IN SILICO ANALYSIS OF HYPERMETHYLATION IN CPGISLANDS OF *UCHL1* GENE'S PROMOTER IN NASOPHARYNGEAL CARCINOMA

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ABSTRACT

Background and Objective: The methylation of Ubiquitin C-Terminal Hydrolase L1 (*UCHL1*) gene has been reported in many human cancers including nasopharyngeal carcinoma (NPC). In Vietnam, the methylation of UCHL1 gene's promoter in NPC has not been demonstrated yet. In this study, a systematic literature revision was carried out to summarize the current evidences about the frequencies of *UCHL-1* gene's promoter methylation in NPC for further application in Vietnamese population.

Methods: A systematic literature analysis was conducted based on the comprehensive studies. Moreover, many bioinformatic tools such as Methprimer, TFsearch, IDT OligoAnalyzer 3.1 were used to predict the CpG islands, transcriptional factors, and to pick up the MSP (Methylation-Specific PCR) primers.

Results: Total of three previous studies were summarized and accessed for eligibility from literature research. As the results, the average weight methylated frequencies were 72.4% and 13.0% for NPC and non-cancerous samples, respectively. The significant association between *UCHL-1* promoter methylation and NPC with the OR of 10.459 (95% CI = 4.915 − 22.254, p < 0.001) and RR of 4.117 (95% CI = 1.958 − 6.645, p < 0.0001) based on the random effects model, was observed. Moreover, we were successful in predicting the CpG islands as well as identifying transcriptional factor binding sites which served as "hot spot" for ideal primer pick up and located in gene promoter.

Conclusion: The methylation of *UCHL-1* gene promoter was significantly associated and contributed to NPC developmentin which it could be further applied in evaluation of *UCHL-1* gene promoter status in Vietnamese population.

Keywords: Methylation; MSP; NPC and *UCHL-1* gene.

1. Introduction

The DNA methylation that refers to a covalent modification of Cytosine ring at the 5' position of CpG dinucleotide by adding a methyl group in the 5th Carbon of the ring by S-adenosyl methionine has been identified as the common mechanism of tumor suppressor genes (TSGs) silenced, involves in many human diseases (Yuanyuan et al., 2014; Le et al., 2017). It is noted that the CpG dinucleotides are concentrated in the upstream promoter regions of many genes, therefore, the promoter methylation of genes involved in many cell progresses such as DNA repair, cell-cycle regulation, signal transduction has already confirmed in various tumor types, including nasopharyngeal carcinoma (NPC) (McCabe, Caudill 2005; Tsao et al., 2014; Le

et al., 2017). A serie of TSGs are silenced by promoter CpG methylationidentified in NPC, including *UCHL1*, play a key role in NPC pathogenesis (Kwong et al., 2007; Ayadi et al., 2008; Chan et al., 2010; Li et al., 2010; Tian et al., 2013).

Concerning to NPC, NPC has a striking and geographic distribution, which gravitatestoward China and Vietnam (Pathmanathan et al., 1995; Da Costa et al., 2015; Dai et al., 2016). Notably, Vietnam is considered as one of most panic areas which iscontributed to these indices, due to 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). The major obstacle to early diagnosis and screening of NPC are the different access due to the deeply seated location of nasopharynx, as well as the unclear presenting symptoms, such as hearing loss, nosebleeds, headache, trouble opening the mouth, etc.,.(ref). Consequently, to achieve favorable treatment and increasing of patient's survival, early diagnosis and screening are necessary to be established. The major etiological factors proposed for nasopharyngeal cancer includes viral infection, genetic susceptibility, the aberrant methylation of CpG islands belonged to the TSGs' promoter, and environment factors (Tsao et al., 2014). Herein, we summarize some recent studies on the CpG aberrant methylation, as well as the prediction of CpG islands in *UCHL1* gene for further using methylated genes as a molecular marker. This may provide a useful biomarker for future diagnosis and prognosis in Vietnamese cancer patients.

2. Marterials and Methods

2.1. Search strategy, selection criteria, eligibility and data extraction

Firstly, a systematic literature analysis was conducted in the Medline database, using PubMed, was carried out for the *UCHL-1* promoter methylation in NPC,

published before October 2017. The following keywords: Nasopharyngeal carcinoma, *UCHL1*, methylation, epigenetics, and nasopharyngeal cancer were used for literature research. For these articles, the type of study separating prospective studies from others, mainly based on case – control studies was extracted. When data were available, the range and average methylation frequencies of candidate gene in both case – control were reported. 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 systematically enrolled into our current *in vitro* studies to have general vision in techniques permitting the highly specific and sensitive identification of CpG methylation.

