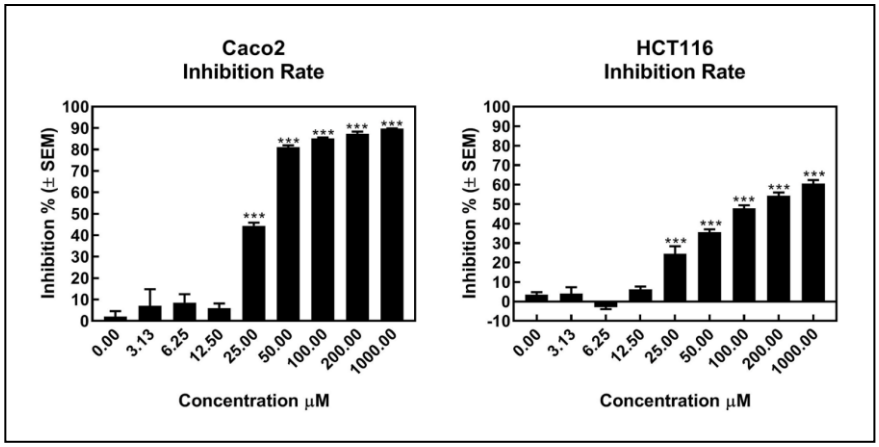

Fig. 1.Chemo sensitivity of colorectal cancer cell line to iAsIII. The sign (***) is indicative of P value < 0.001

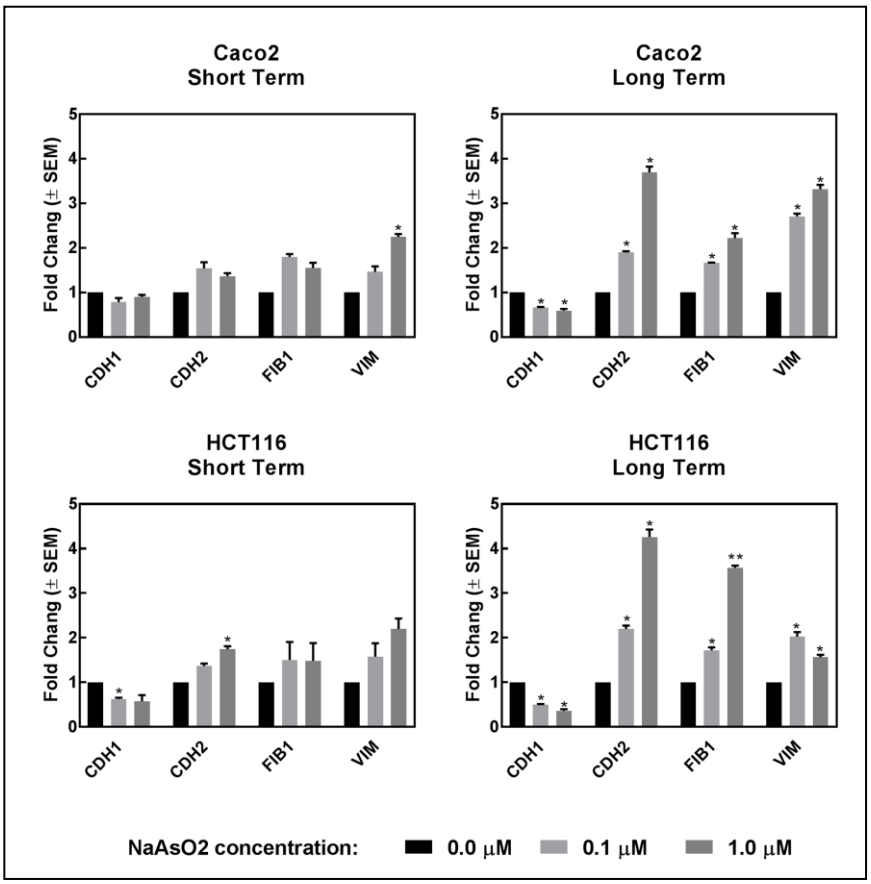

Fig. 2. The expression pattern of EMT genes in Caco2 and HCT116 cell lines under short-term and long-term treatment with iAsIII. *GAPDH* was used as the reference gene for data normalization. The sign (*) is indicative of P value < 0.05, and (**) is indicative of P value <0.01

