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European Journal of Cancer 39 (2003) 524–531

European Journal of Cancer

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# The association of E-cadherin expression and the methylation status of the *E-cadherin* gene in nasopharyngeal carcinoma cells

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Received 11 February 2002; received in revised form 12 August 2002; accepted 3 September 2002

**Abstract**

Loss of E-cadherin (E-cad) has been associated with progression and poor survival in nasopharyngeal carcinoma (NPC). In this study, we investigated the role of methylation on *E-cad* inactivation in NPC cell lines, as well as in NPC tissue samples. Using 6 NPC cell lines, we found that methylation of the *E-cad* 5′ CpG island promoter region was correlated with the loss of both mRNA and E-cad protein expression in these cell lines. In addition, using 29 NPC and 10 non-malignant nasopharyngeal samples, we also observed 5′ CpG methylation of the *E-cad* gene in 52% (15 out of 29) NPC samples, but in only 10% (1 out of 10) of the non-malignant nasopharyngeal tissues. Our findings indicate that 5′ CpG island methylation of the *E-cad* gene may play an important part in the inactivation of *E-cad* in NPC. Our results also suggest that reducing the methylation of the *E-cad* gene may be a potential therapeutic strategy for NPC.


*Keywords:* Nasopharyngeal carcinoma; E-cadherin; Methylation

## 1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the common carcinomas in Southeast Asia, although it is rare in other parts of the world [1]. Previously, we have shown that downregulation of E-cadherin (E-cad) is a common event in this cancer which accounts for more than 90% of the cases [2], and this rate is also rather high compared with rates reported for other tumours [3,4]. A marked decrease in E-cad expression has also been associated with an advanced stage of disease and poor survival in NPC patients [2]. Reduction or loss of E-cad has also been observed in many poorly differentiated carcinomas such as breast, colon, bladder, breast and prostate [5]. Loss of E-cad has been related to tumour aggressiveness and the ability to invade and metastasise to distant sites in cancers such as breast, gastric, hepatocellular and prostate [6–10]. However, the mechanism involved in the downregulation of E-cad in tumour cells is poorly understood.

E-cad is a 120-kDa transmembrane glycoprotein which is involved in mediating cell–cell adhesion between adjacent epithelial cells in various tissues [11] and cellular adhesiveness may be a critical step in the ability of epithelial tumour cells to invade and metastasise. Recently, more and more evidence suggests that E-cad plays an important role in the development and behaviour of cancer cells. *In vitro* studies showed that restoration of *E-cad* into tumour cells led to the reduction of tumour cell invasiveness and suppression of metastasises both *in vitro* and in animal model systems [12–14]. Therefore, *E-cad* has been implicated as an "invasion-suppressor" gene. The *E-cad* gene is located on chromosome 16q in a region that is frequently affected by allelic loss in several tumour types [15–17]. However, inactivation of *E-cad* through mutation was only found in diffused-type cancers such as gastric carcinoma and lobular carcinoma of the breast, which have complete loss of cell-cell adhesiveness

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0959-8049/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0959-8049(02)00494-X

