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Identification and validation of the hypermethylation biomarkers on *ZMYND10* promoter in Nasopharyngeal Carcinoma biopsies from Vietnamese patients.

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Abstract

Silencing of tumor suppressor gene, which caused by the DNA hypermethylation, an epigenetic modification, has been reported to be involved in human various cancers, including nasopharyngeal carcinoma. The aims of this study was to identify and evaluate the hypermethylation frequency of the *ZMYND10* promoter, which located at 3p21.3, in nasopharyngeal biopsies from Vietnamese patients by Nested Methylation Specific PCR (Nested MSP). In current study, fifty tumor biopsies and ten healthy samples, which were obtained from Cho Ray hospital, were enrolled into study. As the results, the hypermethylation frequency of *ZMYND10* gene promoter was more frequent in tumor biopsies. In detail, the hypermethylation frequency of *ZMYND10* gene promoter were 92.0% (46 of 50 samples), and 20.0% (2 of 10 samples) for in NPC biopsies and uncancerous specimens. A trend toward positive association was found between hypermethylation of *ZMYND10* gene and nasopharyngeal carcinoma (*p* < *0.0001*). In addition, these analyses exhibited the high OR (Odds ratio) was 46.00 (p = 0.001). In conclusion, our data suggested that the hypermethylation of *ZMYND10* gene promoter is a significant in nasopharyngeal carcinoma in Vietnamese patients.

Keywords: ***ZMYND10*, nasopharyngeal carcinoma, hypermethylation, tumor suppressor gene.**

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I. Introduction

Involvement of the 3p21.3 disorder, such as the hypermethylation of tumor suppressor gene, in various cancers, including nasopharyngeal carcinoma (NPC), has been reported previously (Kok et al., 1997; Ji et al., 2005; Hesson et al., 2007). Among several genes located at the 3p21.3 region, such as *RASSF1A*, *ZMYND10*, *PR2L*, *101F6*, *PL6*, etc., many studies have been suggested that *Zinc-finger*, *MYND-type containing 10* (*ZMYND10*), commonly called ZMYND10, spans 4.5 kbs on 3p21.3, expression has been identified to be frequently downregulated in human NPC due to promoter hypermethylation, which detected in 66 – 74% of primary NPC, leading to development of NPC (Liu et al., 2003; Qiu et al., 2004; Hesson et al., 2007; Cheng et al., 2015). These alterations have been detected in NCP biopsies as well as the NPC cell lines (Liu et al., 2003; Qiu et al., 2004; Cheng et al., 2015). The function of ZMYND10 protein has been considered as inhibitor of colony formation of cancer cells, and found it could be activated by environmental stresses such as heat sock and its regulated by E2F (Qiu et al., 2004; Ayadi et al., 2008).

Nasopharyngeal carcinoma (NPC) is a prevalent malignant tumor of nasopharynx has considered remarkably distinctive geographic and ethnic contribution, gravitating toward Southern Asia, especially in China and Vietnam (Sham et al., 1990; Chang et al., 2006). According to statistics of Globocan (2012), the high prevalence of NPC cases was observed in reached to 4,931 cases (ASR = 5.4/100,000) and deaths was 2,885 cases (ASR = 3.3/100,000) In Vietnamese population. For the past few years, many studies have been demonstrated that multiple risk factors, including Epstein-Barr virus infection, genetics/or epigenetic changes and environmental factor have been suggested to be strongly linked to NPC (Hildesheim, Levine, 1993). Moreover, there is growing evidence demonstrating that the prevalent epigenetic changes, the hypermethylation of CpG islands in promoter regions of genes, the abnormalities at 3p21.3 region, contribute to many cell processes in cell-cycle regulation, apoptosis, signal transduction, cell adhesion, etc. which

leading to the inactivation or less expression of these TSGs involves in many human tumorigenesis, including NPC (Herman et al., 1996; Santini et al., 2001; Challouf et al., 2012).