The expression patterns of EMT genes

Comparative expression of the genes *CDH1*, *CDH2*, *FIB1* and *VIM* before and after short-term (36 h) and long-term (20 days) treatment with iAsIII (0.1 μM and 1 μM) in Caco2 and HCT116 cell lines is shown in Fig.2. Expression of *VIM* in Caco2 cell line increased significantly after short-term treatment with 1 μM iAsIII. Long-term treatment of Caco2 cell line with 0.1 μM and 1 μM concentrations of iAsIII, significantly affected the

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expression of EMT genes. *CDH1* expression was decreased while the expression of *CDH2*, *FIB1* and *VIM* genes was increased.

Short-term treatment of HCT116 cell line with 0.1 µM iAsIII led to a decrease in the expression of *CDH1* gene, while *CDH2* gene expression increased significantly after short-time treatment with 0.1 and 1 µM iAsIII, respectively. Long-term treatment of HCT116 cell line with 0.1 and 1 µM iAsIII, revealed a similar pattern of down-regulation and up-regulation of EMT genes as that in Caco2 cell line.

MSI status

MSI status of Caco2 and HCT116 cell lines before and after short-term and long-term treatment with 0.1 µM and 1 µM iAsIII are shown in Fig.3 and Fig.4. In Caco2 cell line, the size of NR21, NR27, BAT25, BAT26 and MONO27 microsatellites without treatment and with short-term and long-term treatment by iAsIII were 110 bp, 84 bp, 126 bp, 104 bp, and 157 bp, respectively. In HCT116 cell line, NR21, BAT25, BAT26 and MONO27 microsatellites were 103 bp, 119 bp, 92 bp, and 148 bp, respectively, and for NR27 there were of 72 bp and 77 bp.

***SEPT9* methylation status**

Short-term treatment of Caco2 and HCT116 cell lines with iAsIII revealed 100% PMR of *SEPT9* (Fig. 5). PMRs were reduced as much as 4.4% and 12.1% in long-term treatment of Caco2 with 0.1 µM and 1 µM iAsIII, respectively ($P < 0.05$). PMRs of *SEPT9* in HCT116 cell line under long-term treatment with 0.1 µM and 1 µM iAsIII reduced as much as 4.9% and 10.9%, respectively ($P<0.05$).