[9,18,19]. This suggests that E-cad dysfunction in these tumours may be due to mutations at the gene level. However, in the majority of cancers in which E-cad expression is altered, mutations in the *E-cad* gene are rare or absent. Mechanisms underlying the reduced or absent expression of E-cad in these tumours are poorly understood. Recently, two possible mechanisms have been proposed. In breast cancer, defects in signalling pathways or *trans*-acting transcription factors that regulate E-cad expression have been suggested to be the primary mechanisms responsible for the loss of E-cad expression [20]. This may be due to alterations in factors that interact with sequence elements in the proximal 108bp of the *E-cad* promoter in this cancer [21]. However, so far, evidence of transcriptional defects have only been shown in breast cancer cells. Therefore, CpG methylation around the promoter region is considered to be another possible mechanism of *E-cad* gene inactivation in human tumours. In normal tissues, CpG islands are generally unmethylated and extensive methylation of the promoter region CpG islands is associated with transcriptional silencing of imprinted alleles and genes in the inactive X-chromosomes [22]. In neoplasia, aberrant DNA methylation of the promoter region CpG island has been found to be responsible for the inactivation of tumour suppressor genes such as *Rb*, $p16^{INK4a}$, $p15^{INK4a}$ [23]. A CpG island has been identified within the 5′ promoter region of the *E-cad* gene [24,25] and *in vitro* studies using various carcinoma cell lines showed a clear correlation between aberrant hypermethylation of the CpG island with decreased E-cad expression [26–28]. In addition, treatment of E-cad-negative cells with the demethylation agent 5-azacytidine resulted in the re-expression of the gene [26–28]. This strongly suggests that hypermethylation is important in the inactivation of *E-cad* gene in human tumour cells. In addition, CpG methylation around the promoter region correlated significantly with reduced E-cad expression in hepatocellular carcinomas, gastric carcinomas and oral squamous cell carcinomas [9,10,29], and has been suggested to participate in carcinogenesis. Furthermore, a recent study showed that in papillary thyroid carcinomas, a hypermethylated *E-cad* promoter was detected in over 80% of the cases that had no E-cad expression [30]. Evidence so far indicates that in some tumour types, hypermethylation appears to be a major mechanism to inactivate E-cad function.

The aim of this present study was to continue our previous investigations by studying if CpG methylation played a role in the downregulation of E-cad in NPC. Using a panel of six NPC cell lines, we examined the methylation status of the 5′ CpG island promoter region of the *E-cad* gene using methylation-specific polymerase chain reaction (PCR) (MSP).

## 2. Materials and methods

### 2.1. Cell culture conditions and sample collection

Six NPC cell lines CNE1 [31], CNE2 [32], CNE3 [33], HK1 [34], SUNE1[35], HKM1 (established from a lymph node metastasis of a NPC patient in Queen Mary Hospital, Hong Kong, a gift from Dr L Cao, Dept. of Microbiology, University of Hong Kong) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Inc.) supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum at 37 °C. Normal nasopharyngeal epithelial cells were derived from non-neoplastic nasopharyngeal tissues taken by routine operation in the Department of Surgery, Queen Mary Hospital in Hong Kong and cultured in MCDB151 medium containing 0.1 mM calcium and 1% (v/v) dialysed fetal calf serum. 29 NPC samples were collected from patients in Queen Mary Hospital, Hong Kong, after confirmation of diagnosis.

### 2.2. Immunofluorescence staining

Cells were grown on cover slips and fixed in cold methanol/acetone (1:1) for 10 min. The fixed cells were washed three times with phosphate-buffered saline (PBS) and blocked in swine serum (20% (v/v)) for 30 min. After washing with PBS, the cells were incubated with anti-E-cad antibody (1:100, Boehringer Mannheim, USA) at 4 °C overnight and then washed three times with PBS. They were then incubated with Rhodamine-conjugated secondary antibody for 1 h and washed again with PBS. Then the cover slips were sealed and observed under the fluorescence microscope.

### 2.3. Reverse transcriptase (RT)-PCR analysis of E-cad expression

Total RNA was isolated and first strand cDNA was synthesised using Superscript™ II RNase H- reverse transcriptase (Gibco BRL). Briefly, 2 μg total RNA was mixed with 1 μl oligo $(dT)_{12–18}$. The volume was brought to 12 μl using sterile distilled water. The mixture was then heated to 70 °C and incubated for 10 min followed by a quick chill on ice. 4 μl 5× first-strand buffer, 2 μl 0.1 M dithiothreitol (DTT) and 1 μl 2.5 mM deoxynucleoside triphosphate (dNTP) mix (2.5 mM each of deoxyadenosine triphosphate (dATP), deoxygunosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP), pH 7.0) were added to the mixture. After 2 min incubation at 42 °C, 1 μl (200 units) Superscript™ II was added and the mixture was incubated at 42 °C for 50 min. The reaction was stopped by incubation at 70 °C for 15 min. The expression pattern of the *E-cad* gene was studied using HEC17 (5′-GTAACCGATCAGAATGAC-3′) and HEC24

(5′CGTGGTGGGATTGAAGAT-3′) primers [36]. The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was amplified as a control for the abundance of the mRNA. The following primers were used: 5′-CTCAGACACC-ATGGGG-3′ and 5′-ATGATCTTGAGGCTGTTGT-CATA-3′. PCR was performed using 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The initial denaturation step was performed at 94 °C for 5 min and the final extension step was performed at 72 °C for 10 min. PCR products were verified by running a 1% (w/v) agarose gel (with 0.5 μg/ml ethidium bromide) at 100 V for 1 h.

