PERGAMON

European Journal of Cancer 39 (2003) 517–523

European Journal of Cancer

www.ejconline.com

E-cadherin expression is silenced by DNA methylation in cervical cancer cell lines and tumours

C.-L. Chen[a], S.S. Liu[b], S.-M. Ip[b], L.C. Wong[b], T.Y. Ng, H.Y.S. Ngan[b,*]

[a]*Department of Obstetrics and Gynecology, First Hospital of Peking University, Beijing, China*
[b]*Department of Obstetrics and Gynecology, Queen Mary Hospital, The University of Hong Kong, Hong Kong*

Received 1 October 2001; received in revised form 25 January 2002; accepted 31 May 2002

Abstract

A previous study showed E-cadherin expression was lost in some cervical cancer cell lines and tumours. This study was designed to clarify the significance of DNA methylation in silencing *E-cadherin* expression. We examined promoter methylation of *E-cadherin* in five cervical cancer cell lines and 20 cervical cancer tissues using methylation-specific PCR (MSP) and bisulphite DNA sequencing. The correlation of *E-cadherin* methylation and expression together with methyltransferase (*DNMT1*) were further studied. We found that hypermethylation of *E-cadherin* was involved in five cervical cancer cell lines and 40% (8/20) of cervical cancer tissues. E-cadherin protein was lost in 6/8 (75%) samples and 3/5 (60%) cell lines with promoter methylation. *E-cadherin* methylation was significantly correlated with increased *DNMT1*. Using an antisense *DNMT1* oligo to transfect into SiHa HeLa C33A cell line, E-cadherin protein was re-expressed. We concluded that loss of *E-cadherin* expression was in part correlated with DNA methylation and *DNMT1* expression in cervical cancer.

Keywords: E-cadherin; Methylation; Cervical cancer; MSP; Methyltransferase; Antisense oligonucleotide

1. Introduction

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent interactions between adjacent epithelial cells [1]. Decreased or loss of E-cadherin expression is a common finding in many human epithelial cancers including colon and prostate cancer especially in poorly differentiated, advanced-stage carcinomas [2,3]. The E-cadherin-mediated cell adhesion system is inactivated by multiple mechanisms. It may be inactivated as a result of genetic alteration, reduced gene expression or changes of other cadherin–catenin complexes [1]. It has been reported that aberrant hypermethylation of CpG islands in the *E-cadherin* promoter region [4–7], together with alterations in chromatin structure and transcription factor activity [4,5], may conspire to suppress E-cadherin expression [4,8]. However, in most cancers with reduced or absent E-cadherin gene and protein expression, mutations in *E-cadherin* are rarely detected [1].

Epigenetic silencing of tumour suppressor or related genes such as the tissue inhibitors of metalloprotease [9], DNA repair proteins hMLH-1 [10], p16 and p15 [11] had been studied by CpG island methylation. It is known that DNA methylation results from a methyl transfer reaction by DNA methyltransferases. So far, several DNA methyltransferases are identified including DNMT1, DNMT2, DNMT3A and DNMT3B. DNMT1 is the predominant mammalian DNA methyltransferase which is considered to have both maintenance and *de novo* methylation activity [12]. Elevated DNMT1 activity, mRNA and protein were investigated in some human cancers such as colon cancer and hepatocarcinoma [13,14]. Over-expression of *DNMT1* mRNA was significantly associated with CpG island methylator phenotype (CIMP) [15]. Using the methylation inhibitor 5-aza deoxycytidine, 5-azacytidine [5], or antisense methyltransferase oligonucleotide [16], the methylation state of the CpG island can be changed resulting in activation of some of the silenced genes [5]. DNMT3A and DNMT3B also have *de novo* methylation function, however, they are not associated with CIMP [15]. There are no reports of DNMT2 because it is not essential for

* Corresponding author. Tel.: +852-2855-4684; fax: +852-2855-0947.
E-mail address: hysngan@hkucc.hku.hk (H.Y.S. Ngan).

0959-8049/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0959-8049(02)00175-2

global *de novo* or maintenance methylation of DNA in embryonic stem cells [17].

