

Aberrant promoter methylation may be responsible for the control of CD146 (MCAM) gene expression during breast cancer progression*

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The CD146 (also known as MCAM, MUC-18, Mel-CAM) was initially reported on in 1987, as a protein crucial for melanoma invasion. Recently, it has been confirmed that CD146 is involved in progression and poor overall survival of many other cancers, including breast cancer. Importantly, in independent studies, CD146 was reported to be a trigger of epithelial to mesenchymal transition in breast cancer cells. The goal of our current study was to verify possible involvement of an epigenetic mechanism behind regulation of the CD146 expression in breast cancer cells, as it has been previously reported for prostate cancer. First, we analysed the response of breast cancer cells, varying in the initial CD146 mRNA and protein content, to an epigenetic modifier, 5-aza-2-deoxycytidine, and subsequently the methylation status of CD146 gene promoter was investigated, using direct bisulfite sequencing. We observed that treatment with a demethylating agent led to induction of CD146 expression in all analysed breast cancer cell lines, both at the mRNA and protein levels, which was accompanied by an elevated expression of selected mesenchymal markers. Importantly, CD146 gene promoter analysis showed aberrant CpG island methylation in 2 out of 3 studied breast cancer cell lines, indicating epigenetic regulation of the CD146 gene expression. In conclusion, our study revealed for the first time that aberrant methylation may be involved in expression control of CD146, a very potent EMT inducer in breast cancer cells. Altogether, the data obtained may provide basis for novel therapies, as well as diagnostic approaches enabling sensitive and very accurate detection of breast cancer cells.

Key words: CD146 (MCAM, MUC-18), EMT, epigenetic silencing, DNA methylation

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Abbreviations: 5'-Aza-dC, 5-aza-2-deoxycytidine; BS, direct bisulfite sequencing; EMT, Epithelial to Mesenchymal Transition; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor type 2; HPRT1, hypoxanthine Phosphoribosyl transferase 1; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; VIM, vimentin; TNBC, triple-negative breast cancer; TSS, transcription start sites

INTRODUCTION

Death of breast cancer patients is mainly caused by metastasis, transforming the locally confined disease into a disseminated and usually incurable one (Felipe Lima *et al.*, 2016). Metastasis can be defined as spreading of cancerous cells into distinct organs, followed by formation of a secondary tumor site. At the molecular level, this process is accompanied by actin cytoskeleton rearrangement and attenuation of cell-cell and cell-extracellular matrix adhesive interactions, which altogether resembles the Epithelial to Mesenchymal Transition (EMT), a morphogenetic process observed during development (Brabletz *et al.*, 2018; Nieto *et al.*, 2016). EMT is driven by mesenchymal transcription factors, including Snail, Slug, Zeb, and Twist, which are responsible for altering the epithelial transcriptional profile into mesenchymal one (Nieto *et al.*, 2016; Felipe Lima *et al.*, 2016). Breast cancer cells undergoing EMT become highly motile and invasive, which is especially apparent in the most aggressive estrogen-negative, progesterone-negative, human epidermal growth factor receptor-negative subtype (ER-/PR-/HER2-), defined as a triple-negative breast cancer (TNBC) (Khaled & Bidet, 2019; Felipe Lima *et al.*, 2016). TNBC diagnosed patients have a relatively poor prognosis and cannot be subjected to endocrine therapy or therapies directed against human epidermal growth factor receptor type 2 (HER2) (Schneider *et al.*, 2008). Interestingly, recent publication and meta-analysis, covering a high number of solid tumors, revealed a significant association of CD146 protein expression, EMT and poor survival of cancer patients (Zeng *et al.*, 2017). Importantly, independent studies reported that CD146 is highly expressed in TNBC and in metastatic breast cancer, which is contrary to normal tissue and benign tumors (de Kruijff *et al.*, 2018; Garcia *et al.*, 2007; Jang *et al.*, 2015; Zabouo *et al.*, 2009). Moreover, preclinical *in vitro* and *in vivo* studies revealed that aberrantly overexpressed CD146 is sufficient to induce acquisition of mesenchymal phenotype in breast cancer cells (Zeng *et al.*, 2012; Imbert *et al.*, 2012). Nevertheless, although overexpressed CD146 is considered an important oncogene in breast carcinogenesis, there is still lack of information about potential regulation of the CD146 gene expression in breast cancer cells. Despite the fact that CD146 is overexpressed in cancer cells, its amplification or mutation has been excluded so far (Wang & Yan, 2013). In the study presented here, by using breast cancer cell lines as a model, we revealed that CD146 gene promoter is aberrantly methylated in breast cancer cells and the

Table 1. Sequences of primers used for selected gene expression analyses.