### 2.4. Methylation study of the 5′ CpG island of the E-cad. gene

Methylation in the promoter region of the *E-cad* gene was determined by chemical modification of unmethylated, but not methylated, cytosine to uracil using bisulphite and subsequent PCR using primers specific for either methylated or modified unmethylated DNA. The detailed experimental procedures were published previously in Ref. [30]. Briefly, for bisulphite modification, 1 μg of genomic DNA was denatured by adding freshly prepared NaOH (final concentration 0.3 M) and incubated for 10 min at 37 °C. Thirty microlitres of freshly prepared 10 mM hydroquinone (pH 5.0) and 2 M sodium metabisulphite (pH 5.0) were added and mixed. The sample was incubated under mineral oil at 50 °C for 16 h. The modified DNA was purified by the High Pure PCR Product Purification Kit (Boehringer Mannheim, Indianapolis, IN, USA) and eluted in 94 μl of $H_2O$. Six microlitres of 5 M NaOH was added and incubated at room temperature for 5 min, followed by an ethanol precipitation. The DNA was resuspended in 50 μl of tris-EDTA (TE) buffer (pH 8.0). Methylation-specific PCR was performed using primers spanning the transcription start site of *E-cad*, previously referred to as island set 3. Primer sequences for the unmethylated reaction were 5′-GGT AGG TGA ATT TTT AGT TAA TTA GTG GTA-3′ and 5′-ACC CAT AAC TAA CCA AAA ACA CCA-3′; and for the methylated reaction were 5′-GGT GAA TTT TTA GTT AAT TAG CGG TAC-3′ and 5′-CAT AAC TAA CCG AAA ACG CCG-3′. Unmethylated or methylated DNA provided by the Oncor methylation kit (USA) was used as positive and negative controls. The PCR reaction contained (in a final volume of 10 μl) 1 × PCR buffer (20 mM Tris–HCl (pH 8.4) and 50 mM KCl), dNTPs (100 μM), primers (0.4 μM each), bisulphite-modified DNA (approximately 15 ng), 0.125 Units of Taq DNA polymerase (BRL Life Technologies) with 0.125 units of Platinum Taq antibody (BRL Life Technologies), and 2 or 1.5 mM of magnesium chloride for unmethylated and methylated primers, respectively. Forty cycles of PCR was carried out in an automated thermal cycler at 95 °C for 15 s, 59 °C for 20 s and 72 °C for 30 s, followed by an extension step at 72 °C for 7 min. The PCR products were visualised on 3% (w/v) agarose gel stained with ethidium bromide under ultraviolet (UV) illumination.

### 2.5. Western blotting analysis

To extract proteins from the cultured cells, the monolayers were washed once with PBS and lysed in 200 μl of lysis buffer (50 mM Hepes pH 7.5; 100 mM NaCl; 1 mM ethylene glycol tetra acetate (EGTA); 1% (v/v) Triton X-100; 1 mM DTT; 1 mM $CaCl_2$; 1 μg/ml aprotinin; 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA). 50 μg protein was separated on a 10% (w/v) sodium dodecyl sulphate-polyacryl-amide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane. The membrane was then blocked at room temperature with 5% (v/v) non-fat dry milk in tris buffered saline/tween 20 (TBST) for 1h and incubated with anti-E-cadherin (1:2500, Boehringer Mannheim, USA) and secondary antibodies (1:5000). Specific immunoreactive bands were identified using the enhanced chemiluminescence (ECL) kit (Amersham) on autoradiographic films. The same membrane was stripped using 0.2% (w/v) NaOH for 5 min and reprobed with anti-actin antibodies (1:500, Santa Cruz, USA).

