Investigation of the Role of CpG Methylation in Epithelial Mesenchymal Transition Master Genes in a Chemoresistant Ovarian Cancer Cell line

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Abstract

Ovarian cancer is one of the commonly diagnosed cancers among women. Chemoresistant is an essential reason for treatment failure and high mortality. Emerging evidence connects epithelial mesenchymal transition (EMT) like changes and acquisition of chemoresistance in cancer. DNA methylation influences cellular processes including EMT. Here, we investigate EMT like changes in cisplatin-resistant A2780 ovarian cancer cells (A2780cis), and we study the DNA methylation role in EMT master genes regulation. Cell viability assay was carried to test the sensitivity of A2780, and A2780cis human cancer cell lines to cisplatin compared to other cancer cell lines. Differential mRNA expression of EMT markers using qPCR was conducted to investigate EMT like changes. The role of CpGs methylation in gene expression regulation was investigated by 5-azacytidine (5-aza) treatment. DNA methylation changes in EMT genes were identified using Methylscreen assay between A2780 and A2780cis cells. A2780cis maintains its cisplatin tolerance ability and exhibits phenotypic changes congruent with EMT. Methylscreen assay and qPCR study revealed DNA hypermethylation in promoters of epithelial adhesion molecules *CDH1* and *EPCAM* in A2780cis compared to the cisplatin-sensitive parental cells, these changes were concomitant with gene expression down-regulation. DNA hypomethylation associated with transcription up-regulation of the mesenchymal marker *TWIST2* was observed in the resistant cells. Azacytidine treatment confirmed the DNA methylation role in the regulation of gene expression of *CDH1*, *EPCAM* and *TWIST2* genes. A2780cis cell line undergoes EMT like changes, and EMT master genes are regulated by DNA methylation. A better perception of the molecular alterations which correlate with chemoresistance may lead to therapeutic benefits such as chemosensitivity restoration.

Introduction

Ovarian cancer is one of the deadliest gynecologic cancer in females ¹. Patients with ovarian cancer have a poor prognosis, and a high mortality rate ². The low survival rate of ovarian cancer is due to metastasis and acquiring of chemotherapies resistance ³. Cisplatin (cis-diamminedichloroplatinum [II]) is a platinum compound used to treat many solid tumors including ovarian cancer ^{4,5}. Cisplatin induces DNA lesions and activates several signaling pathways such as DNA repair mechanisms ⁶. When DNA damage fails to be repaired, cells undergo apoptosis and die ⁷. Drug resistance is the main obstacle that limits treatment effectiveness ⁸. The mechanisms that underlie platinum drug resistance are multifactorial. Several cellular processes have been identified as responsible for the resistant phenotype, including alterations in drug influx or efflux, DNA repair, cell cycle and apoptosis ^{9,10}. Recent evidence suggests that epithelial-mesenchymal transition (EMT) processes may have a role in the development of chemoresistance. EMT is a cellular procedure where epithelial cells miss their cell-cell adhesion, cell polarity and gain metastasis capability ¹¹. The molecular signatures of EMT are loss of epithelial cell markers *CDH1* and mesenchymal markers up-regulation including EMT transcription factors *SNAIL* and *TWIST* ^{12,13}. EMT like changes has been reported in various chemoresistant cancer such as gastric cancer ¹⁴ non-small cell lung cancer ^{15,16}, nasopharyngeal carcinoma cells ¹⁷, breast cancer cells ^{18,19}, and ovarian cancer cells ^{20,21}.

Multiple epigenetic events, such as DNA methylation, histone modifications, and non-coding RNA, have been described to contribute to the acquisition of chemoresistance^{22–24}. DNA methylation is the most well-known epigenetic mechanism that occurs at CpGs islands that influence cellular processes by regulating gene expression²⁵. Commonly, gene promoter hypermethylation is associated with reduced expression, while hypomethylation increases gene expression^{26,27}. DNA methylation changes at CpG islands associated with transcriptional

silencing have been stated in cisplatin-resistant cancer cell lines^{28–30}. For example; DNA methylation of several genes including (*ARMCX2*, *COL 1A1*, *MDK*, *MEST*, *MLH1*, *KLF4*, *ST3GAL5*, *SYNE1*, *CXCL8*, *HERC5*, *FOSL1*, and *ARRDC4*) was linked to ovarian cancer initiation and chemotherapy resistance^{31,32}. A study in ovarian and breast cancer cell lines with doxorubicin tolerance described methylation changes in genes that contributed to chemoresistance and identified hyper-methylation of *CDH1*, *BRCA1*, *SULF2* and *DNAJC15* besides hypo-methylation of *APC*, *ABCB1* and *HIC1* genes²³. Based on these considerations, the need to study the linkage between EMT and DNA methylation regulation has become pivotal to understand the chemoresistant phenotype.