In cervical cancer, previous data showed that the expression of E-cadherin at both the mRNA and protein levels was reduced especially in invasive types [18–21]. An immunochemical staining study demonstrated that in intra-epithelial neoplasia (CIN), the presence and localisation of cytoplasmic E-cadherin were significantly correlated with cervical CIN grade. Loss of membranous E-cadherin expression was detected in 4/7 metastatic deposits, as well as in three cervical cancer cell lines (HeLa, SiHa, C33A) [22]. These data suggested that E-cadherin might be an important factor in cervical cancer. To understand the role of E-cadherin and the mechanisms of DNA methylation in the inactivation of E-cadherin in cervical cancer cell lines and tissues, we analysed the E-cadherin expression and methylation status by western blot analysis, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), methylation-specific PCR (MSP), bisulphite DNA sequencing and with a methyltransferase activity assay. To further understand the role of DNA methyltransferase in E-cadherin inactivation, we designed an antisense methyltransferase oligo and a mismatch control oligo to transfect into cervical cancer cell lines.

2. Materials and methods

2.1. Cell culture and treatment with 5-aza-deoxycytidine

Cervical cancer cell lines (HeLa, SiHa, C33A, Caski and C4I) were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Minimum Essential Medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FBS). Selected cell lines were seeded at 20% confluence and incubated with a final concentration of 3 μM 5-aza-deoxycytidine (5-Aza CdR) (Sigma) for 24 h and washed with phosphate-buffered solution (PBS) twice then changed into medium without drugs. The media were changed every other day until confluence.

2.2. Antisense methyltransferase oligo treatment

The antisense methyltransferase oligo was designed to hybridise to the 5′-untranslated region [23]. Antisense sequence (5′-**AAG**CATGAGCACCGTT**CTCC**-3′); Control sequence (5′-**AATG**ATCAG**G**ACC**C**TT**GTCC**-3′) with six mismatch bases. The two oligos were 2′-methylphosphorothioate modified with eight 2′-*O*-methyl modification at the first and the last four bases. Cells were treated with these oligos at doses from 0 to 70 nM in the presence of Lipofectin (5 μg/ml) (Life Technology). Cells were seeded in 40% confluence in the first day, then transfected with antisense or control oligo for 5 h without FBS in the following 2 days then washed with PBS twice and changed into medium with FBS. The cells were harvested 24 h later. For continuous transfection, cells were transfected with antisense oligo or control oligo every day and were split every second day.

2.3. Cervical cancer tissues and normal cervical tissues

Twenty cervical cancer samples and 10 normal cervical tissues were collected during operation in the Department of Obstetrics and Gynecology, the University of Hong Kong. The samples were quickly frozen in liquid nitrogen, DNA was extracted using proteinase K/phenol-chloroform protocol. RNA and protein were extracted by Tripure isolation reagent (Boehringer Mannheim). Among the 20 cervical cancer patients, 5 of them were stage IB, 5 were stage IIA or IIB, and the remaining 10 were stage IIIB.

2.4. Western blot

Western blot analysis of cervical cancer cell lines was performed as previously described in Ref. [4]. 50 μg protein was loaded on 8% polyacrylamide gels and samples were electrophoresed at 100 volts for 2 h at room temperature. Protein was transferred to nitrocellulose membranes by electroblotting. After the blocking of nitrocellulose membranes in 5% non-fat dry milk for 1 h, E-cadherin protein was detected by incubating at 4 °C overnight with a mouse monoclonal antibody against human E-cadherin (Transduction Lab) at a 1:2000 dilution, followed by incubating this mixture with a rabbit anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) (Amersham) at room temperature for 1 h at a 1:1000 dilution. Bands were visualised by enhanced chemiluminescence (ECL) (Amersham) and exposed to Kodak BioMax MR film for 20–30 s. The same nitrocellulose membrane was stripped and incubated with β-actin monoclonal antibody (Sigma) at a 1:2000 dilution for 1 h which acted as a control for loading and blotting.

2.5. Methylation analysis by MSP and bisulphite sequencing

1 μg of DNA was modified by CpGenome DNA modification kit (Intergen). Methylation of the E-cadherin 5′ CpG islands was analysed by methylation-specific PCR (MSP). The primers used were previously described, and span the transcription start site of *E-cadherin* [24]. The primers were as follows: *E-cadherin* methylated sense primer: TTAGGTTAGAGGGTTATCGC GT; antisense primer: TAACTAAAAATTCACCTAC CGAC; *E-cadherin* unmethylated sense primer: TAAT TTTAGGTTAGAGGGTTATTGT; antisense primer: CACAACCA ATCAACAA CACA. The PCR was

started with a hot start at 95 °C for 5 min before the addition of 1.25 units of Taq polymerase, followed by 35 cycles of 95 °C 30 s, 57 °C 30 s, 72 °C 30 s, and finally 72 °C for 5 min for the methylated primer. The annealing temperature was 53 °C for the unmethylated primer. The PCR products for methylated and unmethylated *E-cadherin* were 116 and 97 base pairs, respectively. A human placenta DNA was used as an unmethylated positive and methylated negative control. A breast cancer DNA (known to be *E-cadherin*-methylation positive from the Department of Surgery) was selected as a methylation-positive control. Bisulphite genomic sequencing was performed on an ABI PRISM 310 system after PCR and purification and using the sense methylated and unmethylated primers.