Gene	Length (bp)	Primer Sequences
CD146	438	forward 5'-CCAAGCAACCTCAGCCATG-3' reverse 5'-CTGACTCCAGTCTGGGA-3'
HPRT1	504	forward 5'-TGGCGTCGTGATTAGTGATG-3' reverse 5'-TATCCAACACTTCGTGGGGT-3'
SNAI1	307	forward 5'-GGATCTCCAGGCTCGAAAGG-3' reverse 5'-CATTCTGGGAGAAGTCCGAG-3'
TWIST1	177	forward 5'-CCGTGGACAGTGATCCAG-3' reverse 5'-CCTTCAGTGGCTGATTGGC-3'
ZEB1	140	forward 5'-TCCTCGAGGCACCTGAAGAGG-3' reverse 5'-CAGAGAGGTAAGCGTTTATAGCC-3'
SNAI2	372	forward 5'-GCTACCAATGGCCTCTCTC-3' reverse 5'-TTGCCGAGATCTTGAAC-3'
CADHERIN 1	342	forward 5'-GCCAAGCAGCAGTACATTCTACAGC-3' reverse 5'-GTGGTCTTCTCAGTCAAATCC-3'
CADHERIN 2	370	forward 5'-GCGTCTGTCCACTCATAGGAGG-3' reverse 5'-GTGCCATTAGCCAAGGGAATCAGC-3'
MMP2	499	forward 5'-TGATGGAGAGGCAGACATCA-3' reverse 5'-ATACTTCTGTCCGGTCTGT-3'
MMP9	98	forward 5'-CTGAGAACCAATCTCACCGACA-3' reverse 5'-AGATTCGACTCTCCACGCA-3'

demethylating agent, 5-aza-2-deoxycytidine, can trigger its expression. According to our knowledge, this is the first study suggesting a role of epigenetics behind the *CD146* expression in breast cancer cells. Noteworthy, this finding not only sheds new light on regulation of *CD146* expression during breast carcinogenesis, but also provides an important rationale for novel therapeutic strategies in future. If confirmed with primary samples, it may contribute to a significant improvement of the diagnostic process, allowing early and very sensitive detection of breast cancer cells.

MATERIALS AND METHODS

R2 Database and statistical analysis of publically available data. The R2 database (<http://r2.amc.nl>) is a simple to use website-related tool for analysis and data visualization created at the Department of Oncogenomics in the Academic Medical Center (AMC) in Amsterdam, Netherlands. It gives an opportunity to perform different analyses based on well annotated datasets. In case of the study presented here, we selected tool “Correlate Genes” in the panel of 10 breast cancer data sets in order to check correlation between *CD146* and the panel of mesenchymal markers. Bonferroni corrected p-value of the Spearman correlation coefficient was used to show that the panel of mesenchymal marker encoding genes significantly correlated with *CD146* in breast cancer patients. Since the 9 mesenchymal markers were compared with *CD146*, p value below 0.0055 was considered as significant (0.05/9). The ten breast cancer data sets chosen for analysis included two sets (GSE7396 and GSE46563) composed of only lymph node negative patients and eight data sets (GSE1456, GSE12276, GSE2109, GSE3494, GSE102484, GSE29271, GSE69031, GSE36771) in which lymph node negativity was not the criterion for patient selection. Since the number of genes correlated with *CD146* was lower in data sets with confirmed lymph node negativity in comparison to other data sets, we applied cluster analysis (nearest neighbor, Euclidean distance) algorithm to verify if these two data set are indeed different from all the

others (Statistica, TIBCO Software Inc.). For the purpose of analysis, the non-significant Spearman correlation coefficient was set as zero.

