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Brief Report

Epigenteic Alteration of *DOK7* Gene CpG Island in Blood Leukocyte of Patients with Gastric Cancer and Intestinal Methaplasia

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Background: Intestinal metaplasia (IM) is a benign lesion with no serious concern for patients' health. On the other hand, gastric cancer (GC) is a malignant lesion that has to be differentially diagnosed from benign intestinal metaplasia. Epigenetic modifications have been suggested to play an important role in cancer initiation and development, and they have been investigated as a reliable biomarker tool even for early cancer diagnosis. Whole blood leucocytes (WBC) are potentially the most accessible tissue for cancer early diagnosis, especially for GC, which is hard to diagnose in the early stage.

Objective: This study aims to investigate the methylation status of *DOK7* gene CpG island in blood leukocytes of patients with IM and GC compared to normal control groups.

Material and Method: DNA was extracted from the whole blood of 30 IM patients, 30 GC patients, and 34 normal controls samples, and MSRE-PCR was utilized to evaluate the loci methylation status.

Results: Significant hypermethylation of *DOK7* gene CpG has been observed in GC 88.1 % ($p < 0.001$) and IM 66.0 % ($p = 0.03$) in comparison to the normal control group 56.8%. A cut−off upper than 84.5 % of hypermethylation is considered as a presence of gastric cancer malignant lesions.

Conclusions*:* This is the first reported on hypermethylation in *DOK7* CPG in blood leukocytes of patients with GC and IM and establishing a laboratory blood based test that may be useful as a novel biomarker test in the early diagnosis and screaning of GC and IM.

Keywords: DOK7, Epigenetic, Methylation, MSRE, MSRE-PCR

1. Background

As an age-related disease, Gastric Cancer is one of the most common and fatal cancer worldwide because it has been diagnosed in late stages. GC incidence is highly dependent on the region and culture and is observed more in men than in women. It accounts for 783,000 deaths each year, as the third most deadly cancer worldwide.

The gradual accumulation of genetic and epigenetic factors would lead to gastric carcinogenesis. DNA hypomethylation is a major mechanism of tumor-related gene activation, particularly in oncogenes (1). Breast and gastric cancer are initiated from the same epithelial tissue origin, which may be similar in their development mechanisms (2). *DOK7* gene has been reported to play a role in breast cancer development in a twin study using whole blood from 15 twin pairs discordant for breast cancer and high-resolution (450K) CpG methylation sites in microarray analysis. A validation cohort of 21 twin pairs determined the docking protein *DOK7* as a candidate for blood-based cancer diagnosis (3). Furthermore, hypermethylation of *DOK7* CpG Island has been reported in primary breast cancer tissues and cell lines (4).

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2. Objective

This study has aimed to identify the methylation alterations of *DOK7* CpG in blood leukocytes of patients with IM and GC compared to normal controls using designed methyl sensitive restriction enzyme PCR (MSRE-PCR). This test can accurately determine the hypermethylation of CpG and is usable as a lab diagnostic test with less cost and hassle than another method such as the bisulfite treatment method. This study was performed based on the hypothesis that *DOK7* hypermethylation could occur in GC and IM in blood leukocytes of some cancer patients.

3. Material and Methods

3.1. Differentially Methylated CpG (DMC) Region of Chosen Genes and Designing Primers

The "UCSC Genome Browser" (UCSC Genome Browser Home) was used to gain the CPG islands' sequences. The CPG islands' methylation assessment was utilized by "CpGPlot/CpGreport" (EMBOSS Cpgplot < Sequence Statistics < EMBL-EBI). We considered CpG islands with a high frequency of CpG dinucleotides, the Obs.Exp^{-1} value greater than 0.6, and the GC content were higher than 50% characteristics.

A restriction endonuclease recognition site was determined by the "NEBcutter analysis tool" (NEBcutter V2.0), and specific methylation-sensitive restriction enzymes *SmaI* have been chosen. Primer sequences were designed by "Primer3Plus" (https://primer3plus.com/) on either side of the selected restriction enzyme recognition sites.