### 2.6. Immunohistochemistry staining

Detailed experimental procedures on sample collection, processing and immunostaining of the E-cad protein were described in one of our previous studies [2]. Differential E-cad expression was defined in three categories: strong (approximately more than 75% cells showed positive staining), moderate (approximately 25–50% cells showed positive staining) and weak (approximately 0–25% cells showed positive staining). Moderate and weak staining was considered as decreased E-cad expression in NPC cells.

## 3. Results

### 3.1. E-cad expression in NPC cell lines

The expression of E-cad protein was studied in six NPC cell lines using Western Blotting analysis. As shown in Fig. 1a, a 120kD E-cad protein was detected in NPC cell lines CNE2, SUNE1 and HK1 but was absent in the CNE1, CNE3 and HKM1 cell lines. Immunofluorescence staining of E-cad protein also showed positive (and negative) signals for the same cell lines (Fig. 1b). This suggests that downregulation of E-cad was relatively common in the NPC cell lines (50%, three out of six).

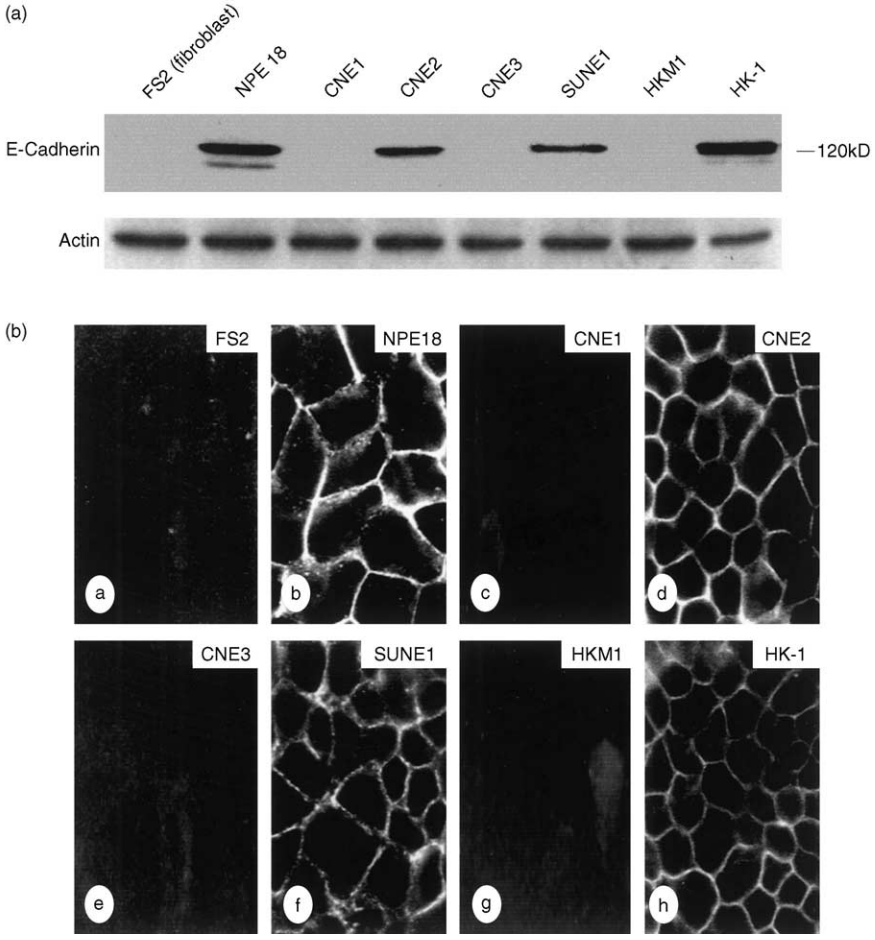


Fig. 1. Expression of E-cad in Nasopharyngeal carcinoma (NPC) cell lines CNE1, CNE2, CNE3, SUNE1, HKM1 and HK1. A fibroblast cell line (FS2) and primary cultured non-malignant nasopharyngeal epithelial cells (NPE18) were used as negative and positive controls, respectively. (a) Western blotting analysis of 120kD E-cad protein expression in the NPC cell lines; (b) immunofluorescence staining of E-cad expression in the NPC cell lines. Photos were taken under 4000× magnification using a fluorescence microscope. The nature of the additional band below 120D is obscure but may represent a degradative form of E-cadherin.