However, *ZMYND10* promoter hypermethylation has not been investigated in NPC specimens from Vietnam, thus, in order to determine whether promoter hypermethylation profile in Vietnam, the data on *ZMYND10* hypermethylation was evaluated and investigated in a series of NPC samples which were collected from Vietnamese NPC patients, were enrolled in current study to develop method for prognosis and early diagnosis of NPC based on the detection of *ZMYND10* methylation status.

II. Materials and Methods

A. Samples collection

Twenty biopsy samples were collected from nasopharyngeal cancer patients, in Cho Ray Hospital, Ho Chi Minh City, Vietnam. All of those biopsies were collected from patients, which was obeyed to ethical approval for study human samples, and patients agreed with purpose of the study. All the samples were submitted to histopathological diagnosis and confirmed NPC. In addition, ten nasopharyngeal swab samples, which were collected from healthy donors, used as negative-nasopharyngeal carcinoma control.

B. DNA extraction, bisulfite modification

Total of genomic DNA was isolated from biopsy or swab samples by phenol/chloroform method. Cells obtained from samples were lysed in lysis buffer (10 mM Tris-HCl pH = 8, 10 mM EDTA, 150 mM NaCl, 2% SDS) containing Proteinase K (0.1 mg/ml). Then, total of genomic DNA was isolated and purified by using standard phenol-chloroform and ethanol precipitation. The bisulfite conversion of 2 µg genomic DNA was performed using EpiTect Bisulfite Kits (Qiagen). The final precipitation was eluted in a volume of 20 µl and stored at -20^{o}C for further studies.

C. Nested-Methylation specific polymerase chain reaction

The methylation status of each promoter in samples were examined by two-steps nested PCR. In current study, firstly, the primers of stage 1 PCR were used for preceding amplification which recognize the bisulfite-modified template, notably, do not discriminate between methylated and unmethylated sequences. The sequence of the forward and reverse primers of stage 1 were 5'TTGGGAATTTAAATATTATG3', and 5'AACAACAATTCCAAATCTC3', respectively (Ayadi et al., 2008). In stage 2 PCR, two pairs of primer were used to amplify the regions of interest. One pair recognized a sequence in which CpG sites were methylated (unmodified by bisulfite treatment). Other pair recognized a sequence in which CpG sites were unmethylated (modified to UpG treatment). The sequence of the methylated forward and reverse primers were 5' GCGGGTTAGAGATTCGTTC3', and 5'TCGAAACCGAAATCCGACG3', respectively. The sequence of the unmethylated forward and reverse primers were 5'GGTGGGTTAGAGATTTGTTT3', and 5'ATATCAAAACCAAAATCCAACA3', respectively (Ayadi et al., 2008). Each stage of PCR was performed in a total of 15 µl containing 3 µl bisulfite-modified template DNA (in case of stage 1 PCR) or 3 µl stage 1 PCR product (in case of stage 2 PCR), 0.75 unit iTaq DNA polymerase (Biorad), 0.5 µM each primer, 7.5 µl MyTaqTM Mix (Bioline). Thermal cycling was initiated at 95^{o}C for 5 min, followed by 40 cycles of denaturation at 95^{o}C for 30 sec, annealing at the X^{o}C for 30 sec, extension at 72^{o}C for 30sec, and a final extension at 72^{o}C for 10 min (Note: X^{o}C was the specific annealing temperature for each specific methylated or unmethylated primer, X^{o}C = 50^{o}C, 58^{o}C and 64^{o}C for Stage 1, methylated and unmethylated primers, respectively). Finally, The PCR products of the methylated and unmethylated were separated on 2% agarose gel and visualized by ethidium bromide staining. MSP products were sequencing to confirm the specificity of primers, examine the efficiency of bisulfite modification and the hypermethylation status of target gene.

D. Statistical analysis

Statistical analyses were performed by Medcalc® Version 12.7.0.0. The average frequency of methylation was calculated. The association between hypermethylation of *ZMYND10* and NPC were examined by using Chi-square test. A p-value ≤ 0.05 was considered statistically significant. Moreover, the association between hypermethylation of *ZMYND10* and risk of NPC was estimated by computing OR, RR and 95% confidence intervals (CI).

