RESEARCH ARTICLE

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Metformin Reshapes the Methylation Profile in Breast and Colorectal Cancer Cells

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Abstract

With no sharp cure, breast cancer still be the major and the most serious life-threatening disease worldwide. Colorectal is the third most commonly occurring cancer in men and the second most commonly occurring cancer in women. In the present investigation, colon cancer cells (CaCo-2) and breast cancer cells (MCF-7) were treated with elevated doses of metformin (MET) for 48h. Cell count was assessed using trypan blue test, and the cytotoxicity was evaluated using MTT assay. Methylation-specific PCR was performed on the bisulfite-treated DNA against two tumor suppressor genes; *RASSF1A* and *RB*. Results indicated that: in breast cancer, the cell count was decreased significantly (P>0.005) after being treated with 5, 10, 20, 50, and 100 mM of MET. The elevated concentration had increased reduction percentages on the MCF-7 cells, as 5 mM and 100 mM have yielded 35% and 93.3% reduction in cell viability, respectively. Colon cancer cells have responded to the doses of MET differently, as for the 5 mM and the 100 mM, it gave 88% and 60% reduction in cells viability, respectively. Cytotoxicity assay revealed that 5 mM and 100 mM of MET caused breast cancer cells to loss 61.53% and 85.16% of its viability, respectively, whereas colon cancer cells have responded to the 5 mM and 100 mM of MET by reducing the cells viability with 96.91% and 96.24%, respectively. No *RB* promoter methylation was detected in colon cells, while *RASSF1A* was partially methylated. In the MCF-7 breast cancer cells, both *RASSF1A* and *RB* were partially methylated.

Keywords: Metformin- breast- colon- cancer- methylation- *RASSF1A*- *RB*

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Introduction

Breast cancer (BC) incidence is rising worldwide with an increase in aggressive neoplasia in young women (Chajès and Romieu, 2014), with 1 million new cases causing 375, 000 deaths worldwide per year. Breast cancer is the leading cause of cancer death in women in both developing and developed countries, and it is considered a major public health concern with tremendous socioeconomic implications (Ginsburg, 2013; Rivera-Franco and Leon-Rodriguez, 2018). Breast cancer incidence and mortality rates are increasing in the Arab world and the involved women are often diagnosed at advanced stages of the disease (Donnelly et al., 2013; Karim et al., 2015). Meanwhile, the incidence rates of BC being increased during the last 24 years in the Arab region, with high incidence rates in Egypt, Tunisia, Saudi Arabia, Syria, and Palestine, as it constitutes 13-42% of all female cancers (El Saghir et al., 2007; Saggu et al., 2015). While the incidence of breast cancer in the Middle East region is lower than in other western countries, it has substantially increased in the last quarter century (Tarabeia et al., 2007). Furthermore, the diagnosis of breast cancer in this region often occurs at a later stage in the progress of the disease and in a higher proportion of women in their thirties and forties (Tarabeia et al., 2007; Al-Saad et al., 2007; Miller, 2010) than in industrialized nations. Breast cancer that presents at a younger age is generally more aggressive with a possibly poorer prognosis (Cancello et al., 2010; Gnerlich et al., 2009; Kheirelseid et al., 2011). About 50% of the breast cancer cases and 60% of the deaths are estimated to occur in developing countries (Mackay et al., 2006). Nevertheless, there is a large difference in breast cancer incidence among Caucasian, Hispanic, African, and Asian women with Caucasian women being the highest and Asian women being the lowest (Hea and Chenb, 2013).

Colorectal cancer is a major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidence. It is the third most common cancer worldwide and the fourth most common cause of death (Haggar and Boushey, 2009). Colorectal cancer is one of

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the most common human malignancies with a high rate of mortality. Most colorectal cancers are due to lifestyle factors and increasing age, with only a small number of cases due to genetic causes. The majority of patients are diagnosed at an advanced stage so that chemotherapy is required, with the incidence and mortality being high among young adults (Grivicich et al., 2007; Marley and Nan, 2016; Bhandari et al., 2017).