The present study aimed to assess the morphological and transcriptional changes in cisplatin resistant A2780 ovarian cancer cell line in relation to EMT, and comparing these changes with different types of cisplatin-resistant cell lines. Our study focused on CpG methylation changes that regulate EMT master genes in A2780 chemoresistant cells.

Results

Cancer cells display different morphological phenotypes

We observed the morphological characteristics of the different cancer cell lines sensitive or resistant to chemotherapy during exponential growth, i.e. shape, size and growth pattern of the cells during culture. A2780 cells formed clusters with small round-shaped cells consistent with an epithelial phenotype. In contrast, A2780cis cells were larger, more elongated and were frankly polygonal (Fig. 1A).

These observations were compared with the morphological of MDA-MB-231 and T98G cell lines as they were known to be resistant to chemotherapy^{33–35}. Figure 1B shows that MCF7 cells have small squamous epithelial appearance, whereas MDA-MB-231 cells have spindle-like morphology. U-87 MG and T98G cells exhibit spindle like morphology, T98G cells showed a larger and more elongated cell shape compared to U-87 MG (Fig. 1C). These observations suggest that this dissimilar cellular shape is possibly associated with alteration in the EMT process.

Dissimilar shaped cancer cell lines have different cisplatin tolerance capacity

MTT assay was performed to assess the effect of cisplatin on different human cancer cell lines. Cells were treated with increased concentrations of cisplatin for 24 hrs. Concentration-dependent effect of cisplatin on different cells was observed. IC₅₀ value in A2780cis cells was 4 times higher than the parental

cell line's IC_{50} , which suggested that the A2780cis cells were more resistant to cisplatin-induced cytotoxicity compared with A2780 cells and had still maintained cisplatin resistance (Fig. 2A). Results in Fig. 2B show that the IC_{50} value for MDA-MB-231 cells is six times greater than IC_{50} for MCF7 cells. Figure 2C shows the differences in cisplatin sensitivity between U-87 MG and T98G cell lines, the difference in IC_{50} values was the least among other cell line models used in this study. IC_{50} in T98G cells was 1.3 times higher than U-87 MG's IC_{50} . The IC_{50} results were summarized in (Fig. 2D).

MDR1 Expression increases in the resistant variant A2780cis cell line

To determine whether cisplatin resistance in the A2780cis cells is associated with increased expression of ABC transporters genes, mRNA expression level of the following genes was assessed: *MDR1* (multidrug resistance protein 1 gene), *MRP1* (MDR-related protein 2) and *MRP2* (MDR-related protein 2) using qRT-PCR. Figure 3 showed that there was a statistically significant increase of *MDR1* transcript level (5.8 fold) in A2780cis cell line compared to the parental cell line. On another hand, *MRP1* and *MRP2* expression did not significantly change in A2780 and A2780cis cell lines.

Cisplatin resistant cancer cells have molecular changes consistent with EMT

To determine whether the gaining of cisplatin resistance promotes specific molecular alterations corresponding with EMT in ovarian cancer, qRT-PCR was performed to investigate EMT-related biomarkers expression. Results showed that the expression of epithelial markers, *CDH1* and *EPCAM*, was significantly reduced by 0.02 and 0.013 fold, respectively in the A2780cis cells compared with A2780 cells. The expression of mesenchymal markers, *SNAIL*, and *TWIST2*, were higher by 4.9 and 20.3 fold respectively in the A2780cis cells compared with the parental cell line (Fig. 4A). Based on these observations, A2780cis cells may be considered to have a mesenchymal like phenotype.

We also compared these changes in transcriptional levels in MDA-MB-231 and T98G cells, to figure if the changes observed in A2780cis are common in other cancer cells resistant to therapy. The same molecular changes of EMT markers observed in A2780cis cells were detected in MDA-MB-231 in comparison with MCF7, *CDH1* and *EPCAM* genes were decreased 0.5 and 0.006 fold respectively. These decreases were associated with up-regulation of EMT genes *SNAIL* and *TWIST2* (2.6 and 40732 fold) (Fig. 4B). The same molecular changes were observed in EMT markers; *CDH1* and *EPCAM* were down-regulated (0.046 and 0.2 fold, respectively) in T98G the more resistant cell line, whereas *SNAIL* and *TWIST2* (88.5, 76 fold respectively) were up-regulated (Fig. 4C). We notice a significant overlap in expression profiles of EMT genes between resistant cancer cell lines, proposing a shared mechanism associated with resistance to therapy.

