TÜBİTAK

Original Article

Turk J Med Sci
2012; 42 (Sup.2): 1379-1387

E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1204-30

Analysis of promoter methylation of *Dickkopf1 (DKK1)* gene in breast cancer

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Aim: Breast cancer is the most common malignancy in women worldwide. The Dickkopf1 (DKK1) gene product is a potent inhibitor of the Wnt pathway. DKK1 methylation status and its protein expression were examined in normal and cancer tissues of breast cancer patients.

Materials and methods: We examined methylation changes in 83 tumor and adjacent normal tissues, and also in 9 normal breast tissues from unaffected women (no breast cancer history) as controls. DKK1 promoter methylation was assessed by methylation-specific polymerase chain reaction. Immunohistochemical staining was used to investigate DKK1 protein expression in the 9 controls and in 70 out of the 83 tumors as well as their adjacent normal tissues. Immunohistochemical results were categorized as positive or negative for DKK1.

Results: DKK1 gene methylation was detected in 3 out of the 9 (33%) normal breast tissue samples of unaffected women. The DKK1 gene was hypermethylated in 58 out of the 83 (70%) tumor samples and 51 out of the 83 (61%) adjacent normal tissue samples. In immunohistochemistry, 33% of normal breast tissue from unaffected women, 31% of the tumors, and 17% of the adjacent normal tissues in the breast cancer patients were found to be DKK1-positive. DKK1 promoter methylation and protein expression were not associated with age, tumor size, lymph node status, histological grade, estrogen/progesterone receptor status, or HER2 positivity.

Conclusion: Our results indicate that there is a concordant DKK1 methylation change between normal and cancerous breast tissue.

Key words: Breast cancer, Dickkopf1, DNA methylation

Introduction

The Wnt signaling pathway plays an important role in determining cell fate and regulating cell proliferation. Dysregulation of this pathway causes developmental defects and cancer (1). When Wnt ligands bind to their Frizzled receptors, Dishevelled (Dsh) protein blocks β-catenin degradation. Stabilized β-catenin accumulates in the cytoplasm and enters the cell nucleus. β-Catenin interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family, thereby activating Wnt target genes (2). The Wnt signaling pathway is regulated by antagonists. Wnt antagonists can be classified into 2 groups and both of them prevent ligand–receptor interactions. The first group includes the secreted Frizzled-related protein (sFRP) family, Wnt inhibitory factor (WIF)-1, and Cerberus, which bind directly to Wnts. The second group comprises members of the Dickkopf (DKK) family, which bind to the Wnt receptor complex (3). The DKK family consists of 4 members (DKK1 to DKK4) and a DKK3-related protein named Soggy. DKK1 is the founding and most studied member of the family (4). DKK1 binds to the extracellular domain of LRP5/6 and prevents the

Received: 06.04.2012 – Accepted: 16.05.2012
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formation of active Wnt-Frizzled-LRP5/6 receptor complexes (5). Additionally, transmembrane proteins Kremen1 and Kremen2 bind to DKK1 and form a ternary complex with DKK1-LRP5/6, triggering endocytosis. Consequently, the DKK1-LRP5/6 complex is removed from the plasma membrane and the Wnt signaling pathway is blocked (6).

Epigenetic silencing of tumor-related and tumor suppressor genes in association with promoter CpG island hypermethylation is a frequent event in tumor cells (7). These genes are not only hypermethylated in tumor cells but also in normal epithelium surrounding a neoplasm. In other words, the presence of cancer causing changes in apparently normal tissue surrounding a neoplasm can be defined as a field defect (8).

Our aim was to identify and compare the methylation changes of the *DKK1* gene promoter methylation status. We also identified the protein expression of the DKK1 gene in tumor tissue of invasive ductal breast cancer patients as well as adjacent normal tissue (with no sign of tumor) of breast cancer patients, and breast tissue of healthy women (with no history of breast cancer). Furthermore, our objective was to determine if there was a relationship between the methylation pattern and the protein level and the clinicopathologic characteristics of the patients.