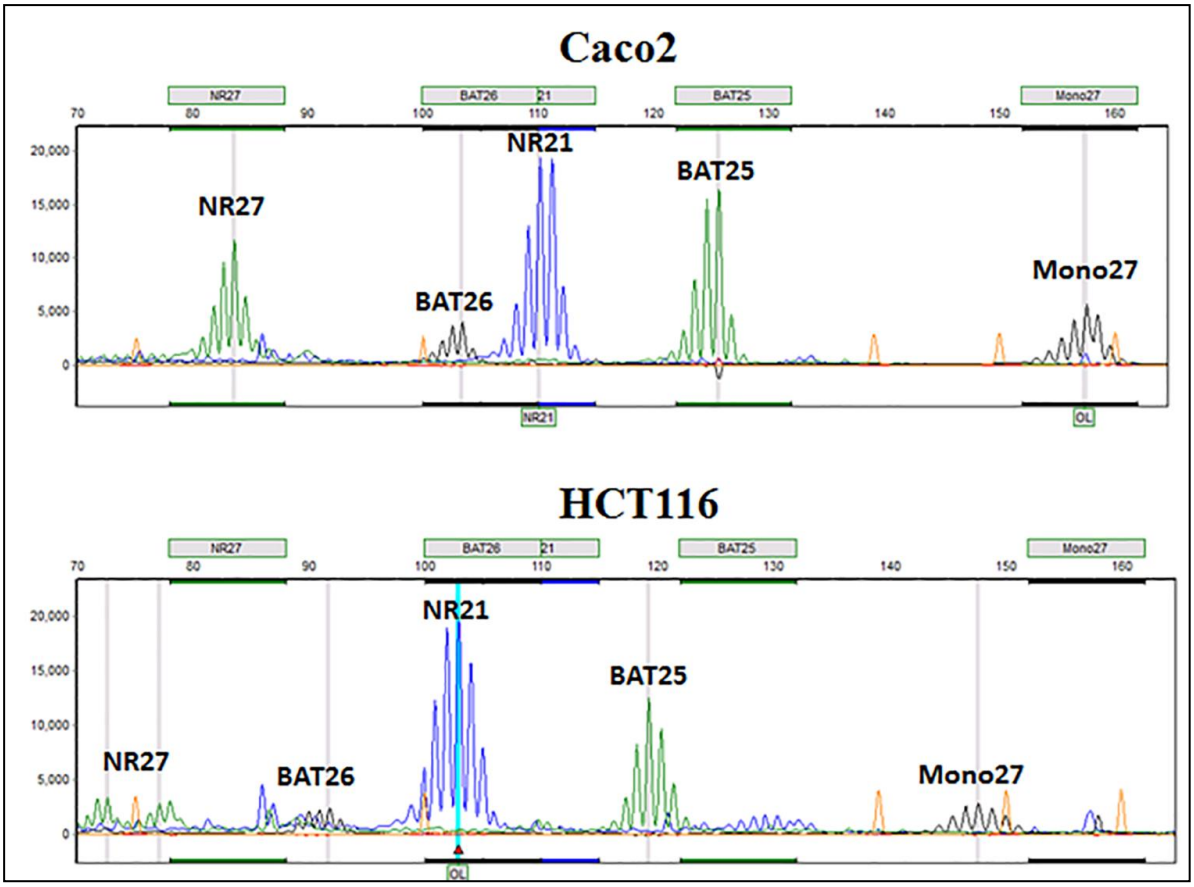

Fig. 3. The status of five microsatellites (NR21, NR27, BAT25, BAT26, and Mono27) in Caco2 and HCT116 colorectal cancer cell lines before treatment with iAsIII.

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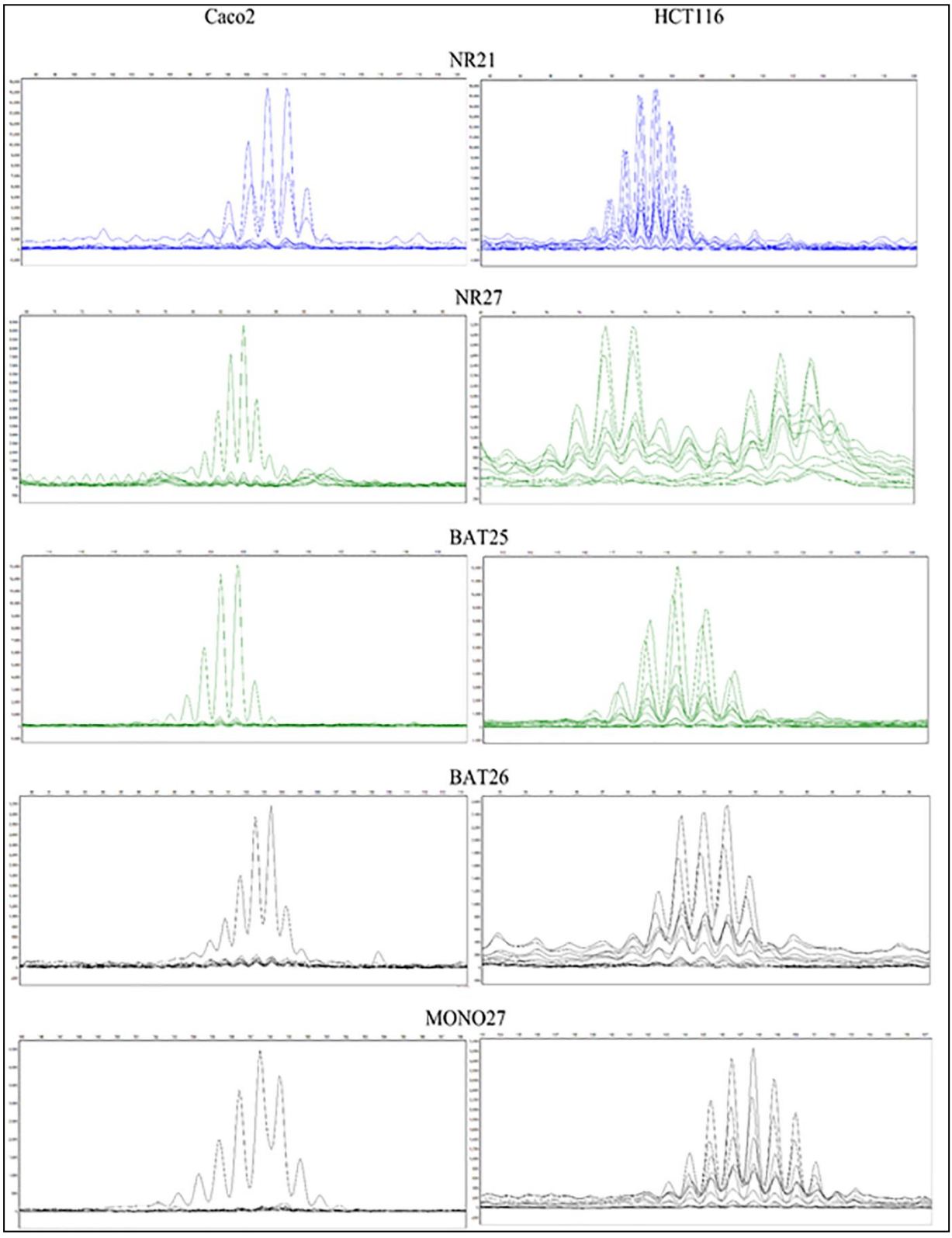

