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Folic acid enforces DNA methylation-mediated transcriptional silencing of *PTEN*, *APC* and *RARbeta2* tumour suppressor genes in breast cancer

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ABSTRACT

Folate, one of the most studied dietary compounds, has recently become the main topic of debates on food fortification. Although low folate levels may be associated with increased risk of cancer development, simultaneously several reports indicate a detrimental effects mediated by high folate concentrations. Using the methylation sensitive restriction analysis (MSRA) and *real-time* RT-PCR we tested the effect of folic acid on DNA promoter methylation and expression of *PTEN*, *APC* and *RARbeta2* tumour suppressor genes in MCF-7 and MDA-MB-231 breast cancer cell lines with different invasive capacity. The tested genes encode proteins involved in regulation of oncogenic intracellular signaling pathways. The results show that the increasing concentrations of folic acid lead to a dose-dependent down-regulation of tumour suppressor genes which may be linked to the increased DNA methylation detected within their promoter regions. The effects were more remarkable in non-invasive MCF-7 cells where we also observed 30% up-regulation of *DNMT1* expression at the highest folate concentration used. Our findings show that caution need to be used when introducing folic acid supplementation since it may lead to cancer progression.

1. Introduction

Folic acid is a water-soluble vitamin B9 present in a variety of foods including lentils, okra, beans, asparagus, spinach, broccoli, and avocado. Since adequate folate intake was shown to reduce the risk of cancer [1], cardiovascular disease [2] and protect from neural tube defects [3], supplementation for women intending to become pregnant and dietary fortification have been introduced in multiple countries. Folate constitutes one of the coenzymes of one-carbon metabolism [4]. After dietary intake, it is converted to tetrahydrofolate that is involved in remethylation of homocysteine to methionine which is a precursor of S-adenosylmethionine (SAM), primary methyl group donor for most methylation reactions, including DNA [5,6]. DNA methylation in normal cells is implicated in oncogene repression, the control of expression of genes crucial for cell proliferation, differentiation, and normal development as well as in parental imprinting, X chromosome inactivation, and chromosomal integrity [7,8].

Animal studies and clinical observations from the last decade suggest that folate plays a dual role in carcinogenesis depending on the timing, dose and individual conditions, for example the age [9–11]. It is well known that folate deficiency leads to increase in cancer risk by disturbing homeostasis of one-carbon metabolism, thereby leading to perturbation of SAM synthesis and subsequent alterations in DNA methylation. Numerous studies showed a causal role of folate deficiency in the development of different types of cancer, such as colon and rectum, esophagus, gastric, pancreatic and breast cancer [1,11–15]. Intervention trials conducted in order to assess the effect of folic acid supplementation on the risk of cancer development delivered inconsistent results with concluded decreased risk of colorectal cancer [16,17]. On the other hand, the literature data from the last decade demonstrate that high doses of folate may increase cancer risk and promote cancer progression [17–19].

DNA methylation is an epigenetic DNA modification which participates in regulation of gene expression without changes in underlying DNA sequence. Alterations in DNA methylation patterns have been reported in many malignancies and have been shown to be implicated in cancer initiation and progression. The hallmarks of cancer cells are hypermethylation and silencing of tumour suppressor genes [20–25], hypomethylation and activation of oncogenes and pro-metastatic genes as well as global DNA hypomethylation [26].

In our previous studies with MCF-7 cells, we observed differences in DNA promoter methylation states depending on folic acid