5-azacytidine treatment up-regulates expression of EMT-related genes in A2780 cell line

To identify the epigenetically regulated genes from the gene set that expressed differentially between A2780 cell line and the resistant variant, we analyzed gene expression by qPCR after azacytidine treatment. As shown in Fig. 5 azacytidine treatment significantly increased the expression of *CDH1*,

EPCAM, *SNAIL*, and *TWIST2* genes, by 7.8, 9.26, 5.24, and 4 fold respectively. This suggests that DNA methylation may be essential in the regulation of the expression of these genes in this cell line.

Acquisition of cisplatin resistance in A2780 cell line induced aberrant DNA Methylation in EMT-related genes

Methylscreen assay was used to determine the DNA methylation profile in genes that differentially expressed between A2780 and resistant variant cells and that expression increased after azacytidine treatment. PCR primers were designed to amplify genomic DNA at the TSS associated CpG islands of these genes. The PCR amplicons ranged from 151 bp to 523 bp in length. These amplicons contained different site numbers for HhaI/HpaII, AclI and MCRBC. Size of DNA fraction amenable to digestion (analytical window) determine assay sensitivity and it was represented by ΔC_t between the Rsd and R0 reactions and it ranged from 3.2 to 13.6.

The kinetic profiles obtained from each of the four assays (*CDH1*, *EPCAM*, *SNAIL*, *TWIST2*) obtained from A2780 and A2780cis DNA are displayed (Figs. 6B, 7B, 8B, 9B). The data depict results from cell lines; each genome's amplification is color coded by their digestion treatment: mock restriction (black), MSRE restriction (blue), MDRE restriction (red) and a DD (green). The charts display the result of each assay as a percentage of each portion of DNA according to its methylation state, i.e., the unmethylated fraction, intermediate methylated and hypermethylated fraction (Figs. 6C, 7C, 8C, 9C). Results from *CDH1* assay revealed a 13.47 % hypermethylation in the densely methylated portion after the acquisition of cisplatin resistance. The methylation of the region from (-306 to -82 bp) which contains [7 AclI, 3 HpaII and 7 MCRBC] restriction sites increased from 24.76 % to 39.11 % in A2780 and A2780cis, respectively (Fig. 6). On the other hand, Fig. 7 shown that the CpGs in region (-463 to -296 bp) of *EPCAM* gene that contains [8 AclI, 4 HhaI, and 7 MCRBC] restriction sites in A2780 DNA was 73.15 % unmethylated, 0 % intermediate methylated and 26.85 % densely methylated. In A2780cis the unmethylated portion value was decreased and CpGs were gained methylation by 23.67 %. Methylscreen assay of *SNAIL* gene revealed that (-688 to -165 bp) region is unmethylated in A2780 cell line and there are no significant differences in methylation between A2780cis and its parental cell line (Fig. 8). The methylation analysis of *TWIST2* gene revealed that the region from (-328 to -177 bp) that contains [6 AclI, 3 HhaI, and 4 MCRBC] restriction sites was 3.37 % hypermethylated, 88.98 % intermediate methylated and 7.65 % unmethylated. In A2780cis the intermediate methylated portion value was decreased compared to A2780 and the fraction of unmethylated DNA was 21.76 % in A2780cis (Fig. 9).

Discussion

Ovarian cancer ranks as one of the most common causes of cancer deaths among females¹. Patients suffering from ovarian cancer have a poor prognosis, with a low survival rate³. High mortality of ovarian cancer is mainly due to metastasis and the evolution of resistance to chemotherapies². Cisplatin is a cornerstone of the treatment regime for many solid tumors including ovarian cancer^{4,5}. However, its clinical effectiveness is influenced by acquiring tumor cells chemoresistant⁸. Many studies concentrated on the molecular mechanisms mediating the development of cisplatin resistance and lots have been

characterized including decreased cellular uptake of the drug, increased drug efflux, enhanced DNA damage repair capacity, and anti-apoptotic signaling^{9,10}.