Epigenetic alterations are one of the most common molecular alterations in human neoplasia. In particular, aberrant promoter methylation occurs in numerous genes in cancer development and progression (Kim and Paik, 2010). It was suggested that these CpG island methylation of tumor-related genes are an early event in breast cancer progression (Park et al., 2011). Meanwhile, epigenetic profiling represents a promising approach to discover novel disease-specific markers (Esteller, 2007), and might play a key role in most kinds of cancer, both in the early and late stages of disease. One of the most powerful epigenetic mechanisms is DNA hyper-/hypo-methylation (Szyf and Paknesha, 2004) that plays an important role in multi-stages of breast cancer and are considered the main epigenetic modification occurring in the early stages of carcinogenesis (Li et al., 2010).

Metformin (chemically designated as 1,1-Dimethylbiguanide hydrochloride) is a drug of first choice for the treatment of type II diabetes as it functions to and its primary inhibit hepatic gluconeogenesis, but a clear mechanistic understanding of its effects has remained elusive (Hundal et al., 2000; Kirpichnikov et al., 2002; Castillo-Quan and Blackwell, 2016).

Several reports suggested that metformin slows cancer cell growth and protects against multiple human cancers (Pryor and Cabreiro, 2015; Bruno et al., 2015; Rodriguez-Lirio et al., 2015; Wu et al. 2016), although the majority of available clinical data on the anti-cancer potential of metformin are based on observational studies (Gadducci et al., 2016). However, Garcia et al., (2017) observed no statistical significant association between metformin use and overall survival in a matched cohort of 360 ovarian cancer patients.

Metformin regulates mitonuclear communication and modulate the epigenetic landscape in pre-cancerous cells, and this might guide the development of new metabolic-epigenetic strategies for cancer prevention and therapy (Cuyas et al., 2016; Liang et al., 2017). However, the molecular mechanisms underlying the anticancer properties of metformin remain elusive (Zhong et al., 2017).

In the present study, we are aiming to investigate the role of metformin in modulating the methylation pattern (s) of two tumor suppressor genes; *RASSF1A* and *RB* in colorectal and breast cancer cells.

Materials and Methods

Cell line maintenance

MCF-7 breast cancer cells and CaCo-2 colorectal cancer cells were purchased from the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt. Adherent cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin mix (Invitrogen Life Technologies). Cells were seeded in 12-well U-bottom microplates (Nunc, Denmark) and incubated for 24 h at 37 °C in a fully humidified atmosphere of 5% CO_2 before being treated with metformin.

Metformin doses

Metformin was kindly provided by Dr. Aya Salem, College of Biotechnology, Misr University for Science and Technology. Cells (1.8×10^4 cell/mL) were treated with metformin (dissolved in water) in final concentrations of 5, 10, 20, 50, and 100 mM for 48h.

Cell counting

Trypan blue test was employed in the present study to count the cells after being treated with metformin. Briefly, cells were harvested with 0.25% trypsin (Invitrogen, USA) and resuspended again in 1.5 mL fresh RPMI 1640 media. About 50 µL of the cell suspension was mixed with an equal volume of trypan blue dye (Sigma Aldrich, Germany) for 2-4 min. at room temperature. An appropriate volume was loaded on hemocytometer slide, covered with glass coverslip, and read under an inverted microscope. The average of four readings for each sample was taken, and the cell count was calculated according to the following equation:

Number of cells/mL = average cell count x2 x 10^4.

Cytotoxicity assay

The cytotoxic/cytostatic effects of metformin *in vitro* on both MCF-7 breast cancer cells and CaCo-2 colon cancer cells was tested with a rapid colorimetric assay using MTT assay and compared with the untreated controls. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolving in DMSO (Denizlt and Lang, 1986). To evaluate cell viability, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h. Then the media were replaced with 150 µL of DMSO, and the complete dissolving of formazan crystals was achieved by repeated pipetting of the solution. Optical density was then determined at 540 nm by an ELISA plate reader.

The cytotoxic effect of metformin was expressed as the relative viability (% control). To calculate the percentages of cell viability, the following equation was applied:

Relative viability = Experimental absorbance – background absorbance/absorbance of untreated cells – background absorbance X 100.

DNA extraction

Total DNA was extracted using the Quick-DNA Plus (Zymo research, USA) according to the kit's instructions. DNA was extracted from treated and untreated cells, and stored in -20°C until being used.

