J. Biochem. 2011;149(1):49–54 doi:10.1093/jb/mvq128

JB THE JOURNAL OF BIOCHEMISTRY

Demethylation of E-cadherin gene in nasopharyngeal carcinoma could serve as a potential therapeutic strategy

Received July 9, 2010; accepted September 22, 2010; published online November 8, 2010

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E-cadherin has been proven to be widely downregulated and tightly associated with tumour invasion and metastasis in multiple human cancer types. Recent research demonstrated that aberrant methylation around gene promoter region attributes to E-cadherin silencing. However, the detailed information about this epigenetic inactivation in nasopharyngeal carcinoma (NPC) is rare. The aim of this study was to probe more into the basic mechanism of E-cadherin methylation in NPC and elucidate the application of demethylating agents to restore E-cadherin expression. To address this question, we initially studied E-cadherin methylation status in NPC primary tumours and cell lines by methylation-specific PCR, and compared it with E-cadherin expression. Methylated E-cadherin was detected in 13 of 20 (65%) NPC clinical specimens and 2 of 2 (100%) NPC cell lines (HNE-1 and CNE-2), which was inversely correlated with E-cadherin expression. The detailed methylation profile at individual CpGs within CpG island of E-cadherin promoter region was confirmed by bisulphite sequencing. E-cadherin gene could be demethylated and reactivated in HNE-1 and CNE-2 cells upon treatment with 5-aza-dC, a DNA demethylating agent. Our findings indicate that frequent aberrant methylation of E-cadherin may play an important role in downregulation of E-cadherin, and demethylation therapy could serve as a promising strategy for NPC patients. Furthermore, a high frequency of E-cadherin methylation (9/20, 45%) in peripheral blood of NPC patients suggests its potential clinical application as an early diagnostic or predictive marker.

Keywords: bisulphate sequencing/demethylation/E-cadherin/nasopharyngeal carcinoma.

Abbreviations: BGS, bisulphite genomic sequencing; NPC, nasopharyngeal carcinoma.

Nasopharyngeal carcinoma (NPC) is a commonly occurring cancer that generally has an unfavourable prognosis despite the availability of multimodal therapies. Early diagnosis is a major factor adversely affecting the effect of treatment. Recent studies has revealed that downregulated expression of E-cadherin, a cell-adhesion molecule locating at the 16q22 locus, contributes to tumour invasion and metastasis and may serve as a prognostic indicator in different types of cancers including NPC *(1, 2)*. As a tumour suppressor gene, E-cadherin expression is frequently inhibited by diverse mechanisms *(3)*. In many cases, E-cadherin expression is inhibited by transcriptional repression. For instance, snail, a zinc finger protein that recognizes E-box motifs in target promoter, could suppress E-cadherin expression as a transcriptional repressor *(4)*. Many other transcription regulators such as Twist and SIP1, also can serve as efficient repressor to downregulate E-cadherin *(5, 6)*.

Alternatively, it has been widely established that E-cadherin expression is frequently inhibited by epigenetic modification. Increasing evidence revealed that aberrant methylation of CpG island plays a crucial role for inactivation of E-cadherin during tumourigenesis in many kinds of cancers *(7)*. CpG island is a region of the genome possessing high content of cytosine and guanine repeats, which are often clustered at the 5′-ends of genes. Methylation of CpG islands around gene promoter regions is being recognized as one of the alternative and perhaps common mechanisms of silencing tumour-suppressor genes *(8)*. Although methylated E-cadherin has been proven to occur widely in human NPC via methylation-specific PCR (MS-PCR) analysis, the detailed methylation profile at individual CpGs within E-cadherin promoter region remains unclear *(9–11)*. Moreover, the function of demethylating agents, which could reactivate the silenced E-cadherin in many other types of cancer cells, has not been elucidated in NPC *(12)*. Here, we investigated the correlation between E-cadherin methylation and inactivation in NPC primary tumours as well as in NPC cell lines, with emphasis on the accurate methylation profile of E-cadherin CpG island and the expression-inducing effect of 5-aza-dC. Because hypermethylated DNA has high specificity to differentiate cancer from normal tissues, the potential of E-cadherin methylation of the circulating tumour DNA in peripheral blood as a diagnostic or predictive marker for NPC was also evaluated in this study.

