IN SILICO ANALYSIS OF HYPERMETHYLATION OF E-CADHERIN GENE PROMOTER IN NASOPHARYNGEAL CARCINOMA

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ABSTRACT

Background: DNA hypermethylation changes in CpG islands of promoter region of *E-cadherin* (*E-cad*) gene, one of the tumor suppressor genes, have been described to be involved the formation and progression of human nasopharyngeal carcinoma, which is the most common and highly incident cancer of head and neck cancer in Asian countries, especially in Vietnam.

Purpose: In Vietnam, there is still no research about *E-cad* promoter methylation in NPC, thus, in current report, a systematic literature revision was carried out to summary the current evidences about the frequencies of *E-cad* gene promoter on NPC for further applied in Vietnamese population.

Methods: A systematic literature analysis was conducted based on the comprehensive search of observational studies. Moreover, CpG islands of candidate gene and transcriptional factors were predicted by using many bioinformatics tools, such as Methprimer, TFsearch, etc.

Results: Total of 9 previous published studies were identified and accessed for eligibility from the literature research and enrolled into systematic revision. The variants of *E-cad* hypermethylation frequency ranked from 11.0% to 64.55% were observed. Moreover, the average weight frequencies of methylated and unmethylated E-cad gene promoter were 55.46% and 40.78%, respectively. Moreover, by several bioinformatics tools, we were successful in predicting the CpG islands as well as identifing transcriptional factor binding sites, served as "hot spot" for ideal primer pick up, located in candidate gene promoter.

Conclusion: Based on these data, it suggested that the hypermethylation of *E-cad* gene promoter was a significant characteristic of NPC, in which, it could be further applied in evaluation of *E-cad* gene promoter status in Vietnamese population.

Keywords: *E-cad* gene; hypermethylation; Nasopharyngeal carcinoma.

1. Introduction

Nasopharyngeal carcinoma (NPC), a nonlymphomatous squamous cells carcinoma raising from the epithelial lining of the nasopharynx, is considered as a highly invasive and malignant tumor of the nasopharynx, the uppermost region of the pharynx (Sham *et al*. 1990). NPC has a striking geographic and ethic distribution, gravitating toward Southeast Asia, especially in China and Vietnam. According to Globocan (2012), in the world, the total of number of NPC was 86,691 cases (Age-standardized rate – ASR = 1.2/100,000), while the number of death was 50,831 (ASR = 0.7/100,000). Among these cases, Vietnam was one of countries contributed the most to these indices, because the total number were

4,931 cases (ASR = 5.4/100,000) and deaths were 2,885 cases (ASR = 3.3/100,000) (Globocan, 2012), when compared to Europe (incidence = 4172 cases; death = 2134 cases), Africa (incidence = 3031 cases; death = 2069 cases), etc. The etiology of NPC has been considered multiple factors, including viral infection, genetic factors and environmental variables (Lo et al. 2004; Tsao et al. 2014). Recent previous studies suggested that NPC is associated with the accumulation of many epigenetic alterations on the particular chromosomal regions and genes, including the alterations involve both tumor suppressor genes (TSGs) and proto-oncogenes on multiple cellular pathways, which further contribute to the malignant cancer hallmarks (Liu et al. 2004; Dai et al. 2016). The prevalent epigenetic change is the hypermethylation of CpG islands in promoter regions of genes, has been identified as the common mechanism of TSGs silenced, contributing to many cell processes in cell-cycle regulation, apoptosis, signal transduction, cell adhesion, etc., which involves in tumorigenesis, including nasopharyngeal carcinoma (Kwong et al. 2002). The hypermethylation of a variety of TSGs' promoters, such as *RASSF1A* (*Ras association domain family 1A*), *RARβ* (*retinoic acid receptor β*), *E-cad* (*E-cadherin*), *Blu* (*MYND type containing 10*), *DAPK* (*Death-associated protein kinase*), etc. have been reported to be associated with loss of tumor suppressor protein expression in NPC cells, consequently, led to tumorigenesis. *E-cad* (*E-cadherin, CHD1*), locates at 16q22.1, a member of TSGs, has been proven to be an important role in the formation and maintenance of normal architecture and functions of epithelial tissues (Takeichi, 1991). Its encoded protein, E-cad protein, is a 120-kDa transmembrane glycoprotein, localized in lateral cell – cell contacts and enriched in the zonula adherens junctions, which is involved in mediating the cell adhesion between adjacent epithelial cells in various tissues (Takeichi, 1991, 1995). E-cad mediates Ca^{2+}-dependent hemophilic binding and a cytoplasmic domain that interacts with the catenin polypeptide alpha and beta (or plakoglobin) and p120ctn. This complex interacts with the actin cytoskeleton and physically links cells to each other (Gottardi et al. 2001). Numerous studies have suggested that the E-cadherin adhesion system is dysregulated in several human carcinoma progression and metastatic spread of tumors (Bringuier et al. 1993; Siitonen et al. 1996; Zheng et al. 1999; Tamura et al. 2000; Tsao et al. 2003). Zheng et al. (1999) proposed that the downregulation of E-cad was the common event in NPC which accounted for more than 90% of the case of NPC. Other research was carried out by Tsao et al. (2003), they also reported that 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. It suggests that the transcriptional silencing by 5' CpG island methylation in the promoter region of E-cad could be an important event and associates with advanced stage of NPCs.

