

## RESEARCH ARTICLE

# Alu Methylation in Serum from Patients with Nasopharyngeal Carcinoma

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### Abstract

**Background:** Nasopharyngeal carcinoma (NPC) is a common cancer in Southern China and Southeast Asia. Alu elements are among the most prevalent repetitive sequences and constitute 11% of the human genome. Although Alu methylation has been evaluated in many types of cancer, few studies have examined the levels of this modification in serum from NPC patients. **Objective:** To compare the Alu methylation levels and patterns between serum from NPC patients and normal controls. **Materials and Methods:** Sera from 50 NPC patients and 140 controls were examined. Quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu) was applied to measure Alu methylation levels and characterize Alu methylation patterns. Amplified products were classified into four patterns according to the methylation status of 2 CpG sites: hypermethylated (methylation at both loci), partially methylated (methylation of either of the two loci), and hypomethylated (unmethylated at both loci). **Results:** A comparison of normal control sera with NPC sera revealed that the latter presented a significantly lower methylation level ( $p=0.0002$ ) and a significantly higher percentage of hypomethylated loci ( $p=0.0002$ ). The sensitivity of the higher percentage of Alu hypomethylated loci for distinguishing NPC patients from normal controls was 96%. **Conclusions:** Alu elements in the circulating DNA of NPC patients are hypomethylated. Moreover, Alu hypomethylated loci may represent a potential biomarker for NPC screening.

**Keywords:** Alu element - methylation - nasopharyngeal cancer - serum marker

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### Introduction

DNA methylation is an epigenetic modification that is essential for determining an individual's phenotype and regulating gene expression. DNA methylation involves the addition of a methyl (CH<sub>3</sub>) group at the carbon-5 position of the cytosine ring and is frequently observed at CpG sites (Ramsahoye et al., 2000). In general, CpG sequences are distributed as interspersed repetitive sequences (IRS) and dispersed throughout the genome. DNA methylation participates in the regulation of many cellular processes, including embryonic development and differentiation. Furthermore, aberrant DNA methylation is implicated in several diseases (Robertson, 2005; Conerly and Grady, 2010). Changes in the DNA methylation status also contribute to a higher risk of carcinogenesis, including hypermethylation of tumor suppressor genes (Das and Singal, 2004) and genome-wide hypomethylation (Kitkumthorn and Mutirangura, 2011). Genome-wide hypomethylation can promote carcinogenesis, primarily by lowering the rate of replication-independent DNA double-strand breaks and consequently promoting genomic instability (Pornthanakasem et al., 2008; Kongruttanachok et al., 2010; Thongsroy et al., 2013).

Alu elements are members of the short-interspersed nuclear element (SINE) family. These elements are distributed throughout the genome, with over 1 million copies per genome, and they account for approximately 11% of the human genome. Therefore, a reduction of the Alu methylation level results in genome-wide hypomethylation. The methylation level of Alu elements is known to decrease during the aging process (Jintaridith et al., 2013) and has been observed in osteoporosis in post-menopausal women (Jintaridith et al., 2013). Alu hypomethylation has also been observed in cancers of the breast, colon, stomach, liver, lung, ovaries, urinary bladder, prostate gland and oral cavity (Choi et al., 2007; Rodriguez et al., 2008; Watts et al., 2008; Yoo et al., 2008; Bollati et al., 2009; Choi et al., 2009; Daskalos et al., 2009; Lee et al., 2009; Park et al., 2009; Cho et al., 2010; Xie et al., 2010; Hou et al., 2010; Kwon et al., 2010; Xiang et al., 2010; Yoshida et al., 2011; Puttipanyalears et al., 2013). Moreover, it has been suggested that the Alu methylation level is reduced, corresponding to the severity of oral cancer (Puttipanyalears et al., 2013), mucoepidermoid carcinoma of the salivary gland (Sirivanichsunton et al., 2013) and lymph node metastases of head and neck cancers (Kitkumthorn et al., 2012).

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Nasopharyngeal carcinoma (NPC) is a neoplasm that arises from the mucosal epithelium of the nasopharynx. In 2012, approximately 87,000 newly diagnosed cases were observed worldwide (GLOBOCAN, 2012). Southern China and Southeast Asia frequently report the highest incidence rate (Tiwawech et al., 2003). The 2-year survival rate is only 50% for patients with advanced NPC in stage III or IV. However, early detection of NPC is associated with a 2-year survival rate up to 90% or more (Sun et al., 2014). Currently, diagnosis is based on the patient's signs and symptoms and the radiographic and pathological features; however, most of these characteristics are only detected at a late stage (Raab-Traub et al., 1987). Consequently, searching for surrogate marker especially circulating serum DNA is considered for NPC screening.