Cell lines and cell culture. MCF7 and T47D breast cancer cell lines, as well as human fibroblast (Hs27) cells, were purchased from the American Type Culture Collection (Rockville, MD, USA). MDA-MB-231 breast cancer cell line was purchased from Sigma-Aldrich (St. Louis, MO, USA). All cells were grown in RPMI 1640 medium (Gibco Life Technology), supplemented with 10% fetal bovine serum (FBS), (EURx, Gdansk, Poland), 2 mmol/L L-glutamine and 100 units/mL penicillin G and Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). For demethylation study, MCF7, T47D and MDA-MB-231 cells were cultured with and without 10 μ M 5-aza-2-deoxycytidine (#PA-03-1241-P, Pol-AURA, Poland) for 6 days, and on day 4 the cell culture media were exchanged for fresh ones, with and without 5-aza-2-deoxycytidine, respectively.

RNA isolation and cDNA synthesis.

Total RNA was isolated from harvested cells using Gene Matrix Universal RNA kit (EURx, Gdansk, Poland). NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine concentration and quality of the isolated RNA. Total RNA (1 μ g) was used for cDNA synthesis according to the manufacturer's protocol (EURx, Gdansk, Poland).

Reverse transcription polymerase chain reaction (RT-PCR). For PCR reaction, the Color OptiTaQ PCR Master Mix (2x) (EURx, Gdansk, Poland) was used according to the manufacturer's protocol. Sequences of reverse and forward primers used in this study are presented in Table 1. For *CD146*, *HPRT1*, *SNAI1*, *TWIST1*, *ZEB1* and *SNAI2*, the PCR reactions were carried out as previously described (Dudzik *et al.*, 2019). For *CADHERIN1* and *CADHERIN2*, the conditions were as following: initial denaturation at 95°C for 5 min; followed by 30 cycles: 30 s at 95°C, 30 s at 58°C and 30 s at 72°C; and final extension at 72°C for 10 min. For *MMP2* and *MMP9* the following conditions were applied: initial denaturation at 94°C for 3 min; followed by 30 cycles: 45 s at 94°C, 60 s at 59°C and 60 s at 72°C; and final extension at 72°C for 5 min. PCR product was visualized on 1.5% agarose gel stained with ethidium bromide and photographed with Bio-Rad ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, USA). *HPRT1* was used as internal control to ensure equal sample loading. In case of MDA-MB-231 cells, the RT-PCR analysis of *CD146* was performed according to a modified, adjusted PCR protocol in which 25 cycles were used instead of 30 cycles, whereas all other conditions remained unchanged.

The *CD146* and *HPRT1* primers were manufactured by IBB PAN (Warsaw, Poland). All other primers were purchased from Sigma-Aldrich (St. Louis, MO, USA). The images of the gels were captured using Bio-Rad ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, USA) and subsequently analyzed by means of publicly available ImageJ software. All values were normalized to the *HPRT1* signal.

Western blot. The cells were lysed and protein concentration was determined as previously described (Kocemba-Pilarczyk *et al.*, 2018). Protein lysates (30 μ g)

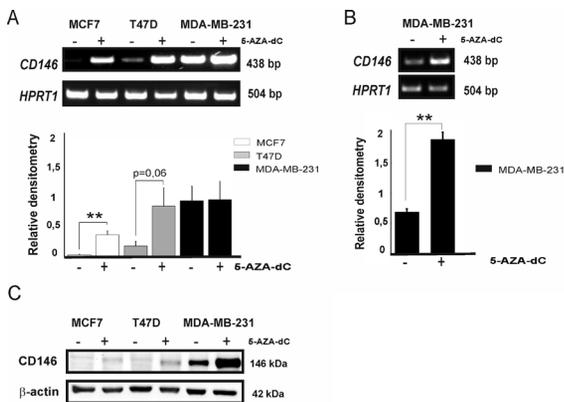


Figure 1. The effect of demethylating agent, 5-aza-2-deoxycytidine, on expression of CD146 in breast cancer cell lines at the mRNA and protein level.