In Vietnam, there is still no research about *E-cad* promoter methylation in NPC, thus, in current report, we summarized the current evidences about the hypermethylation frequencies of *E-cad* gene promoter on NPC in various previous published studies via a systematic literature revision. The identification of candidate gene may serve as targets for the further experiment and screening test for NPC in Vietnamese population.

2. Materials and methods

Literature search, eligibility and data abstraction

A systematic literature analysis was conducted based on the comprehensive search of observational studies. Previous published

reports in peer-reviewed journals were obtained from the following databases using validated search strategies: Ovid MEDLINE database, ISI Web of Science, Science Citation Index Expanded, PUBMED, PUBMED CENTRAL, etc. The following keywords were used for literature research: Nasopharyngeal carcinoma, *E-cadherin*, *CHD1*, hypermethylation, etc. Other published reports were found in the reference lists of the retrieved articles and preceding reviews on this topic. The literature search was conducted up to October, 2015. For these articles, we also extracted the type of study separating prospective studies from others, mainly based on case – control studies. When data were available, we reported the range and average hypermethylation frequencies of candidate gene in both case – control studies. Average hypermethylation frequencies was calculated by following formula:

$A = \frac{\sum Wi.ai}{\sum Wi}$ (Note: W: weight of study i; a: percentage of study i)

Additionally, the detection of methylated gene in NPC is technically feasible by numerous techniques invented for the mapping of Cytosine methylation, thus, kinds of method were systematic enrolled into our current *in vitro* studies to have general vision in techniques permit the highly specific and sensitive identification.

CpG island prediction

For the primer selection, CpG islands have to be predicted with the promoter sequence of the target gene. The promoter of E-cad sequence was collected from Genecards® Human database (http://www.genecards.org/) with following keywords: CHD1 within Prod ID S722792. Several bioinformatics programs such as: MethPrimer (www.urogen.org), TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) were applied used to predict CpG islands and transcriptional factor binding sites located in CpG islands in promoter regions of E-cad gene.

3. Results

Characteristics of included studies

Overall, total of 9 previous published studies were identified and accessed for eligibility from the literature research and enrolled into systematic revision by using many keywords described above. The characteristics of those studies were shown in Table 1.

Table 1

Characteristics of the studies included in this literature search

Studies	Country	Method	Number of case/Type	
			Case	Control
Chang et al. (2003)	Hong Kong	MSP	30 (biopsy)	6 (biopsy and body fluid)
			43 (MTF)	
			37 (swab)	
			43 (PBB)	
Wong et al. (2003)	Hong Kong	MSP	30 (biopsies)	5 (biopsies)
Tsao et al. (2003)	China	Real-time-MSP	21 (metastatic lymph node)	-
Wong et al. (2004)	China	Real-time-MSP	41 (plasma)	43 (plasma)
Tan et al. (2006)	China,	MSP	19 (tumor	-

Studies	Country	Method	Number of case/Type	
			Case	Control
	Malaysia		biopsies)	
Ayadi et al. (2008)	Tunisia	MSP	44 (biopsy)	3 (tissue)
Niemhom et al. (2008)	Thailand	MSP	38 (PET)	15 (PET)
Marsit et al. (2008)	Boston	MSP	340 (tissue)	-
Challouf et al. (2012)	Tunisia	MSP	36 (biopsy paraffin)	19 (biopsy)

Note: PET: paraffin-embedded tissues; MTF: Mouth and throat rinsing fluid; PBB: peripheral blood plasma and buffy coat; -: no reported.