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concentration in cell culture media. The present studies focus on evaluation whether folic acid supplementation can affect promoter methylation and transcriptional activities of three tumour suppressor genes, *PTEN*, *APC* and *RARbeta2*, in human breast cancer cell lines, and whether folic acid may be used in epigenetic therapy of breast cancer. In order to test our hypothesis, we estimate the effects of folic acid on DNA promoter methylation and expression of the above tumour suppressor genes as well as *DNMT1* expression in MCF-7 and MDA-MB-231 breast cancer cell lines with distinct invasive and metastatic potentials. The tested genes encode proteins that participate in down-regulation of intracellular oncogenic signaling pathways. PTEN is involved in regulation of PI3K/Akt and Ras/MAPK/AP-1 pathways, whereas APC controls Wnt-1/beta catenin cascade. The action of RARbeta is mostly mediated by its receptors. The ligand/receptor complex acts as a transcriptional factor binding to responsive elements within genes regulating cell cycle, differentiation and apoptosis. The three selected tumour suppressor genes are often epigenetically silenced in cancer tissues and cell lines [20–25]. For instance, *PTEN* and *APC* promoter hypermethylation with concomitant reduction of expression on mRNA level was detected in breast tumours and breast cancer cell lines [22,23,25]. Similarly, promoter hypermethylation of *RARbeta2* was associated with partial or complete suppression of the gene transcriptional activity in breast cancer [24].

The results of the present studies, which focus on the evaluation of folic acid effects on methylation and expression of the selected tumour suppressor genes, reveal that rising folate concentrations may promote breast cancer progression. Our findings should also be taken into consideration when one wishes to investigate the role of DNA methylation in vitro as the presence of folic acid in culture medium may affect final outcome.

2. Materials and methods

2.1. Reagents, cell culture, RNA and DNA isolation and purification

Reagents for RNA and DNA purification and folic acid calcium salt were purchased from Sigma–Aldrich Co (Poland), endonuclease HpaII and Eco72I from Fermentas (Lituania). Folic acid was dissolved in water at the concentration 1 mg/ml (1.96 mM).

Human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231, from American Type Culture Collection, ATCC (LGC Standards) and European Collection of Cell Cultures, ECACC (Salisbury, UK) were cultured for 96 h in EMEM medium (MEM Eagle with Earle's BSS, without L-glutamine, Lonza) and L15 medium (Leibovitz's L15 medium without L-glutamine, Lonza), respectively. These media were supplemented with: 2 mM L-glutamine; 0.01 mg/ml bovine insulin (only for MCF-7 cells) (Sigma–Aldrich, St. Louis, MO, USA); 10% (and for MDA-MB-231 cells – 15%) fetal bovine serum (FBS); 1 U/ml penicillin, and 1 μg/ml streptomycin (Gibco, Scotland, UK). Cells were grown for 96 h at 37 °C in a humidified atmosphere of 5% CO_2, except for MDA-MB-231 cells which were incubated without CO_2. Media used for both cell lines contained 1 mg/l of folic acid (control samples, CFA, control folic acid concentration). For the experiments, the concentration of folic acid was increased to 4 mg/l (LFA, lower folic acid concentration) and 8 mg/l (HFA, higher folic acid concentration).

Cell viability was estimated with trypan blue (Sigma–Aldrich) exclusion test. Additionally, the viability of cells were confirmed and completed by applying flow cytometry analysis (FACSCalibur flow cytometer, Becton Dickinson), using annexin V/propidium iodide assays, according to the manufacturer's protocol.

Cellular DNA from the breast cancer cell lines was isolated after 20 h of incubation with proteinase K, followed by extraction using phenol: chloroform: isoamyl alcohol (25:24:1) mixture (Sigma–Aldrich) according to the manufacturer's protocol. Pure DNA was diluted in TE buffer and stored at −20 °C.

Total RNA from the tested cells was isolated using TRIZOL (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Isolated RNA was dissolved in water containing 1% DEPC (ribonuclease inhibitor) and stored at −70 °C.