III. Results

A. Status of the promoter hypermethylation of ZMYND10

The frequency of the promoter methylation of *ZMYND10* in 50 nasopharyngeal biopsy samples and 10 non-cancer samples were examined by nested-MSP. Overall, the promoter frequencies for *ZMYND10* in NPC samples

and non-cancerous samples were 92.0% (46 of 50 samples), and 20.0% (2 of 10 samples), respectively. Conversely, the promoter unmethylation frequencies were 6.0% (3 of 50 samples), and 80.0% (8 of 10 samples) in NPC samples and healthy samples, respectively. In addition, the p = 0.0001 (lower than 0.05) indicated the methylation of *ZMYND10* in NPC samples was found to be significant higher than in non-cancerous samples.

The MSP products of samples hypermethylation and/or unmethylation in the promoter of *ZMYND10* were observed in electrophoresis with visualized by ethidium bromide staining and showed in Fig. 1. According to Fig. 1, the MSP products of *ZMYND10* in clinical samples were observed in the band of 231 bps and 235 bps length in case of methylation and unmethylation, respectively. The sequencing of samples hypermethylated promoter region of representative sample revealed a conversion of unmethylated Cytosine, but not methylated Cytosine (Fig. 2). By sequencing, comparison between the non-bisulfite modified (Fig. 2a) and bisulfite modified (Fig. 2b), all methylated Cytosines were unchanged, which were marked by square symbols. Otherwise, all the unmethylated Cytosines were totally changed into Thymine, which were indicated by triangle symbols, in bisulfite sequence. Additionally, three methylated CpG sites were observed in methylated reverse primer, which were according to the primer designed.

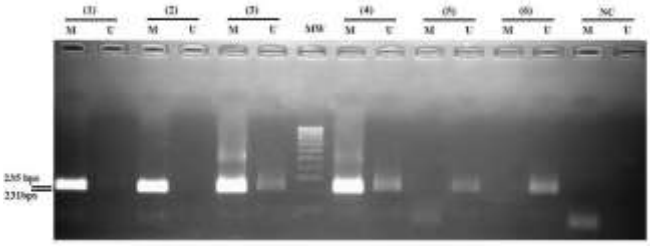

Figure 1. Methylated promoter of *ZMYND10* gene was analyzed on some clinical samples by nested-MSP. (1) (2) (3) (4) NPC biopsy samples; non-cancerous sample; (5),(6) non-cancerous samples; NC: negative control; MW: molecular weight 100 bp ladder.

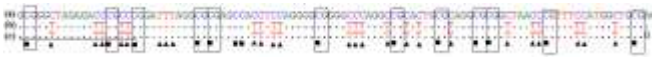

Figure 2. Sequencing profile of methylated ZMYND10 segment (Stage 2 PCR). CG sites were in flamed; Forward primer sequence is underlined; (a) DNA sequence was without bisulfite modified (Accession number: AC02481); (b) DNA sequence was bisulfite modified by Methprimer; (c) The ZMYND10 sequencing by using the forward primer.

B. *Odds ratio, relative risk for promoter hypermethylation in ZMYND10 gene*

In this study, the odds ratio and relative risk values were computed between NPC biopsy samples and non-cancerous samples. The results show that, odds ratio, relative risk values were 46.0 (95%CI = 1.3281 – 15.9325, p = 0.001) and 4.6 (95%CI = 7.1893 – 294.3263, p = 0.02), respectively.

IV. *Discussion*

Nasopharyngeal carcinoma is one of the commonly occurring cancers among Asian region, including Vietnam with the high prevalence (Globocan, 2012). Within the ambiguous symptoms such as hearing loss, nosebleeds, headache, trouble opening the mouth, etc. thus, most patients present with the stage III or IV cancer when diagnosed (Epstein, Jones, 1993; Hao et al., 2004), thus, it could be a challenge in early diagnosis. Therefore, to achieve favorable treatment and increasing of patient's survival, early diagnosis and prognosis are necessary to be appropriate managed.