In this study, we demonstrated that the A2780cis cell line still has the tolerance capacity to cisplatin and its resistance accompanied by an increase in the expression of *MDR1* (Fig. 3). This marker seems to be a universal cellular response marker to chemotherapy in various cancer. A high expression level of *MDR1* was detected in the cisplatin, paclitaxel and doxorubicin resistant variant of A2780 cells and different resistant cell lines derived from ovarian cancer^{36–39}. Several studies have shown conflicting results about the differential expression of *MRP1* and *MRP2* genes between sensitive and resistant ovarian cancer cell lines, and the ability of these changes to represent the chemoresistance^{38,40}. Recently, epithelial-mesenchymal transition (EMT) was implicated as a core mechanism mediating drug resistance⁴¹. EMT is defined as a biological mechanism characterized by loss of cell adhesion, as well as loss of cell polarity and gain motility¹¹. EMT results in changes in cell morphology associated with alterations in epithelial and mesenchymal markers expression⁴². EMT like phenotype has been reported in chemoresistant variant cell lines generated upon multiple rounds of chemotherapy treatment such as; cisplatin resistant ovarian cancer cells (TOV-112D, MDAH, OVSAHO, SKOV-3/DDP, OVCAR3/DDP and A2780CP)^{43–46}, A2780/PTX, NOS-PR, TAOV-PR, and SKOV-PR paclitaxel resistant ovarian cancer cells^{20,21}. Also, chemotherapeutic resistance promotes EMT like changes in other cancer cell lines, including non-small cell lung cancer^{15,16}, gastric cancer¹⁴ nasopharyngeal carcinoma cells¹⁷, and breast cancer cells^{18,19}. In this present study, we demonstrate that the resistant variant A2780cis cell line underwent EMT. This was confirmed by noticing a morphological change from small round shaped to elongated and polygonal shaped cells and changes in molecular markers of EMT; significant reduction in *CDH1* and *EPCAM* and upregulation of the transcription factors, *SNAIL* and *TWIST2* (Fig. 1 and Fig. 4).

Multiple studies have shown that naïve cancer cell lines and clinical tumor samples can be divided according to their mesenchymal/epithelial phenotype, this sorting could determine the sensitivity to chemotherapy in various cancers including ovarian, breast, and lung cancers^{47–52}. From this observation, we compared the EMT like changes detected in A2780cis with changes in breast and glioma cell line models known as resistant cancer cells^{33–35}. We used MDA-MB-231 and T98G cancer cells as other models of cell lines to figure if the acquired changes observed in A2780cis are common in other cancer cells, resistant to therapy. We detect the same molecular alteration consistent with morphological in cisplatin resistant MDA-MB-231 cells compared with the sensitive cells MCF7. The same transcripts changes in *CDH1*, *EPCAM*, *SNAIL* and *TWIST2* genes also detected in chemoresistant T98G cell line in comparison with the less cisplatin tolerance cells U-87-MG (Fig. 2). This indicates the importance of *CDH1*, *EPCAM*, *SNAIL* and *TWIST2* genes in EMT mechanism associated with cisplatin resistance.

CDH1 is Ca²⁺ dependent adhesion molecule that binds by its extracellular domain to *CDH1* on the adjacent cell creating a bridge between the cell's cytoskeletons⁵³. Many studies revealed that *CDH1* down-regulation may be associated with cancer cells resistant to chemotherapy that can be attributed to the EMT mechanism activation^{23,54}. Acquisition of paclitaxel chemoresistant induces EMT phenotypic

changes and *CDH1* in down-regulation in NOS-PR and A2780/PTX ovarian cancer cell line^{20,21}. EPCAM is an epithelial cell surface transmembrane glycoprotein that mediates homophilic cell-cell adhesion without Ca^{2+} dependent^{55,56}. In ovarian cancer cells, *EPCAM* upregulation is connected to a more favorable prognosis and more effective platinum-based therapy⁵⁷. Galle et al, found *EPCAM* expression is down-regulated in addition to *CDH1* in resistant variant cancer cells due to EMT process activation³⁰. EMT transcription factors such as *SNAIL* are considered as direct repressors of *CDH1* as they bind to E-boxes existing on the *CDH1* promoter^{58–60}. Hojo, et al, observed that ovarian cancer cell lines OVCAR8 and COV318 with high Snail/*CDH1* showed more motile and cisplatin resistant phenotypes than OVSAHO and Kuramochi cell lines that have low *SNAIL/CDH1*⁴⁷. *TWIST2* is considered as a direct repressor of *CDH1*, it can bind directly on E-boxes existing on the *CDH1* promoter to suppress its expression and it can repress *CDH1* expression indirectly through activation of other signaling pathways⁶¹. Studies show that *TWIST2* expression is a prognostic indicator for overall survival and disease-free survival and its overexpression correlates with poor prognosis and is associated with *CDH1* down-regulation giving mesenchymal cell phenotype on ovarian cancer tumors^{62,63}. Wang et al, demonstrate that *TWIST2* plays a critical role in the cisplatin resistance of ovarian cancer. They found that *TWIST2* expression up-regulated in resistant variant C13K ovarian cancer cell line compared to the cisplatin sensitive ovarian cancer cell line OV2008⁶⁴.