2.2. Methylation gene analysis

The methylation status of *PTEN*, *RARbeta2* and *APC* promoters was estimated using methylation-sensitive restriction analysis (MSRA) according to Iwase's method [27]. The MSRA analysis included four steps: (i) digestion of cellular DNA with endonuclease that recognizes only non-methylated sequence, (ii) PCR amplification of digested DNA, (iii) electrophoretic analysis of amplified promoter fragments, and (iv) densitometric quantitative analysis of the band intensity. Genomic DNA (0.5 μg) was incubated with 20 U of HpaII or Eco72I restriction enzymes at 37 °C overnight. HpaII recognizes non-methylated C$^{\downarrow}$CGG sequence located within *PTEN* and *RARbeta2* promoter fragments, whereas Eco72I cuts non-methylated CAC$^{\downarrow}$GTG sequence within *APC* promoter fragment. Two controls of digestion reaction, a sample without an enzyme and MspI-digested sample, were incubated in the same conditions. After incubation, control and digested DNA were amplified in PCR using the following primers for the selected promoter fragments: *PTEN* (GenBank accession no. AF143312; chr:10q23.3; amplicon length 214 bp [22,23]: (forward) 5′-cagccgttcggaggattattc-3′ and (reverse) 5′-gggcttcttctgcaggatgg-3′; *RARbeta2* (GenBank accession no. X56849; chr:3p24; amplicon length 295 bp [24]: (forward) 5′-ctcgctgcctgcctctctgg-3′ and (reverse) 5′-gcgttctcggcatcccagtc-3′; *APC* (GenBank accession no. U02509; chr:5q21-q22; amplicon length 317 bp [25]: (forward) 5′-ctaggcaggctgtgcggttg-3′ and (reverse) 5′-cggtttaagacagtgcgagg-3′.

The reaction mixture for PCR was prepared as described previously [28], and was carried out in Tpersonal Thermal Cycler (Biometra, Goettingen, Germany) at 95 °C for 5 min, cycled for 1 min at 94 °C, 1 min at annealing temperature (61.1 °C, 58.4 °C and 61.1 °C, for *PTEN*, *RARbeta2* and *APC* promoter fragments, respectively) and 1 min at 70 °C (30 cycles), followed by a 10 min extension at 72 °C. The amplified PCR products were fractioned on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under UV illumination. For densitometric analysis of band intensities the Quantity One software (Bio-Rad Laboratories Ltd., UK) was used. Methylation level in each sample was calculated based on densitometric analysis and expressed as a percentage of undigested DNA after the comparison of band intensities for digested and undigested DNA. The percentage of methylation inhibition was evaluated by comparison of methylation level in control cells that grew in the presence of 1 mg/l folic acid and in cells treated with folic acid at concentration 4 or 8 mg/l.

2.3. cDNA synthesis and real-time PCR (QPCR)

Total RNA was isolated using TRIZOL® (Invitrogen, Life technologies, Carlsbad, USA) and cDNA was synthesized using: 2 μg of total RNA; 6 μl of random hexamers, 5 μl of oligo$(dT)_{15}$ (Promega, Madison, USA) and ImProm-II reverse transcriptase (Promega) according to manufacturer's protocols.

All QPCR reactions were carried out in a Rotor-Gene TG-3000 machine (Corbett Research, Australia). The reaction mixture prepared according to manufacturer's protocol comprised the following primers: *PTEN* (forward) 5′-cgaactggtgtaatgatatgt-3′ and (reverse) 5′-catgaacttgtcttcccgt-3′; *RARbeta* (forward) 5′-ttcaagcaagcctcacatgtttcca-3′ and (reverse) 5′-aggtaattacacgctctgcaccttag-3′; *APC* (forward) 5′-tgcgagaagttggaagtgtgaaagcattg-3′ and (reverse) 5′-tgacaaattccataaggcactcaatacgc-3′; *DNMT1* (forward)

5′-accgcccctggccaaagccattg-3′ and (reverse) 5′-agcagcttcctcctcctttattttagctgag-3′. After an initial 2 min denaturation step at 94 °C, amplification consisted of 50 cycles were performed under the following conditions: 30 s at 94 °C, 15 s at annealing temperature (50 °C and 56 °C for *PTEN* and *RARbeta*, respectively, 60 °C for *APC* and *DNMT1*), and 30 s of elongation at 72 °C. The relative expression of each tested gene was normalized to the geometric mean of four housekeeping genes, *RPS17* (40S ribosomal protein S17), *RPLP0* (60S acidic ribosomal protein P0), *H3F3A* (H3 histone family 3A) and *BMG* (beta 2-microglobulin), according to Pfaffl's method [29].

