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Study of DOK4 gene expression and promoter methylation in sporadic breast cancer

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The analysis of DNA methylation patterns in circulating cell-free DNA in body fluids is an analyte of great interest in clinical research. Downstream of Kinase (DOK) proteins represent a multigenic family of adaptors that includes negative regulators of immune cell signaling and plays important roles in signaling, cellular growth, and survival. The aim of the present study was to investigate the pattern of promoter methylation and expression of the *DOK4* gene in breast cancer, and its possible association with histoclinical characteristics. The mRNA expression of the *DOK4* gene was evaluated in 164 breast tissues using Real-Time RT-PCR. The promoter methylation of this gene was evaluated in breast tissues and plasma free nucleic acids by MS-PCR. The associations of gene expression and DNA methylation with histoclinical characteristics of the patients were studied. The data indicated that *DOK4* mRNA expression was significantly downregulated in breast tumors compared with normal control in all the stages. About 40% of breast tumors showed *DOK4* promoter methylation, which somehow confirms the *DOK4* promoter methylation rate in the plasma. The data revealed that the reduction in *DOK4* expression in all breast cancer groups could well endorse this gene as a candidate possible marker for future investigations on breast cancer management. The *DOK4* methylation pattern in breast tissue was correlated with plasma free nucleic acids but the state of *DOK4* promoter methylation alone may not be sufficient to differentiate between the two cancerous and normal groups.

Key words: DOK4, methylation, breast cancer, MS-PCR, expression

Breast cancer, a complex disease, is a result of intricate genetic and epigenetic changes that ultimately lead to changes in cellular processes such as proliferation, apoptosis, angiogenesis, and acquisition of invasive phenotype. The most common type of cancer among Iranian women is ductal carcinoma breast cancer, which comprises about 77.8% of invasive breast cancer cases [1].

The liquid biopsy is a potential alternative for tissue biopsy. Developing noninvasive strategies to early detect cancer based on the analysis of extracellular nucleic acids in biological fluids has huge importance. DNA can be released from normal and cancer apoptotic cells into the circulating blood system. The use of circulating DNA to provide a non-invasive, personalized genomic snapshot of a patients' tumor has huge potential. As far as the comparable increase of DNA concentration in plasma was also found in patients with other disorders, the increased plasma circulating DNA concentration itself is not specific enough for cancer detection [2]. So finding the informative biomarkers expressing tumors in circulating blood may have great importance in cancer management. Epigenetic alternations such as gene promoter methylation may be considered as a novel cancer biomarker with prognostic, diagnostic, or predictive value in the variety of cancers [3]. One of the frequent molecular alterations in human neoplasia [4], including breast cancer [5] may be the change in the status of DNA methylation express. These epigenetic alterations may induce neoplastic process by transcriptional silencing of tumor suppressor genes or inducing oncogenes and may be responsible for initial steps of induction of tumor cell proliferation [4]. Therefore, gene methylation pattern analysis could be considered with profound significance in the early detection of cancer.

The first three members of the Dok family including Dok-1, 2, and 3 compose separate subfamily known as Dok-A. This family of proteins has repeatedly been reported to influence and inhibit activation of the Ras/Erk pathways, a

part of the downstream target of the tyrosine kinases. While the inhibitory function of Dok-A family of proteins through interaction with multiple inhibitory effector proteins such as Ras inhibitor p120R as GAP is well understood, there is not sufficient information regarding other Dok proteins such as Dok-4, 5, and 6 which encompass a distinct subfamily (i.e. the Dok-B subfamily). As well, it was found a tissue dependent expression of the Dok family of proteins such that Dok-5 and 6 expression is essentially restricted to the neural tissues, while Dok-4 is ubiquitously expressed with a preference for the neural, endothelial, as well as epithelial tissues [6, 7].

The prognostic role of DOK family adapters was reported in acute myeloid leukemia [8]. Also, the Dok-4 protein acts as an inhibitor of tyrosine kinase signaling [9] but also activates mitogen activated protein kinase (MAPK), tumor necrosis factor alpha, and ROS mediated production of mitochondria in the lung cancer. The DOK4 gene expression is decreased in hypoxia [10].