Materials and Methods

Cell lines and culture conditions

Two human NPC cell lines, HNE-1 and CNE-2, were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 10% foetal bovine serum (Gibco), 10 mM glutamine (Invitrogen, Carlsbad, CA, USA), 100 U/ml of penicillin (Invitrogen), and 100 μg/ml of streptomycin (Sigma, St Louis, MO, USA) in 25-cm^2 tissue flasks at 37°C in a humidified atmosphere containing 5% CO_2.

Clinical specimens

With informed consent, 20 primary tumour biopsies and 20 peripheral blood samples of NPC patients were collected from Affiliated Hospital of Bethune Military Medical College. Five normal nasopharyngeal epithelia from healthy volunteers served as negative controls. All tumour specimens were confirmed pathologically. Samples were stored at liquid nitrogen for transportation at −70°C until use.

RNA extraction and reverse transcription PCR analysis

Total cellular RNA was extracted from cells and frozen stored tissues with TRIzol reagent (Invitrogen) and then cDNA was prepared from 3 μg of RNA with the random hexamers and avian myeloblastosis virus reverse transcriptase (Invitrogen) according to the manufacturers' instructions. The cDNA was subjected to PCR for 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. The amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining. β-Actin was amplified as an invariant housekeeping gene internal control. The primer sequences for E-cadherin and β-actin expression are summarized in Table I.

Western blot analysis

Whole cell and tissue lysates were prepared in RIPA lysis buffer (50 mM Tris–Cl pH 7.4; 150 mM NaCl, 0.05% SDS, 0.1% Tween-20, 0.1% NP40). Approximately 50 μg of total proteins for each sample was separated on an 12% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and incubated overnight in PBS containing 5% bovine serum albumin (BSA, Sigma) at 4°C. After briefly washing with 0.1% Tween-20 (Sigma) in PBS, the membrane was incubated with polyclonal rabbit E-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 5000. The membrane was washed again and incubated in goat anti-rabbit horseradish peroxidase-conjugated IgG (Amersham, Arlington Heights, IL, USA) at a dilution of 1 : 5000. The expression of E-cadherin protein was detected using the enhanced ECL kit (Amersham). To verify equal loading of the samples, the same membrane was stripped using 0.2 M NaOH for 5 min and incubated with the mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham).

DNA extraction and purification

DNA extraction from cell lines, primary tumour biopsies and peripheral blood was through the use of a QIA amp DNA mini kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions. For DNA extraction for blood samples, the peripheral venous blood of patients was collected by EDTA-containing tubes and 200 μl of each sample was used for genomic DNA extraction. The blood samples were treated with proteinase K (0.5 mg/ml) and DNA isolation and purification was performed according to the protocol as recommended by the manufacturer.

Bisulphite treatment and methylation analysis

For analysis of methylation status, 1 μg of genomic DNA from each sample was modified by sodium bisulphite using the EpiTect Bisulphite Kit (Qiagen) according to the manufacturer's instructions. The modified DNA then was subject to MSP and bisulphite genomic sequencing (BGS) to investigate the methylation status of E-cadherin gene. MSP was performed with 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, after an initial incubation at 94°C for 3 min. As described in Fig. 1A, the MSP primers were designed upstream the transcription start site of E-cadherin and coincidental with the maximum density of CpG sites. The sequences of PCR primers specific for methylated and unmethylated alleles of E-cadherin and the sizes of expected PCR products are summarized in Table I. For BGS, the CpG island of E-cadherin gene from −416 to +15 bp with respect to the ATG site was amplified from bisulphite-converted DNA (Fig. 1A). Amplification with a total of 35 cycles was carried out in which each cycle involved 1 min each of 94°C denaturation, 51.5°C of annealing and 72°C of extension followed by a final 72°C extension for 7 min. Primer sequences for BGS are listed in Table I. The purified PCR product was cloned into the pGM-T vector using a TA cloning kit (Invitrogen). We determined the sequence of the promoter in four independent colonies in each case and examined the average methylation levels by determining the number of methylated cytosine residues.

Restoration of E-cadherin expression by demethylating agent of 5-aza-dC

For demethylation, HNE-1 and CNE2 cells were treated with a final concentration of 20 μM 5-aza-2′-deoxycytidine (5-aza-dC, Sigma) for 72 h, respectively. RT–PCR, western blot and MSP analyses were performed to confirm the expression of E-cadherin.

