Establishing protocol for detecting methylation of Ubiquitin carboxyl terminal hydrolase 1 gene's promoter in nasopharyngeal carcinoma

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ABSTRACT

Background: The methylation of *Ubiquitin Carboxyl Terminal Hydrolase 1* (*UCHL1*) gene's promoter has been reported as the etiological factor of nasopharyngeal tumorigenesis. Purpose: This study is designed to establish a protocol for detecting methylation of *UCHL1* gene's promoter in nasopharyngeal carcinoma (NPC) in a Vietnamese population. Materials and methods: 10 samples of NPC biopsy tissues and 10 samples of non-cancerous swabs were collected from the local hospital. Chloroform/Phenol method and Nested-MSP assays were established to detect methylation of a target gene. Results: The isolated DNA reached purity and high concentration which were confirmed by the method of absorbance measurement at 260nm and 280nm. Additionally, the Nested-MSP products of methylation or unmethylation were analyzed and visualized in the agarose gel with the band of 169bps and 210bps, respectively. By sequencing, it was confirmed that the two sets of primer could distinguish the status of methylation and unmethylation of *UCHL1* gene's promoter. Conclusion: Our data suggested that the current protocol could successfully identify the status of methylation and/or unmethylation of *UCHL1* gene's promoter.

1. Introduction

Multiple factors, including (1) infection of Epstein-Barr Virus (EBV); (2) environmental elements; (3) genetic disorders, have been identified as the etiological factors of nasopharyngeal carcinoma (NPC) (Lao, Nguyen, & Le, 2021; Tsao et al., 2014). Events of epigenetics, one of the genetic disorders, have been reported that plays striking roles in many human cancers, including NPC (Herceg & Hainaut, 2007; Dai, Zheng, Cheung, & Lung, 2016; Lao, Nguyen, et al., 2021). DNA hypermethylation (or DNA methylation) is the best-studied epigenetic alteration (Herceg & Hainaut, 2007; Lao, Truong, et al., 2021). The DNA methylation refers to the transfer a methyl group from S-adenyl methionine to the bases of 5- carbon of Cytosine which is located at 5' to a Guanosine base in a CpG dinucleotide by DNA methyltransferase (DNMT) (Herceg & Hainaut, 2007; Moore, Le, & Fan, 2013; Lao, Nguyen, et al., 2021; Lao, Truong, et al., 2021) (Figure 1). Importantly, aberrant methylation of DNA is significantly related to a wide variety of human cancers, including NPC (Herceg & Hainaut, 2007; Dai et al., 2016; Lao, Nguyen, et al., 2021; Lao, Truong, et al., 2021). For the past few decades, many efforts have been pointed out aberrant methylated CpG islands located at promoter regions of tumor suppressor genes leading to its inactivation and participations in various processes of nasopharyngeal tumorigenesis (Han et al.,

2020; Lao, Nguyen, et al., 2021; Lao, Truong, et al., 2021; Sharma, Kelly, & Jones, 2010; Tian et al., 2013). It is necessary to find out the epigenetic target gene or panel of genes, which could be used as markers of early diagnosis, risk evaluation as well as cancer prevention (Herceg & Hainaut, 2007). The diagnosis and therapy of NPC will both benefit from increased attempts to understand the molecular processes underlying the disease's progression (Zhao et al., 2020). To the large extent, the gene of *Ubiquitin Carboxyl Terminal Hydrolase 1* (*UCHL1*), the Tumor Suppressor Gene (TSG) in NPC, appears to fulfill criteria being the potential biomarker for NPC diagnosis (Li et al., 2010; Zhao et al., 2020).