Reports indicate that the knocking down of endogenous *DOK4* expression in Madin-Darby Canine Kidney (MDCK) cells results in an upregulation of TCF target genes, including IEGs Egr-1 and Fos, as well as upregulation of the *cyclin D1*. Furthermore, enhanced cell proliferation despite a reduced Erk activation would be a consequence of the *DOK4* gene knocking down [6]. As the RET substrate, *DOK4* activates GDNF/RET signaling pathway [10, 11]. GDNF acts as RET ligands and *DOK4* regulates the GDNF family, resulting in the activation of several signaling pathways including Ras/ERK, PI3K, PLPCγ, and JNK pathways [11].

Considering the important role of *DOK4* in proliferation events, in the present study we aimed to evaluate the prevalence of epigenetic silencing and expression of *DOK4* gene via promoter methylation in the Iranian patients with sporadic ductal breast cancer and monitor the correlation of promoter methylation pattern in plasma and tissue in order to propose possible non-invasive indicator for cancer management.

Patients and methods

Patients. The tissue samples used in the present study were from patients with sporadic ductal carcinoma breast cancer referred to the Cancer Institute of Tehran University of Medical Sciences. In this case-control study, we tried to evaluate *DOK4* gene promoter methylation status along with gene expression in 67 samples of invasive ductal carcinoma and the normal adjacent tissues, and 10 normal control breast tissues retrieved from unaffected individuals who underwent surgery due to cosmetic purposes. In addition, 67 plasma samples belonging to breast cancer patients, plus 30 normal plasma samples were also included in the study. The study was approved by the Ethical Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB) based on the Declaration of Helsinki. All individuals signed informed consent to participate in the study. Tumor staging was performed according to the tumor, node, and metastasis (TNM) classification. The blood samples were collected before surgery. In addition, patients who had recurrent breast tumors, or had chemo/radiotherapy before surgery, or showed the breast and/or ovarian cancers in the first- and second-degree relatives were excluded from the study. Tumor samples were obtained following surgical resection and their pathological features were examined on macro dissection of the samples. Tumor tissues were transferred to liquid nitrogen and stored until use.

The demographic and clinical features of the study population are summarized in Table 1.

RNA extraction and quantitative real-time RT-PCR. Total RNA was extracted from tissue samples using Tri Pure Isolation Reagent (Roche Applied Science, Germany) as instructed by the manufacturer. RNA was analyzed by Thermo Scientific NanoDrop™ 1000 Spectrophotometer to check its purity and concentration, and electrophoresed to confirm its integrity. One μg of the RNA was used for cDNA synthesis through the application of Fermentas Revert AidTM H Minus First Strand cDNA Synthesis Kit (Ferments, Canada). The synthesized cDNA was then checked spectrophotometrically to estimate its concentration. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out on a rotor gene 6000 Corbett detection system using the SYBR Green PCR kit (TAKARA, Japan). The cDNA

Table 1. Characteristics of breast cancer patients and controls.

	Patient N (%)	Control N (%)
Number	67	30
Age (years)		
Mean	47.2±12.6	48.5±16.4
Range	27–84	25–80
Stage at diagnosis		
Stage I	15 (22.4%)	
Stage II	21 (31.3%)	
Stage III	23 (34.3%)	
Stage IV	8 (12%)	
Lymph node status		
N0	30 (44.8%)	
N+	37 (55.2%)	
Distance metastasis		
yes	8 [2 bone, 6 lung] (12%)	
No	59 (88%)	
Hormone receptor status (IHC)		
ER positive	54 (80%)	
ER negative	13 (20%)	
PR positive	52 (77.6%)	
PR negative	15 (22.4%)	
HER-2 status (IHC)		
+ + +	23 (34.3%)	
Negative	44 (65.7%)	
Triple-negative breast cancer	8 (12%)	
Non-triple negative breast cancer	59 (88%)	

of normal breast sample was used as a positive control for gene expression. The thermal cycling condition was composed of an initial activation step for 5 minutes at 95 °C followed by 40 cycles of denaturation step for 30 seconds at 95 °C, annealing step for 30 seconds at 60 °C and extension step for 15 seconds at 72 °C. No template control (NTC) consisting of H_2O was included in each run. The *β-actin* gene was used as a normalizer. The sequences of the primers designed for specific genes are listed in Table 2. Melting curve analysis was performed to verify the specificity of PCR products.