Bisulfite conversion

Bisulfite modification is the most widely used of all the pre-treatment options available for DNA methylation analysis. The extracted DNA was subjected to bisulfite conversion using EZ DNA Methylation Kit (Zymo research, USA). Bisulfite conversion involves the deamination of unmodified cytosines to uracil, leaving the modified bases 5-mC and 5-hmC unconverted. Treatment of denatured DNA with sodium bisulfite leads to deamination of unmethylated cytosine residues to uracil, leaving 5-mC or 5-hmC intact. The uracils are amplified in subsequent PCR reaction as thymines, whereas 5-mC or 5-hmC residues are amplified as cytosines. We followed the kit's instructions with minor modification in terms of the time needed for incubation of DNA.

Methylation-specific PCR

Methylation-specific PCR was performed to detect the methylation status of two tumor suppressor genes; *RASSF1A* and *RB*. The reaction was performed in StepOne Plus (ABI). The primer sequences used in this study are presented in Table 1.

The thermal cycling conditions used for the two genes were as follows: For *RB*, 1 cycle at 95°C for 5 min, followed by 39 cycles of 95°C for 45 sec, 63°C for 60 sec and 72°C for 60 sec, with a final extension cycle of 72°C for 10 min. For *RASSF1A*, 1 cycle at 95°C for 5 min, followed by 39 cycles of 95°C for 30 sec, 58°C for 45 sec and 72°C for 45 sec, with a final extension cycle of 72°C for 5 min. MSP products for methylated and unmethylated promoters were separated on 2% agarose gels after being stained with ethidium bromide.

Statistical analysis

T-test was used in the present study to identify whether the differences between treated and untreated cell counts significant. Comparisons with p-values less than 0.05 were considered significant.

Results

Cell count

In the present study, the role of metformin as an anticancer agent was evaluated. Colon and breast cancer cells were treated with elevated doses of metformin for 48 h before being harvested. Cell count was performed using the trypan blue assay, and the obtained results indicated that, for breast cancer cells, the higher the concentration of metformin the lower count of viable cells (Figure 1). Metformin induced the apoptotic machinery (Saber et al., 2016), as it demonstrated an anti-proliferative activity in MCF-7 cells that was both time- and concentration-dependent (Queiroz et al., 2014). Metformin inhibited 70%, of MCF-7 cell viability at a final concentration of 25 mM (Queiroz et al., 2014; Lee et al., 2014). The present study revealed 35, 50, 91.6, 91.6, and 93.3% inhibition of the viability of cells for the concentrations 5, 10, 20, 50, and 100 mM metformin, respectively. These results were in accordance with several researches (Zhuang and Miskimins, 2011; Ganjali and Ganjali, 2013; Ariaans et al., 2017), where the metformin

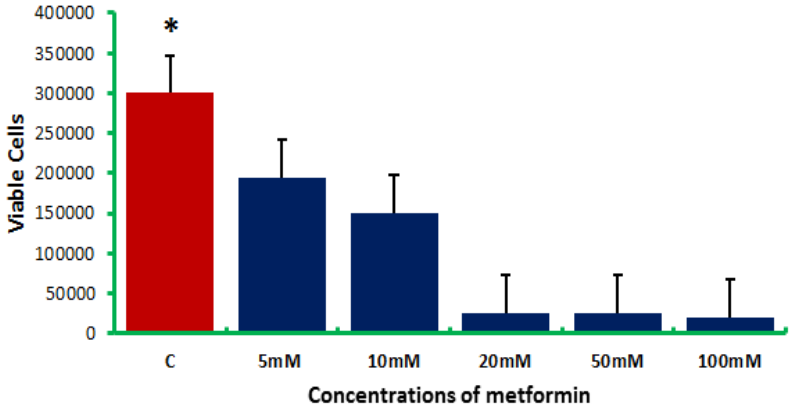

Figure 1. Breast Cancer Cell (MCF-7) Counts after being Treated with Elevated Doses of Metformin

Table 1. The Methylated and Unmethylated Primer Sequences of *RASSF1A* and *RB* used in the Present Study