3.2. Study Design and Participants

In this study, 94 samples were analyzed, including 30 patients with GC and 30 patients with IM, and 34 normal control samples. All participants in this study have signed the consent form for using their clinical samples, and the study was confirmed by the Ethical Committee of the National Institute of Genetic Engineering and Biotechnology with the code number I.R.NIGEB.EC1398.12.3.A. The patient's clinicopathological features are presented in **Table 1**, including age, sex, and disease stage.

3.3. Genomic DNA Isolation

DNA was extracted using a DNA extraction Kit (GeneAll Biotechnology, Korea) and stored at -20 °C. The quality and quantity of DNA were determined using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and electrophoresis of the DNA sample using 1% agarose gel.

3.4. Analysis of CpG Island Methylation Alteration Using MSRE-PCR

The *MSRE-PCR* method was used for CpG methylation analysis using methyl sensitive restriction enzyme (MSRE). In this analysis, the unmethylated enzyme recognition site was cleaved, and the methylated site was undigested by methyl sensitive RE. In this case, the SmaI MSRE was selected to digest *DOK7* unmethylated CpG in its recognition site. In this way, a digestion reaction containing 50 ng of extracted DNA, 2.5 units of *SmaI* (Takara, Japan), 1 µL of tango buffer, and dH_2O up to 10 µL total volume was prepared and incubated over night at 37 °C according to the manufacturer's instruction. An undigested reaction tube was prepared to control each DNA sample, similar to the digestion reaction except RE. In this control tube, 0.5 µL dH_2O was added instead of RE as a 100% hypermethylated control sample for final comparison after gel electrophoresis on the agarose gel. A 100% hypomethylated DNA control was also used as a negative control to confirm proper *SmaI* digestion.

Table 1. Demographic data for all studied participants

Factors		*Patients (n=94)*	*Age range*
Gender	Female Male	45 49	
Diagnosis	Gastric Cancer	30	37–82
	Intestinal Metaplasia	30	41 - 81
	Normal	34	39-78

Each PCR reaction consisted of 1 µL of digestion reaction as template contained 10 ng of digested DNA, 10 pmol of each forward (TGGAAGAGTAGGTGGCTGGT) and reverse (TTCCACAAGCACAGCTCAACC) primers, and 10 µL of 2×Taq master mix Kit (Ampliqon, Denmark) and dH2O to reach 20 µL total volume reaction mixture. PCR condition consisted of 30 cycles, each cycle consisted of three-step denaturation (95 °C for 40"), annealing (60 °C for 40") and extension (72 °C for 60"), with an initial denaturation at 95 °C for 5' and a final extension at 72 °C for 7'. PCR products (386 bp) were run on the 1.5% agarose gel, stained with ethidium bromide, and visualization under UV transilluminator.

Each treated sample with RE was compared with its undigested one as 100 % methylated control using gel analyzer software to identify the accurate DNA methylation percentage. The intensity of the treated samples amplified products has a direct relationship with the methylation level. The methylation intensity was calculated by gel analyzer software (GelAnalyzer 19.1).

3.5. Methylation Quantification Using MSRE-qPCR

Real-time PCR was applied to quantify methylation alteration in the *DOK7* gene. For each sample, digested and undigested DNA samples were amplified. All PCRs were performed in a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Australia). Real-time PCR was performed with the following constituents: 1 µL of the digested DNA solution, 5 µL of 2× SYBR Green PCR Master Mix (Takara, Japan), 10 pmol of forward and reverse primers, and dH_2O up to 10 µL total valium. Real-time PCR was performed in 45 cycles, including strings denaturation (95 °C for 30"), annealing (60 °C for 30") and extension (72 °C for 30"), initial denaturation at 95 °C for 5'. ΔCt values were obtained as the difference between ΔCt on cycle threshold for each sample. The formula %Methylation =100($e^{-0.7(\Delta Ct)}$) was used to obtain methylation fraction (5).