DNA extraction and bisulfite modification. To study promoter methylation, genomic DNA was extracted from tissue and plasma specimens using the Cinnapure DNA extraction kit and viral nucleic acid extraction kit, respectively, (Sinaclon, Tehran, Iran) following manufacturer's protocol. Quantity and quality of the extracted DNA were evaluated by a spectrophotometer (Nanodrop 2000, Thermo Scientific, USA). For each sample, 1 μg of genomic DNA was used for sodium bisulfite modification.

Methylation specific PCR. The extracted DNA was treated with bisulfite to convert unmethylated cytosine to uracil prior to a methylation-specific polymerase chain reaction (MS-PCR) using Epi Tect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions. The modified DNA was amplified using MS-PCR primers (Table 2), which specifically recognize either the unmethylated (product size 143 bp) or methylated (142 bp) *DOK4* promoter sequence after bisulfite conversion. MS-PCR reaction was performed in 40 cycles. The condition for *DOK4* gene amplification was 10 minutes at 95 °C, followed by amplification cycles including 30 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C for the extension, in addition to final elongation of 10 minutes at 72 °C. After PCR, electrophoresis was performed on a 1.5% agarose gel. The MS-PCR reaction was performed in a final volume of 10 μl in a hot start reaction. In this study, MS-PCR was carried out using Methylation Specific PCR Kit (Epi Tect MSP, Qiagen Co.). The positive control of methylated and unmethylated purchased from Epi Tect, Qiagen Co.

The target promoter region selection was done using Genomatix (http://www.genomatix.de/en/index.html) and the primer pairs were designed using the Meth Primer program (http://www.urogene.org/methprimer/index.html). The promoter region starting from –1500 to +200 and transcriptional start site was obtained from the database of Transcriptional Start Site (DBTSS) (http://dbtss.hgc.jp) for *DOK4* amplification. The Transcriptional Start Site position of the *DOK4* gene was identified as 57486224-57486723 in the genomic region.

Statistical analysis. Data were analyzed using SPSS 16.0 (SPSS Inc. Chicago, USA). The Mann-Whitney U test and Kruskal-Wallis test were performed for numerical data and the Chi-square test was used to analyze the relationship between parameter data. Correlation and consistency were analyzed using Pearson correlation analysis. Numerical data are presented as the mean ± standard deviation (SD). Differences were considered statistically significant if $p<0.05$.

Results

***DOK4* gene expression in breast tumors compared with control groups.** The obtained results shown in Figure 1 indicate a significantly lower mean of expression for the *DOK4* gene in the breast tumor compared to normal adjacent and the normal control breast tissues ($p<0.0001$). The mean of *DOK4* expression was lower than 0.5 fold change compared with both normal and normal adjacent control group, which is considered as the downregulation. The statically significant difference was observed in *DOK4* gene expression between normal adjacent and the normal control groups. The *DOK4* gene expression was lower in the normal adjacent compared with the normal control group ($p<0.05$). The range of *DOK4* expression is 0.1–1.2 and 0.1–1 fold change compared with normal and normal adjacent groups, respectively.

***DOK4* gene expression in different breast cancer categories based on cancer stages and histopathological characteristics.** The mean of *DOK4* gene expression in all the four stages of breast cancer was significantly downregulated compared with normal control ($p<0.0001$). As shown in Figure 2, there were no significant differences between the different four breast cancer stage groups ($p>0.05$).

There were no significant differences in *DOK4* gene expression between different breast tumor nodal involvement and hormone receptors situations such as LN+/LN– and PR+/PR–, and triple-negative/non triple-negative, but

Table 2. Sequences of the primers.