Gene name	Primer status	Primer sequence (5′ to 3′)	Ref.
RASSF1A	Unmethylated Forward	GGTTGTATTTGGTTGGAGTG	Matthaios et al., 2016
	Unmethylated Reverse	CTACAAACCTTTACACACAACA	
	Methylated Forward	GTTGGTATTCGTTGGGCGC	
	Methylated Reverse	GCACCACGTATACGTAACG	
RB	Unmethylated Forward	GGGAGTTTTGTGGATGTGAT	
	Unmethylated Reverse	ACATCAAAACACACCCCA	Liu et al., 2012
	Methylated Forward	GGGAGTTTCGCGGACGTGAC	
	Methylated Reverse	ACGTCGAAACACGCCCCG	

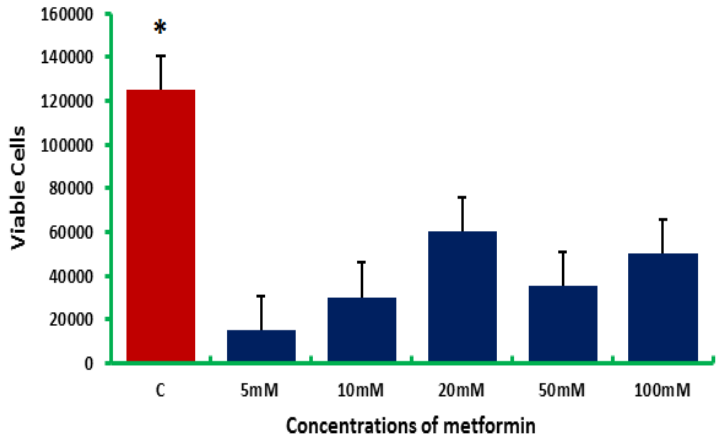

Figure 2. Colon Cancer Cell (CaCo-2) Counts after being Treated with Elevated Doses of Metformin

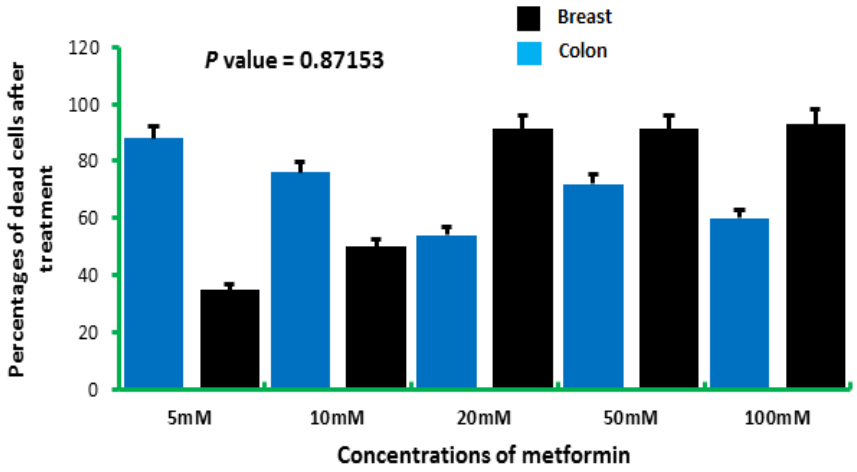

Figure 3. Colon Cancer Cell (CaCo-2) Counts after being Treated with Elevated Doses of Metformin

was found to inhibit CRC, breast, hepatic cancer cells growth.

For colon cancer cells, metformin also induced cell death as indicated by trypan blue assay (Figure 2). In CaCo-2 colon cancer cells, the most effective metformin dose was, surprisingly, 5 mM, as it resulted in 95% inhibition of the cell viability compared to control. Metformin is a potent growth inhibitor (Ma et al., 2011), as it was indicated that 10 mM of metformin have resulted in 80% reduction in HCT-116 colorectal cancer cells (du Potet et al., 2009). Our results showed that the reduction in the cell viability was dose-dependent up to a final concentration of 20 mM. The same profile was reported by Kim et al., (2018), who indicated that