DNA methylation is one of the best described mechanisms of epigenetic that regulate gene expression. Aberrant DNA methylation is observed in cancers in CpG dinucleotides clustered around the TSS of genes, called CpG islands, leading to gene expression dysregulation⁶⁵. Upon initiation of EMT, DNA methylation of the genome selectively undergoes CpG site methylation changes, which regulate transcription of EMT-related genes^{23,30,66,67}. In our study, we investigate the role of methylated CpG islands in the modulation of gene expression of EMT regulated genes in A2780 cancer cell lines. DNA methyltransferase inhibitor 5-aza induces gene expression of *CDH1*, *EPCAM*, *SNAIL*, and *TWIST2* which indicate that these genes may be regulated by DNA methylation. Chang et al, used gene expression profiling after cancer cells treatment with 5-azadeoxycytidine, they identified genes that were dysregulated in cisplatin resistant cancer cells and reactivated by the DNA methyltransferase inhibitor⁶⁸. Here, we found that epithelial gene promoters *CDH1* and *EPCAM* became significantly more methylated in A2780cis compared to the parental cell line. These promoter methylation changes correlate with significant gene expression down-regulation. Boettcher et al, profiled DNA methylation of 800 selected CpG islands and identified hypermethylation in *CDH1* CpG islands in breast and ovarian doxorubicin resistance cancer cells²³. *EPCAM* overexpression has been linked to promoter hypomethylation *EPCAM*-negative cells treated with a DNA methyltransferase inhibitor prompted *EPCAM* expression in various cancer types including ovarian cancer^{69–71}. A recent study reported consistent methylation changes across multiple cancer cell lines differed in chemoresistant. Specifically, hypermethylation of epithelial marker genes such as *CDH1* and *EPCAM* promoters and hypomethylation of mesenchymal marker genes such as *SNAIL* in resistant versus parental cell lines³⁰. Analysis of *SNAIL* promoter region predicted a CpG island surrounding the TSS, we examined the DNA methylation status in the genomic region (-688 to -165bp), we cannot observe any methylation in

CpGs located in this region of *SNAIL* promoter in A2780 and its resistant variant cells, although there were changes in *SNAIL* expression between the two cell lines and after azacytidine treatment. Literature has described changes in the histone modifications regulating *SNAIL* gene expression⁷². Single study described changes in the methylation of CpG island located in the first intron after 1000 pb from TSS in EMT models of cancer cells⁷³. Maybe the differential methylation in *SNAIL* gene could be identified in the intron region in A2780 cell line. CpGs island of mesenchymal transcription factor *TWIST2* promoter showed DNA hypomethylation in A2780cis compared to the parental cells, this hypomethylation coincides with gene expression down regulation due to EMT activation. *TWIST2* methylation changes were observed in various cancer such as colorectal cancers, prostate cancer, and chronic lymphocytic leukemia, this epigenetic event might be the underlying mechanism for *TWIST2* transcriptional regulating^{74,75}.

In conclusion, we have shown in this study that, the gain of cisplatin resistance in cancer cells is accompanied by EMT-like changes at the morphological and molecular levels. We show that DNA methylation changes of *CDH1*, *EPCAM* and *TWIST2* genes underlie the resistant induced EMT in ovarian cancer cell lines. Further evaluation is needed in future clinical studies to determine potential EMT associated epigenetic biomarkers for resistant phenotypes.

Methods

Cell Culture

Human ovarian cancer cell lines A2780 (cisplatin sensitive human epithelial ovarian cancer cell line) and A2780cis (the resistant variant), human breast cancer cell lines MCF7 and MDA-MB-231, and human glioblastoma cancer cell lines U-87 MG and T98G were purchased from European Collection of Authenticated Cell Cultures (England). A2780 and A2780cis cell lines were grown in complete RPMI-1640 medium containing 10 % FBS, 2 mM glutamine, 0.1 mg/ml each of penicillin and streptomycin. To maintain A2780cis resistance to cisplatin, 1 μ M of cisplatin (Sigma-Aldrich, St. Louis, USA) was added to the media every 3 passages. MCF7 and MDA-MB-231 cell lines were grown in EMEM containing 10 % FBS, 2 mM glutamine, 1 % non-essential amino acids (NEAA), 0.1 mg/ml each of penicillin and streptomycin. U-87 MG and T98G cell lines were cultured in EMEM, 2 mM Glutamine, 10 % FBS and 1 % Sodium Pyruvate (NaP). All cell cultures were kept in 5 % (v/v) CO₂ humidified atmosphere at 37 °C (Binder, Germany). Morphological phenotypes of cell lines were assessed when the cell density was up to 70 % confluence using Eclipse TS100 inverted light microscope (Nikon, Japan).