Amplicon length (bp)	Annealing Temperature (°C)	Sequence 5'–3'	Gene name
117	60	F: AAGAACGTGAGGCTGCTGAAC R:GGCGATGTTCTGGGAACCA	DOK4
161	60	F: GAGACCTTCAACACCCCAGC R: AGACGCAGGATGGCATGG	β-actin
142	60	F: GGTGTTAGGGCGTTAGGGTAGAATC R:ACTCCGACTAAAACTCCGC	*DOK4 - MET*
143	60	F: AGGGTGTTAGGGTAGAATTGTGTGTT R:AACTAAAACTCCAACTAAAACTCCAC	DOK4 - UNMET

in ER and HER2 negatives, the *DOK4* expression was lower than ER and HER2 positive ones ($p<0.01$).

***DOK4* promoter methylation status in breast tissues and plasma.** The methylation status of breast tissues comprised of the tumor, normal adjacent, and normal control are summarized in Table 3. As the data indicated, about 45% of breast tumors showed methylation of the *DOK4* promoter region, which is significantly higher than normal and normal adjacent control, 1 and 17%, respectively. But considering the methylation frequency in cancerous breast tissues, there were no significant differences between methylation statuses that were *DOK4* methylated and unmethylated promoter region.

The data revealed that about 40% of breast cancer patients showed the *DOK4* promoter methylation in their plasma sample, which is significantly higher than normal control, which was about 3%.

The data showed that 44.8% of breast tumors were methylated in the promoter region of the *DOK4* gene, interestingly the approximately same results were observed in the plasma samples of these patients (41.8%). The data indicated the positive correlation between plasma and breast tumor tissues in *DOK4* promoter methylation status. The Pearson correlation r was 0.993. In other words, the methylation status of breast tumors was traceable in plasma. A comparison of methylated *DOK4* promoter frequency in different breast cancer groups based on histopathology situations revealed no significant difference between studied groups (Figure 3).

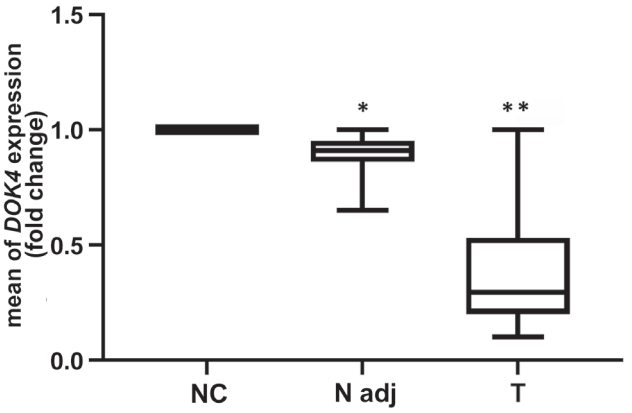

Figure 1. The mean expression of the *DOK4* gene in the breast tumor and normal adjacent tissues in comparison with the normal control (NC: Normal control tissue, N adj: Normal adjacent tissue, T: tumor). *p<0.05, **p<0.0001

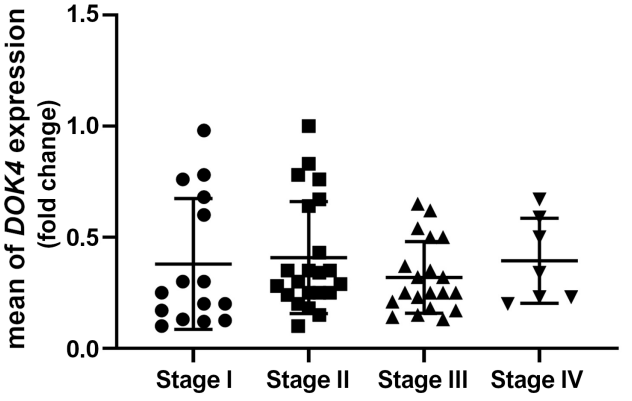

Figure 2. The mean expression of the *DOK4* gene in tumor tissue with different stages of cancer.