Fig. 4. Results of microsatellite instability in Caco2 and HCT116 colorectal cancer cell lines after treatment with iAsIII. Charts of short-term and long-term treatments are overlapped..

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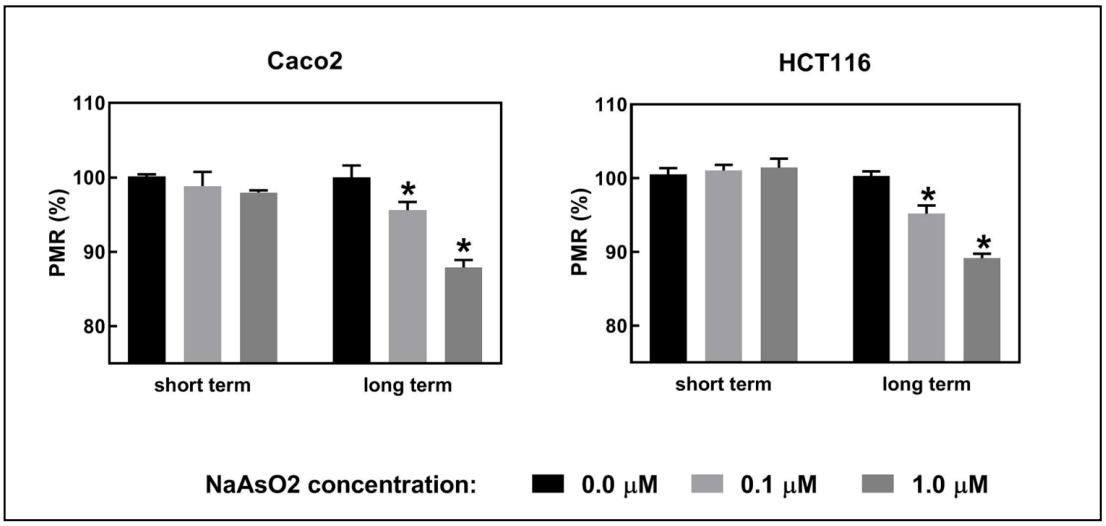

Fig. 5. Methylation status of SEPT9 in Caco2 and HCT116 colorectal cancer cell lines treated with iAsIII. The sign (*) is indicative of P value <0.05.