(A) **upper panel:** Representative results of RT-PCR analysis for CD146 expression in breast cancer cell lines cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. **Lower panel:** Graph presenting densitometry analysis of three independent experiments. Mean values \pm S.E.M. (standard error of the mean) after normalization to HPRT signal are shown. $**p < 0.01$, by student's *t*-test, *p*-value between 0.05 and 0.1 was considered as an indication of a trend. (B) **upper panel:** Representative results of RT-PCR analysis for CD146 expression in MDA-MB-231 breast cancer cell line cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. **Lower panel:** Graph presenting densitometry analysis of three independent experiments. Mean values \pm S.E.M. after normalization to HPRT signal are shown. $**p < 0.01$, by student's *t*-test. (C) Western blot analysis of CD146 protein expression level in breast cancer cell lines cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. Representative experiment of three independent ones is shown.

with CD146 in all analysed data sets, regardless of the lymph node status. In order to verify if two lymph node negative data sets are different from the others, cluster analysis was performed. This approach further confirmed that two lymph node negative data sets clustered together as being different from all the others. Thus, the results of our analysis clearly demonstrated, based on transcriptomic data, the existence of association between CD146 expression, mesenchymal profile and the progression of breast cancer.

The effect of 5-aza-2-deoxycytidine on expression of CD146 in breast cancer cell lines

In order to check whether *CD146* expression is regulated at the transcriptional level by an epigenetic modifier, 5-aza-2-deoxycytidine, as it has been shown previously in prostate cancer cell lines (Dudzik *et al.*, 2019), two epithelial breast cancer cell lines (MCF7 and T47D) (Dai *et al.*, 2017) and a mesenchymal one (MDA-MB-231) (Dai *et al.*, 2017) were cultured with and without 5-aza-2-deoxycytidine (10 μ M) for 6 days, as previously described, followed by *CD146* expression analysis at the mRNA and protein level. As expected, basal expression of *CD146* was low in the MCF7 and T47D cancer cell lines with epithelial characteristics, and high in the MDA-MB-231 cell line with mesenchymal expression profile. As shown in Fig. 1, *CD146* expression was apparently induced in the MCF7 and T47D cells, whereas in MDA-MB-231 its high basal expression disabled correct assessment of expression difference between the control and 5-aza-2-deoxycytidine treated cells. Thus, in order to verify if high *CD146* expression in MDA-MB-231 is still further inducible, we performed an expression analysis according to an adjusted, modified

PCR protocol, as described in the Materials and Methods section. Interestingly, in MDA-MB-231, the *CD146* expression was also significantly induced, regardless of the high basal level and the mesenchymal characteristics of these cells (Fig. 1B). As for the protein analysis (Fig. 1C), the demethylating compound had significantly induced CD146 expression in all analysed cell lines. This effect was clearly visible in the epithelial lines with very low basal expression of CD146, as well as in the MDA-MB-231 cells, where the relatively high basal expression was still apparently inducible, in accordance with the data obtained at the mRNA level. Altogether, this data suggest that epigenetic silencing plays an important role in the control of CD146 expression in breast cancer cells with epithelial characteristics (MCF7 and T47D), whereas in mesenchymal breast cancer cell line (MDA-MB-231) expression of CD146 is apparently not fully unleashed and still partially trapped by the epigenetic mechanism.

The effect of 5-aza-2-deoxycytidine on induction of the mesenchymal profile

To determine if *CD146* gene expression increase is associated with a mesenchymal profile induction, we analysed the influence of an epigenetic modifier, 5-aza-2-deoxycytidine (10 μ M), on expression panel of mesenchymal markers: Slug (*SNAI2*), Twist1 (*TWIST1*), Zeb1 (*ZEB1*), Snail (*SNAI1*), N-cadherin (*CADHERIN 2*), Vimentin (*VIM*), matrix metalloproteinase-2 (*MMP2*) and matrix metalloproteinase-9 (*MMP9*), and an epithelial marker E-cadherin (*CADHERIN 1*). The mesenchymal markers subjected to this analysis were selected based on the expression correlation studies performed in breast cancer patients (Table 2). As shown in Fig. 2A, treatment of breast cancer cell lines with the demethylating agent resulted in gene expression changes at the mRNA level for several mesenchymal markers, such as *SNAI1*,