2.6. RT-PCR

2 μg of total RNA was reverse-transcribed using 200 U Superscript II RT (GIBCO) in a total volume of 20 μl. PCR was done using 1 ul of the cDNA. The primers used were as following: P1F 5′-CCTTCCTCCCAATACATCTCCC-3′ and P1R 5′-TCTCCGCTCCTTCTT CATC-3′ for *E-cadherin* with 432bp band, *β-actin* was also performed at the same time using primers: P2F 5′-TCACCAACTGGGACGACATG-3′ and P2R 5′-AC CGGAGTCCATCACGATG-3′ for a 242 bp band. The RT-PCR was performed at 56 °C and 30 cycles. The cycles were selected after doing the standard curve from 20, 24, 28, 32, 36 and 40 cycles (Fig. 1a). The expression level was determined by UVP Gel work 1.0. The ratio of the band volumes of *E-cadherin* and *β-actin* represented the mRNA level. The primers for *DNMT1* were as follows: P3F 5′-GTGGCAGTGGACGGAGCAAG-3′, P3R 5′-AACC AGTGGGCGTGAAACAT-3′. This *DNMT1* was performed together with *β-actin* using an annealing temperature of 62 °C, 30 cycles and resulted in a 361 bp band. Each RT-PCR was performed twice.

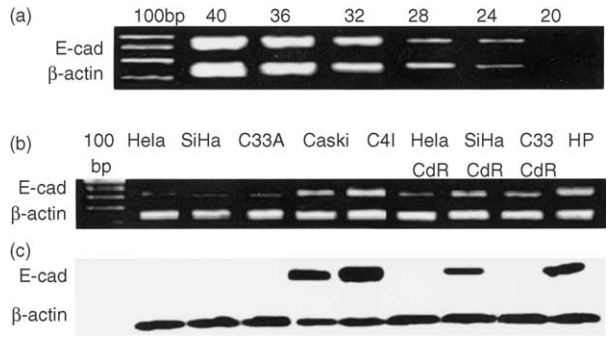


Fig. 1. *E-cadherin* expression in cervical cancer cell lines: (a) standard curve of RT-PCR for *E-cadherin* and *β-actin*. (b) Semi-quantitative RT-PCR. *E-cadherin* mRNA expression was determined in five cervical cancer cell lines before and after 5-aza-deoxycytidine (CdR) treatment by semi-quantitative RT-PCR. Human placenta (HP) was used as a control. (c) Western blot of E-cadherin and β-actin.

2.7. DNA methyltransferase activity assay

DNA methyltransferase activity assay [13] was performed to test DNA methyltransferase level after antisense oligo treatment as well as the methyltransferase level in the cancerous and normal cervical tissues. 2 μg of protein lysate diluted to 15 μl was mixed with 2 μl (0.5 μg) poly [d(I-C).d(I-C)] (Amersham) and 3 μCi S-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-SAM) (Amersham) in a total volume of 20 μl. This solution was incubated at 37 °C for 2 h, then stopped by 300 μl stop solution containing 1% sodium dodecyl sulphate, 2 mM ethylene diamine tetra acetic acid (EDTA), 5% butanol, 125 mM sodium chloride, 0.25 mg/ml carrier salmon testis DNA and 1 mg/ml proteinase K. After incubation for 30 min at 37 °C, DNA was purified by phenol: chloroform extraction and ethanol precipitation. RNA was removed by 0.3 M sodium hydroxide. The final solution was spotted on Whatman GF/C filters and washed in 5% trichloroacetic acid (TCA) and 70% ethanol, then placed in 5 ml scintillation fluid and counted in a Beckman scintillation counter. All reactions were performed in duplicate and included a positive control (same protein lysate) and a negative control (without poly [d(I-C).d(I-C)]. Results are shown in disintegrations per minute (DPM) per 2-hour incubation minus the negative control.