Materials and methods

Samples

Formalin-fixed paraffin-embedded (FFPE) archival matched tumor/macroscopically normal samples of 83 patients (median age: 54.2 years; range: 31–83 years) who were diagnosed with invasive ductal breast cancer between 2006 and 2010 were obtained from the Department of Pathology, Faculty of Medicine, Selçuk University, Konya, Turkey. These samples were collected after approval of the study by the institutional human ethics committee.

Tumor histology was determined according to the 2003 criteria of the World Health Organization, while disease stage was assessed according to the Union for International Cancer Control. Tumors were graded according to Bloom and Richardson. Each tumor and normal breast tissue sample was histopathologically confirmed for the presence or absence of cancer cells. Tumor samples containing more than 70% tumor cells were selected. Estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2) statuses were determined by immunohistochemistry.

We collected 9 normal breast tissue samples from patients and none of these samples showed pathological changes.

Extraction of genomic DNA

Tumor and normal tissues were deparaffinized and rehydrated in a decreasing alcohol series prior to DNA extraction by use of the PureLink Genomic DNA Mini Kit (Invitrogen, USA). The extracted genomic DNA was finally eluted in 100 µL of Tris–HCl buffer (10 mM; pH 9.0).

Bisulfite modification and methylation-specific PCR

Sodium bisulfite conversion of genomic DNA was carried out using the DNA Methylation Detection Kit (BioChain, USA) according to the manufacturer's instructions. *DKK1* promoter methylation status was analyzed using methylation-specific polymerase chain reaction (MSP). We used the primers for the methylated and unmethylated promoter regions as previously reported (9). The PCR conditions for unmethylated primers were 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C; and 7 min of final extension at 72 °C. The PCR conditions for methylated primers were 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C; and 7 min of final extension at 72 °C. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Immunohistochemistry

DKK1 protein expression was investigated by immunostaining of formalin-fixed, paraffin-embedded tissue sections. Sections of 5 µm in thickness were cut, dried, deparaffinized, and dehydrated in graded ethanol solutions. Antigen retrieval was performed by heating in a citrate buffer (pH 6.0). We used a Histostain-Plus Kit (Invitrogen) for immunostaining. Samples were incubated for 55 min with primary DKK1 antibody (mouse monoclonal, LifeSpan, USA; dilution 1:150), washed, and incubated for 20 min with the biotinylated secondary antibody. Diaminobenzidine (Invitrogen)

was used for antibody detection. Normal human placenta was used as a positive control. Cases that showed at least 10% staining were considered positive.

Statistical analysis

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., USA). To study statistical associations between clinicopathological factors and DKK1 expression or *DKK1* methylation status, contingency tables and Fisher's exact test (2-sided) were used. We also used Pearson's chi-square test. P-values less than 0.05 were considered statistically significant. The Kappa statistic was used to assess concordance between the normal and tumor tissues of breast cancer patients.

Results

We examined *DKK1* gene methylation in 83 primary breast cancer tissues as well as in adjacent normal tumor-free tissues and in 9 patients without cancer history with the MSP method. The clinicopathological characteristics of the patients are shown in Table 1.

***DKK1* gene methylation in normal breast and tumor tissues**

We determined the presence of *DKK1* gene methylation both in the primary tumors and normal tissues. In 70% (58/83) of the cancer samples and in 61% (51/83) of the normal tissues, *DKK1* promoter methylation was determined (Figure 1). On the other hand, the rate of methylation in the 9 normal breast tissues belonging to women without cancer was 33%.

Concordant gene methylation in normal and cancerous breast tissue

Concordant methylation changes in normal and cancerous breast tissues were present in 63% of cases for *DKK1* (Table 2).

DKK1 protein expression in primer breast tumor, normal tissue, and normal breast tissue of unaffected women

DKK1 protein expression was investigated with immunohistochemical methods in 70 primary tumor samples and normal tissues that did not contain tumors (n = 70) and at the same time in 9 normal breast tissues (with no history of cancer). While the

Table 1. Clinicopathological characteristics of patients.