2.4. Statistical analysis

Data were assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Each value represents the mean ± SD of three independent experiments. The results were considered as statistically significant when $P < 0.05$.

3. Results

3.1. Folic acid effects on viability of MCF-7 and MDA-MB-231 cells

As measured by the trypan blue exclusion test, it was observed that folic acid did not affect growth and viability of the cells in both cell lines (data not shown).

The cell viability profiles according to flow cytometry are presented in Fig. 1. Folic acid at 4 and 8 mg/l concentrations increased the number of apoptotic cells by 12–14% in MCF-7 cell line. Over 32% of all apoptotic cells showed active caspase-3 which indicates the significant involvement of caspase-dependent apoptotic pathway. The incubation with the tested compound did not induce apoptosis in invasive MDA-MB-231 cells.

3.2. Promoter methylation and expression levels of the tested genes in the control cells

DNA methylation level of the tested fragment within *PTEN* promoter in control MCF-7 and MDA-MB-231 cells that grew in the presence of 1 mg/l folic acid was estimated to be approximately 34% and 70%, respectively. *APC* promoter fragment was methylated at 62% and 41%, whereas *RARbeta2* at 42% and 57% in MCF-7 and MDA-MB-231 cells, respectively (Fig. 2A and B). *Real-time* PCR revealed that invasive MDA-MB-231 cells show a significantly lower expression of all tested genes by 33%, 74% and 73%, respectively, for *PTEN*, *RARbeta2* and *APC* in comparison with MCF-7 cells (Fig. 3). Although, *DNMT1* expression was similar in both cell lines (Fig. 3), lower *PTEN* and *RARbeta2* expression was associated with higher DNA methylation within promoters of these genes in MDA-MB-231 cells as compared with MCF-7 cells (Figs. 2 and 3).

3.3. DNA methylation and expression of PTEN, APC and RARbeta2 after treatment with 4 and 8 mg/l folic acid

3.3.1. MCF-7 cells

In non-invasive and ER-positive MCF-7 breast cancer cells, the increasing concentrations of folic acid led to elevation of DNA methylation of all tested promoter fragments with the highest change in *PTEN* promoter (over 142% at 4 mg/l and 144% at 8 mg/l folic acid, Fig. 2A). We also observed a concomitant decrease in expression of the tested genes on mRNA level. The most robust down-regulation by 58% was detected for *RARbeta* after challenge with 8 mg/l folic acid (Fig. 2A). Furthermore, the alterations in methylation and expression levels after the treatments were associated with up to 30% increase in *DNMT1* expression as measured by QPCR (Fig. 4A).