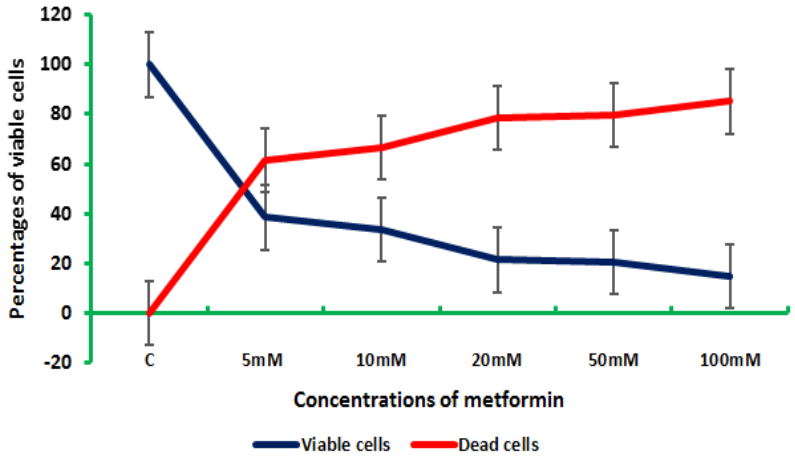

Figure 4. The Overall Cell Viability of Breast Cancer Cells (MCF-7) after being Treated with Elevated Doses of Metformin as Assessed by MTT Assay

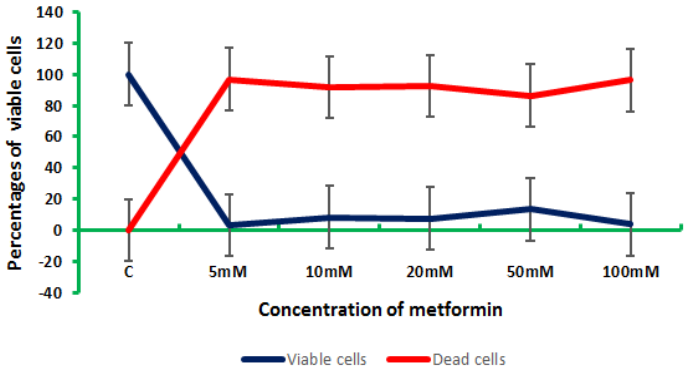

Figure 5. The Overall Cell Viability of Colon Cancer Cells (CaCo-2) after being Treated with Elevated Doses of Metformin as Assessed by MTT Assay

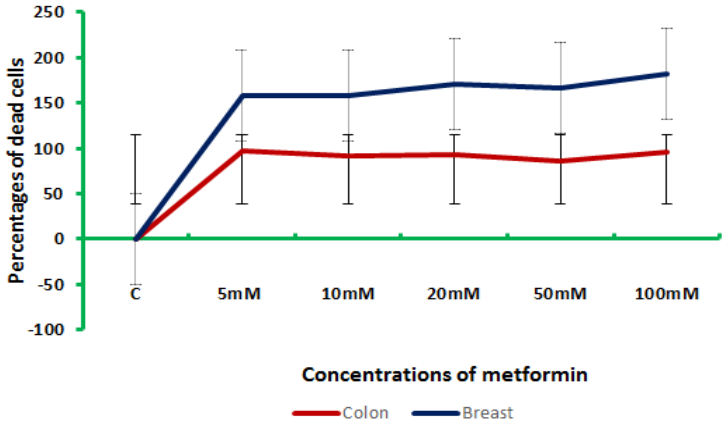

Figure 6. The Percentages of Dead and Viable Colon and Breast Cells after being Treated with Elevated Doses of Metformin