In this study, we aimed to evaluate circulating tumor DNA by observing the Alu methylation levels in sera from NPC patients compared to normal controls.

## Materials and Methods

### Participants

The study protocol was reviewed and approved by the Ethics Committee of the National Cancer Institute, Bangkok, Thailand. Written informed consent was obtained from all participants. Fifty patients (39 males and 11 females with a mean age of 50 years, range 27-78 years) with histological confirmation of nasopharyngeal cancer between 2009 and 2011 were recruited for the study. One hundred and forty age-matched normal controls (80 males and 60 females with a mean age of 50 years, range 29-69 years) were enrolled in the study as controls. The health status of the controls was confirmed by direct interview and by reviewing the results of an annual medical check-up to ensure that there was no history of any malignancies.

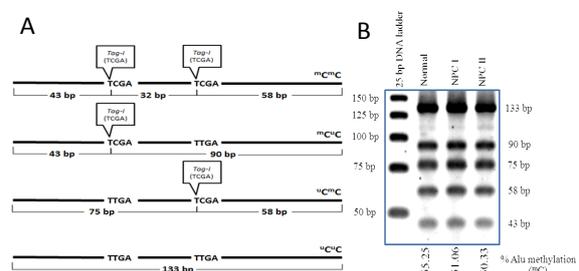
### Specimen collection and DNA extraction

Six milliliters of blood was collected from each participant. The blood samples were centrifuged at 1,600x g for 10 minutes to collect the serum. Then, the serum was transferred to new polypropylene tube and centrifuged at 14,000x g for 15 minutes. The supernatant was discarded, and the DNA in the remaining pellet was isolated using Tris/SDS and proteinase K and incubated at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. The isolated genomic DNA was eluted and then treated with bisulfite. Bisulfite modification of the genomic DNA was performed using previously published methods (Chalitchagorn et al., 2004). Briefly, a total of 1 µg of DNA from each sample was denatured in 0.22 M NaOH at 37°C for 10 min. After the addition of 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 3 M sodium bisulfite (pH 5.0), the samples underwent an additional incubation at 50°C for 16-20 h. Then, the DNA was recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) following the manufacturer's protocol. The DNA samples were eluted from the columns with distilled water and precipitated with sodium acetate and 100% ethanol as described previously. *Quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu)*

The quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu) technique is designed to detect 2 CpG loci among the thousands of Alu elements using one set of conserved primers. The qCOBRA-Alu, bisulfite-treated DNA was amplified using primers with the following sequences: Alu forward 5'- GGRGRGGTGGTTTARGTTTGTAA-3' and Alu reverse 5'- CTAACCTTTTATATTTTAA TAAAAACRAAATTCACCA-3'. The Alu sequence primers were based on the nucleotide sequences of the Alu Sx subfamily (Batzer MA, 1999). The PCR amplification of qCOBRA-Alu was performed according to the following cycling conditions: pre-denaturation at 95 °C for 15 min followed by 45 cycles of denaturation at 95 °C for 45 sec, annealing at 63°C for 45 sec, and extension at 72 °C for 45 sec, with a final extension at 72 °C for 7 min. After amplification, the Alu PCR products (133 bp in length) were digested with 2 U of the Tag1 restriction enzyme (Fermentas International Inc., Burlington, Canada). Each reaction was incubated overnight at 65 °C, and then, DNA fragments were separated on 8% polyacrylamide gels. Afterthat, the gel was stained using the SYBR green nucleic acid gel stain (Gelstar, Lonza, Rockland, ME, USA). The intensity of the DNA fragments was measured using a Phosphoimager with Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). For normalization of the inter-assay variation between each experiment, we used DNA templates from the HeLa, Jurkat and Daudi cell lines as controls. The methylation levels of each cell line were standardized. Therefore, the results of all experiments were adjusted using the same control methylation levels.

