Original Article

Frequent Methylation of Genes Encoding Wnt Pathway Antagonists: Secreted Frizzled-Related Protein 1 and Dickkopf 3 in Invasive Breast Cancer

Mahnaz M. Kazi[1], Hemangini H. Vora[2], Kinjal K. Gajjar[1], Toral P. Kobawala[1], Nandita R. Ghosh[1]

[1]Tumor Biology Lab, Cancer Biology Department, [2]Immuno-Hematology Lab, Cancer Biology Department, The Gujarat Cancer and Research Institute, Ahmedabad, Gujarat, India

Abstract

Introduction: Wnt signaling pathway is often dysregulated in the pathogenesis of various malignancies, including breast cancer. This might be related to methylation of the genes encoding antagonists of this signaling pathway. **Aim:** The aim of the study was to analyze the methylation status of the promoter regions of Wnt antagonists-secreted frizzled-related protein 1 (sFRP1) and Dickkopf 3 (DKK3) and to determine their correlation with clinicopathological parameters and survival outcome in patients with primary invasive ductal breast cancer. **Materials and Methods:** The methylation status of *sFRP1* and *DKK3* was analyzed in 160 breast tumor samples using methylation-specific polymerase chain reaction. Statistical analysis was performed using SPSS software. $P \leq 0.05$ was considered as statistically significant. **Results:** The promoter region of *sFRP1* and *DKK3* genes was found to be methylated in 76% and 64% of total invasive ductal breast cancer patients, respectively. The promoter methylation in *sFRP1* and *DKK3* genes was significantly associated with larger tumor size, positive lymph nodes, advanced stage, and perinodal extension of breast tumors. Further, *sFRP1* methylation was associated with human epidermal growth factor receptor 2-positive tumors while *DKK3* methylation was associated with Grade 3 tumors. Survival analysis demonstrated that *sFRP1* methylation was correlated with reduced overall survival in breast cancer patients. **Conclusion:** Promoter methylation of Wnt pathway antagonists is frequent in breast cancer ultimately leading to probable upregulation of the pathway in these tumors. Hence, *sFRP1* and *DKK3* methylation may be used as a valuable biomarker in clinical breast cancer management.

Keywords: *Breast cancer, methylation, survival, Wnt pathway*

Introduction

Breast cancer is a frequently diagnosed cancer contributing to 24.2% of total cancer cases and 13% of cancer deaths among females, worldwide. It is also the most common cancer among women in India with an estimated 162,468 (27.7%) new cases diagnosed and 87,090 (12.19%) deaths, according to GLOBOCAN 2018. The increasing trend of breast cancer owes to its clinically, molecularly, and biologically heterogeneous nature. This leads to disparate clinical behaviors and outcomes in breast cancer patients despite common histopathological features at diagnosis.[1] Hence, the aberrations at the genomic and molecular level results in dysregulated signaling pathways and thereby breast cancer initiation and progression are required to be explored.

In addition, breast carcinogenesis being a multistep process involves a combination of both genetic and epigenetic alterations.[2] The most common and well-defined epigenetic alteration is 5'-cytosine methylation that occurs within CpG islands in gene promoter regions and affects gene expression.[3] The genes affected by such alteration are mostly the tumor suppressor genes involved in regulation of several cellular pathways such as cell cycle, DNA repair, and growth factor signaling or cell adhesion involved in several cancer development including breast cancer.[4] Wnt signaling is one such developmental pathway that is predominantly disrupted, most importantly in breast cancer.[5,6] The dysregulation of Wnt signaling pathway due to epigenetic aberrations owes to the promoter methylation of genes encoding pathway antagonists, such as secreted frizzled-related protein (sFRP), Dickkopf (DKK), and Wnt-inhibitory-factor

How to cite this article: Kazi MM, Vora HH, Gajjar KK, Kobawala TP, Ghosh NR. Frequent methylation of genes encoding Wnt pathway antagonists: Secreted frizzled-related protein 1 and Dickkopf 3 in invasive breast cancer. Clin Cancer Investig J 2019;8:106-13.

Address for correspondence:
Dr. Nandita R. Ghosh, Tumor Biology Lab, Department of Cancer Biology, The Gujarat Cancer and Research Institute, NCH Compound, Asarwa, Ahmedabad - 380 016, Gujarat, India.
E-mail: nandita.ghosh@gcriindia.org

Access this article online

Website: www.ccij-online.org

DOI: 10.4103/ccij.ccij_14_19

Quick Response Code:

which are reported to play a significant role in malignant behavior of breast cancer.

Methylation of *sFRP1* gene leads to loss of its antagonistic effect on Wnt ligand, eventually resulting in elevated levels of β-catenin.[7] In breast cancer patients, it is shown to be associated with reduced overall survival (OS).[8,9] Moreover, methylation of *DKK3* gene results in downregulation of its protein expression which thereby causes anomalous Wnt signaling and poor survival in several human malignancies, prominently breast cancer.[10] Thus, the functional loss of *sFRP1* and *DKK3* genes contributing to Wnt pathway activation leads to dysregulation of cell proliferation and differentiation.[11] Based on these facts, in the present study, we aimed to evaluate the frequency of the promoter methylation of *sFRP1* and *DKK3* genes in patients with primary breast cancer. Further, the relation of promoter methylation status of these genes with various clinicopathological parameters is analyzed as well as their prognostic and predictive value is determined.