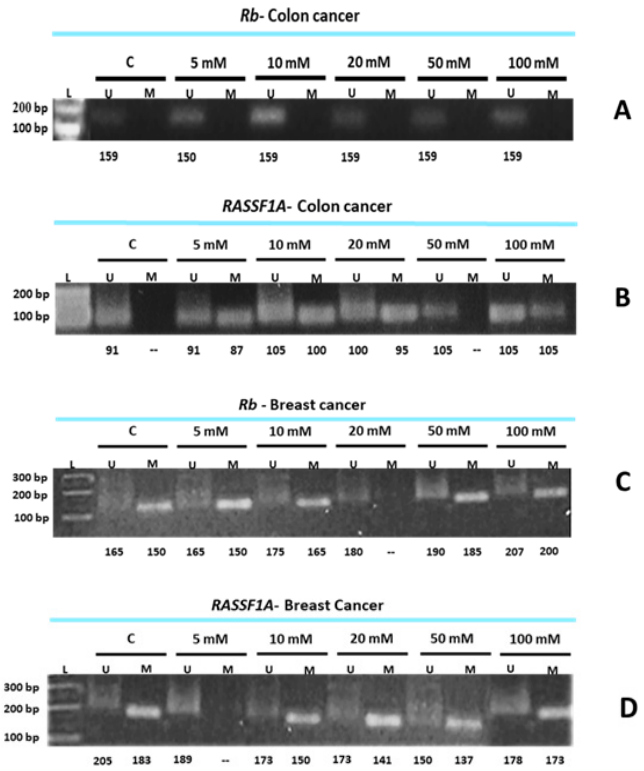

Figure 7. Methylation Detection of *RASSF1A* and *RB* via MSP. A, Colon cancer cells (CaCo-2) treated with elevated doses of MET and subjected to MSP to detect *RB* prompter methylation; B, Colon cancer cells (CaCo-2) treated with elevated doses of MET and subjected to MSP to detect *RASSF1A* prompter methylation; C, Breast cancer cells (MCF-7) treated with elevated doses of MET and subjected to MSP to detect *RB* prompter methylation; D, Breast cancer cells (MCF-7) treated with elevated doses of MET and subjected to MSP to detect *RASSF1A* prompter methylation.

5 mM, 10 mM, and 20 mM of metformin have resulted in 78.3%, 63.5%, and 41.9% reduction in colon cancer cell viability, respectively. For that, metformin could be a useful adjuvant agent, with the greatest benefits seen in colorectal cancer (Coyle et al., 2016).

Discussion

Meanwhile, different concentrations of metformin had different effects on the viability of both colon and breast cancer cells. Figure (3) represents the effect of the elevated concentrations of metformin on colon and breast cells *in vitro*.

For the metformin concentrations 5 and 10 mM, the highest mortality was reported in colon cancer cells, while in 20, 50, and 100 mM, the highest mortality was reported in breast cancer cells. This might indicate that colon cancer cells were more sensitive to the lower concentrations of metformin compared to breast cancer cells. Metformin benefits are a controversial issue, as some researches indicated that there are no sufficient data available to conduct analyses on the impact of metformin dose and duration (Coyle et al., 2016), while others reported its potential effect on breast cancer *in vitro* (Hadad et al., 2011; Niraula et al., 2012) and *in vivo* (Soffer et al., 2014) and in colorectal cancer (Hosono et al., 2010).

Cell viability assay

The viability of both colon and breast cancer cells were assessed using MTT assay as a sensitive and accurate way to assess cell viability *in vitro* (Hundie et al., 2016).

For breast cancer cells (Figure 4), the elevated doses of metformin have resulted in a significant ($P>0.005$) reduction in the cell viability. The most effective dose of metformin was 100 mM as it yielded an 85.1% reduction in the cell viability compared to control (calculated as 100% viability). Several studies indicated the effectiveness of metformin in reducing the overall cell survival by increasing reactive oxygen species, which induce DNA damage and apoptosis (Marinello et al., 2016), or by inducing apoptosis in a concentration- and time-dependent manner *via* decreasing the ATP production (Gao et al., 2016).

For colon cancer cells, results indicated that 5 mM of metformin resulted in the highest cell mortality (96.91%) followed by the concentration 100 mM (96.24%) (Figure 5). This might indicate that colon cancer cells were sensitive even to lower doses of metformin. It was indicated elsewhere (Safari et al., 2015; Mogavero et al., 2017) that lower concentrations of metformin *i.e.,* 5 and 10 mM were capable to inhibit the cell growth of colorectal cancer cells *in vitro*.

However, colon and breast cells were responding differently to the elevated concentrations of metformin. For the concentration 5 mM, colon cancers cells were severely affected with a rate of reduction of cell viability reached 96.915, while breast cancer cells exhibited a 61.53% reduction of the viability of cells for the same concentration. The concentrations 10, 20, 50, and 100 mM yielded 91.72, 92.55, 86.46, and 96.24%, respectively for colon cells, while breast cells gave 66.84, 78.57, 79.67, and 85.16% for 10, 20, 50, and 100 mM, respectively (Figure 6).