### 3.2. *E-cad mRNA expression in NPC cells*

To investigate the mechanism involved in the downregulation of E-cad in the NPC cells, we used RT-PCR to assess *E-cad* transcription. Using primers flanking exons 7–8 of the *E-cad* gene [36], we found that normal NP cells (NPE18), as well as CNE2, SUNE1 and HK-1 cell lines, showed bands for *E-cad* mRNA (Fig. 2), which correlates with the expression of E-cad protein in these cell lines (Fig. 1). The cell lines CNE1, SUNE1 and HKM1, in agreement with the protein analyses, failed to show detectable levels of *E-cad* mRNA. This indicates that defects at the transcriptional level may be responsible for the downregulation of E-cad in these NPC cell lines.

### 3.3. *Methylation status of the* E-cad *promoter 5′CpG island in NPC cells*

As loss of E-cad expression has frequently been correlated with hypermethylation of the 5′ CpG island in many cancers [26,27,30], we next examined the methylation of the *E-cad* CpG island in these NPC cell lines using MSP [37]. As shown in Fig. 3, cell lines (CNE1, CNE3 and HKM1), which showed a lack of E-cad expression, were found to be heavily methylated at this transcription start site, a region that has been shown to correlate best with transcriptional silencing [37]. In the

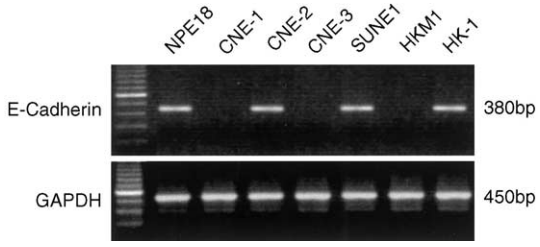


Fig. 2. Reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis of *E-cad* mRNA expression in NPC cell lines and primary cultured non-malignant NPE18 cells. *E-cad* gene was amplified as previously described in Ref. [36], *GAPDH* gene was also amplified as an internal control. The size of the *E-cad* and *GAPDH* RT-PCR products is indicated.

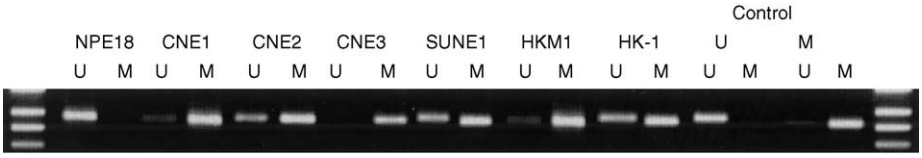


Fig. 3. Methylation-specific PCR (MSP) analysis of methylation status of the *E-cad* 5′ island in NPC cell lines and non-malignant NPE18 cells. Unmethylated (U) and methylated DNA (M) (Oncor, USA) was used as controls. Bisulphite-treated genomic DNA was PCR-amplified using two primers for the transcription start site [one amplifies methylated DNA (M) and the other that amplifies unmethylated DNA (U)].

non-malignant nasopharyngeal epithelial cells (NPE118) and other three NPC cell lines that expressed E-cad, either no (NPE18) or partial methylation was observed (CNE2, SUNE1, HK-1) (Fig. 3). These results indicate an association between the methylation status of the 5′ CpG transcription start site and the expression of E-cad.