Results

Frequent downregulated expression of E-cadherin in NPC

E-cadherin expression was examined in 20 primary NPC biopsies and five nomal nasopharyngeal epithelia by RT–PCR. Figure 1A depicts a representative RT–PCR result which demonstrated that showed undetectable or decreased transcription of E-cadherin in 16 of 20 (80%) primary NPC tumours compared with its expression in normal nasopharyngeal tissues. Downregulated expression of E-cadherin was also detected in two NPC cell lines (HNE-1 and CNE-2) using RT–PCR and western blot, with normal nasopharyngeal tissue used as a positive control in which E-cadherin is expressed at a high level (Fig. 1B).

Table I. PCR primer sequences.

Primers	Sequences	Product size
E-cadherin-forward	5′-TTAAGGGGTCTGTCATGGAAGGT-3′	109 bp
E-cadherin-reverse	5′-GTGTAAGCGATGGCGGCATTGTA-3′	
β-actin-forward	5′-GAACCCCAAGGCCAACCGCGAGA-3′	149 bp
β-actin-reverse	5′-TGACCCCGTCACCGGAGTCCATC-3′	
MSP-methylated-forward	5′-TAATTTTAGGTTAGAGGGTTATCGC-3′	213 bp
MSP-methylated-reverse	5′-CTCACAAATACTTTACAATTCCGAC-3′	
MSP-unmethylated-forward	5′-ATTTTAGGTTAGAGGGTTATTGTGT-3′	215 bp
MSP-unmethylated-reverse	5′-CAAACTCACAAATACTTTACAATTCCA-3′	
BGS-forward	5′-AGTGTAAAAGTTTTTTTTGATTTTA-3′	431 bp
BGS-reverse	5′-ACTCCAAAAACCCATAACTAACC-3′	

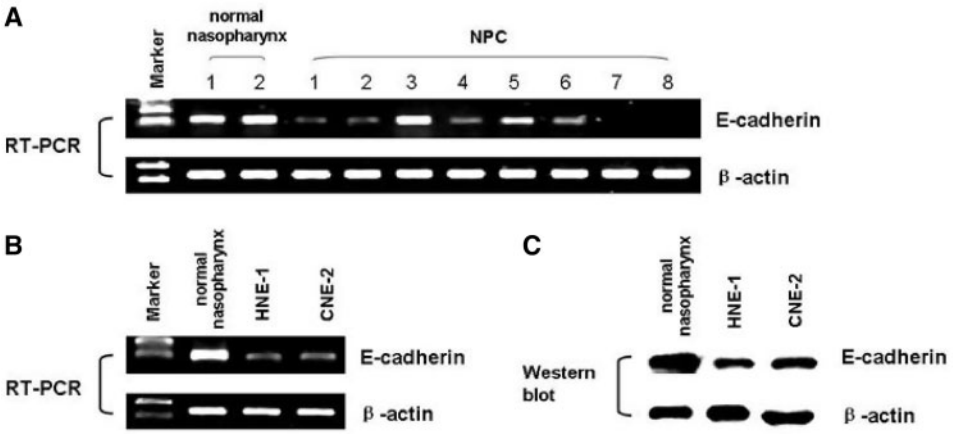

Fig. 1 Reduced expression of E-cadherin in NPC biopsies and cell lines. Total RNA isolated from primary NPC biopsies (A) and NPC cell lines (B) was subjected to RT–PCR analysis with E-cadherin specific primers. Normal nasopharyngeal tissue was used as a positive control and β-actin was used as an internal loading control. (C), total cell lysates from HNE-1 or CNE-2 cells was collected and the expression of E-cadherin protein was determined by western blot analysis. β-actin was used as an internal loading control.