Cell Viability Assay

Cells were plated into 96-well-plates (1×10⁴ cells/well) for MTT assay and allowed to attach O/N. Different concentrations of cisplatin were used to treat cells for 24 hrs. MTT solution (Roche, Germany) was added to each well and incubated for 4 hrs at 37 °C. Then absorbance values were measured at 550 nm using Multiskan Ascent absorbance plate reader (Thermo Labsystems, Germany). Cell viability determined as following:

Cell viability (%) = (average OD value of experimental group/average OD value of control group) *100 %

Gene expression analysis by qRT-PCR

Total RNA was extracted from A2780, A2780cis, MCF7, MDA-MB-231, U-87 MG, T98G cell lines and from A2780 treated with 5-azacytidine (5-aza) using the RNeasy Mini kit (Qiagen, Germany). First-strand cDNA synthesis was carried out from 3 µg total RNAs were reverse transcriptase to cDNAs using M-MLV reverse transcriptase (Invitrogen, USA) for 2 hrs at 37 °C. To calculate the relative expression of EMT regulating genes; (*CDH1*, *EPCAM*, *SNAIL 1*, *TWIST2*), and ABC transporters (ATP-binding cassette transporters) genes; (*MDR1*- multidrug resistance protein 1 gene, *MRP1*- MDR1-related protein 1, *MRP2*- MDR-related protein 2), quantitative real-time PCR was performed using Maxima™ SYBR™ Green/ROX 2x qPCR Master Mix (Thermo Scientific, USA) for 40 amplification cycles using [StepOne™ Real-Time PCR System](#) (Applied Biosystems, USA). Relative transcript fold changes were calculated using the $\Delta\Delta C_t$ method with *GAPDH* as a reference gene. All reactions were run in triplicate. Primers sequences are detailed in Table 1.

5-Azacytidine treatment

In order to select the candidate genes for the methylation study, A2780 cells were cultured and treated with 0.1 mM 5-Azacytidine (Sigma-Aldrich, USA). Culture medium was removed every 24 hrs and replaced by a fresh medium containing 0.1 mM 5-aza. Treated and mock treated cells were collected after 7 treatment days and total RNAs were extracted as described above.

DNA extraction

Genomic DNAs from A2780 and A2780cis cells were extracted using the QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Isolated DNAs were quantified using NanoVue Plus (GE Healthcare Life Sciences, Germany).

Methylscreen assay

Quantitative PCR-based methylation analysis (Methylscreen assay) was performed to analyze DNA methylation of genes that have differential expression between A2780 and the resistant variant cells and that expression increased after 5-aza treatment. Methylscreen assay is based on combined restriction digestion of DNA with methylation sensitive and methylation dependent restriction enzymes, MSRE and MDRE respectively ⁷⁶. Genomic DNA of A2780 and A2780cis cells were divided into four parts and treated with different digestions: (1) Rs: two methylation-sensitive enzymes MSRE (HhaI + AclI) or (HpaII + AclI) depending on the frequency of their restriction sites within the studied fragments, which are cutting only unmethylated DNA, (2) Rd: one methylation-dependent restriction enzyme McrBC (MDRE), which is cutting only methylated DNA or (3) Rsd: both MSRE and MDRE enzymes (double digest, DD), and (4) R0: neither MSRE nor MDRE (mock control). Each 50 µl reaction contained 1 mg of gDNA, 1x CutSmart Buffer, 100 µg/mL BSA, 1 mM guanosine-5'-triphosphate, 3 % glycerol and 10 U of each enzyme used in restriction reaction, 50 % glycerol was used instead of enzymes in mock reaction in order to keep

restriction digest cocktail homogeneity. Digestions were incubated at 37 °C for 6 hrs followed by inactivation of the enzymes at 65 °C for 20 min. The enzymes, CutSmart Buffer, BSA, and guanosine-5'-triphosphate were purchased from New England Biolabs, USA. Restricted samples were analyzed by qPCR with locus-specific PCR primers and SYBR Green dye. An *in-silico* analysis was performed using EMBOS Cpgplot sequence analysis tool (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) from European Bioinformatics Institute (EMBL-EBI) to identify the CpG sites associated with the proximal promoter and transcription start site (TSS) for four genes. Sets of locus-specific PCR primers were designed to amplify gDNA at proximal CpG located within 1000 bp ($\pm$) of the transcription start site for each gene. Primers sequences, genomic loci, numbers of CpGs nucleotides and number of restriction sites contained in amplified amplicons are listed in Table 2. The PCR amplification was performed in 20 ml volume with 10 ml Maxima™ SYBR™ Green/ROX 2x qPCR Master Mix (Thermo Scientific, USA), 300 nM of each primer and 2 ml (40 ng) of digested template DNA using the qPCR System. The PCR conditions were as follows: 95 °C for 10 min, and 45 cycles of 95 °C for 1 min and temperature for optimized annealing for 1 min. Amplification for each sample was performed in triplicate in a 48-well plate. All primer pairs were tested to identify the annealing temperature for optimal efficiency and melting curve analysis was conducted after the reaction to verify the amplification of the desired products.