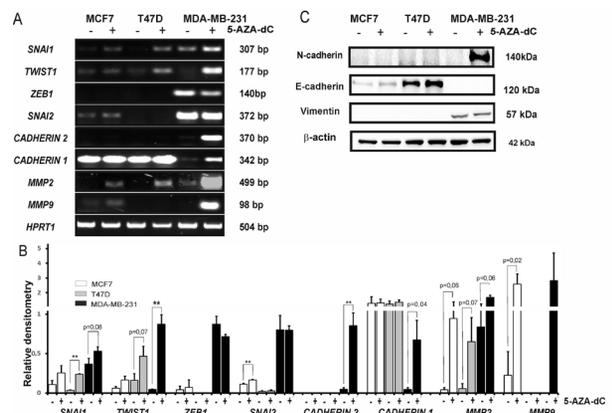


Figure 2. The effect of demethylating agent, 5-aza-2-deoxycytidine, on expression of selected mesenchymal markers and E-cadherin, in breast cancer cell lines at the mRNA and protein level.

(A) Representative results of RT-PCR analysis of mesenchymal markers and E-cadherin expression in breast cancer cell lines cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. (B) Densitometric quantification of mRNA expression of selected EMT-related genes in breast cancer cell lines cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. Data are shown as mean values \pm S.E.M. of 3 independent experiments after normalization to respective HPRT1 levels. $**p < 0.01$, by student's *t*-test, *p*-value between 0.05 and 0.1 was considered as an indication of a trend. (C) Western blot analysis of N-cadherin, E-cadherin and Vimentin protein expression in breast cancer cell lines cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h. Representative experiment of two independent ones is shown.

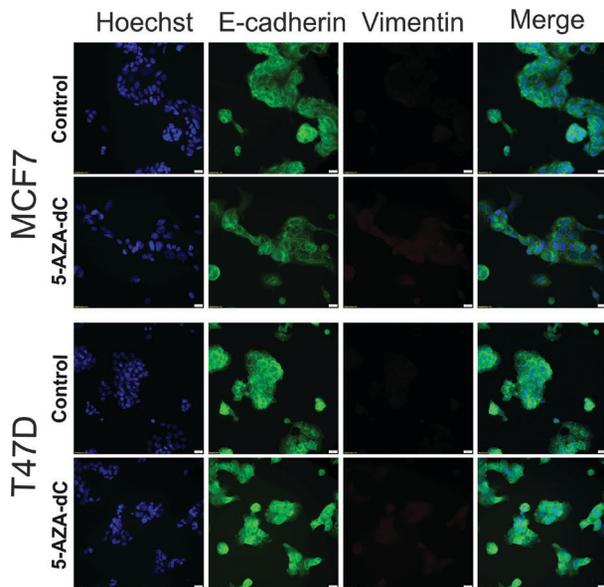
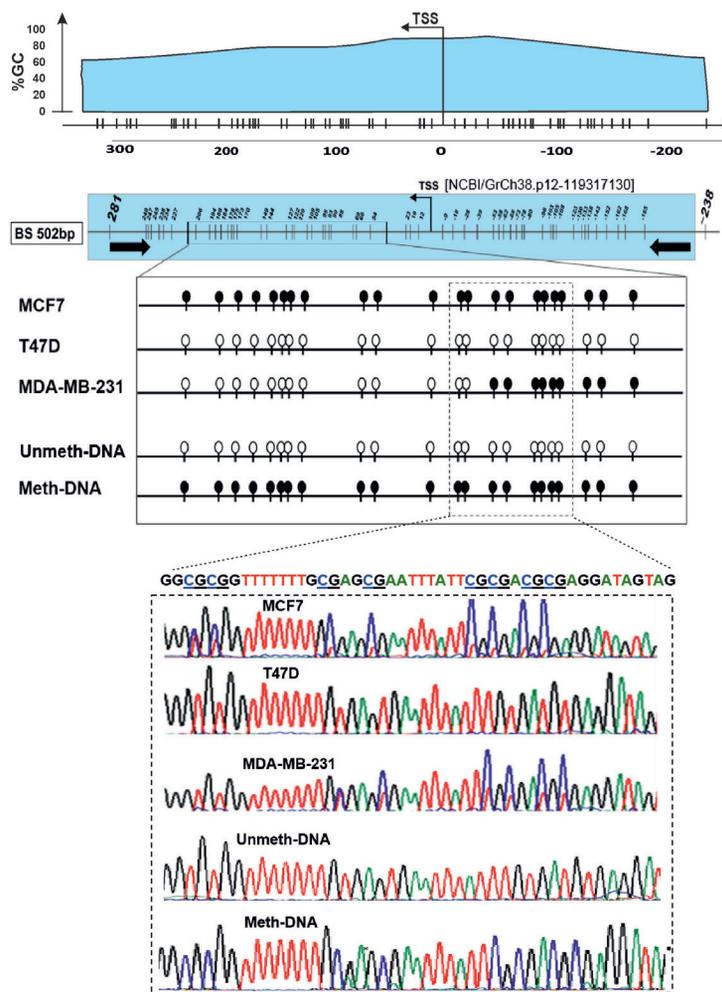