During our search of articles in the many electronic databases, the frequencies of candidate gene was observed in a wide range of methylation in various studies. As shown in Table 1, according to systematic analysis, nasopharyngeal biopsy tissue was the majority of sample sources used in methylation detection. Additionally, the results showed that, for the methylation analysis, MSP method, counting for 77.78% (7 of 9 studies), was the majority technique for mapping methylated candidate gene status in NPC. Figure 1 showed the frequencies of candidate gene were observed, based on the previous studies, in a wide range of methylation for reported gene between various studies, ranked from 11.0% to 64.55%. Additionally, the average weight frequencies of methylated and unmethylated E-cad gene promoter were 55.46% and 40.78%, respectively, in NPC, were computed. Additionally, the low hypermethylation frequency was observed in normal tissue, counting for 11.0% (Figure 1).

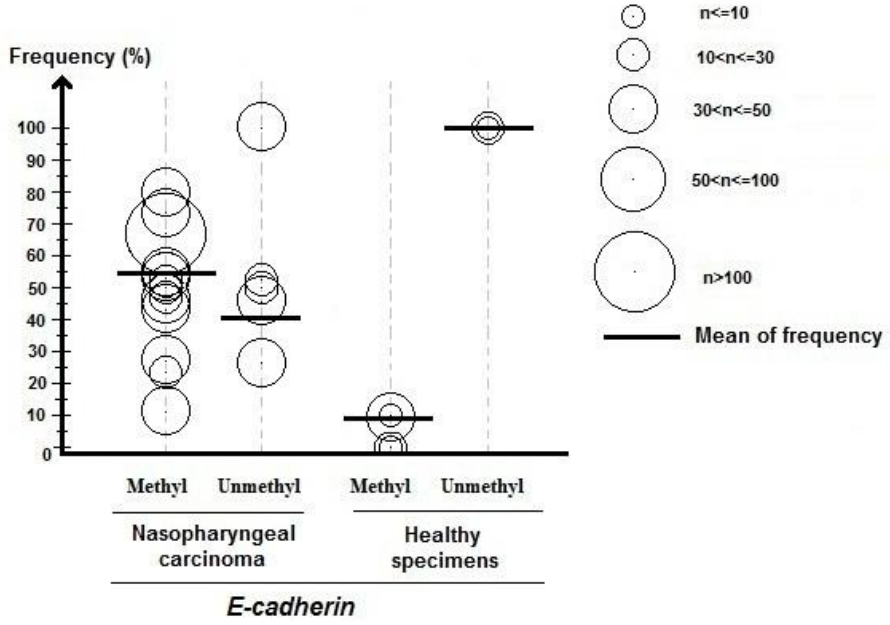

Figure 1. Methylation frequencies of *E-cad* gene promoter were reported NPC and non-cancerous samples. The center of the circle indicated the reported frequency. The size of circle indicated the size of the study. Horizontal lines indicated the mean weighted average methylation frequency for each candidate gene based on all previous studies

Prediction of CpG islands and primer selection

The CpG sites in candidate gene promoter and transcriptional factor binding sites were identified by using Methprimer, TFsearch, MethBlast. The CpG islands of *E-cad* and transcriptional factor binding sites located in CpG islands were also identified, showed in Figure 2.