Previous studies have been shown that DNA hypermethylation plays an important role in human cancer development, including NPC. In particular, according to Lo and Huang (2012), alteration of the 3p21.3 loci are highly prevalent in NPC with a combination of loss of heterozygosity (LOH) and aberrant promoter hypermethylation. In current study, we examined the methylation status of *ZMYND10*, which map to this 3p21.3 region, in NPC biopsy samples by Nested-MSP method. The combination both nested-PCR and MSP shows an advantage in the hypermethylation analysis by increased MSP sensitivity approximately 50-fold (Palmisano et al., 2000). In our study, a high frequency of aberrant methylation in *ZMYND10* promoter (96.0%) in NPC biopsy sample was found, which showed higher frequency when compared to 66.0% – 74.0% in the Chinese NPC (Liu et al., 2003; Qiu et al., 2004), and 34.1% in the Tunisian NPC samples (Ayadi et al., 2008), and a low frequency of methylation in candidate gene promoter (20.0%) in non-cancerous samples, which was according to the previous studies. Despite the size of the sample, we still found the correlation between methylation of *ZMYND10* gene promoter and NPC. The p-value (p = 0.0001) pointed out the strongly significant statistical association between aberrant methylation of *ZMYND10* gene and NPC. In fact, the function of *ZMYND10* protein, its encoded protein, has been reported as inhibitor of colony formation of cancer cells, and found it could be activated by environmental stresses such as heat sock and its regulated by E2F. (Qiu et al., 2004). Moreover, tumor suppressor *ZMYND10* inhibits proliferation of NPC cells by regulation of cell cycle, c-Jun N-terminal kinase and the cyclin D1 promoter (Zhang et al., 2012). Recently, growing evidences proved that the loss of *ZMYND10*

expression was downregulated correlated with promoter hypermethylation (Liu et al., 2003; Qiu et al., 2004; Zhang et al., 2012). Therefore, it strongly suggested that *ZMYND10* might be one of the important TSG candidates at this locus in NPC. The hypermethylation of *ZMYND10* was significantly associated with an approximately 76.0-fold increase in NPC than compared to non-cancerous samples (OR = 76.0, 95%CI = 6.0019 – 962.3636, p = 0.0008). Concerning to RR value, it indicated that the risk of nasopharyngeal tumorigenesis significantly increased 1.78 times in the case within aberrant methylation of *ZMYND10* gene promoter, leading to the inactivation of *ZMYND10* (RR = 1.78, 95%CI = 1.0332 – 3.0625, p <0.04). Therefore, due to these results, we believed that aberrant methylation of *ZMYND10* gene is a significant in nasopharyngeal carcinoma in Vietnamese patients, and the Nested-MSP method for *ZMYND10* hypermethylation detection in NPC biopsy samples could to be considered as the promising biomarker that could be potentially used for diagnosis and prognostic purposes in Vietnam.

It is noted that the discovery of hypermethylated TSGs, has been detected not only in primary tumors, but also in serum, sputum, bodily fluids, saliva, etc. Therefore, as a next stage of our study, we intend to analyze the methylation status of *ZMYND10* gene in several non-invasive samples to develop the non-invasive biomarkers which will be easily applied in clinic, to prognosis and early diagnosis of NPC in Vietnamese population.

v. Conclusion

In summary, the results of this study showed a higher prevalence of *ZMYND10* promoter hypermethylation in NPC biopsy samples, counting for 96.0%. On the contrary, the low frequency of *ZMYND10* promoter hypermethylation was found in healthy samples. Additionally, the significant correlation between candidate gene hypermethylation and human nasopharyngeal tumourigenesis, as well as the odds ratio and relative risk were found in the significant correlation, counting for 46.0 and 4.6, respectively were reported. And, the screening, which based on the detection of *ZMYND10* promoter hypermethylation, will be an auspicious characteristic for early prognosis and diagnosis of NPC. In further study, the present findings require extension to numbers of many sources of sample in order to find out the potential non-invasive tumor markers for diagnosis and prognostic purposes in Vietnam.

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