### 3.4. *E-cad* methylation in NPC Tissue samples

Having established that NPC cell lines lacking E-cad expression correlated to the hypermethylation of the *E-cad* gene promoter region, we next determined whether methylation was common in primary NPC tissues. 29 NPC samples were studied and 10 non-malignant nasopharyngeal tissues were also used as a comparison. We found that 5′ CpG methylation was found in 15 out 29 (52%) NPC samples, while 1 out of 10 (10%) was methylated in the non-malignant tissue samples (Table 1; Fig. 4). These results support our evidence obtained in the NPC cell lines that methylation of the *E-cad* promoter region is common in NPC (more than 5 times that of the non-malignant nasopharyngeal tissue samples). Using an immunocytochemical method, we found that all of the non-malignant nasopharyngeal tissues, but one, showed a positive staining of E-cad (90%), while only 24% of NPC samples expressed this protein at moderate levels. The one non-malignant sample which showed weak E-cad expression also showed CpG island methylation (Table 1). However, among the 22 NPC samples that showed decreased E-cad expression, 68% (15/22) correlated with the presence of CpG island methylation. These results suggest that while *E-cad* promoter methylation may play an important part in the downregulation of E-cad protein commonly observed in NPC cells, other mechanisms may also contribute to the decreased expression of E-cad in NPC.

## 4. Discussion

E-cad expression is decreased or lost frequently in NPC and such loss has been correlated with an advanced stage and poor survival of NPC patients [2]. Therefore, downregulation of E-cad in NPC may be an important prognostic factor. Our current study provides the first evidence that loss of E-cad expression in NPC cells was associated with the CpG island methylation around the promoter region of the *E-cad* gene (Figs. 1–3). Similar findings have also been documented in primary breast, prostate, gastric, oral squamous cell and hepatocellular carcinomas [10,26,27,38]. In addition, we found that methylation of *E-cad* promoter was also common in NPC tumour samples (greater than 50%) (Table 1). Our results provide further evidence to support the suggestion that aberrant CpG methylation around the promoter region may play an important role in *E-cad* inactivation in human tumours.

Using six NPC cell lines, we found that *E-cad* 5′ CpG island methylation was evident in all of them while there was no evidence of methylation in the non-malignant cells NPE18 (Fig. 3). This indicates that methylation of the *E-cad* promoter region is a common event in NPC cell lines. It is possible that the detectable levels of E-cad protein and transcripts in the CNE2, SUNE1 and HK1 cell lines might be due to the presence of an unmethylated *E-cad* promoter region in these cell lines (Fig. 3), as although methylation was evident in these cells they were less heavily methylated than the other 3 cell lines that lacked E-cad expression. Our results agree with previous findings in breast and prostate cancer cell lines where only complete methylation of the *E-cad* promoter correlated with the loss of both E-cad protein and mRNA expression [28,30]. We also noted that both HK1 and SUNE1 cell lines showed comparable levels of *E-cad* mRNA (Fig. 2) and hypermethylation of the promoter (Fig. 3), but the E-cad protein levels in SUNE1 cells were much lower compared with the HK1 cells (Fig. 1a). At present, we do not know the mechanism responsible for this differential E-cad expression in these two cell lines. It is possible that, in addition to transcriptional inactivation by hypermethylation, inactivation of the E-cad protein at a posttranslational level, such as a decreased half life or protein degradation through the ubiquitin–proteasome pathway, may be responsible for the decreased E-cad protein expression in the SUNE1 cells.

In addition, using 29 NPC and 10 normal nasopharyngeal samples, we demonstrated that methylation of the *E-cad* CpG island was found in 15 out of 29 (52%) NPC samples and 1 out of 10 (10%) samples in the normal nasopharynx. This suggests that methylation of the 5′ CpG island of *E-cad* is not only frequent in NPC cell lines, but also a frequent event in NPC tumours.