By Methprimer, two CpG sites in promoter of *E-cad* was identified to be located in Nu. 465 to Nu. 619 within 155 bps length, and Nu. 687 to Nu. 826 within 140 bps length of 883-bps length promoter. The results of transcriptional factor prediction showed many transcription factor binding sites were identified, such as AP1, OCT1, GATA1, ER, STAF, etc. with the S score ranked from 0.7372 to 0.8958. These sites are significant regions for picking up the MSP primer since the aberrantly hypermethylated Cytosine residue in CpG, that lead to transcriptional inactivation. As shown in Figure 2, the methylated/unmethylated primers were picked up to cover the CAAT, STAF factor. Additionally, many transcriptional factors, such as P53, CAP, AP1, etc. were included in the product that yielded by methylated/unmethylated primers. Parameters of primer pairs, including Bisulfite sequencing, MSP methylated/unmethylated primers, were shown in table 2 such as melting temperature, length, GC-base pair ratios, Gibbs Free Energy (Δ G) for secondary structures (hairpin, self-dimer, and heterodimer).

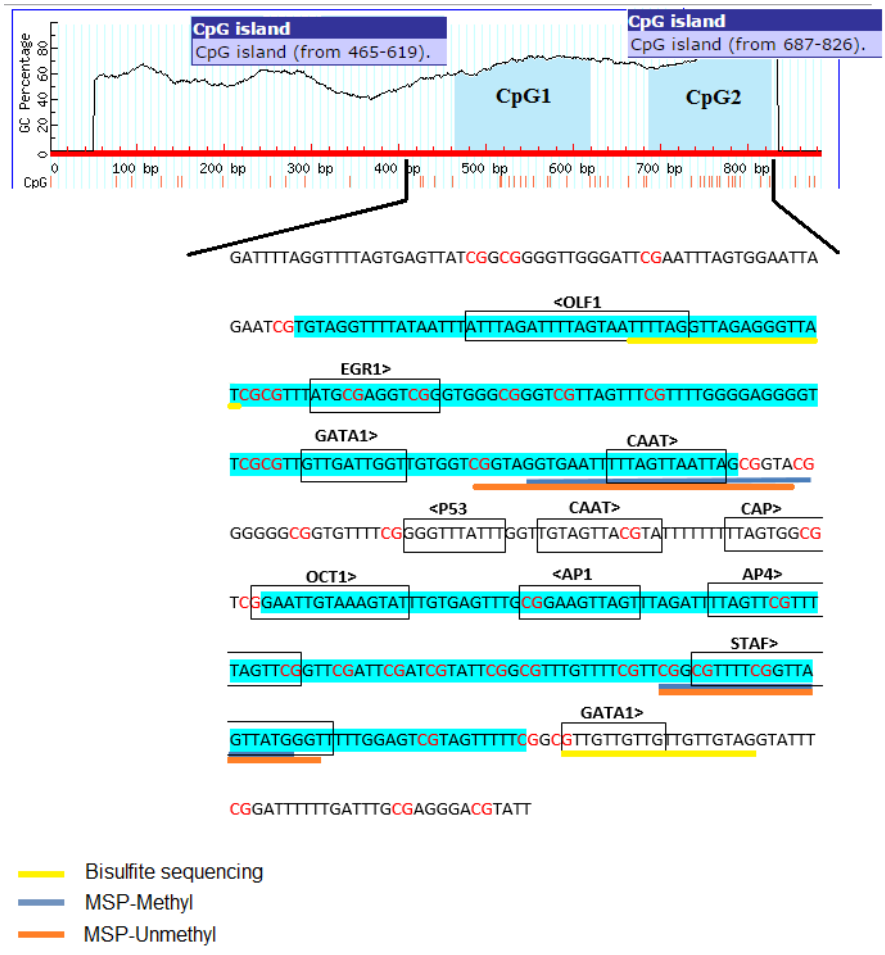

Figure 2. CpG islands in *E-cad* gene promoter and the transcription binding sites located in CpG site. Note: Red Characters: CpG, Blue region: CpG islands

Table 2

Primer sequences and parameters

Primer	Sequences (5'-3')	L (bp)	Tm (^{O}C)	% GC	(1)	(2)	(3)
Bisulfite sequencing	Forward: TTTTAGGTTAGAGGGTTAT	19	44,6	31,6	2,28	-1,47	-3,29
	Reverse: CTACAACAACAACAACAAC	19	47,1	36,8	-	-0,96	
MSP methylated	Forward: GGTGAATTTTTAGTTAATTAGCGGTAC	27	52,8	33,3	0,42	-7,29	-8,75
	Reverse: CATAACTAACCGAAAACGCCG	21	54,3	47,6	0,63	-3,61	
MSP unmethylated	Forward: GGTAGGTGAATTTTTAGTTAATTAGTGGTA	30	53,5	30	0,42	-7,29	-8,75
	Reverse: ACCCATAACTAACCAAAAACACCA	24	54,9	37,5	-	-1,47	