### Alu methylation analysis

An illustration of the qCOBRA-Alu technique and an example of gel electrophoresis are shown in Figure 1. The COBRA Alu loci were categorized into four groups based on the methylation status of 2 CpG dinucleotides in the 5' and 3' regions of the Alu sequence. These four groups were defined as follows: two unmethylated CpGs (<sup>u</sup>C<sup>u</sup>C); two methylated CpGs (<sup>m</sup>C<sup>m</sup>C); 5'-methylated



**Figure 1. Determination of the Alu Methylation Pattern Using the COBRA-Alu Method.** **A)** The detected Alu sequence contains two CpG dinucleotides. The amplified products were digested with Tag1 and presented 4 patterns: hypermethylation; <sup>m</sup>C<sup>m</sup>C (43, 32 and 58 base pair (bp) fragments), partial methylation; <sup>m</sup>C<sup>u</sup>C (43 and 90 bp fragments), partial methylation; <sup>u</sup>C<sup>m</sup>C (75 and 58 bp fragments) and hypomethylation; <sup>u</sup>C<sup>u</sup>C (133 bp fragment). **B)** Representative gel image from the COBRA-Alu assay. Lane 1: 25-bp markers. Lane 2: DNA from normal controls, Lanes 3-4: DNA from NPC patients

and 3'-unmethylated CpGs ( ${}^m\text{C}^u\text{C}$ ); and 5'-unmethylated and 3'-methylated CpGs ( ${}^u\text{C}^m\text{C}$ ). We calculated the Alu methylation level and percentage of Alu loci in each group based on the intensity of the COBRA-digested Alu products. The DNA fragments derived from enzymatic digestion of the COBRA-Alu products were separated into 6 fragments of 133, 90, 75, 58, 43 and 32 bp, which represented different methylation states. The  ${}^u\text{C}^u\text{C}$  methylation state was represented by the 133-bp fragments. The  ${}^m\text{C}^m\text{C}$  methylation state was represented by the 90-bp fragment. The  ${}^u\text{C}^m\text{C}$  methylation state was represented by the 75-bp fragment. The  ${}^m\text{C}^m\text{C}$  and  ${}^u\text{C}^m\text{C}$  methylation states were represented by the 58-bp fragment. The  ${}^m\text{C}^m\text{C}$  and  ${}^m\text{C}^u\text{C}$  methylation states were represented by the 43-bp fragment. Finally, the  ${}^m\text{C}^m\text{C}$  methylation state was represented by the 32-bp fragment. The number of CpG dinucleotides was calculated by dividing the intensity of each band by the number of double-stranded bp of DNA sequence as follows: A=intensity of the 133-bp fragment divided by 133; B=intensity of the 58-bp fragment divided by 58; C=intensity of the 75-bp fragment divided by 75; D=intensity of the 90-bp fragment divided by 90; E=intensity of the 43-bp fragment divided by 43; and F=intensity of the 32-bp fragment divided by 32. After calculating the number of CpG dinucleotides and substituting this value in the formula, the Alu methylation levels were calculated as follows: Alu methylation level percentage ( $\%{}^m\text{C}$ )= $100 \times (E+B) / (2A+E+B+C+D)$ ; percentage of  ${}^m\text{C}^m\text{C}$  loci ( $\%{}^m\text{C}^m\text{C}$ )= $100 \times F / (A+C+D+F)$ ; percentage of  ${}^u\text{C}^m\text{C}$  loci ( $\%{}^u\text{C}^m\text{C}$ )= $100 \times C / (A+C+D+F)$ ; percentage of  ${}^m\text{C}^u\text{C}$  loci ( $\%{}^m\text{C}^u\text{C}$ )= $100 \times D / (A+C+D+F)$ ; and percentage of  ${}^u\text{C}^u\text{C}$  loci ( $\%{}^u\text{C}^u\text{C}$ )= $100 \times A / (A+C+D+F)$ .

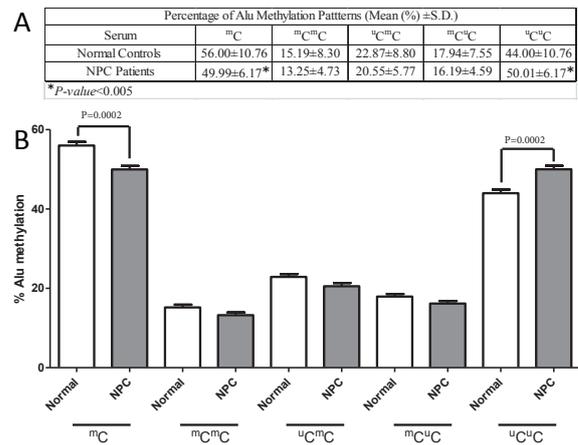
#### Statistical analysis

The statistical analysis was performed using SPSS software for Windows version 17.0 (SPSS Inc., Chicago, IL). An independent sample t-test was performed to calculate significant differences between the normal controls and the NPC patients. A P value < 0.05 was considered statistically significant. A receiver-operating characteristic (ROC) curve analysis was performed to test the ability of the qCOBRA-Alu method to distinguish between the sera of the normal controls and the NPC patients.