2.8. Statistics

Statistical analyses were performed using unpaired Student *t*-test, a P value of <0.05 was considered a significant statistical difference. The correlation between *E-cadherin*mRNA and *DNMT1* both mRNA and methyltransferase activity in the methylated and unmethylated *E-cadherin* groups were assessed by the Spearman correlation test.

3. Results

3.1. Loss of E-cadherin expression in cervical cancer cell lines and tissues is in part due to DNA methylation

Among five cervical cancer cell lines (SiHa, HeLa, C33A, Caski and C4I), three of them (SiHa, HeLa, C33A) had no E-cadherin protein and a low mRNA level (Fig. 1). MSP analysis showed that *E-cadherin* was hypermethylated in these three cell lines without unmethylated bands. The other two cell lines Caski and C4I harboured both methylated and unmethylated alleles (Fig. 2). Bisulphite DNA sequencing demonstrated that all the 10 CG sites were methylated in the five cell lines (Fig. 3).

Promoter methylation was found in 40% (8/20) of cervical cancer tissues. E-cadherin protein was lost in 75% (6/8) of the cervical cancer tissues with promoter

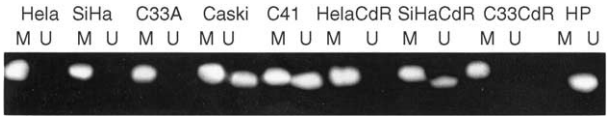
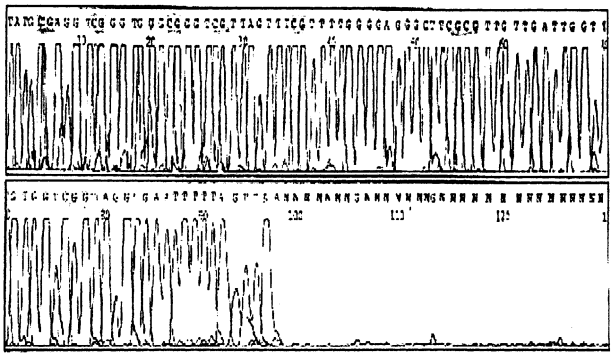


Fig. 2. Methylation analysis of *E-cadherin* using MSP in cervical cancer cell lines. Methylation analysis was determined in five cervical cancer cell lines before and after 5-aza-deoxycytidine (CdR) treatment. Human placenta (HP) was used as a control for unmethylated band. M, methylated; U, unmethylated.



Genomic DNA sequences: (116bp)
CCAGGCTAGAGGGTCAC**CGCG**TC-
TATG**CG**AGGC**CG**GGTGGG**CG**GGC**CG**TCAGCTC**CG**CCCTGGGGA
GGGGTC**CGCG**CTGCTG
-ATTGGCTGTGGC**CG**GCAGGTGAACCCTCAGCCA

Bisulphite modification sequences:
TTAGGTTAGAGGGTTATAT**CGCG**TT-
TATG**CG**AGGT**CG**GGTGGG**CG**GGT**CG**TTAGTTT**CG**TTTTGGGGA
GGGGTT**CGCG**TTGTTG
-ATTGGTTGTGGT**CG**GTAGGTGAATTTTTTAGTTA

Bisulphite modification sequencing in cervical cancer cell lines showed that C changed to T, if it was not methylated as shown in underlined. 10 CG dinucleotides remained CG suggesting that all the CGs were methylated.

Fig. 3. DNA bisulphite sequencing in cervical cancer cell lines. Genomic DNA sequences (116 bp): ccaggctagagggtcac **cgcg** tc-tatg **cg** aggc **cg** ggtggg **cg** ggc **cg** tcagctc **cg** ccctggggagggtc **cgcg** ctgctg-attggctgtggc **cg** gcaggtgaaccctcagcca. Bisulphite modification sequences: ttaggtta-gagggttat **cgcg** tc-tatg **cg** aggt **cg** ggtggg **cg** ggt **cg** ttagttt **cg** ttttggggaggggtt **cgcg** ttgttg- attggttgtggt **cg** gtaggtgaatttttagtta Bisulphite modification sequencing in cervical cancer cell lines showed that C changed to T, if it was not methylated as shown in underlined. 10 CG dinucleotides remained CG suggested that all CGs were methylated.

methylation. The mRNA level of *E-cadherin* was lower in the methylated (8/20; 40%) group than unmethylated (12/20; 60%) group. This difference was statistically significant.