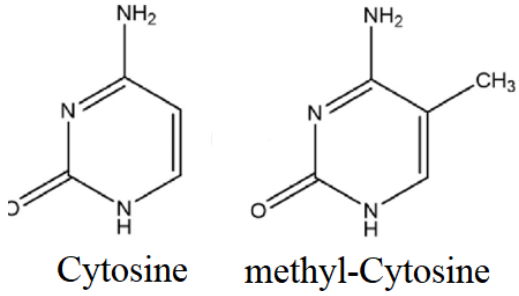

Figure 1. Structure of cytosine and methyl-Cytosine

Protein of Ubiquitin Carboxyl Terminal Hydrolase 1 (UCHL1), encoded by *UCHL1*, assembly, chr4: 41,256,413-41,268,455, (GRCh38/hg38), belongs to ubiquitin C-terminal hydrolase subclass of deubiquitinating enzymes (Nijman et al., 2005). Protein UCHL1 has been described as thiol protease which hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin (NCBI: Gene ID: 7345, updated on 6-Sep-2022). UCHL1 was first reported in the study by Gong et al. (2006), and they claimed that UCHL1 played a key role in Alzheimer's disease (Gong et al., 2006). In their study, UCHL1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory (Gong et al., 2006). Interestingly, it has been reported that UCHL1 functions in many cancers as either a tumor suppressor gene or an oncogene (Herceg & Hainaut, 2007; Hussain et al., 2018; Goto et al., 2015; Li et al., 2010; Yu et al., 2008). It is emphasized that aberrant methylation of the *UCHL1* promoter leads to the downregulation of *UCHL1* gene's expression, as well as promotes cell proliferation via the pathway of regulating the stability of p53 and MDM2 in and NPC through the complex of p53/MDM2/ARF (Li et al., 2010). Additionally, the aberrantly methylated promoter of *UCHL1* was also reported in many previous studies (Li et al., 2010; Loyo et al., 2011; Tian et al., 2013). Thus, in the case of NPC, *UCHL1* functions in as TSG, and its aberrant methylation has been confirmed as the key role driving nasopharyngeal tumorigenesis. As far as we are aware, no research has been done in the Vietnamese community to characterize the methylation of the *UCHL1* gene promoter in NPC. Herein, aiming to further analyze of methylation status of *UCHL1* gene promoter in NPC, we established the protocol for detecting methylation of *UCHL1* gene's promoter in NPC biopsy samples collected from Vietnamese NPC patients.

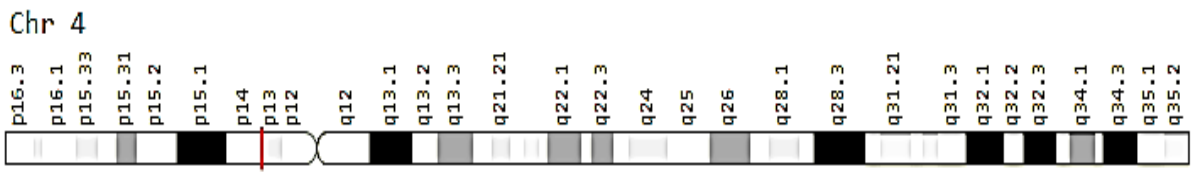

Figure 2. Location of *UCHL1* locates on Chromosome 4

2. Materials and methods

2.1. Sample collection, ethics approval

Ten samples of NPC biopsy tissues, confirmed by immunohistochemistry, and ten non-cancerous swab samples, were obtained from the Cho Ray Hospital, Ho Chi Minh City, Vietnam. All samples enrolled in current study were approved by Cho Ray Hospital, Ho Chi Minh City, Vietnam (The approval number: 516/BVCR-HDDD).

2.2. DNA extraction, bisulfite modification

Total DNA was isolated from both case and control samples by using method of Phenol/Chloroform (pH = 8), then, precipitated and purified by Ethanol. The quality of the DNA isolated by measuring using a nano photometer at the wavelength of 260nm, and 280nm.

The bisulfite modification assays were performed by using 500ng purified DNA via the guideline of EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific Inc.). Finally, the precipitation was eluted in 20µL and stored at -20°C for next experiments.

2.3. Two-stage nested-methylation specific PCR assay

Aiming to establish protocol for detection of *UCHL1* gene's promoter methylation, two-stage nested-methylation specific PCR assay (Nested-MSP) was performed by using pairs of following primers (Table 1).

Table 1

The primer sequences used

Stage	Primers	Sequences (5'-3')	References	X°C
I	Seq-UCHL1-F	CGTATTATTTCGCGTTGCGTACGG	Tian et al. (2013)	55
	Seq-UCHL1-R	ACTCGACTCCCCCTCCCTCGACCT		
II	M-UCHL1-F	TTTATTTGGT**CGCG**AT**CG**TT**C**		57
	M-UCHL1-R	CATCTT**CGCG**AAA**CG**CC**CG**AC		
	U-UCHL1-F	GTT**TG**TATTTATT**TG**GT**TGTG**AT**TG**TT**T**		57
	U-UCHL1-R	T**CA**CCT**CA**AAATTAATCTC**CA**T**CA**ACT		

Note: F: Forward primer, R: Reverse primer, Seq: outer sequencing primer (Stage-I), M: methylated, U: Unmethylated (Stage-II), CpG islands in bold and underlined characters
Source: The researcher's data analysis

Each stage of PCR or MSP was performed in a volume of 15µL containing 3µL bisulfite-modified template DNA (in case of stage I PCR) or 3µL stage 1 PCR product (in case of stage II MSP), 0.75 unit iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA), 0.5µM each primer, 7.5µL MyTaqTM Mix (Bioline Reagents Ltd., London, UK).