## Results

#### Comparison of serum Alu methylation status

In this analysis, we compared the frequencies of each Alu methylation level and pattern between sera from normal controls and NPC patients. The results shown in Figure 2 indicate that the overall methylation levels ( $\%{}^m\text{C}$ ) in the normal controls were higher than those in the NPC patients ( $p=0.0002$ ). Moreover, when the same comparison was performed using the  $\%{}^u\text{C}^u\text{C}$  values, this difference was significantly higher in the NPC patients ( $p=0.0002$ ). In contrast, the other three methylation patterns were slightly less prevalent in the NPC patients, although the difference was not statistically significant ( $\%{}^m\text{C}^m\text{C}$ :  $p=0.1189$ ,  $\%{}^u\text{C}^m\text{C}$ :  $p=0.0847$  and  $\%{}^m\text{C}^u\text{C}$ :  $p=0.1239$ ).



**Figure 2. The Percentages of Each Alu Methylation Pattern in the Serum between Normal Controls and NPC Patients. A)** Detailed description of the methylation level **B)** Histogram representation

#### Receiver Operating Characteristic (ROC) curve analysis of the ${}^m\text{C}$ and ${}^u\text{C}^u\text{C}$ methylation patterns

A ROC analysis was performed to assess the ability of the Alu methylation pattern to distinguish between serum from normal controls and serum from NPC patients. Our data showed that  ${}^m\text{C}$  and  ${}^u\text{C}^u\text{C}$  had the greatest diagnostic value, and therefore, these methylation patterns were chosen for this analysis. Both of these markers showed AUC values at 0.676. At a 58.58% cutoff, the sensitivity and specificity of  ${}^m\text{C}$  were 94.00% and 42.90%, respectively. The cut-off value of  ${}^u\text{C}^u\text{C}$  was 40.21%, and the sensitivity and specificity values were 96.00% and 40.00%, respectively. These results imply that both of these Alu methylation patterns are potentially useful for screening purposes. Furthermore, when we combined these two markers ( ${}^m\text{C}$  or  ${}^u\text{C}^u\text{C}$ ), the specificity was not improved and there was a slight reduction in the sensitivity for NPC detection (90.00% sensitivity and 65.74% specificity).

## Discussion

Alu elements are widely distributed throughout the genome, and previous studies have demonstrated Alu hypomethylation is correlated with aging and many types of cancer (Jintaridith and Mutirangura, 2010; Kitkumthorn and Mutirangura, 2011) To prevent interference related to the effect of aging, we therefore selected participants with age-matched controls. In this study, we used qCOBRA-Alu instead of the more commonly used pyrosequencing technique. Unlike pyrosequencing, qCOBRA-Alu can be used to measure Alu methylation levels and to classify Alu methylation patterns (Jintaridith and Mutirangura, 2010; Jintaridith et al., 2013; Puttipanyalears et al., 2013). Moreover, methylation patterns were shown to be more sensitive for detecting early carcinogenesis, for example, in mucoepidermoid carcinoma of salivary gland (Sirivanichsunthorn et al., 2013) Furthermore, for screening usage, we recently demonstrated that the number of Alu hypomethylated loci, particularly  ${}^u\text{C}^u\text{C}$ , could be useful for tumor DNA detection in an oral rinse and in

peripheral blood mononuclear cells from patients with oral cancer (Puttipanyalears et al., 2013). We then applied this approach in NPC, and the findings were consistent with the hypothesis that the Alu methylation level in NPC patients was significantly decreased compared with normal controls.

The results of this study also indicated the importance of Alu methylation patterns. The frequencies of the <sup>18</sup>C and <sup>14</sup>C patterns were significantly different between the NPC patients and the normal controls. We performed an analysis of the ROC curve to evaluate the sensitivity and specificity of this test. Interestingly, a very high level of sensitivity was observed for the <sup>14</sup>C level, followed by the <sup>18</sup>C level. Therefore, measurement of serum Alu methylation by qCOBRA-Alu is a potentially useful tool for NPC screening. In summary, we propose that <sup>14</sup>C level may suggested to be a potential biomarker for screening and follow-up of NPC patients.

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