Discussion

The plasma cfDNA phenomenon in cancer patients has been studied extensively in recent years. The level of plasma cfDNA could be considered as a universal marker indicating malignancy [2]. Numerous cancer-specific alterations, such as methylation, allelic imbalances, and mutations have been found in blood cfDNA [12, 13]. It has been also reported that cfDNA levels monitoring in peripheral blood can be considered as the biomarker indicating therapy responses in different cancer types [14]. In addition to quantitative changes, cfDNA in tumor cells may possess qualitative changes such as mutations, microsatellite instabilities, and methylation [15, 16]. Gene promoter methylation is a well-known gene expression regulation mechanism. Aberrant gene promoter methylation in cfDNA has been reported as a noninvasive biomarker for detection, differential diagnosis, prognosis, and therapy responses in cancers [17].

The Dok-4 protein acts as a tyrosine kinase signaling inhibitor. As well, the protein activates the mitogen activation protein kinase (MAPK), tumor necrosis factor alpha, and ROS-mediated generation of the mitochondria. A reduction of *DOK4* gene expression in lung cancer has been reported. The *DOK4* gene is also expressed in T cells and non-immune cells as the C-Ret substrate [10].

Considering the important role of this gene in the signaling pathways of the tyrosine kinase, including the two receptors tyrosine kinases RET and Tie2, lipid phosphatase Ship1 [6], and MAPK, we investigated the expression of this gene in the breast cancer which has been reported as the leading cause of cancer death in woman worldwide. The results revealed that the *DOK4* gene expression has been decreased in most tumor tissues of Iranian breast cancer patients. The *DOK4* expression was reduced in 75% of studied breast tumor tissues. Compared to the normal surrounding tissue, the lower level of expression in the tumor confirms the suppressor effect of the *DOK4* gene in breast cancer. The lowest expression of the *DOK4* gene was observed in the ER and HER2 negative groups focusing on the hormone receptor status of the studied breast cancer tumors. The results also showed that the mean of *DOK4* gene expression was reduced in the four stages of breast cancer, while, there were no significant differences between stages. The data was also revealed that in the normal adjacent samples the *DOK4* expression was significantly lower than the normal control group. This lower expression in normal tumor margin tissues may be due to

Table 3. Grouping of the methylated and unmethylated *DOK4* gene promoter based on the types of applied specimens.

Sample type	Total number	DOK4 promoter methylated (%)	DOK4 promoter unmethylated (%)	Both methylated and unmethylated promoter (%)	p-value X^2 test
BC/plasma	67	28 (41.8%)	39 (58.2%)	0 (0%)	
N/plasma	30	2 (3.33%)	28 (93.33%)	0 (0%)	*
BC/tumor	67	30 (44.8%)	36 (53.7%)	1 (1.5%)	
N adj/tissue	67	17 (25.4%)	45 (67.2%)	5 (7.4%)	*
NC/tissue	10	1 (10%)	9 (90%)	0 (0%)	*