3.2. *Increased* DNMT1 *mRNA and activity in methylated* E-cadherin *group*

The *DNMT1* mRNA and enzyme activity were elevated in cervical cancer tissues to approximately 2- and 4-fold compared with normal cervical tissues, respectively ($P<0.05$) (Table 1). The differences of the *DNMT1* mRNA level and activity were also significant between the methylated and unmethylated *E-cadherin* cancerous groups (Table 2; Fig. 4).

In the cervical cancer cell lines, *DNMT1* mRNA and activity were higher in the cell lines that lacked E-cadherin expression. E-cadherin expression had a negative coefficient correlation with both *DNMT1* mRNA and enzyme activity (Spearman $r=-0.5742$, $P=0.007$; and $r=-0.44$, $P=0.046$, respectively). There was no correlation of either *E-cadherin* or *DNMT1* expression with clinical stage and pathological differentiation in this study.

3.3. *Inhibition of* DNMT1 *by methylation inhibitor or antisense* DNMT1 *oligo can upregulate* E-cadherin *expression in cervical cancer cell lines*

As DNA methyltransferase was upregulated in cervical cancer, especially the methylated *E-cadherin* cervical cancer tissues and cell lines, we designed an antisense (AS) *DNMT1* oligo and a control oligo with six mismatch bases to abrogate *DNMT1*. The *DNMT1* was significantly downregulated in the three cell lines in the AS groups in a dose-dependent manner after 2 days transfection (Fig. 5). The upregulation of *E-cadherin* mRNA was found on the third day in all cell lines, and E-cadherin protein was present in SiHa cell line on the sixth day after continuous transfection (Fig. 6). E-cadherin protein and mRNA were upregulated in a dose-dependent manner after AS oligo transfection in concentrations of 20, 40 and 70 nM. (Fig. 6a and b). Simultaneously, MSP showed that the unmethylated band appeared in SiHa cell line after AS oligo treat-

Table 1
E-cad herin, *DNMT1* mRNA and DNMT1 activity in cervical cancer and normal cervical tissues

	No	*E-cadherin* mRNA	Methylated alleles No.%	*DNMT1* mRNA	DNMT1 activity DPM ($\times 10^3$)
Cervical Ca	20	0.86±0.34	8/20 40	1.06±0.35	1.80±0.85
Normal Cx	10	0.96±0.24	0/10 0	0.49±0.35	0.45±0.30
		$P>0.05$		$P=0.0003$	$P<0.0001$

Ca, cancer; Cx, cervix.

ment, no unmethylated band was shown in the control oligo groups (Fig. 6c). The *E-cadherin* mRNA was also increased in a time-dependent manner in the AS oligos group within 48 h in three cervical cancer cell lines and down to approximately the previous levels after 2–4 weeks (Fig. 7).

4. Discussion

In this study, we provided evidence that promoter methylation of *E-cadherin* was involved in five cervical cancer cell lines and 40% (8/20) of cervical cancer tissues. Loss of *E-cadherin* gene expression was correlated

Table 2
E-cadherin and *DNMT1* mRNA and activity in *E-cadherin* methylated and unmethylated groups

Group	No.	*E-cadherin* mRNA	*DNMT1* mRNA	DNMT activity DPM ($\times 10^3$)
Methylated	8	0.52±0.08(a)	1.30±0.07(c)	2.44±0.30(e)
Unmethylated	12	1.08±0.05(b)	0.90±0.09(d)	1.36±0.15(f)
		$P<0.0001$	$P=0.002$	$P<0.0001$

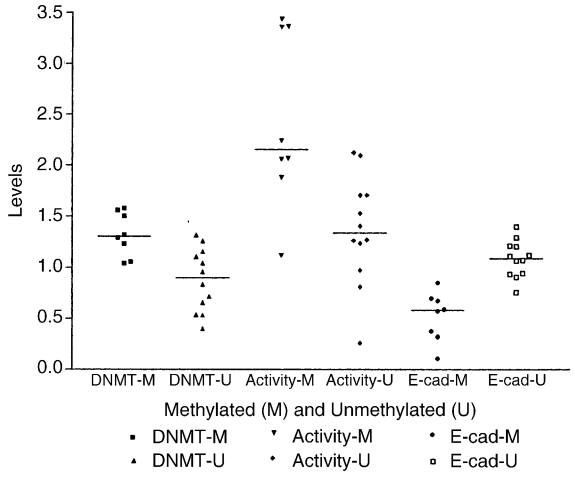


Fig. 4. *DNMT1* mRNA, DNMT1 activity and *E-cadherin* mRNA in methylated and unmethylated *E-cadherin* groups in cervical cancer tissues. The methylated *E-cadherin* group (M) $n=8$, and unmethylated group (U) $n=12$. Data shown in Table 2.