Criteria		Number	%
Age (mean ± SD)		54.2 ± 1.3	
Tumor status	pT1	18	21.7
	pT2	52	62.6
	pT3	13	15.7
	pT4	0	0
Lymph node status	pN0	22	26.5
	pN1	21	25.3
	pN2	20	24.1
	pN3	20	24.1
Histological grade	G1	5	6.0
	G2	67	80.7
	G3	11	13.3
Immunohistochemical staining			
Estrogen receptor	Negative	28	33.7
	Positive	55	66.3
Progesterone receptor	Negative	35	42.2
	Positive	48	57.8
HER2	Negative	34	41
	Positive	49	59

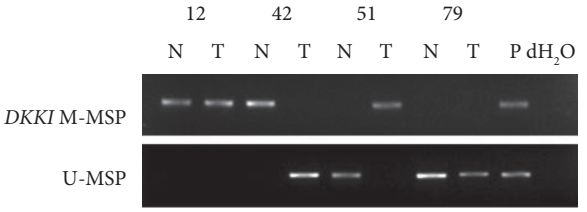

Figure 1. Analysis of methylation of *DKK1* gene in primary breast cancers. T indicates tumor tissue. N indicates normal breast tissue from the same patients. M-MSP indicates that methylation-specific primers were used in MSP. U-MSP indicates that unmethylation-specific primers were used in MSP. P indicates the positive control. dH_2O indicates no DNA added.

tumor samples were positive for DKK1 in 31% (22/70) of cases, this rate in tumor-free normal tissues was determined as 17% (12/70). Normal breast tissues showed a 33% incidence of positive DKK1 expression (Figure 2).

Methylation and protein expression correlation with clinicopathological characteristics in tumor tissue

DKK1 promoter methylation and DKK1 protein expression were not associated with clinicopathological factors (age, tumor size, lymph node status, histological grade) and some of the proteins in the etiology of breast cancer such as ER, PR, and HER2 receptors (Tables 3 and 4).

Discussion

In this study, we sought to identify and compare the methylation changes of *DKK1* gene promoter methylation status. Our data demonstrate that the *DKK1* gene is highly methylated in normal (61%) and cancer tissues (70%) of breast cancer patients. *DKK1* promoter methylation changes are also concordant between these tissues and the presence of field defects.

Breast cancer is the most common malignancy in women and generally appears by a multistep process from normal epithelium to invasive cancer (10). Tumor development requires the accumulation of various genetic changes including mutation or loss of tumor suppressor genes and amplification of oncogenes (11). Epigenetic changes occurring as genetic changes accumulate during the multistep carcinogenetic process (12,13). As a general rule, hypermethylation and hypomethylation can lead to carcinogenesis (14). DNA promoter methylation in CpG islands frequently occurs during breast carcinogenesis and is an early stage in this process (15). Genes necessary for cell cycle regulation, differentiation, and apoptosis are often silenced during the breast cancer progression by promoter hypermethylation (16).

Field defects, which can be defined by at least some of the genetic alterations found in invasive cancers, are also present in the morphologically normal epithelium (17). The presence of field defects has been detected in breast cancer as well as in many other cancers (18). Loss of heterozygosity was indicated in the morphologically normal tissue adjacent to breast cancer (17). Different studies have shown methylation changes in the normal breast tissues adjacent to primary breast carcinomas. Tumor cells also exhibit the same changes (19). Van der Auwera et al. (20) showed that the histologically normal breast tissue of breast cancer patients shared the same abnormal DNA methylation changes as the tumors of the same patients. These changes in normal breast tissues that have not shown any microscopic evidence of malignancy are not enough to transform them; however, in the future, as other genetic and epigenetic changes accumulate, they may lead to cancer (20). Lewis et al. (15) confirmed that promoter hypermethylation may accumulate increasingly during the transformation of premalignant lesions into invasive cancers.

Table 2. Concordance between the methylation status of normal and cancerous breast tissues (n = 83).

Gene	T+ N+	T– N+	T– N-	T+ N–	Kappa	P-value
DKK1	43 (52%)	8 (10%)	17 (20%)	15 (18%)	0.39	<0.001

Abbreviations: T, tumor; N, normal.