2.2. CpG island prediction

For picking primer, CpG islands were necessary to be predicted with the promoter sequence of candidate gene. The promoter sequence and information of *UCHL1* gene were downloaded from Ensemble database (https://asia.ensembl.org/index.html). Several bioinformatics programs such as: Methprimer (www.urogen.org), TFsearch (http://cbrc3.cbrc.jp/papia/howtouse/howtouse _tfsearch.html) were applied forpredicting CpG islands and transcriptional factor binding sites located in CpG islands in promoter regions of *UCHL1* gene. For evaluation of MSP primers, primerphysical characteristics were computed by IDT OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer).

3. Results

3.1. Systematic literature analysis

After exclusion of studies that did not meet the inclusion criteria, up to now, only three studies were carried out for the analysis of the *UCHL1* promoter aberrant methylation in NPC. The frequencies of *UCHL1* promoter were observed in different range of methylation. Overall, the mean weighted average of methylated UCHL1 were

72.40% and 13.00% in NPC samples and non-cancerous samples, respectively (Table 1). These results indicated that the frequency of *UCHL1* promoter in NPC samples was higher than normal samples. Concerning to methods, the MSP assay was the majority technique for mapping methylated candidate gene status in NPC. Additionally, *UCHL1* promoter methylation was associated with NPC risk with OR of 10.459 (95% CI = 4.915 – 22.254, *p < 0.001*) and RR of 4.117 (95% CI = 1.958 – 6.645, *p < 0.0001*) based on the random effects model (Table 2).

Table 1

Studies included in the systematic review

Studies	Year	Country	Method	SCS	SC	MT	MTF (%)	MC	MCF (%)
Li et al	2010	China	MSP	Tissue	Tissue	34/41	82.93	3/9	33.33
Loyo et al	2011	HongKong	qMSP	Biopsy	Biopsy	32/50	64.00	2/28	7.14
Tian et al	2013	China	MSP	Serum	Serum	24/37	64.86	8/41	19,52
Average weight frequency (%)							72.40		13.00

Note: SCS: Source of cancer sample; SC: Source of control; MT: Methylation Tumor; MC: Methylation control; MSP: Methylation specific PCR; MTF: Methylation frequency in NPC samples; MCF: Methylation frequency in non-cancerous samples; qMSP: quantitative Methylation specific PCR.

Table 2

Odds ratio and Relative risk of *UCHL1* gene promoter using Random effects model based on previous studies

Studies	OR	95% CI	P	W (%)	RR	95% CI	P	W (%)
Li et al	9.714	1.948 – 48.455		22.08	2.49	0.977 – 6.333		28.99
Loyo et al	23.11	4.906 – 108.871		23.74	8.96	2.319 – 34.618		13.85
Tian et al	7.62	2.730 – 21.241		54.19	3.32	1.709 – 6.467		57.16
Total	10.459	4.915 – 22.254	*< 0.001*	100.00	4.117	1.958 – 6.645	*< 0.001*	100.00