Methylation detection via MSP

Promoter methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years (Ohtani-Fujita et al., 1997; Wajed et al., 2001; Das and Singal, 2004; Mamrut et al., 2013). In the present investigation, bisulfite-treated DNA was subjected to methylation-specific PCR to amplify two tumor-suppressor genes; *RASSF1A* and *RB*. Results indicated that the methylation patterns of both cancer cells under investigation (MCF- and CaCo-2) exposed to metformin were reshaped (Figure 7).

Metformin had no effect on the methylation status of *RB* promotor region in colon cancer cells (Figure 7A), since the loss or inactivation of the *RB* gene is infrequent in colorectal carcinomas, and the reduced *RB* expression in these cells is probably due to a cellular regulatory mechanism (Ali et al., 1993). It seems that the function of *RB* gene in colorectal carcinoma is often preserved (Collard et al., 2012), and this might explain the insensitivity of *RB* to metformin as an anti-cancer agent. *RB* methylated primers generated no bands in the colon control cells, and this might indicate that this gene is normally unmethylated in this type of cancer.

The methylation of *RASSF1A* promoter is frequent in colorectal cancer, although it appears significantly more frequently methylated in metastasis than in the organ itself (Schirosi et al., 2016), and in cancer tissues than in benign, adjacent, and normal tissues (Wang et al., 2014). It seems that the methylation pattern was more editable after treating colon cancer cells with metformin as indicated in the present study and in other studies (Fernandes et al., 2013). However, some studies on CRCs found no *RASSF1A* promoter methylation (van Engeland et al., 2002).

Breast cancer cells have different methylation profiles in *RB* and *RASSF1A* genes after been treated with metformin (Figure 7B). Aberrant methylation is a common feature across many types of cancers, and these hallmarks are shared by almost all solid tumors, but some epigenetic marks most often found in distinct types of tumors, *e.g., RB* in retinoblastoma (Sakai et al., 1991). *RB* promoter region appeared to be partially unmethylated in the control cells, while the methylated primers were able to generate a defined band (Figure 7C) although with variable molecular sizes. It is believed that *RB* promoter methylation is crucial, and seems to facilitate, *via* multiple mechanisms, the tumorigenesis process (Ertel et al., 2010; Witkiewicz and Knudsen, 2014). However, the unmethylated primers were also able to generate a less intense band, and this might indicate that the promoter region of this gene was partially methylated.

The same profile was obtained in the case of *RASSF1A* (Figure 7D). Meanwhile, several studies have indicated the correlation between *RASSF1A* hypermethylation and breast tumorigenesis (Dammann et al., 2001; Jezkova et al., 2017) and tumor progression (Geng and Wu, 2016). Others indicated that *RASSF1A* could serve as a potential

prognostic biomarker (Kioulafa et al., 2009; Xu et al., 2012).

In conclusion the role of metformin (MET) as an anticancer agent was evaluated. Breast cancer cells (MCF-7) and colon cancer cells (CaCo-2) was challenged with different doses of MET *i.e.,* 5, 10, 20, 50, and 100 mM. Trypan blue assay revealed a significant decrease in the cell count of both colon and breast cancer cells. The same was indicated by the cytotoxicity assay (MTT), as it revealed a decrease the cell viability after treating the cell lines with elevated doses of MET. Promoter hypermethylation was assessed in both *RASSF1A* and *RB* as two tumor-related genes. *RASSF1A* was shown to be involved in the apoptosis process, as a level of hypermethylation was detected in colon cancer cells. *RB* was found to be less responding to the treatment, although the colon cancer cell count decreased upon treatment, but this might be attributed to another mechanism of cell death apart from *RB*-mediated one. In breast cancer cells, both *RB* and *RASSF1A* were proved to be involved in inducing cell death via hypermethylation of these genes, which might be correlated with apoptosis.

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Author participation

HS: put the idea and write the manuscript
SEA: conducted the practical experiments
OAMS: revised the manuscript and statistics
MAM: participated in the revision of the manuscript
ME: took the final revision of the manuscript

Conflict of interests

The authors declare no conflict of interests.

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