For the amplification assay, thermal cycles were initiated at 95°C for 05 minutes, followed by 35 - 40 cycles, including denaturation at 95°C for 30 seconds, annealing at X°C for 30 seconds, extension at 72°C for 30 seconds, and finally, extension at 72°C for 10 minutes. (Note: X°C was the specific annealing temperature for each primer, Table 1). For methylation/unmethylation analysis, the final stage of Nested-MSP products was separated on 1.5% or 2.0% agarose gel and visualized by GelRed. Representative products were sequenced aiming to confirm the examine the efficiency of bisulfite modified, specificity of primers, and the status of *UCHL1* gene's methylation.

3. Results

3.1. The quality of DNA extraction

Chloroform/Phenol (pH = 8)-based extraction and Ethanol-based precipitation were used to isolate DNA retrieved from biopsy samples and swab samples. The purification values and concentration of obtained DNA were presented in Table 2.

Table 2

Results of DNA extraction

	Purification (A_{260}/A_{280})	**Concentration (µg/ml)**		**Purification (A_{260}/A_{280})**	**Concentration (µg/ml)**
T1	1.96	670.0	S1	1.86	377.5
T2	1.88	532.5	S2	1.93	460.0
T3	1.86	460.0	S3	1.77	432.5
T4	1.88	360.0	S4	1.84	400.5
T5	2.00	312.5	S5	1.83	330.5
T6	1.93	302.5	S6	2.00	502.5
T7	1.83	407.5	S7	1.81	300.5
T8	1.95	365.0	S8	1.95	306.5
T9	2.00	317.5	S9	1.89	355.0
T10	1.82	307.5	S10	1.91	380.0

Note: T: Nasopharyngeal cancer biospy sample; S: non-cancerous swab sample

3.2. Nested-MSP analysis

In the current study, Nested-MSP assays were carried out to evaluate the methylation status of *UCHL1* gene's promoter in both cancerous and non-cancerous samples according to the above describing protocol. The Nested-MSP products of methylation or unmethylation were analyzed and visualized in the agarose gel with the band of 169bps and 210bps, respectively (Figure 3). For confirming the specificity of methylation or unmethylation, amplified products were sent to sequencing by the Sanger method (Figure 4).

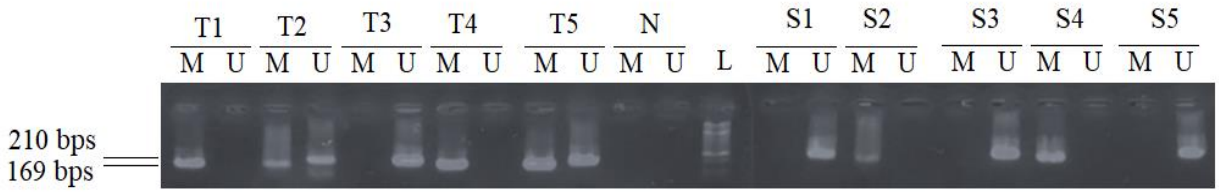

Figure 3. Electrophoresis analysis of samples

T: Nasopharyngeal cancer biopsy sample; S: non-cancerous swab sample; M: Methylated; U: Unmethylated; L: Molecular ladder (50bps)

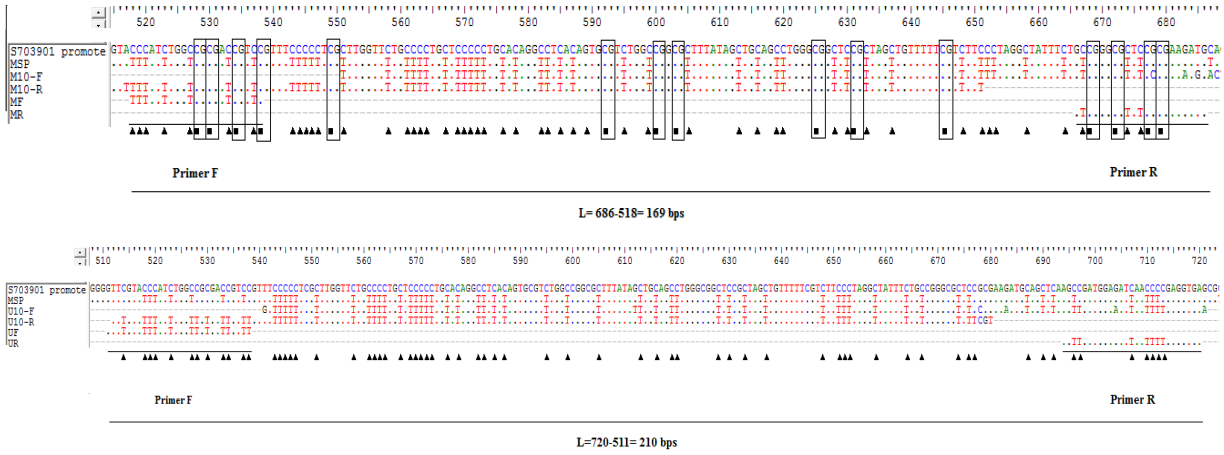

Figure 4. Sequencing profile of (A) methylation, and (B) unmethylation of *UCHL1* gene's promoter in representative sample