3.6. Statistical Analysis

Results expressed as means ± standard deviation (SD), and all statistical analyses were performed using IBM SPSS version 26 (SPSS, Inc., Chicago, IL, USA) and Prism (GraphPad Software, San Diego, CA) version 9.0.0. The statistical significance of the difference between groups was determined using one-way ANOVA to determine the three groups' differences. The receiver operating characteristics (ROC) was utilized to identify the sensitivity and specificity of the test. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Clinicopathological Parameters

The present study analyzed the association between *DOK7* CpG island methylation status and GC and MI clinicopathological characteristics. The participants' age range was among normal, IM, and GC groups as 39-82, 41–81, and 37–82 years old. (**Table 1**)

4.2. Methylation Changes Obtained Using MSRE-qPCR Analysis

This study obtained hypermethylation of *DOK7* CPG in IM and GC groups compared to healthy individuals. Our assessments indicate that the *DOK7* CPG island was hypomethylated in normal samples (mean = 56.8 %) compared with IM (mean = 66 %) and GC (mean = 88.07 %) patients.

The difference between the hypermethylation of the *DOK7* CPG in MI and GC versus normal controls was significant ($p< 0.001$), where it was more hypermethylated in GC patients. The ROC statistical analysis revealed sensitivity %73.33 and specificity %97.06, with a hypermethylation cut−off of approximately 84.5 % in the patients' group and area under the ROC curve of 0.9495. (**Fig. 1 and 2**)

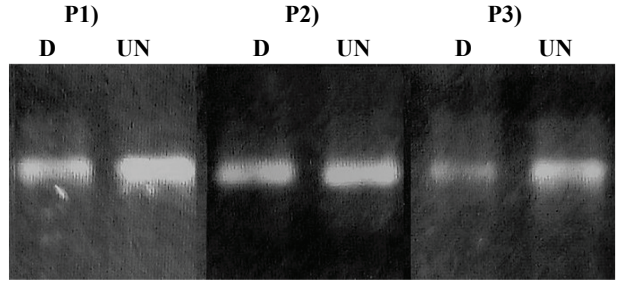

Figure 1. Methylation status of DOK7 desired region after digestion and MSRE-PCR of DNA samples treated (D) sample compared untreated (UN) control. The samples derive from the same experiment, and the gels were processed in parallel. **P1)** Metaplasia **P2)** Gastric Cancer **P3)** Normal for DOK7.