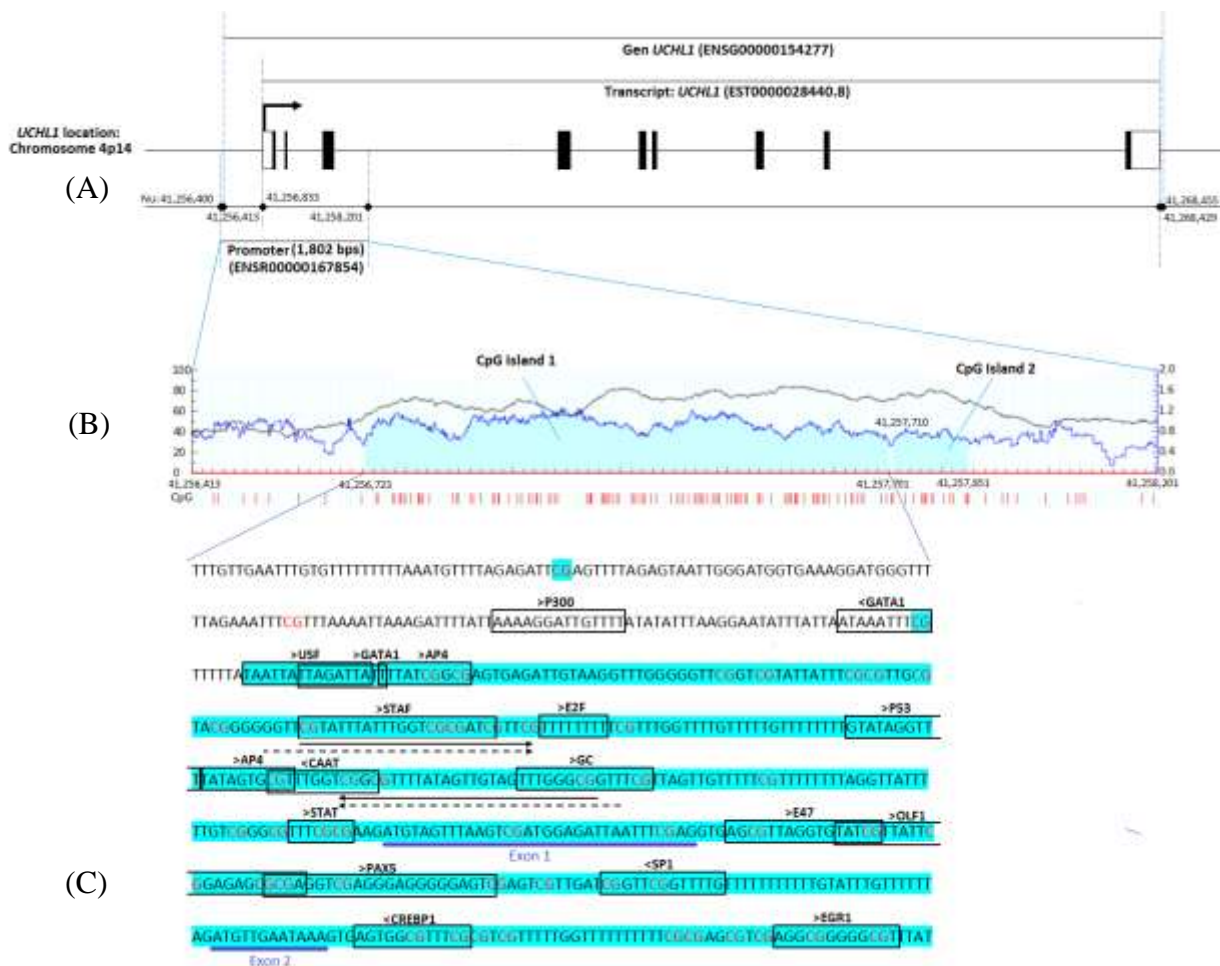### *3.2. Prediction of CpG islands*

Figure 1. (A): Schematic structure of *UCHL1* gene (locus: 4p13) and its promoter and exons (9 exons); (B): CpG Islands located in the promoter regions; (C) The predictive transcription factors and MPS primers identified in the CpG1. Line arrow: methylated primer; Dash arrow: unmethylated primer.

The *UCHL1* gene's sequence, promoter and transcript were collected from Ensembl database via accession number: ENSG00000154277, ENSR00000167854 and EST0000028440.8, respectively. *UCHL1* gene locates in 4p11-14, Nu. 41,256,833 to Nu. 41,268,455. The promoter of *UCHL1* gene locates in Nu. 41,256,413 to Nu. 41,258,201 (Fig. 1A). The CpG sites located in *UCHL1* genepromoter were identified by using MethPrimer. As the result, two CpG sites in promoter of candidate gene were identified to be located in Nu. 41,256,723 to Nu. 41,257,701 (yielded 979 bps) and Nu. 41,257,710 to Nu. 41,257,851 (yielded 142 bps) (Fig. 1B). In current study, CpG Island 1

(CpG1) was chosen to be searched for transcriptional factors and picked up the MSP primers. The results of transcription factor prediction showed many transcription factor binding sites were identified, such as STAF, CAAT, E2F, GC (Fig. 1C). Notably, the CpG1 covered exon 1 (Nu. 41,256,977 to Nu. 41,257,009), exon 2 (Nu. 41,257,115 to Nu. 41,257,126) and a partial exon 3 (Nu. 41,257,609 to Nu. 41,257,737). For MSP primer, the forward methylated/unmethylated primer and reverse methylated/unmethylated primer were picked up as covered five CpG sites, and three CpG sites, respectively (Fig. 1C). The primer sequences were showed in Table 3.