*p<0.0001

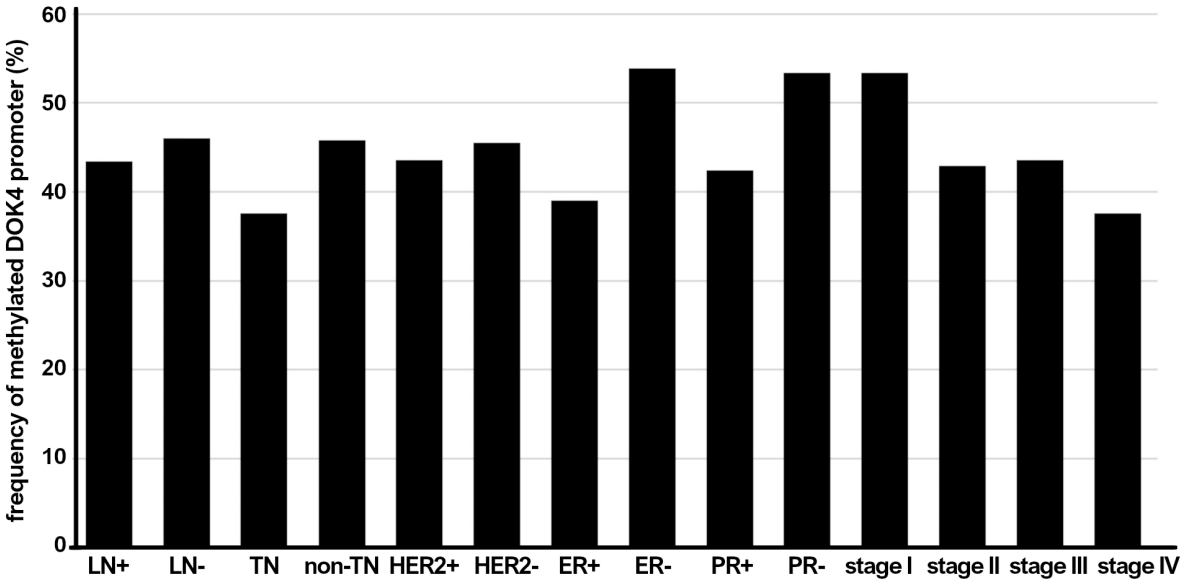

Figure 3. The frequency of *DOK4* promoter methylation in different groups of studied individuals with respect to a hormone receptor, lymph node involvement, and various stages of breast cancer.

starting cancer molecular signals, which were not detectable as pathological events under the microscope. It was reported that different gene expression in normal adjacent regions of the tumor compared with normal tissues from unaffected individuals might be due to epigenetic control of those genes expression [18]. The present investigation has shown the frequency of *DOK4* promoter hypermethylation was about 42% and 45% in breast tumor and plasma samples of the breast cancer patients whereas in the normal breast tissue and plasma about 90% of samples showed *DOK4* promoter non-methylated status. These data stated there was an almost similar pattern of *DOK4* promoter methylation in plasma and breast tissue.

The Dok-4 proteins are highly conserved in the course of evolution and have important roles. In response to insulin and the insulin-like growth factor that causes phosphorylation of tyrosine, the Dok-4 is associated with important kinases, including RET, Src, Fyn, and Jak2 [10]. The role of the *DOK4* gene is tumor suppression and inhibition of tyrosine kinase and MAPK signaling, and the reduction in expression leads to triggering of these pathways in breast cancer. Tyr1062 acts as the key to the RET signaling. The tyrosine phosphorylation in this position plays the role of the docking site for the multiple intracellular proteins [19]. Following the RET activation by the GDNF, the tyrosine located at position 1062 spontaneously undergoes phosphorylation, which then recruits the Dok-4 adaptor proteins or Shc. The phosphorylated Shc stimulates the Grb2/SOS/Ras/ERK cascade, thereby induces transient activation of the ERK. Alternatively, activation of the *DOK4* leads to increased GTP-bound Rap1 levels, which induces sustained activation of ERK and neuronal differentiation including neurite outgrowth [11]. Due to the fact that the *DOK4* gene acts as a regulator of GDNF and RETS substrates, a reduction in the expression of this gene leads to the progression of the signaling pathway of Shc and Ras activation and cell proliferation.

Hooker et al. have shown that knocking down of the endogenous Dok4 in MDCK cells results in the upregulation of the TCF target genes and cyclin D1, as well as enhanced cell proliferation [6].

The data retrieved from the present study revealed that the *DOK4* gene may act as a tumor suppressor in breast cancer and its downregulation may be preceded to tumorigenesis. Also, the data indicated that the promoter methylation is not the only reason for the *DOK4* downregulation in breast cancer tumors and may suggest that the observed downregulation could be attributed to the other genetic or epigenetic factors, such as miRNAs, histone modification, or chromatin remodeling.

The positive correlation between plasma and tissue methylation pattern of the *DOK4* gene promoter suggests that the methylation study in plasma free nucleic acids extracted from the tumor may be a more suitable alternative for cancer methylation study.

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