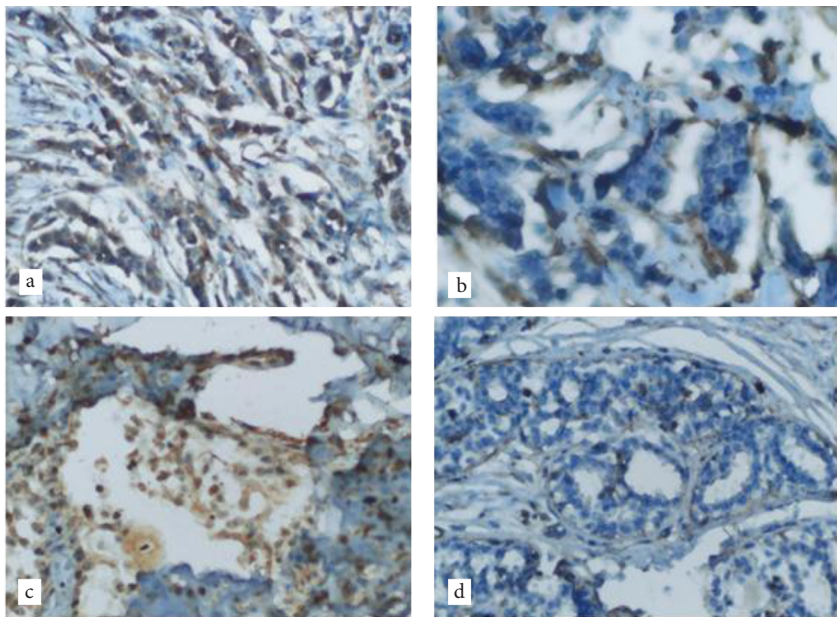

Figure 2. DKK1 protein expression by immunohistochemical staining: a) DKK1-positive tumor cell, b) DKK1-negative tumor cell, c) DKK1-positive normal tissue, d) DKK1-negative normal tissue (200×).

We identified 33% of methylation in the *DKK1* gene of normal breast tissue of women with no history of breast cancer. In various studies, in tissues with similar properties of methylation, *DAPK*, *TWIST*, and *RASSF1A* genes (20) and *RARB2* (9%), *APC* (26%), *H-cadherin* (17%), and *RASSF1A* (37%) genes (15) were reported.

Different studies have also proven that the abnormal methylation of specific genes has contributed to the formation of field effects in breast cancer (21). Yan et al. (19) showed that methylation changes in breast cancer can be found in the area within a 4-cm diameter from the primary tumor. Tumor and adjacent normal tissues have often been hypermethylated, and it was shown that *RASSF1A*, *HIN1*, and *APC* genes have been widely methylated (tumor: 82.5%, 75%, 52.5% and normal tissue: 85.2%, 70.4%, 44.4%, respectively) (21). In addition to that, *APC*, *CDH1*, *CTNNB1*, *ESR1*, and *GSTP1* have been methylated in both normal tissues and tumors (22). The promoter domains of *APC*, *CDH1*, and *CTNNB1* were methylated at a higher frequency in tumors than in normal breast tissues adjacent to tumors (22). The methylation frequencies of *TIMP3*, *ESR1*, and *GSTP1* were found to be similar in normal and neoplastic breast tissues of cancer patients (22). Yeo et al. (23) found that *RASSF1A* promoter was hypermethylated at 95% in breast tumor tissues and at 92.5% in paired nontumorous tissues. The excess methylation of normal tissues with no sign of tumor that are in the vicinity of the tumor can be explained by the existence of a few tumor cells in those tissues that could not be detected pathologically by immunohistochemical staining (23).

DKK1 promoter hypermethylation has been sought in various cancer types and it has been shown that *DKK1* was frequently methylated. *DKK1* methylation was determined in 12% (7/58) of primary colorectal cancers and 48% (15/31) of primary stomach cancers (24). Maehata et al. (25) found *DKK1* methylation in esophagus, stomach, and colon cancers at 36.4% (4/11), 25% (2/8), and 35% (7/20), respectively. It was shown that *DKK1* promoter was methylated in 16%–32% of patients with acute myeloid leukemia (9,26,27). Belshaw et al. (28) found the same *DKK1* methylation in the normal mucosa and tumors of 19 patients with colon cancer and observed no relation between normal mucosa and tumors. Suzuki et al. (29) determined *DKK1* hypermethylation in 19% (15/78) of primary breast tumors.

Table 3. Relationship between *DKK1* promoter methylation and clinicopathological characteristics.