Figure 3. The effect of demethylating agent, 5-aza-2-deoxycytidine, on cell morphology and expression of E-cadherin and Vimentin in epithelial breast cancer cell lines (MCF7 and T47D), assessed by immunofluorescence.

Immunofluorescence staining of E-cadherin and vimentin in breast cancer cell lines (MCF7 and T47D) cultured in the absence and presence of 10 μ M 5-aza-2-deoxycytidine for 144 h.



The analysis of CD146 gene promoter methylation in breast cancer cell lines

Since presence of the CpG island in *CD146* gene promoter has been already described and its proper location reported by our group (Kocemba *et al.*, 2016), we subjected the *CD146* promoter region, including the CpG island encompassing the transcriptional start site in exon 1, to methylation analysis using direct bisulfite-sequencing (BS) of PCR products. DNA isolated from human fibroblasts and *in vitro*

Figure 4. Pattern of CpG island methylation in selected region of the *CD146* gene promoter.

Schematic representation of the CpG island area analyzed in the *CD146* gene promoter. Arrows mark the positions of bisulfite sequencing primers. Each of the CpG dinucleotides is presented as a vertical line. The figure shows a representative fragment containing representative 22 CpG sites out of 50 analyzed ones. Electropherograms of bisulfite modified DNA from a representative *CD146* gene promoter CpG island fragment in MCF7, T47D, MDA-MB-231 is shown together with DNA isolated from human fibroblasts and *in vitro* methylated DNA used as an unmethylated and methylated control DNA, respectively. White circles indicate unmethylated CpG sites, black circles indicate the presence of methylation in CpG sites detected by direct Sanger sequencing of the bisulfite-modified DNA.

SNAI2, *TWIST1*, *MMP2*, *MMP9* and *CADHERIN 2*, whereas densitometry analysis of three independent experiments (Fig. 2B) confirmed significant changes/or indication of the trend for *SNAI1* (T47D, MDA-MB-231), *TWIST1* (T47D, MDA-MB-231), *SNAI2* (MCF7), *CADHERIN 2* (MDA-MB-231), *MMP2* (MCF7, T47D, MDA-MB-231) and *MMP9* (MCF7). As for the epithelial marker, *CADHERIN 1*, the basal mRNA expression was high in cell lines with epithelial characteristics and low in MDA-MB-231. Of note, in all three independent experiments, we observed an increase of *CADHERIN 1* expression at the mRNA level in the MDA-MB-231 cells, regardless of the induction of mesenchymal marker expression (*SNAI1* and *TWIST1*) in these cells, which are the well known transcription factors inhibiting the *CADHERIN 1* gene promoter. Concerning protein analysis, E-cadherin was not visibly induced in MDA-MB-231, opposite to N-cadherin, whose expression was reproducibly enhanced after treatment with 5-aza-2-deoxycytidine, in two independent experiments. As for vimentin, it was apparently expressed only in the MDA-MB-231 cell line and not further induced by treatment with 5-aza-2-deoxycytidine. Undoubtedly, however, the fact that an epigenetic mechanism appears to control expression of such an important oncogene in breast cancer seems to be of high importance. To further determine if 5-aza-2-deoxycytidine, resulting in *CD146* induction, may as well trigger the changes in morphology of epithelial breast cancer cells and/or induce the protein expression changes at the level of a single cell, we performed an immunofluorescence analysis of E-cadherin and Vimentin in the MCF7 and T47D cells cultured for 6 days with and without 5-aza-2-deoxycytidine. As determined in Fig. 3, we neither observed an apparent changes in cell morphology, nor, in accordance with the Western blot results, an alteration in the E-cadherin and Vimentin cell content.