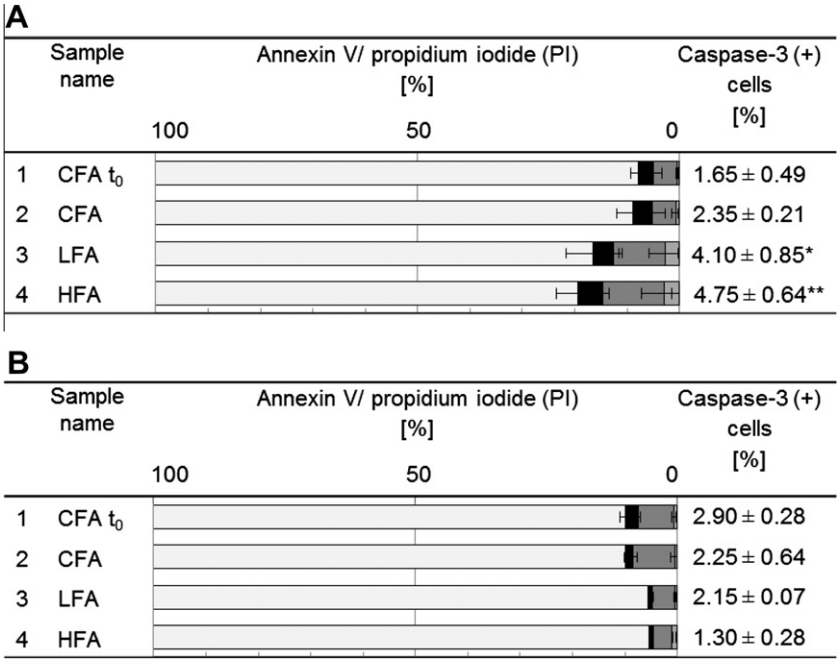

Fig. 1. Flow cytometry data analysis for non-invasive MCF-7 (A) and invasive MDA-MB-231 (B) cells cultured with folic acid (viable cells – white bars; necrotic cells – black bars; cells in late apoptosis – medium gray bars; cells in early apoptosis – light gray bars). CFA t_0, control cells used for experiments (time 0 h) [1 mg/l]; CFA, control folic acid concentration [1 mg/l]; LFA, lower folic acid concentration [4 mg/l]; HFA, higher folic acid concentration [8 mg/l]. Data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed by ANOVA followed by Tukey's post hoc test. Mean value after treatment was significantly different from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

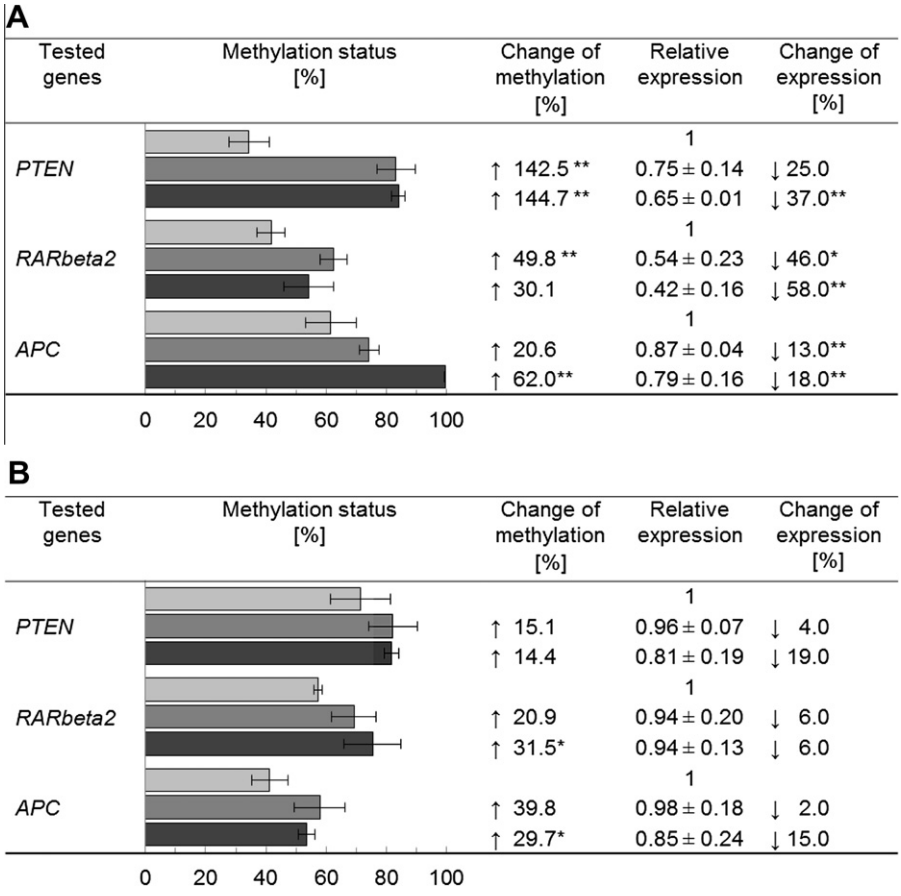

Fig. 2. Folic acid effects on promoter methylation status and gene expression on mRNA level in MCF-7 (A) and MDA-MB-231 (B) cells. Promoter methylation level and relative expression of the tested genes in MCF-7 and MDA-MB-231 cells cultured at 1 mg/l (control, light gray), 4 mg/l (medium gray) and 8 mg/l (dark gray) folic acid were estimated as described in Section 2. Data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed by ANOVA followed by Tukey's post hoc test. Mean value after treatment was significantly different from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