Aberrant expression of E-cadherin correlates with promoter methylation in NPC

Treatment with sodium bisuphite converts the unmethylated cytosine residues in the genomic DNA to uracils, whereas the methylated cytosines remain unaltered. Thus, the promoter methylation status was analysed by MSP using methylation- or unmethylation-specific primers to examine whether the CpG island methylation correlates with the aberrant expression of E-cadherin. Modified genomic DNA isolated from tumour tissues and normal tissues was subjected to MSP. Methylated E-cadherin was detected in 13 of 20 (65%) primary NPC biopsies, whereas no methylation was observed in five normal nasopharyngeal tissues (Fig. 2A, corresponding to Fig. 1A). This result demonstrated downregulated transcription of E-cadherin in the primary NPC tumours in which E-cadherin was methylated compared with the normal nasopharyngeal tissues. Intriguingly, downregulated E-cadherin observed in three NPC biopsiess (*e.g.* Fig. 1A, lanes 7) in which the tumours were devoid of methylation could be caused by other mechanisms such as transcriptional repression. Next, we determined whether methylation of E-cadherin causes its suppression in NPC cell lines. The results showd both methylated and unmethylated alleles occured in HNE-1 and CNE-2 cells, suggesting that partially promoter methylation may account for the reduced expession of E-cadherin (Fig. 2B). These cell lines provided a panel for subsequent analyses to define the mechanism for inactivation of E-cadherin expression by gene promoter methylation.

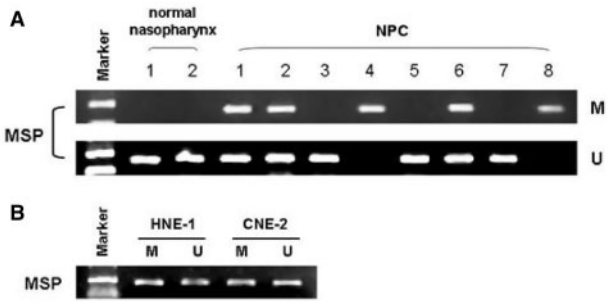

Fig. 2 Representative MSP results of the primary NPC biopsies (A) and NPC cell lines (B). M, PCR reaction by primers which specifically amplify methylated E-cadherin alleles; U, PCR reaction by primers which specifically amplify unmethylated alleles.

Detailed pattern of CpG methylation in the E-cadherin promoter

Exon 1 and the upstream sequence are CpG-rich and show the feature of the CpG island. Figure 3A shows the CpG island on 5′-end of the E-cadherin gene, which harbours 33 CpG sites, in accordance with the published sequence from NCBI. To further explore the methylation details of E-cadherin in NPC, we performed high-resolution BGS analysis with two NPC cell lines; two primary NPC tumour biopsies and the normal nasopharyngeal epithelia tissue. A region of 431 bp surrounding E-caherin CpG island was amplified with bisulphite-modified DNA and the entire desired fragments were sequenced. The presence of cytosines in a CpG context after bisulphate conversion indicates that it is methylated in the genomic DNA of the sample being analysed. These data indeed demonstrated that the majority of the CpG dinucleotides within the analysed region were heavily methylated in the two primary NPC biopsies but were free of methylation in one normal nasopharyngeal epithelia. The results also clearly revealed the minor existence of methylated CpG sites in HNE-1 and CEN-2 cells (Fig. 3B).

Restoration of E-cadherin by treatment with demethylating agent 5-aza-dC

To examine whether promoter methylation was directly responsible for E-cadherin downregulation, HNE-1 and CNE-2 cells were treated with 20 μM of demethylating agent 5-aza-dC. Figure 4A and B exhibit the explicit increase of E-cadherin mRNA and protein expression in HNE-1 and CNE-2 cells