methyated DNA was modified and subsequently used in BS sequencing as unmethylated and methylated control, respectively, to validate our experimental set-up. BS analysis revealed that two (MCF7 and MDA-MB-231) out of the three breast (Fig. 4) cancer cell lines tested (MCF7, T47D and MDA-MB-231) displayed hypermethylation of CpG island in the *CD146* promoter area, whereas T47D was methylation-free. Notably, BS analysis revealed heterogeneous methylation in MCF7 and MDA-MB-231, suggesting clonal variation in the methylation pattern of the CpG island area in these cell lines. Importantly, aberrant methylation detected in the CpG island of the *CD146* gene (Fig. 4) was present in the promoter area encompassing exon one, the region reported previously as crucial for epigenetic silencing of gene expression (Brenet *et al.*, 2011).

DISCUSSION

To our knowledge, this is the first study showing methylation of the *CD146* promoter region in breast cancer cells, and concomitantly suggesting that an epigenetic mechanism may be important in expression control of this relevant metastasis related-oncogene. In our previous paper, using prostate cancer cell lines, we have shown that the *CD146* gene is induced by a demethylating compound, 5-aza-2-deoxycytidine, however, analysis of the CpG island region in those cells did not confirm presence of promoter methylation (Dudzik *et al.*, 2019). Of note, the link between epigenetics and expression of the *CD146* gene was reported previously in prostate cancer patients but the authors of that study incorrectly localized the CpG island in the *CD146* gene promoter (Liu *et al.*, 2008), and in consequence misinterpreted the results suggesting, contrary to the dogma, that presence of the CpG island methylation correlates with increase in the *CD146* gene expression in prostate cancer cells (Liu *et al.*, 2008). This mistake was pointed out in a Letter to the Editor published in *The Prostate* in 2016 (Kocemba *et al.*, 2016), also determining proper localization of the CpG island in the *CD146* gene promoter (Kocemba *et al.*, 2016). So far, in literature, the relation between DNA methylation in the promoter area (regardless of presence of a CpG island) and gene expression inactivation has been well confirmed (Han *et al.*, 2011; Smith & Meissner, 2013). It has been verified that canonical promoter methylation is associated with expression of imprinted genes, the process of X chromosome inactivation and tissue specific gene regulation (Smith & Meissner, 2013; Urbano *et al.*, 2019). On the other hand, CpG islands observed around transcription start sites in 50% of gene promoters are methylation free in non-cancerous cells, regardless of the expression state of the gene of interest (Jones, 2012). However, de novo hypermethylation of canonical CpG islands has been observed in cancer cells in association with inactivation of gene expression (Jones & Baylin, 2007). Although we observed the CpG island methylation of the *CD146* promoter in 2 out of 3 analysed breast cancer cell lines, it does not have to be the only and unique methylated region which controls *CD146* expression, since in the T47D breast cancer cell line and three prostate cancer cell lines published previously (Dudzik *et al.*, 2019), the *CD146* expression was induced by demethylating treatment, while the analysed CpG island was methylation free. Importantly, recent studies revealed that methylation in the upstream and downstream CpG island shores may inhibit gene transcription in cancer cells, whereas the CpG island itself

