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

Arash Moradi 1, Seyed Ahmad Aleyasin 1,*, Kamal Mohammadian 2, Aida Alizamir 3

1Department of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
2Department of Radiation Oncology, Hamadan University of Medical Sciences, Mahdieh center, Hamadan, Iran
3Department of Pathology, Hamadan University of Medical Sciences, Hamadan, Iran.

*Corresponding author*: Seyed Ahmad Aleyasin, Department of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran. Tel: +98-2144787383, Fax: +98-2144787395, E-mail: sogand@nigeb.ac.ir

**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 screening 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).
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 Smal 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 \(-20^\circ{\text{C}}\). 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 Smal 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 Smal (Takara, Japan), 1 \(\mu\)L of tango buffer, and \(dH_2O\) up to 10 \(\mu\)L 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 \(\mu\)L \(dH_2O\) 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 Smal digestion.

| Table 1. Demographic data for all studied participants |
|-----------------|-----------------|------------------|
| **Factors**     | **Patients (n=94)** | **Age range**    |
| Gender          |                  |                  |
| Female          | 45               |                  |
| Male            | 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 dH2O 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(ΔCt)) 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)
5. Discussion

This study examined the methylation alteration of \textit{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, \textit{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 \textit{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 \textit{DOK7} gene mRNA showed a significant reduction in \textit{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 \textit{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 \textit{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.

Funding
Our study was supported by grants number 633 and 667 from National Institute of Genetic Engineering and Biotechnology (NIGEB).

Acknowledgment
This work was carried out at the National Institute of Genetic Engineering and Biotechnology (NIGEB), and the authors would like to express their appreciation to Dr. Behrouz Afshar and the staff of the Novin laboratory, Hamedan, Iran.

References
1. Chen K, Yang D, Li X, Sun B, Song F, Cao W, et al. Mutational landscape of gastric adenocarcinoma in Chinese: implications for prognosis and therapy. Proc Natl Acad Sci U S A. 2015;112(4):1107-1112. doi:10.1073/pnas.1422640112
2. Rossi F, Norell H, Jove R, Beljanski V, Grinnemo K-H. Differences and similarities between cancer and somatic stem cells: therapeutic implications. Stem Cell Research & Therapy. 2020;11(1):489. doi:10.1186/s13287-020-02018-6
3. Heyn H, Carmona FI, Gomez A, Ferreira HJ, Bell JT, Sayols S, et al. DNA methylation profiling in breast cancer discordant identical twins identifies DOK7 as novel epigenetic biomarker. Carcinogenesis. 2013;34(1):102-108. doi:10.1093/carcin/bgs321
4. Pouliot M-C, Labrie Y, Diorio C, Durocher F. The Role of Methylation in Breast Cancer Susceptibility and Treatment. Anticancer Research. 2015;35(9):4569. http://ar.iiarjournals.org/content/35/9/4569.abstract
5. Chen G, Yu H, Satherley L, Zabkiewicz C, Resaul J, Zhao H, et al. The downstream of tyrosine kinase 7 is reduced in lung cancer and is associated with poor survival of patients with lung cancer. Oncol Rep. 2017;37(5):2695-2701. doi:10.3829/or.2017.5538
6. Tahara T, Arisawa T. DNA methylation as a molecular biomarker in gastric cancer. Epigenomics. 2015;7(3):475-486.
7. Fitzgerald LM, Browne EP, Christie KD, Punska EC, Simmons LO, Williams KE, et al. ELF5 and DOK7 regulation in anti-estrogen treated cells and tumors. Cancer Cell Int. 2016;16(8). doi:10.1186/s12935-016-0282-9
8. Yang SM, Li SY, Yu HB, Li JR, Sun LL. Repression of DOK7 mediated by DNMT3A promotes the proliferation and invasion of KYSE410 and TE-12 ESCC cells. Biomed Pharmacother. 2017;90:93-99. doi:10.1016/j.biopha.2017.02.111
9. Hua CD, Bian EB, Chen EF, Yang ZH, Tang F, Wang HL, et al. Repression of DOK7 expression mediated by DNMT1 promotes glioma cells proliferation. Biomed Pharmacother. 2018;106:678-685. doi:10.1016/j.biopha.2018.06.156
10. Meyer AR, Goldenring JR. Injury, repair, inflammation and metaplasia in the stomach. J Physiol. 2018;596(17):3861-3867. doi:10.1113/JP275512
11. Shao L, Li P, Ye J, Chen J, Han Y, Cai J, et al. Risk of gastric cancer among patients with gastric intestinal metaplasia. Int J Cancer. 2018;143(7):1671-1677. doi:10.1002/ijc.31571
12. Boonsongserm P, Aungsuwatcharakon P, Puttipanyalears C, Apornthewan C, Kongruttanachok N, Aksornkitti V, et al. Tumor-induced DNA methylation in the white blood cells of patients with colorectal cancer. Oncol Lett. 2019;18(3):3039-3048. doi:10.3892/ol.2019.10638
13. Ferguson Bennit HR, Gonda A, McMullen JRW, Kabagwira J, Wall NR. Peripheral Blood Cell Interactions of Cancer-Derived Exosomes Affect Immune Function. Cancer Microenviron. 2019;12(1):29-35. doi:10.1007/s12307-018-0209-1
14. Boonsongserm P, Aungsuwatcharakon P, Puttipanyalears C, Apornthewan C, Kongruttanachok N, Aksornkitti V, et al. Tumor-induced DNA methylation in the white blood cells of patients with colorectal cancer. Oncol Lett. 2019;18(3):3039-3048. doi:10.3892/ol.2019.10638
15. Chopra-Tandon N, Wu H, Arcaro KF, Sturgeon SR. Relationships between Global DNA Methylation in Circulating White Blood Cells and Breast Cancer Risk Factors. J Cancer Epidemiol. 2017;2017:705860. doi:10.1155/2017/201705860