	DKK1 methylation		
Criteria	Negative (%)	Positive (%)	P-value
	n = 25	n = 58	
Age (mean ± SD)	53.7 ± 10.2	54.4 ± 14.1	
Tumor status			
pT1	6	12	
pT2	15	37	0.413
pT3	4	9	
pT4	0	0	
Lymph node status			
pNo	8	14	
pN1	6	15	1.000
pN2	7	13	
pN3	4	16	
Histological grade			
G1	2	3	0.415
G2	20	47	
G3	3	8	
Immunohistochemical staining			
Estrogen receptor			
Negative	9	19	1.000
Positive	16	39	
Progesterone receptor			
Negative	11	24	1.000
Positive	14	34	
HER2			
Negative	13	21	0.272
Positive	12	37	

No relation was found between clinicopathologic parameters (age, tumor size, lymph node status, histological grade, and ER, PR, and HER2 receptor positivity) and methylation in our study. Similar results were shown for gastrointestinal and breast tumors (24,29). However, Suzuki et al. (9) showed that DKK1 methylation might be a risk for poor prognosis in core-binding factor leukemia. Tokatlı et al. (30) mentioned that tumor grade and size, lymph node metastases, and expression of hormone receptors provide the only true prognostic and predictive factors related to clinical outcome and response to treatment, but we also supposed that epigenetic change may be one of the prognostic and predictive factors related to clinical outcome and response to treatment.

Collectively, DKK1 protein expression in tumor tissues of patients with breast cancer was higher than that in normal tissues without tumor (31% and 17%, respectively). DKK1 expression varies according to the cancer type. DKK1 expression decreased in colon tumors and *DKK1* functioned as a tumor suppressor gene in this cancer (31). *DKK1* loss may promote

Table 4. Relationship between DKK1 protein expression and clinicopathological characteristics.

	DKK1 protein expression		
Criteria	Negative n = 48 (%)	Positive n = 22 (%)	P-value
Age (mean ± SD)	54.3 ± 13.1	53.7 ± 13.6	
Tumor status			
pT1	7	5	
pT2	30	15	0.644
pT3	11	2	
pT4	0	0	
Lymph node status			
pNo	11	9	
pN1	13	5	0.193
pN2	12	2	
pN3	12	6	
Histological grade			
G1	2	2	0.435
G2	41	16	
G3	5	4	
Immunohistochemical staining			
Estrogen receptor			
Negative	16	9	0.813
Positive	32	13	
Progesterone receptor			
Negative	19	12	0.327
Positive	29	10	
HER2			
Negative	23	8	0.567
Positive	25	14	

cancer progression by increasing the expression of β-catenin/TCF target genes in colon cancer. DKK1 is overexpressed in breast cancer (32,33), hepatoblastoma and Wilms' tumor (34), lung and esophageal cancer (35), ovary carcinoma (36), and glioma (37), suggesting that it plays an oncogenic role in these tumors (38). Recent studies have shown that DKK1 is not only a key inhibitor of the Wnt/β-catenin signal pathway but is also a downstream target of this pathway. Multiple β-catenin/TCF binding sites in the *DKK1* gene promoter allow for *DKK1* activation (31,39). DKK1 upregulation is a consequence of the overactivation of the Wnt/β-catenin signal pathway in breast cancer (33).

We observed no relationship between clinicopathologic features (age, tumor size, lymph node status, histological grade, and ER and PR receptor or HER2 positivity) and protein expression. DKK1 is expressed preferably in hormone-resistant breast tumors (32,33). DKK1 expression is associated with poor prognosis in patients with esophageal squamous cell carcinoma (40) and hepatocellular carcinoma (38). Increased DKK1 protein levels in

prostate cancer metastases are associated with shorter survival rates (41).

In conclusion, our study showed the presence of field defect for the DKK1 gene in breast cancer. The rate of methylation was also increased in tumor tissues in comparison with normal breast tissues of women without a history of breast cancer. This finding suggests that *DKK1* gene methylation plays a role in tumor formation. It will be interesting to explore the effect of epigenetic changes on tumorigenesis. This may result in improvements in our understanding of the breast cancer mechanism.

Acknowledgments

We would like to thank Dr Mehmet Gündüz, Dr Esra Gündüz, and Dr Kadir Demircan for helping to review and edit this manuscript.

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