Note: L: Length; Tm: Melting temperature; Gibbs free energy (kcal/mole) for hairpin loop (1); homodimer (2) and heterodimer (3) structure formations.

4. Discussion

According to the screening and diagnosis of NPC, to date, many clinical screening methods for NPC, including radiological imaging (CT or MRI scan), and endoscopic guided nasopharyngeal biopsy for histological examination are expensive and could not be repeated serially in NPC monitoring (25). Therefore, finding a simple method for screening and early diagnosis of NPC is necessary. For the past few years, in addition to the viral infections, which has been indicated to be strongly related to human NPC, the induction of epigenetic alteration, methylation of TSGs, is now regarded as one of the important mechanisms mediating the cancer development. Aberrant CpG island methylation could occur during the early stage of tumor pathogenesis through chiting many key signaling pathways, even in pre-invasive lesions (11, 31). E-cadherin gene located at 16q22, has been characterized as adherens junction protein, which through homotypic interactions contribute to the maintenance of the epithelial barrier function and regulated several intracellular signal transduction pathways, such as Wnt/β-catenin, P13K/Akt, Rho GTPase and NF-κB signaling (32). Notably, frequently disrupting of E-cad causes the loss of cell adhesion via methylation, leading to the cell metastasis in cancer development, has been reported in human NPC in various countries, including China, Thailand, Boston, etc. (Li et al. 2011). Considering to Vietnam, to date, there was no research was carried on evaluation of the hypermethylation status of E-cad gene promoter in NPC, as well as provide whether

or not an association between patterns of DNA hypermethylation led to risk of NPC. Therefore, in our initial study, we have to integrated the previous knowledge and findings to have an overview of methylation/unmethylation profiles of E-cad gene by calculating of the average weight frequencies of methylation and unmethylation of E-cad gene. Additionally, the CpG sites and transcriptional factors binding sites were predicted to pick up the suitable primers for our experiments carried out on analysis of E-cad methylation profile in Vietnamese population.

The CpG sites in *E-cad* gene promoter and transcriptional factor binding sites were predicted by many bioinformatics tools. Many transcriptional factors, such as AP1, OCT1, GATA1, ER, STAF, etc., which located at the promoter of *E-cad*, were identified by bioinformatics tools. These sites were significant regions for picking up the MSP primer since the aberrantly hypermethylated Cytosine residue in CpG, that lead to transcriptional inactivation. In our study, Nest-MSP was chosen to increase the sensitivity and specificity methylation analysis. In detail, by our analysis, the outer primers covered were numbered 498-516 (forward primer) and 830-848 (reverse primer), which yielded 351-bps length product. The methylated/unmethylated primers were chosen to cover at least one CpG islands belonged to *E-cad* gene promoter. The methylated primers were numbered 599 – 625 (forward primer) and 783-803 (reverse primer), whereas unmethylation-specific primer were numbered 595 – 624 (forward primer)) and 783 – 806 (reverse primer).

Therefore, based on our data, we concluded that the hypermethylation of *E-cad* gene promoter were significant association and contribution to the risk of NPC, might serve as molecular targets for screening test for NPC, which was affirmed again based on our systematic literature analysis, especially, in further study, applied in Vietnamese population.

5. Conclusion

We successfully performed a systematic literature revision and computed average methylation frequencies of E-cad gene was 55.46%. The low methylation frequency was observed in normal tissue, counting for 11.0%. Moreover, Nested-MSP method was the most applied technique in evaluation methylation status of candidate gene. In which, the prediction of CpG islands were carried out and definitely confirmed the specificity and referent MSP primers. These databases will be the useful information for the identification of indicated genes that may serve as targets for the further experiment and screening test for NPC in Vietnamese population■

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