Calculations of DNA Methylation Occupancy

The Ct values from R0, Rs, Rd and Rsd, reactions were used to calculate the initial amount of DNA in each digest before PCR as following:

$$CMs = 2^{-Ct(Rs)}; CRd = 2^{-Ct(Rd)}; CRsd = 2^{-Ct(Rsd)}; CR0 = 2^{-Ct(R0)}.$$

The DNA methylation (%) was calculated as following:

hypermethylated DNA fraction (HM) = $Rs / (R0 - Rsd) \times 100$; unmethylated DNA fraction (UM) = $Rd / (R0 - Rsd) \times 100$; intermediately methylated DNA fraction (IM) = $1 - HM - UM$. If or $\Delta Ct(Rd - R0)$ or $\Delta Ct(Rs - R0) < 1.0$, The DNA methylation (%) was calculate as following: $HM = 1 - UM$, $UM = 1 - HM$ ^{76,77}.

Statistics

GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA) was used to generate graphical figures and to perform statistical analysis. Data are expressed as the mean $\pm$ SD. Statistical significance was defined as $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$.

Abbreviations

EMT: epithelial mesenchymal transition; 5-aza: 5-azacytidine; MSRE: methylation; sensitive restriction enzymes; MDRE: methylation dependent restriction enzymes; HM: hypermethylated DNA fraction; UM: unmethylated DNA fraction; IM: intermediately methylated DNA fraction; TSS: transcription start site.

Declarations

Data Availability

All data generated or analyzed during this study are included in the manuscript.

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Authors' contributions

YA and HM were responsible for the overall study design. YA, HM, CS, and AA performed the data analysis. YA, HM and CS drafted the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests

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Tables

Table 1: Primer sequences used for quantitative real-time PCR in this study.

Gene symbol	Primers sequence (5'-3')
<i>MDR1</i>	F- GAGGGGATGGTCAGTGTTGATGG
	R- ATCGTGGTGGCAAACAATACAGGT
<i>MRP1</i>	F- CTCCTGTGGCTGAATCTGGGC
	R-AGCACTTTGATCCCATTGAGAATTTCG
<i>MRP2</i>	F- CCTGGGAACATGATTCGGAAGCC
	R- GGAGGATTTCCCAGAGCCGAC
<i>CDH1</i>	F- GTGGGCCAGGAAATCACATCCTA
	R- GTTGGCAGTGTCTCTCCAAATCC
<i>EPCAM</i>	F-GCCGCAGCTCAGGAAGAATGTG
	R-CAACTGAAGTACACTGGCATTGACG
<i>SNAIL</i>	F-TGCAGGACTCTAATCCAGAGTTTACC
	R- GGTGGGATGGCTGCCAGC
<i>TWIST2</i>	F-CAAGCTGAGCAAGATCCAGACGC
	R-GGTCATCTTATTGTCCATCTCGTCG
<i>GAPDH</i>	F- ATGACCCCTTCATTGACC
	R- GAAGATGGTGATGGGATTTC

Table 2: Primer sequences used for methylation study using methylscreen method.

Gene symbol	Number of CpGs and their locations	Product size	Number of enzymes restriction sites	Primers sequence (5'-3')
<i>CDH1</i>	17 CpGs -306 to -82	224 bp	7 Acil 1 Hpa1 7 MCrBc	F-CAACTCCAGGCTAGAGGGTCA
				R- ACTTCCGCAAGCTCACAGGTGC
<i>EPCAM</i>	18 CpGs -465 to -276	189 bp	8 Acil 4 Hha1 7 MCrBc	F- CTCCTCGGAGGCCACCAAAGAT
				R- CCGCTGGTGCTCGTTGATGAGT
<i>SNAIL</i>	65 CpGs -688 to -165	523 bp	15 Acil 13 Hha1 19 MCrBc	F-AGAGGGCAGGGGTCTTCA
				R-ATTTCGCGCAGCAGTAGCGCA
<i>TWIST2</i>	15 CpGs -328 to -177	151 bp	6 Acil 3 Hha1 4 MCrBc	F- CCGAAGGGGGAGGCAAACTGA
				R- ACTCTAGCTGGGCTGGGTTGCT

Figures

Figure 1

Cancer cell lines display morphological changes associated with EMT. (A) A2780 cells showed small round-shaped cells consistent with an epithelial phenotype and A2780cis exhibit larger, more elongated shaped cells. (B) MCF7 cells showed squamous epithelial cell shape and MDA-MB-231 exhibit spindle-like morphology. (C) U-87 MG and T98G cells exhibit spindle like. Scale bar=50µm. morphology, T98G showed

a larger and more elongated cell shape compared to U-87 MG. (D) IC50 values for the studied cancer cell line.