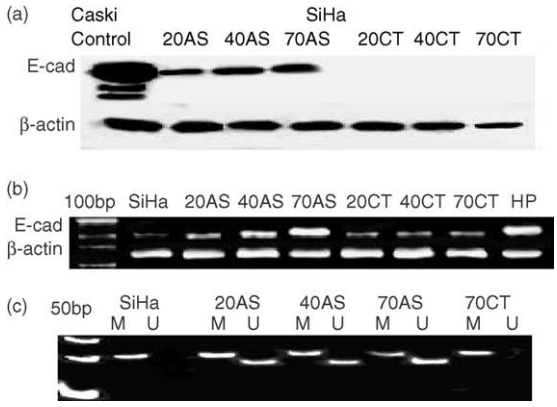


Fig. 6. *E-cadherin* expression after antisense oligo treatment: (a) western blot for *E-cadherin* re-expression after antisense oligo (AS) and control oligo (CT) transfection in SiHa cell line in concentrations of 20, 40 and 70 nM. (b) RT-PCR for *E-cadherin* in the SiHa cell line after AS and CT treatment in concentrations of 20, 40 and 70 nM. (c) MSP of *E-cadherin* in the SiHa cell line after AS and CT oligo treatment.

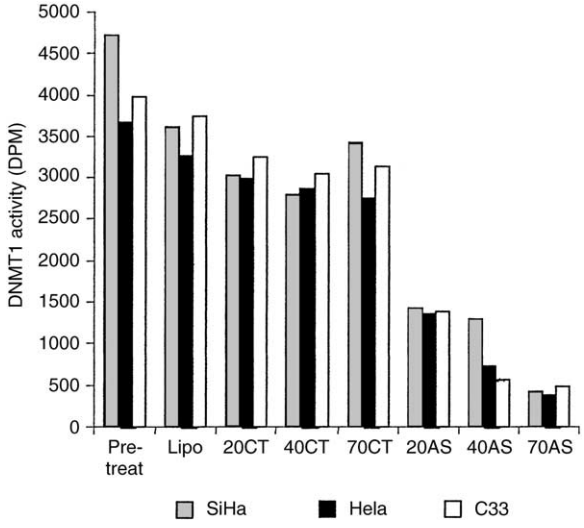


Fig. 5. DNMT1 activity in three cervical cancer cell lines after antisense and control oligo treatment. 2 μg protein lysate was analysed using methyltransferase activity assay in SiHa, HeLa and C33-A cell lines before treatment (pre-treat), lipofectin alone (Lipo), and CT oligo treatment in doses from 20, 40 and 70 nM.

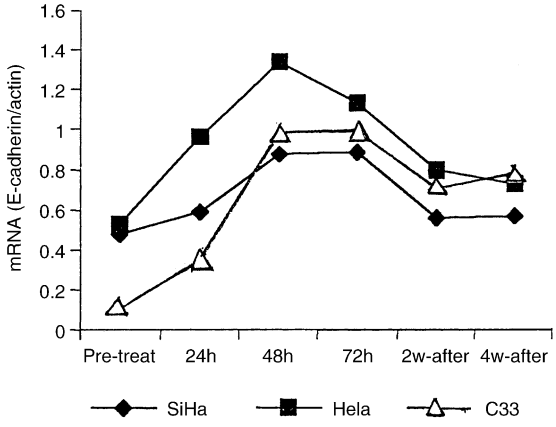


Fig. 7. *E-cadherin* mRNA was upregulated in three cervical cancer cell lines after AS *DNMT1* oligo treatment. Three cell lines (SiHa, HeLa and C33) were treated with antisense oligo for 2 days, cells were collected every day after treatment for 24, 48, 72 h, 2 weeks (2w-after) and 4 weeks (4w-after), respectively.