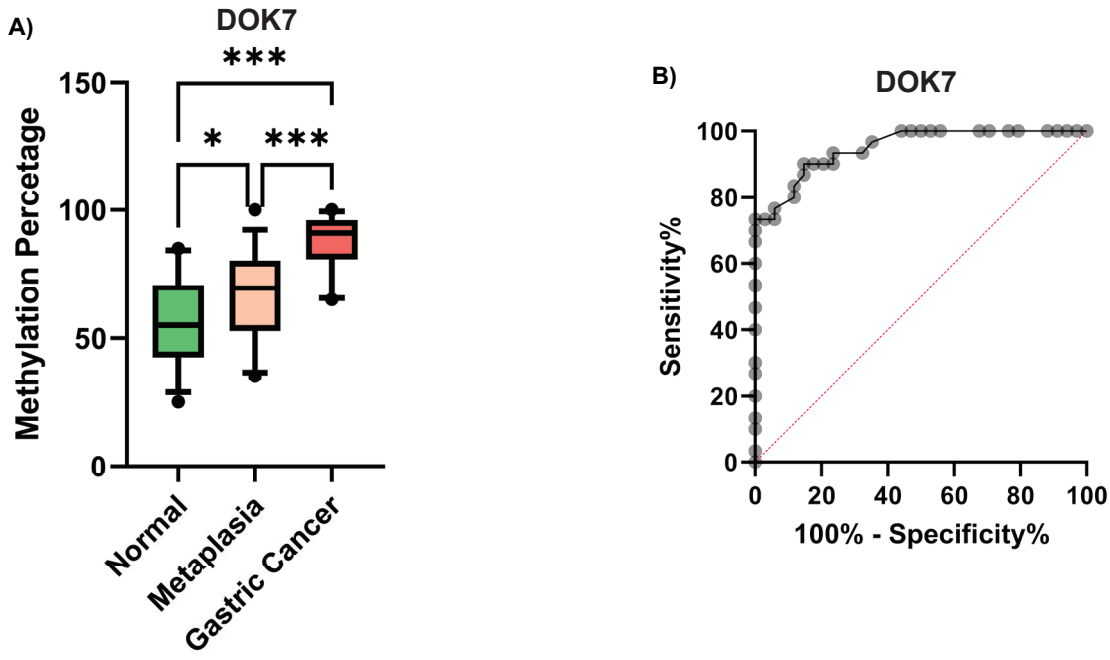

Figure 2. DOK7 methylation status in the DNA obtained from whole blood. **A)** Increased methylation of DOK7 correlated with the presence of various gastric lesions. Each bar represents the mean ± SD. (p-value = 0.05 considered, *: p = 0.03, *** p < 0.001 vs. normal control) **B)** ROC curve of DOK7 methylation (p < 0.001) exhibiting a well-validated gene with high intermediate sensitivity (73.33 %) and high specificity (97.06 %).

5. Discussion

This study examined the methylation alteration of *DOK7* CPG island in DNA extracted from whole blood samples of 30 GC and 30 IM cases compared to 34 normal controls. Significant methylation changes were observed between GC and IM cases and controls whereas, *DOK7* methylation changes were significantly different between GC (p < 0.001) and IM (p = 0.03) in comparison to normal control and even between GC and IM patients itself (p < 0.001). These results suggested that hypermethylation was significantly elevated with malignancy from normal controls to IMs towards GCs cases, respectively.

Genome-wide DNA methylation alterations in circulating WBCs are associated with cancer development and tumor growth (6). Our results from peripheral blood leukocytes follow *DOK7* CPG hypermethylation studies which have been previously reported in tumor samples compared to surrounding normal tissue in breast cancer (7), lung cancer (5), esophageal cancer (8), and glioma (9). Moreover, the same result obtained from the expression analysis of *DOK7* gene mRNA showed a significant reduction in *DOK7* expression in lung cancer tumors and was associated with a poor patient survival rate (5). ROC analysis was employed in this study to compare the predictive accuracy of the methylation status of patients with gastric cancer. Our findings demonstrated that the methylation status of *DOK7* with a cut−off at > 84.50 % could reflect the gastric lesions' malignancy.

In IM, metaplastic cell lineages are expanded in the gastric mucosa (10), and patients with IM are at a higher risk of malignancy emergence (11). The hypermethylation of *DOK7* CpG in IM compared to normal control may play a role in carcinogenesis.

Our result showed the potential usefulness of blood leukocytes might serve as a reliable tissue in cancer development and diagnosis in early diagnosis compared to tumor sample biopsy (12). It may happen through cell-to-cell communication exosomes. Exosomes are secreted from immune and cancerous cells and transfer mRNAs, miRNAs, or proteins to other target cells. They transfer growth factors, cytokines, chemokines, or angiogenic and immunoregulatory molecules in their cargo from cancerous cells to others such as WBCs and play a role in cellular reprogramming and carcinogenesis (13). In colorectal cancer, for example

(CRC), cell exosomes have induced genome-wide DNA methylation changes in the WBCs of CRC patients (14) and other types of cancers (15).

In conclusion, this is the first report revealed a significant alteration in *DOK7* CPG island hypermethylation in blood leukocytes of patients with GC and IM. Most importantly *DOK7* hypermethylation has been detected by establishing a laboratory test on a blood-based assays that may be useful as a novel biomarker test in the early diagnosis and screening of GC and IM.

Competing interests

All authors have read the manuscript and declared that they have no competing interests.

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