can remain methylation free (Rao *et al.*, 2013; Irizarry *et al.*, 2009). Even more, in a recent publication, Skvortsova *et al.* revealed that methylation present in the CpG island shores, particularly in the breast cancer cells, is involved in transcriptional silencing of gene expression. These data clearly confirm that promoter methylation in silenced genes in cancer does not have to be confined only to the CpG island (Skvortsova *et al.*, 2019). To verify exactly which promoter part must be demethylated to unleash the *CD146* expression would be definitely of high importance. Whereas overexpressed *CD146* is considered to be oncogenic in breast cancer cells, as reported by independent studies (Zeng *et al.*, 2012; de Kruijff *et al.*, 2018; Garcia *et al.*, 2007; Zabouo *et al.*, 2009; Imbert *et al.*, 2012), the methylation-based silencing of its expression in these cells seems to be quite surprising. According to our hypothesis, at the beginning of the disease *CD146* may be targeted for aberrant promoter methylation/or it has been already methylated in consequence of tissue specific epigenetic silencing, whereas the loss of methylation in advanced tumour unleashes *CD146* expression, leading to metastasis. The fact that *CD146*-dependent induction of a mesenchymal profile has been already described in the literature (Imbert *et al.*, 2012; Zeng *et al.*, 2012; Zabouo *et al.*, 2009) allows us to speculate that increase in expression of mesenchymal related genes may result from *CD146* induction, triggered by an epigenetic modifier. On the other hand, we cannot exclude that the epigenetic modifier, in parallel to the increase in *CD146* expression, directly induces mesenchymal profile in the breast cancer cell lines, as the EMT induction in consequence of 5-aza-2-deoxycytidine application has been already reported for breast cancer cells (Su *et al.*, 2018). Definitely, more research is needed to elucidate the independent role of *CD146*, expression triggered by an epigenetic modifier, in EMT induction in breast cancer cells. Interestingly, in a mesenchymal cell line (MDA-MB-231), we observed an increase in the epithelial marker, E-cadherin, at the mRNA level, which can be the direct consequence of demethylating agents, since epigenetic silencing of E-cadherin expression by aberrant promoter methylation has been reported previously in cancer cells of mesenchymal characteristics and what is more, exactly in MDA-MB-231. Thus, it can be concluded that our results for *CADHERIN 1* in MDA-MB-231 simply indicate a proper experimental setup for the demethylating treatment. It is also important to mention that Imbert *et al.*, after overexpression of *CD146* in the MCF7 cell line, reported lack of alteration in *TWIST1*, *SNAIL1*, *MMP2* and *MMP9* expression and an increase in *CADHERIN 2* and *VIM* (Imbert *et al.*, 2012), whereas changes in the mesenchymal markers' profile were completely different in our current study, which is most probably the consequence of combined action of *CD146* and the demethylating agent. Undoubtedly, however, showing that expression of *CD146* is controlled by an epigenetic mechanism seems to be the most important message from our study. Moreover, our analysis also suggests coexistence of cells with methylated and unmethylated *CD146* gene promoter, which indicates that alterations in the *CD146* gene promoter methylation may reflect the process of clonal selection during breast cancer progression. In this context, the aspect of epigenetic control of *CD146* expression takes on a particular significance in research centred on application of 5-aza-2-deoxycytidine in treatment of breast cancer patients, and should be considered in planning therapy combined with epigenetic modifiers. It is especially important, since this kind of therapy for breast cancer patients has been

currently investigated (Connolly *et al.*, 2017). Our data undoubtedly requires further verification to potentially reveal the scenario of methylation changes in the CD146 gene promoter during development of breast cancer progression, as we cannot exclude that CD146 acts as a tumour suppressor at the initial stage of carcinogenesis, as suggested by the study of Shih and others (Shih *et al.*, 1997), and turn into an oncogene in the advance stage, when the transcriptional profile of cancer cells is significantly altered. Overall, our study provides strong basis for further research on epigenetic regulation of CD146, which can significantly contribute to novel therapies and/or development of DNA methylation-based assay for sensitive detection of breast cancer cells.

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