with CpG island methylation in 60% (3/5) cell lines and 75% (6/8) of cervical cancer tissues. Our findings corroborate previous studies of E-cadherin expression in cervical cancer [22], in which a reduction of E-cadherin expression was observed in approximately 50% of cervical carcinomas, especially in more advanced cervical cancers. However, these previous reports did not evaluate *E-cadherin* promoter hypermethylation. The high association between absent E-cadherin protein expression in SiHa, HeLa and C33A cell lines and hypermethylation warrants further study on the regulatory control of methylation in cervical cancer. Although loss of E-cadherin expression by DNA methylation has been reported in many cancers and our study only demonstrated 40% of methylation in cervical cancer, it can also partly explain the mechanisms of E-cadherin expression suppression in cervical cancer. In this study, we cannot rule out other causes of suppression of *E-cadherin* gene expression. Genetic or epigenetic alteration such as allelic loss or mutations were not studied in this report. In previous reports, approximately 11–16% of allelic loss was found in cervical cancers [25] and a low frequency of mutation was identified [1]. Another study showed that changes in chromatin structure [8] and alterations of specific transcription factor pathways also regulated *E-cadherin* gene expression [26]. This study demonstrates that hypermethylation of the CpG islands within the 5′ promoter is a prevalent mechanism by which *E-cadherin* expression is inactivated.

E-cadherin methylation is in accordance with an increase of *DNMT1* mRNA and activity of the enzyme in this study. After the MSP analysis and bisulphite DNA sequencing, we demonstrated direct evidence of DNA methylation in cervical cancer cell lines and tissues. We also found a high level of DNA methyltransferase was related to low E-cadherin expression and methylation of the promoter site. The effective antisense methyltransferase oligo in reactivating this gene further identified the role of *DNMT1* in *E-cadherin* gene silencing. Over-expression of *DNMT1* in cervical cancer is compatible with previous reports observed in colorectal cancer, stomach carcinoma and rhabdomyosarcomas [13,27,28]. A recent report showed overexpression of *DNMT1* mRNA was significantly associated with CpG island methylator phenotype (CIMP) in 23% of colorectal cancers and 31% of stomach cancers [15]. However, some studies have failed to demonstrate a correlation between the level of *DNMT1* expression in cancer cells and the state of methylation of the tumour suppressors [29,30]. Our study support the hypothesis that *E-cadherin* gene silencing through DNA methylation may be mediated by an increase of DNA methyltransferase in cervical cancer cell lines and tissues.

To further understand the role of *DNMT1* in *E-cadherin* gene silencing in cervical cancer, we designed an antisense methyltransferase oligo to test the hypothesis that a high level of DNA methyltransferase is the main cause of the hypermethylation in the CpG island and contributes to the loss of *E-cadherin* gene expression and tumour progression. Although DNA methyltransferase inhibitors 5-aza and 5-aza-CdR can produce similar results, these inhibitors are mutagenic and may cause DNA damage. To exert their effect, they must be incorporated into the DNA where they covalently trap the bulky 190 KD DNA methyltransferase, so they may alter gene expression by mechanisms unrelated to the inhibition of DNA methyltransferase [31]. Antisense oligos have been proven to be an effective approach in many areas of cancer research and have been shown to act through an RNase H-dependent cleavage of the target mRNA and then turnover of previously synthesised protein, and lead to a reduction in target protein [23]. In our study, we compared the effect of antisense DNA *DNMT1* oligo and a control oligo with 6 mismatch bases. We found that DNA methyltransferase can be reduced to a very low level on the third day after antisense oligo transfection. *E-cadherin* mRNA level was upregulated in all three cell lines and the protein was present in SiHa cells. This suggested that DNA methyltransferase plays an important role in *E-cadherin* gene silencing and promoter CpG methylation. A previous study also demonstrated that blocking *DNMT1* by antisense oligo can activate the *p16* gene through demethylation [32]. This study suggests that antisense *DNMT1* can reactivate the *E-cadherin* gene in human cancer cell lines.

In summary, reduced E-cadherin expression in cervical cancer cell lines and tissues was in part related to DNA methylation and a high level of DNA methyltransferase. Antisense methyltransferase oligo can abrogate *DNMT1* and increase *E-cadherin* expression. We conclude that DNA methylation plays an important role in *E-cadherin* gene silencing in cervical cancer.

Acknowledgements

This study was supported by a grant from the Committee on Research and Conference, the University of Hong Kong, 10203366.

References

1. Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998, **153**, 333–339.
2. Debruyne P, Vermeulen S, Mareel M. The role of the E-cadherin/catenin complex in gastrointestinal cancer. *Acta Gastroenterol Belg* 1999, **62**, 393–402.
3. Bussemakers MJ, Van Bokhoven A, Tomita K, Jansen CF, Schalken JA. Complex cadherin expression in human prostate cancer cells. *Int J Cancer* 2000, **85**, 446–450.
4. Graff JR, Herman JG, Lapidus RG, *et al*. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995, **55**, 5195–5199.

5. Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA* 1995, **92**, 7416–7419.
6. Kanai Y, Ushijima S, Hui AM, *et al*. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int J Cancer* 1997, **71**, 355–359.
7. Graff JR, Greenberg VE, Herman JG, *et al*. Distinct patterns of E-cadherin CpG island methylation in papillary, follicular, Hurthle's cell, and poorly differentiated human thyroid carcinoma. *Cancer Res* 1998, **58**, 2063–2066.
8. Hennig G, Behrens J, Truss M, Frisch S, Reichmann E, Birchmeier W. Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. *Oncogene* 1995, **11**, 475–484.
9. Bachman KE, Herman JG, Corn PG, *et al*. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* 1999, **59**, 798–802.
10. Herman JG, Umar A, Polyak K, *et al*. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998, **95**, 6870–6875.
11. Toyota M, Ahuja N, Suzuki H, *et al*. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999, **59**, 5438–5442.
12. Bestor TH. Cloning of a mammalian DNA methyltransferase. *Gene* 1988, **74**, 9–12.
13. Issa JP, Vertino PM, Wu J, *et al*. Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J Natl Cancer Inst* 1993, **85**, 1235–1240.
14. Sun L, Hui AM, Kanai Y, Sakamoto M, Hirohashi S. Increased DNA methyltransferase expression is associated with an early stage of human hepatocarcinogenesis. *Jpn J Cancer Res* 1997, **88**, 1165–1170.
15. Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, Hirohashi S. DNA methyltransferase expression and DNA methylation of CPG islands and peri-centromeric satellite regions in human colorectal and stomach cancers. *Int J Cancer* 2001, **91**, 205–212.
16. Ramchandani S, MacLeod AR, Pinard M, von Hofe E, Szyf M. Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1997, **94**, 684–689.
17. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999, **99**, 247–257.
18. Denk C, Hulsken J, Schwarz E. Reduced gene expression of E-cadherin and associated catenins in human cervical carcinoma cell lines. *Cancer Lett* 1997, **120**, 185–193.
19. Jeffers MD, Paxton J, Bolger B, Richmond JA, Kennedy JH, McNicol AM. E-cadherin and integrin cell adhesion molecule expression in invasive and in situ carcinoma of the cervix. *Gynecol Oncol* 1997, **64**, 481–486.
20. Fujimoto J, Ichigo S, Hirose R, Sakaguchi H, Tamaya T. Expression of E-cadherin and alpha- and beta-catenin mRNAs in uterine cervical cancers. *Tumour Biol* 1997, **18**, 206–212.
21. de Boer CJ, van Dorst E, van Krieken H, *et al*. Changing roles of cadherins and catenins during progression of squamous intraepithelial lesions in the uterine cervix. *Am J Pathol* 1999, **155**, 505–515.
22. Vessey CJ, Wilding J, Folarin N, *et al*. Altered expression and function of E-cadherin in cervical intraepithelial neoplasia and invasive squamous cell carcinoma. *J Pathol* 1995, **176**, 151–159.
23. Fournel M, Sapieha P, Beaulieu N, Besterman JM, MacLeod AR. Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. *J Biol Chem* 1999, **274**, 24250–24256.
24. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, **93**, 9821–9826.
25. Krul EJ, Kersemaekers AM, Zomerdijk-Nooyen YA, Cornelisse CJ, Peters LA, Fleuren GJ. Different profiles of allelic losses in cervical carcinoma cases in Surinam and The Netherlands. *Cancer* 1999, **86**, 997–1004.
26. Ji X, Woodard AS, Rimm DL, Fearon ER. Transcriptional defects underlie loss of E-cadherin expression in breast cancer. *Cell Growth Differ* 1997, **8**, 773–778.
27. El Deiry WS, Nelkin BD, Celano P, *et al*. High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc Natl Acad Sci USA* 1991, **88**, 3470–3474.
28. Chen B, Liu X, Savell VH, *et al*. Erratum: increased DNA methyltransferase expression in rhabdomyosarcomas. *Int J Cancer* 1999, **83**, 874.
29. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* 1999, **59**, 2302–2306.
30. Jurgens B, Schmitz-Drager BJ, Schulz WA. Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res* 1996, **56**, 5698–5703.
31. Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R. Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc Natl Acad Sci USA* 1997, **94**, 4681–4685.
32. Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 1997, **277**, 1996–2000.