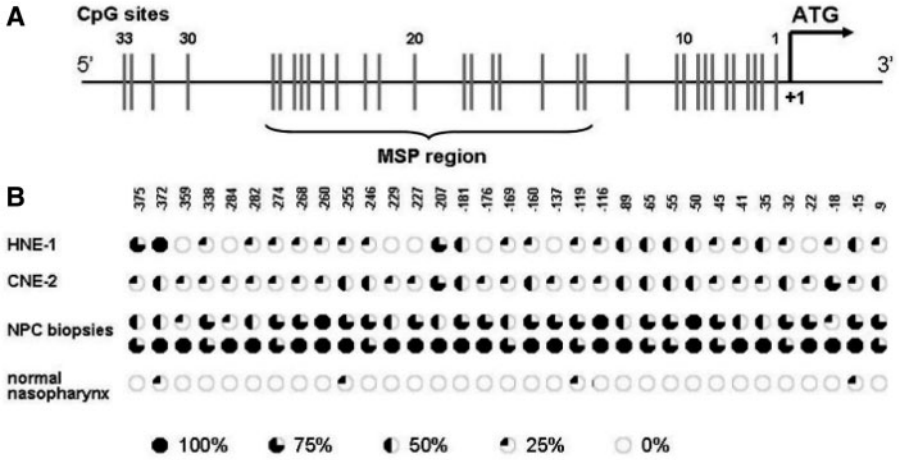

Fig. 3 BGS of E-cadherin CpG island in NPC biopsies and cell lines. (A) A scheme of the E-cadherin promoter region. Thirty-three CpG sites within the E-cadherin gene promoter were included for analysis by bisulphite sequencing. Vertical lines indicate the relative locations of CpG sites to the translation initiation site ATG which was indicated as '+1'. (B) Methylation status of each CpG site within the E-cadherin gene promoter was analysed by bisulphite sequencing PCR. The PCR products were subsequently subcloned into sequencing vector and four clones were sequenced for each sample. Every row represents a single clone. The solid and open square corresponds to methylated and unmethylated cytosines, respectively. Circles were partially filled according to the percentage of methylation of the CpG site represented.

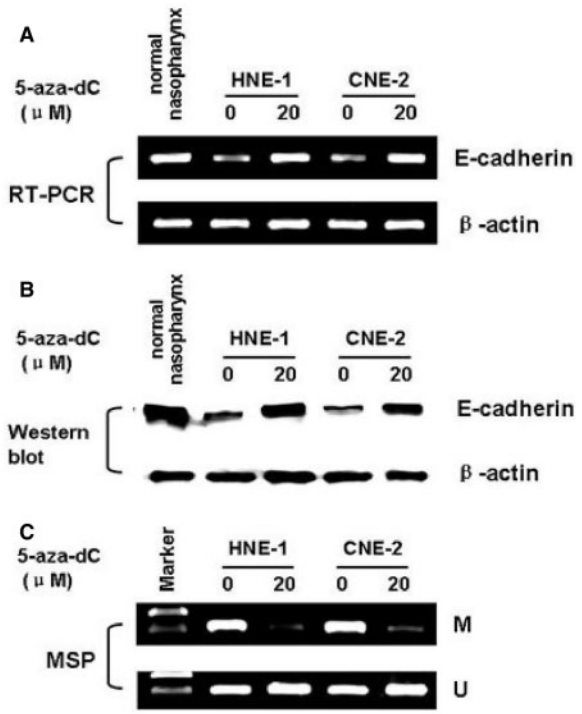

Fig. 4 Recovered expression of E-cadherin after treatment with demethylating agent 5-aza-dC. HNE-1 and CNE-2 cells were treated with 5-aza-dC for 72 h and increased expression of E-cadherin mRNA and protein expression were detected by RT–PCR (A) and western blotting (B). Normal nasopharyngeal tissue was used as a positive control and β-actin was used as an internal loading control. (C) MSP analysis of E-cadherin methylation status in HNE-1 and CNE-2 cells after demethylating treatment. M, methylated; U, unmethylated.

after 5-aza-dC treatment. This result suggests that demethylation of E-cadherin gene by treatment of demethylating agents could lead to its restoration. Moreover, 5-aza-dC treatment also increased unmethylated E-cadherin alleles and decrease unmethylated ones, as determined by MSP (Fig. 4C).

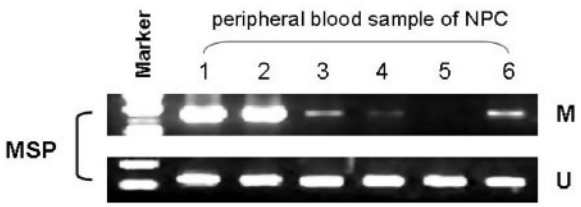

Fig. 5 Representative MSP results using bisulphite-modified peripheral blood DNA of NPC patients. M, PCR reaction by primers which specifically amplify methylated E-cadherin alleles; U, PCR reaction by primers which specifically amplify unmethylated alleles.

Detection of E-cadherin methylation from peripheral blood

To evaluate the potential diagnostic value of methylated E-cadherin, we further explored the feasibility of detecting it using bisulphite-modified peripheral blood DNA of NPC patients. Nine of 20 (45%) cases were found to be methylated, suggesting that methylated promoter DNA of peripheral blood might be potentially useful as tumour marker for prediciton and prognosis of NPC (Fig. 5).