Table 1
Summary of E-cad expression and methylation status in normal nasopharygeal tissue and NPC samples

| Sample | | Presence of *E-cad* methylation | E-cad protein expression | % of methylation | % of E-cad downregulation | % of both methylation/ E-cad downregulation |
|---|---|---|---|---|---|---|
| Normal nasopharyx | 1 | – | Strong[a] | | | |
| | 2 | – | Moderate | | | |
| | 3 | – | Strong | | | |
| | 4 | – | Moderate | | | |
| | 5 | – | Strong | | | |
| | 6 | – | Strong | 10 | 10 | 100 |
| | 7 | + | Weak | | | |
| | 8 | – | Strong | | | |
| | 9 | – | Strong | | | |
| | 10 | – | Strong | | | |
| Nasopharyngeal carcinoma | 1 | – | Moderate | | | |
| | 2 | + | Weak | | | |
| | 3 | + | Weak | | | |
| | 4 | + | Weak | | | |
| | 5 | + | Weak | | | |
| | 6 | – | Moderate | | | |
| | 7 | + | Weak | | | |
| | 8 | + | Weak | 52 | 76 | 68 |
| | 9 | – | Moderate | (15/29) | (22/29) | (15/22) |
| | 10 | – | Weak | | | |
| | 11 | + | Weak | | | |
| | 12 | + | Weak | | | |
| | 13 | – | Moderate | | | |
| | 14 | – | Weak | | | |
| | 15 | + | Weak | | | |
| | 16 | + | Weak | | | |
| | 17 | – | Weak | | | |
| | 18 | – | Moderate | | | |
| | 19 | – | Weak | | | |
| | 20 | + | Weak | | | |
| | 21 | + | Weak | | | |
| | 22 | + | Weak | | | |
| | 23 | – | Weak | | | |
| | 24 | + | Weak | | | |
| | 25 | – | Moderate | | | |
| | 26 | – | Moderate | | | |
| | 27 | – | Weak | | | |
| | 28 | + | Weak | | | |
| | 29 | – | Weak | | | |

[a] Strong: >75% cells positive; moderate: 35–75% cells positive; weak: 0–35% cells positive.

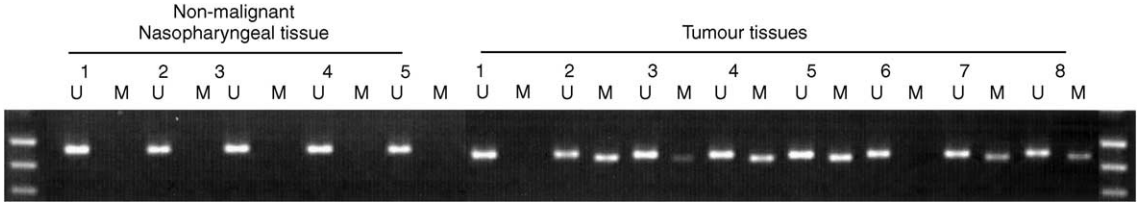


Fig. 4. Representative results of MSP analysis on the *E-cad* 5′ island in normal nasopharynx and NPC tissue samples. Genomic DNA was isolated and treated with bisulphite. Specific primers were used to amplify the methylated (M) and unmethylated DNA (U) by PCR.

This agrees with previous studies on other human cancers such as thyroid carcinoma, primary gastric carcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, breast and prostate carcinomas where 5′ CpG methylation of *E-cad* promoter has been found in between 11 and 83% of tumour samples [10,27,28,30,38]. Evidence from the present study and previous findings strongly suggest that 5′ CpG methylation may play an important part in *E-cad* inactivation in certain tumour types. In the present study, *E-cad* methylation was found in 68% (15 out of 22) NPC samples that showed decreased E-cad expression, indicating

an association between downregulation of E-cad and hypermethylation of the *E-cad* gene. However, the fact that not all of the NPC samples with a weak E-cadherin expression showed evidence of methylation indicates that in addition to 5′ CpG methylation of the *E-cad* promoter region, downregulation of E-cad in NPC may involve other mechanisms.

In summary, the present study provides the first evidence that loss of E-cad expression is associated with methylation of the 5′ CpG island of the *E-cad* promoter region in both NPC cell lines and clinical samples. Our results indicate that 5′ CpG island methylation may play an important role in the downregulation of E-cad in NPC. As loss of E-cad has been correlated with tumour invasion and poor survival, these findings suggest that the restoration of *E-cad* to prevent or reduce metastatic phenotype of NPC cells may be a potential therapeutic strategy.

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