Discussion

Arsenic is one of the worldwide chemical contaminants especially found in drinking water (21). In fact, organic sodium arsenite (iAs) of various concentrations can contaminate different sources of drinking water (29). In this study, we have assessed the molecular effects of daily exposure to iAs in contaminated areas (21,29,30) on CRC cell lines and demonstrated that exposure to iAsIII concentrations below the tolerable intake level noted in WHO guideline (10 µg/L) could alter molecular characteristics of CRC cell lines (21).

Induction of EMT was confirmed by down-regulation of *CDH1* and up-regulation of *CDH2*, *FIB1*, and *VIM* genes in Caco2 and HCT116 cell lines treated with 0.1 µM and 1 µM iAsIII. In one study, EMT was induced in Hela cell line through chronic exposure to a molar dosage of 0.5 µM iAsIII and subsequently approved by measuring the levels of EMT markers including slug, N-cadherin, claudin 1 and snail (31). In another study, upon treatment of T24 human bladder cancer cell line with iAsIII, the levels of N-cadherin and Vimentin increased while the level of E-cadherin decreased (7).

Arsenite could induce epidermal growth factor receptor (EGFR) activity which subsequently phosphorylates and inactivates proliferating cell nuclear antigen (PCNA). Inactivated PCNA can impair mismatch repair system (32), which in its turn, could lead to MSI (33). Our investigation on five microsatellites of Caco2 as sMMR and HCT116 as dMMR CRC cell lines treated with 0.1 µM and 1 µM iAsIII in comparison with non-treated cells did not reveal any alteration of microsatellites status.

The present study is the first one to investigate the methylation status of *SEPT9* promoter as a CRC tumor marker in two iAsIII-treated CRC cell lines. We found that iAsIII could induce *SEPT9* promoter hypomethylation in Caco2 and HCT116, which is correlated with EMT initiation. Furthermore, this effect was dependent on two factors of dosage and time of exposure (only long-term exposure exerts the aforementioned effect). Epigenetic changes in DNA and histone methylation status have been reported as other effects of arsenicals (19,31). In one study, decreased cellular absorbance of methyl 3H was observed upon treatment of Caco2 cell line with 1µM and 2 µM arsenic, which means arsenite

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could cause DNA hypermethylation in Caco2 (34). In another study, the DNA of Hela cells treated with iAs was hypermethylated globally, however, 33% of CpG sites were transiently or permanently hypomethylated (31). Furthermore, in West Bengal of India, individuals who were exposed to arsenic and had skin lesion were examined for the methylation status of mismatch repair genes, which indicated hypermethylation and down- regulation of *MLH1* and *MSH2* genes (19). Another study showed that long-term exposure to 0.5 µM iAsIII up-regulates *LCN2* gene expression through hypomethylation of *LCN2* promoter region after 25 passages in human urothelial cells (35). Long-term treatment of HaCaT cell lines with iAsIII resulted in the depletion of S-adenosylmethionine (SAM) as the source of methyl groups, and could down-regulate the expression of DNA methyltransferse genes, *DNMT1* and *DNMT3A* (36). Previous experiments have revealed that SAM depletion might occur due to arsenic biotransformation through the methylation of arsenic compounds by arsenic-3-methyltransferase, and therefore, induces DNA hypomethylation (37,38).

In summary, our investigation on CRC cell lines demonstrated that iAsIII can have an important role in the induction of EMT, a phenomenon concomitant with *SEPT9* promoter hypomethylation, which is time- and dosage-dependent. However, there was no linkage found between EMT induction and microsatellites status. On the other hand, EMT induction and hypomethylation of *SEPT9* promoter by iASIII could have an impact on the detection of CRC tumors, especially in metastatic CRCs, and might reduce the sensitivity of screening tests developed on the basis of the methylation status of *SEPT9* promoter. Further *in vitro* and *in vivo* investigations are required to unravel the advantages and disadvantages of using "methylated *SEPT9* promoter" as a CRC tumor marker in contaminated areas with inorganic arsenicals.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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