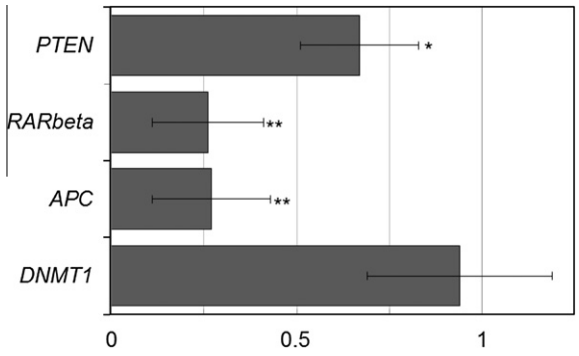

Fig. 3. Relative expression of *PTEN*, *RARbeta*, *APC* and *DNMT1* in MDA-MB-231 cells in comparison with MCF-7 cells. Expression level of each gene in MDA-MB-231 cells was compared with its expression in MCF-7 cells (control) and showed as a fold change. Data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed by ANOVA followed by Tukey's post hoc test. Mean value after treatment was significantly different from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3.2. MDA-MB-231 cells

In the invasive and ER-negative MDA-MB-231 breast cancer cell line, the supplementation of culture media with 4 or 8 mg/l folic acid led to an increase in DNA methylation of the tested promoter fragments although to a lesser extent as compared to MCF-7 cells (Fig. 2B). The most relevant 30% hypermethylation was revealed within *APC* promoter however it was not accompanied by a significant change in gene expression. Interestingly, there was a remarkable difference in the level of *PTEN* promoter hypermethylation (Fig. 2) and *DNMT1* down-regulation between MDA-MB-231 and MCF-7 cells (Fig. 4).

4. Discussion

Human clinical and epidemiological data along with animal studies have suggested that folate may play a protective role in carcinogenesis, particularly in colorectal cancer [16]. However, recent epidemiological studies have indicated that high folate intake, mainly in its synthetic form present in supplements and fortified foods, may increase the risk of breast cancer [30,31]. The large observation study of cancer screening trial cohort performed by Stolzenberg-Solomon showed a statistically significant 32% increase in breast cancer risk in postmenopausal women consuming higher folate level [19].

Because of contradictory results of several epidemiological investigations of the effects of folate intake on breast cancer risk, as well as the premise that folic acid may modify cancer risk through its involvement in regulation of DNA methylation, we have undertaken the present study. Our goal was to elucidate the influence of folic acid on methylation and expression of *PTEN*, *APC* and *RARbeta2* tumour suppressor genes in breast cancer. We established relations between DNA methylation and expression of the tested genes and *DNMT1* expression in non-invasive and invasive cells with different ER status.

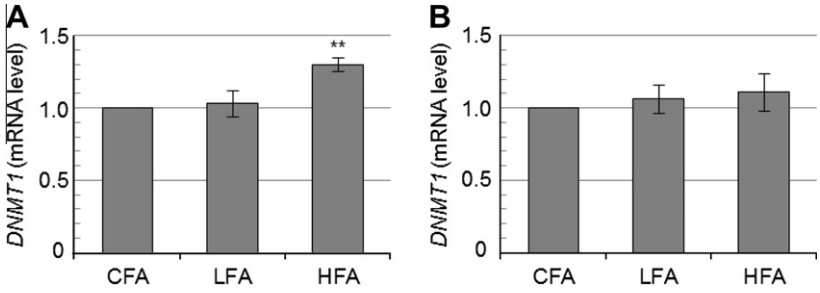

Fig. 4. The effect of folic acid on DNA methyltransferase 1 (*DNMT1*) expression in MCF-7 (A) and MDA-MB-231 (B) cells. Relative expression of *DNMT1* in MCF-7 and MDA-MB-231 cells cultured at 1 mg/l (control, CFA), 4 mg/l (LFA) and 8 mg/l (HFA) folic acid concentrations were estimated as described in Section 2. Data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed by ANOVA followed by Tukey's post hoc test. Mean value after treatment was significantly different from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