S703901 promote: sequence without bisulfite modification (retrieved from Accession number: S703901, NCBI-Genbank), MSP: sequence with bisulfite modification, M10-F, R: sequence of the amplified product by Forward, or Reverse primer

4. Discussion and conclusion

4.1. Discussion

Nasopharyngeal tumorigenesis has been reported as a multistage process involving three main causes, including viral infection, environmental factors, and genetic/epigenetic alterations (Lao, Nguyen, et al., 2021; Tsao et al., 2014). In the current study, we focused on establishing the protocol aiming to detect the methylation status of *UCHL1* gene's promoter. Herein, the method of DNA isolation and Nested-MSP were established on cancerous biopsy samples and non-cancerous samples, collected from the Cho Ray Hospital, Ho Chi Minh City, Vietnam.

The isolated DNA samples were assessed by using the method of absorbance measurement at 260nm and 280nm. As the results, the DNA concentration values were in the range of 302.5 µg/ml to 670 µg/ml with an average concentration value of 403.5 µg/ml, and in the range of 300.5 µg/ml to 502.5 µg/ml with an average concentration value of 384.5 µg/ml for cancerous and non-cancerous samples, respectively. Additionally, the purity values of isolated DNA were in the range of 1.82 to 2, and in the range of 1.77 to 2.00 for cancerous and non-cancerous samples, respectively. Generally, constructing an absorbance ratio between these two absorbance wavelengths can provide an estimate of sample purity (Wilfinger, Mackey, & Chomczynski, 2006). In our isolation, the ratios of A260/A280 of samples were in the range of 1.8 to 2.0 indicating being accepted as pure DNA. Therefore, all isolated samples were accepted as better inputs for further molecular analysis, including bisulfite modification as well as Nested-PCR assay (Olson & Morrow, 2012).

Nested-MSP method was chosen to the evaluation of the methylation status of the *UCHL1* gene's promoter in Vietnamese samples. According to Licchesi and Herman (2009), Nested-MSP method is an alternative method that overcomes the limitations of MSP when it comes to analyse samples with a low quantity of input (Licchesi & Herman, 2009). In detail, the first stage of Nested-MSP is conducted to recognize and amplify a bisulfite-modified template. However, this stage could not discriminate between the methylated and unmethylated alleles. The results of this stage is that the amount of DNA templates will be significantly increased (Divine, Liechty, Crume, & Belinsky, 2006). In the second stage, two separate PCR assays were performed by using two sets of primers: one specific to the methylated template, and another primer for the unmethylated

template (Divine et al., 2006). In current study, the forward and reverse primer for the detection of the methylated template contains three CpG and four CpG, respectively, whereas the forward and reverse primer for the detection of the unmethylated template contain six CpG and four CpG, respectively (Table 1). The Nested-MSP products of methylation and unmethylation were observed good and clear band of 169bps and 210bps in length by electrophoresis analysis, respectively (Figure 3). The methylated and unmethylated sequences were confirmed by sequencing, indicated in Figure 4. According to Figure 4, the sequencing of methylated template in the representative sample showed that all methylated Cytosines were unchanged, which were marked as square symbols (Figure 4A). Otherwise, in the representative template of unmethylation, compared to the methylated template, all the unmethylated Cytosine was changed into Thymine, which was marked as triangle symbols (Figure 4B). Thus, we emphasized that we successfully established the protocol, including DNA isolation, and Nested-MSP assay to detect the methylation or unmethylation of *UCHL1* gene's promoter.

4.2. Conclusion

The methylation of *UCHL1* gene's promoter has been reported as the etiological factor of nasopharyngeal tumorigenesis. Herein, the protocol, including the step of DNA isolation, and the step of Nested-MSP, was successfully established for detecting the methylation and/or unmethylation status of *UCHL1* gene's promoter. With further refinement, this protocol could be applied in the evaluation of *UCHL1* gene's promoter for establishing the potential biomarker for diagnosis of Vietnamese nasopharyngeal carcinoma.

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Availability of data and material

The data generated during and/or analyzed in this study are available from the corresponding author upon reasonable request.

Declaration of interest

The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

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