Figure 2

Cancer cells exhibit different resistance to cisplatin-induced cytotoxicity. (A): A2780 and A2780cis cells. (B): MCF7 and MDA-MB-231 cells. (C) U-87 MG and T98G cells.

Figure 3

qPCR analysis of the MDR genes in A2780cis cells. MDR1 Expression increases in the resistant variant A2780cis cells. The expression levels were normalized to parental cells A2780.

Figure 4

Resistant cancer cells display molecular changes consistent with EMT. Down-regulation in epithelial genes: CDH1 and EPCAM, and up-regulation in mesenchymal marker SNAIL and TWIST2, were assessed using qPCR. (A) A2780cis compared to parental cells A2780. (B) MDAMB-231 cisplatin resistant cells compared to MCF7 cisplatin sensitive cells. (C) T98G cisplatin resistant cells compared to U-87 MG the more sensitive cisplatin cells.

Figure 5

EMT marker gene expression changes after exposure to the demethylating agent 5-azacytidine. qPCR validation of gene expression showed a significant up-regulation of EMT genes in A2780 cells treated with azacytidine compared with control.

A *CDH1* (-306 to -82 bp)

B

C

Figure 6

Methyscreen assay for CDH1 in A2780 and A2780cis cell lines. (A) An illustration showing the studied section of CDH1 gene, CpGs dinucleotide are shown as red arrows, restriction sites of methylation-sensitive enzymes MSRE are indicated as vertical domain, restriction sites of methylation-dependent enzymes MDRE shown as blue arrows. (B) The kinetic profiles obtained from CDH1 assay obtained from A2780 and A2780cis DNA are displayed. Each restricted genome's amplification is color coded by their treatment: mock restriction (black), MSRE restriction (blue), MDRE restriction (red) and a DD (green). (C) Charts display the result of EPCAM assay as a percentage of each portion of DNA.

A *EPCAM*(-465 to -276 bp)

A2780

Figure 7

Methyscreen assay for EPCAM in A2780 and A2780cis cell lines. (A) An illustration showing the studied section of EPCAM gene, CpGs dinucleotide are shown as red arrows, restriction sites of methylation-sensitive enzymes MSRE are indicated as vertical domain, restriction sites of methylation-dependent enzymes MDRE shown as blue arrows. (B) The kinetic profiles obtained from EPCAM assay obtained from A2780 and A2780cis DNA are displayed. Each restricted genome's amplification is color coded by their treatment: mock restriction (black), MSRE restriction (blue), MDRE restriction (red) and a DD (green). (C) Charts display the result of EPCAM assay as a percentage of each portion of DNA.

Figure 8

Methyscreen assay for SNAIL in A2780 and A2780cis cell lines. (A) An illustration showing the studied section of SNAIL gene, CpGs dinucleotide are shown as red arrows, restriction sites of methylation-sensitive enzymes MSRE are indicated as vertical domain, restriction sites of methylation-dependent enzymes MDRE shown as blue arrows. (B) The kinetic profiles obtained from SNAIL assay obtained from A2780 and A2780cis DNA are displayed. Each restricted genome's amplification is color coded by their treatment: mock restriction (black), MSRE restriction (blue), MDRE restriction (red) and a DD (green). (C) Charts display the result of SNAIL assay as a percentage of each portion of DNA.

Figure 9

Methyscreen assay for *TWIST2* in A2780 and A2780cis cell lines. (A) An illustration showing the studied section of *TWIST2* gene, CpGs dinucleotide are shown as red arrows, restriction sites of methylation-sensitive enzymes MSRE are indicated as vertical domain, restriction sites of methylation-dependent enzymes MDRE shown as blue arrows. (B) The kinetic profiles obtained from *TWIST2* assay obtained from A2780 and A2780cis DNA are displayed. Each restricted genome's amplification is color coded by their treatment: mock restriction (black), MSRE restriction (blue), MDRE restriction (red) and a DD (green). (C) Charts display the result of *TWIST2* assay as a percentage of each portion of DNA.