Table 3

The sequences of MSP primers

Primer	Sequence (5' – 3')
M_F	**CG**TATTTATTTGGT**CGCG**AT**CG**TTC
M_R	**CG**CCCAAACTACAACTATAAAA**CG**C**CG**
U_F	GGTT**TG**TATTTATTTGGT**TGTG**AT**TG**TT**T**
U_R	AACC**A**CCCAAACTACAACTATAAAA**CACCA**

Note: F: Forward primer; R: Reverse primer; CpG sites were in bold characters.

4. Discussion

To our knowledge, no any research related to characterize the methylation status of *UCHL1* gene promoter in NPC, has been carried out in Vietnamese population. In our study, we systematically evaluated the promoter methylation profile of *UCHL1* gene, which was considered as an important mechanism of its inactivation in NPC, based on previous studies, in order to have further studied on methylation of *UCHL1* gene in Vietnamese NPC samples. Even though, limit researches were carried out on evaluation of methylation of *UCHL1* gene's promoter, a moderate sized case-control group that had indicated the significant higher methylation in NPC samples (counting for 72.40%, based on the calculation of average weight frequency) than in non-cancerous samples (counting for 13.00%). It could be explained that, previous studies have shown that mechanism by which *UCHL1* functions as tumor suppressor gene is through ubiquitinating oncoproteins and deubiquitinating tumor suppressor genes (Li et al., 2010). Additionally, *UCHL1* has been shown to promote the degradation of cell cycle inhibitor p27^{kip1} and stabilize NF-κB and p53 (Caballero et al., 2002; Shen et al., 2006; Takami et al., 2007; Tokumaru et al., 2008; Li et al., 2010). By collecting the methylation/unmethylation of *UCHL1* frequencies in both NPC samples/non-cancerous, the OR and RR value were calculated, indicating that the *UCHL1* promoter methylation was significantly associated with an increased NPC risk with a pooled OR of 10.459 (*p < 0.0001*) and RR of 4.117 (*p <0.0001*). The OR represents the odds (10.459 times) for cancer risk in case of *UCHL1* promoter methylation occurred, compared to the absence of *UCHL1* promoter methylation. Thus, it suggested that methylation of *UCHL1* promoter would appear the promising methylated gene to obtain an appropriate performance for NPC screening, and further applied in clinical analysis.

Several bioinformatics tools were applied to identify CpG islands located in promoter region, as well as many key transcription factors located in CpG island. In current study, the CpG1 was chosen for methylation analysis, due to the CpG1 covers many CpG sites than CpG2 (Data not shown). Additionally, exon 1, exon 2 and partial exon was covered by CpG1. We confirmed that CpG sites located in several transcription factors, such as STAF (contains four CpG sites), GC (contains two CpG sites), thus, it were chosen for picking MSP primers, including forward primer covers five CpG sites, reverse primer covers three CpG sites, for evaluating the methylation of *UCHL1* gene's promoter. In current study, the MSP

primers were similar to Tian et al., (2013), however, in our methylated primer (forward) and unmethylated primer (both forward and reverse primer) were covered more one CpG site, which located in the transcription binding factor: CAAT, GC, than the research Tian et al. (2013), which may be increased the specificity of evaluation of methylated status of *UCHL1* gene.

Therefore, based on the analysis of previous studies, we confirmed that the methylation of *UCHL1* gene's promoter was significantly associated and contributed to NPC tumorigenesis. Those data will be useful for further experiments related to analyze *UCHL1* gene's promoter methylation status in Vietnamese population, that whether or not could be served as a promising biomarker for prognosis and early diagnosis NPC.

5. Conclusion

A significant association between *UCHL1* promoter methylation and NPC was shown and confirmed by systematical analysis of previous studies. Additionally, CpG island and MSP was successfully identified that covering many transcriptional factors, in which methylation may cause nasopharyngeal tumorigenesis. Further studies will be looked at experiment carried on clinical NPC samples/non-cancerous samples in Vietnamese population■

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