Discussion

NPC is a subtype of head and neck cancers with frequent incidence in Southern China and Southeast Asian countries *(13)*. In spite of improved results with concurrent chemoradiation, significant numbers of patients with locally advanced disease still relapse after treatment and patients with metastatic disease are not cured with conventional treatment. E-cadherin is a cell surface adhesion glycoprotein which contributes to maintenance of tissue integrity in epithelium and is regarded as a tumour suppressor gene. Loss of E-cadherin expression has been shown to be critical for invasion and metastasis of epithelial tumours *(14)*.

DNA methylation is a common epigenetic mechanism involved in silencing the expression of many

tumour suppressor genes. Methylation of the CpG island of tumour suppressor genes has been reported in multiple types of cancers. To determine the inactivation mechanism of E-cadherin in NPC, we studied the methylation status of this gene by MSP analysis. The present study showed a high frequency of promoter hypermethylation of E-cadherin gene in NPC tumours but not in normal nasopharyngeal tissues, which inversely correlated with E-cadherin expression. This result indicates that 5′-CpG island methylation may play an important part in the inactivation of E-cadherin in NPC. Aberrant methylation of E-cadherin was also observed in two NPC cell lines with decreased expression compared with the normal nasopharyngeal tissue. High resolution of methylation status in 5′-CpG island of gene promoter region is indispensable to achieve more accurate information *(15)*. Here, we show the accurate map of the distribution of CpG methylation by bisulphite sequencing in NPC biopsies and cell lines. Methylation of 33 CpG sites was heavily methylated in NPC biopsies, in contrast to the normal nasopharyngeal tissue which showed essentially no methylation. Bisulphite sequencing results from each sample were strongly correlated with the MSP analysis, implicating that our bisulphite treatment of the DNA was complete and the MSP results were reproducible.

The observation of downregulated E-cadherin due to its promoter methylation opens the way for the development of new preventive and therapeutic strategies using demethylating agents. One demethylating agent 5-aza-dC, which inhibits DNA methyltransferase, resulting in the reactivation of methylation-silenced genes, has been used as an anti-cancer agent for patients with chronic myelogenous leukemia in the expectation of gene reactivation *(16, 17)*. Based on its demethylation properties, the NPC cells was treated with 5-aza-dC for restoration of E-cadherin to confirm that methylation directly mediates the downregulation of E-cadherin. This treatment apparently caused a decrease of the methylation status of E-cadherin promoter region in both cell lines, so that significant increase of both E-cadherin expression and mRNA transcription was observed. This finding agrees with the earlier reports on many other types of carcinoma cell lines, in which 5-aza-dC exerted the similar effect on E-cadherin expression *(18–20)*. This result further confirmed that DNA methylation is a major mechanism to regulate E-cadhern expression, implicating demethylation could be considered as one of the candidates to improve therapy of NPC.

Tumour cells can be released from the tumour mass more easily than normal epithelial cells, especially when the normal cell–cell adhesion is subverted. The presence of circulating tumour cells is an important step in the development of distant metastasis. Examination of molecular alterations of tumour cells in body fluids is more convenient for early detection and diagnosis of many cancer types *(21–23)*. Hypermethylated DNA in peripheral blood may serve as potential molecular tumour markers because of its high specificity to differentiate cancer from normal tissues *(24, 25)*. We therefore analysed the possibility of methylated E-cadherin in circulating tumour DNA from NPC patients as a valuable biomarker. A high frequency of methylation (35%) was also noted in 20 NPC peripheral blood samples. The presence of detectable hypermethylated E-cadherin in peripheral blood samples of NPC patients indicates the existence of circulating cancer cells. This result may lead to a more useful application of methylated E-cadherin in NPC detection and diagnosis.

In conclusion, we determined that promoter methylation around the promoter region, which was confirmed by bisulphite sequencing for detailed methylation status of individual CpG site in CpG island, plays an important role in E-cadherin inactivation in human NPC. Given that the E-cadherin gene is now recognized as an invasion suppressor gene, restoration of E-cadherin function by demethylating agents may have clinical value as a potential therapeutic strategy for NPC. Moreover, the possible detection of methylated E-cadherin in peripheral blood suggested that it might be one of the potential useful tumour markers in clinical application. We hope our findings could provide a deeper understanding for E-cadherin inactivation in human NPC, so improved diagnosis and therapy can be facilitated.

Acknowledgements

We thank Dr Ying Ruan for the gift of NPC cells.

Conflict of interest
None declared.

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