Our findings indicate that in both breast cancer cell lines, non-invasive ER-positive MCF-7 cells and invasive ER-negative MDA-MB-231 cells, folic acid at 4 and 8 mg/l concentrations caused an increase in methylation of *PTEN* promoter within the tested fragment and silencing of gene transcription (Fig. 2A and B) as compared to control cells growing in the presence of 1 mg/l folate. The extent of hypermethylation was much higher and statistically relevant in MCF-7 cells although the final *PTEN* methylation level in both cell lines was the same, approximately 80%, after treatment with either 4 mg/l or 8 mg/l folic acid. It needs to be emphasized that before treatments *PTEN* promoter was methylated at 70% and 30% in MDA-MB-231 and MCF-7 cells, respectively, which might explain much weaker effects observed in the invasive cells. The alterations in *PTEN* promoter methylation were associated with diminution of gene expression on mRNA level in both breast cancer cell lines however the change was much lower in MDA-MB-231 cells. Similar changes in promoter methylation and gene expression after exposure to folic acid were detected for *APC* and *RARbeta2* tumour suppressor genes. In both invasive and non-invasive cells, hypermethylation of *APC* and *RARbeta2* promoters was concomitant with these genes transcriptional down-regulation that was statistically significant in MCF-7 non-invasive cells. Folic acid exerted a dose dependent effect on *APC* methylation, particularly in non-invasive cells. The increase in *RARbeta2* promoter methylation in MDA-MB-231 cells was not associated with changes in gene expression. It suggests that transcriptional activity of *RARbeta2* is regulated by another than DNA methylation mechanism at the advanced invasive stage of breast cancer. Interestingly, folic acid at the highest concentration used in the study led to 30% increase in *DNMT1* expression in MCF-7 cells while causing only a slight elevation in MDA-MB-231 cells (Fig. 4A and B).

To our best knowledge, this is the first study focusing on the effects of high folic acid concentrations on methylation and expression of *PTEN*, *RARbeta2* and *APC* tumour suppressor genes in breast cancer. Most of other studies that addressed folate and DNA methylation were undertaken to examine genomic DNA methylation level that is less informative and may be misleading. Genome-wide DNA methylation is dynamic and involves both demethylation of oncogenes and hypermethylation of tumour suppressor genes during carcinogenesis. Hence, evaluating global DNA methylation level averages these alterations in DNA methylation patterns. Moreover, according to Kim's data the final effects of folate on DNA methylation status are highly complex and dependent on a cell type, target organ, state of transformation, age, sex and life style of the individuals [32]. Our results indicate that rising concentrations of folic acid deepen promoter hypermethylation of tumour suppressor genes which was also observed in other studies of our group on K562 human erythroleukemic cell line. In K562 cells, transcriptional silencing of the tested genes was much stronger than in the breast cancer cells that might be partially explained by a high proliferation rate of these cells (unpublished data). Our observation that high concentration of folic acid led to increase in methylation of tumour suppressor genes is consistent with Berner's results for *ESR1*, *p16* and *p15* genes in Caco-2 colon adenocarcinoma cells [33].

Our findings have provided evidence that folic acid may induce apoptotic cell death in non-invasive MCF-7 breast cancer cell line, whereas invasive MDA-MB-231 cells are not responsive to folic acid as a pro-apoptotic agent. Folic acid was reported before as an inducer of apoptosis in human gastric cancer cell lines MKN-45 and MKN-28 [34].

In conclusion, in the present study we demonstrate that folic acid at increasing concentrations impairs transcriptional activities of the tested tumour suppressor genes that is concomitant with increased DNA methylation within their promoters. The highest folate concentration used in our experiments caused induction of *DNMT1* expression. Probably, these observations may be related to the effect of folic acid action on SAM pool.

Our findings confirm other authors' data showing that folic acid supplementation may lead to down-regulation of the tested tumour suppressor genes, what may promote progression of breast neoplasia [9]. It should be taken into account in anticancer therapy where diet enriched with synthetic vitamins is often recommended.

Acknowledgments

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