Materials and Methods

Patients

In the present study, a total of 160 untreated histologically confirmed Invasive Ductal Carcinoma patients of breast registered at The Gujarat Cancer and Research Institute from March 2014 to December 2015 were enrolled. The study was approved by the Institute's Ethics Committee Board, and written consent forms were obtained from all the patients before treatment administration. Detailed clinicopathological history of the patients was obtained from the case files maintained at the medical record department of the institute. Histopathological details such as tumor size, lymph node status, disease stage, Bloom–Richardson score (BR score), histological grade and status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) were evaluated and reported on routine basis by the pathologists of our institute. All patients underwent surgery, and adjuvant treatment decision was based on molecular subtypes of breast cancer patients by clinicians of the institute. The clinicopathological characteristics of the enrolled patients are enlisted in Table 1. Complete follow-up details were obtained for 69% (111/160) patients and were included in OS analysis. Among these, 3% (2/111) patients had persistent disease and so only 68% (109/160) patients were analyzed for relapse-free survival (RFS).

Table 1: Clinicopathological characteristics of breast cancer patients

Characteristics	*n* (%)
Age (years), range	30-95
Median	50
≤50	81 (51)
>50	79 (49)
Menopausal status	
Premenopausal	56 (35)
Postmenopausal	104 (65)
Tumor size	
T1	21 (13)
T2	113 (71)
T3	17 (11)
T4	9 (5)
Nodal status	
Absent	65 (41)
Present	95 (59)
Stage	
Early (I + II)	99 (62)
Advanced (III + IV)	61 (38)
Differentiation grade	
Grade 1	13 (08)
Grade 2	103 (64)
Grade 3	44 (28)
Grade 1 + 2	116 (72)
Grade 3	44 (28)
Lymphatic permeation	
Absent	84 (52)
Present	76 (48)
Vascular permeation	
Absent	140 (88)
Present	20 (12)
Perineural invasion	
Absent	147 (92)
Present	13 (8)
Perinodal extension	
Absent	94 (59)
Present	66 (41)

Bisulfite-modification and methylation-specific polymerase chain reaction

For methylation-specific polymerase chain reaction (MS-PCR) study, breast tumor tissues were collected immediately after surgery and tumor portion selected by the pathologist was snap frozen in the liquid nitrogen and stored at −80°C till further procedure. DNA extraction was performed using phenol: chloroform extraction method and quantified by agarose gel electrophoresis using Lambda DNA-HindIII digest ladder. The isolated genomic DNA was modified by bisulfite treatment using EpiJET Bisulfite Conversion Kit (ThermoScientific, Lithuania, Europe). Manufacturer's protocol was strictly followed. Thereafter, bisulfite modified DNA was amplified by MS-PCR as described by Herman *et al*.[12] The primers used [Table 2] were specific for either the unmethylated or the methylated promoter regions of *sFRP1* and *DKK3* and the PCR reaction was carried out in a 25 µl system using Platinum® PCR SuperMix (Invitrogen, CA, USA) according to the manufacturer's protocol. The reaction conditions for each PCR are described in Table 3.

Statistical analysis

The statistical evaluation of the data was carried out using Statistical Package for Social Sciences (SPSS) software

Table 2: Polymerase chain reaction primers

	Sequence	Product (bp)
sFRP1		
Unmethylated	Forward 5' - GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT - 3' Reverse 5' - CTCAACCTACAATCAAAAACAACACAAACA - 3'	126
Methylated	Forward 5' - TGTAGTTTTCGGAGTTAGTGTCGCGC - 3' Reverse 5' - CCTACGATCGAAAACGACGCGAACG - 3'	135
DKK3		
Unmethylated	Forward 5' - TTAGGGGTGGGTGGTGGGGT - 3' Reverse 5' - CTACATCTCCACTCTACACCCA - 3'	126
Methylated	Forward 5'- GGGCGGGCGGCGGGGC - 3' Reverse 5' - ACATCTCCGCTCTACGCCCG - 3'	120

sFRP1: Secreted frizzled-related protein 1, *DKK3*: Dickkopf 3

Table 3: Reaction conditions for methylation-specific polymerase chain reaction

	Initial denaturation	Denaturation	Annealing	Extension	Final extension
sFRP1 unmethylated reaction	95°C 12 min	95°C 30 s	58°C 30 s 35 cycles	72°C 30 s	72°C 10 min
sFRP1 methylated reaction	95°C 12 min	95°C 30 s	64°C 30 s 35 cycles	72°C 30 s	72°C 10 min
DKK3 unmethylated reaction	95°C 12 min	95°C 30 s	61°C 30 s 35 cycles	72°C 30 s	72°C 10 min
DKK3 methylated reaction (touchdown PCR)	94°C 3 min	94°C 1 min	68°C - 64°C 1 min (8 cycles) 20 cycles (at 64°C annealing temperature)	72°C 30 s	72°C 10 min

PCR: Polymerase chain reaction, *sFRP1:* Secreted frizzled-related protein 1, *DKK3*: Dickkopf 3

version 16 (SPSS Inc, USA). Two-tailed Chi-square test and Spearman's correlation method were used to correlate the promoter methylation status of the molecules with various clinicopathological characteristics of breast cancer patients. Survival analysis was performed using Kaplan–Meier survival function, and the differences in survival were tested for statistical significance using log-rank statistic. $P \leq 0.05$ was considered to be statistically significant.

Results

Secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation in breast cancer

In total breast cancer patients, *sFRP1* promoter region gene was found to be methylated in 76% (122/160) patients and unmethylated in 24% (38/160) patients. Further, *DKK3* promoter region gene was observed to be methylated in 64% (103/160) of breast cancer patients compared to 36% (57/160) of patients with unmethylated *DKK3* promoter. Representative gel images for *sFRP1* and *DKK3* are shown in Figure 1.

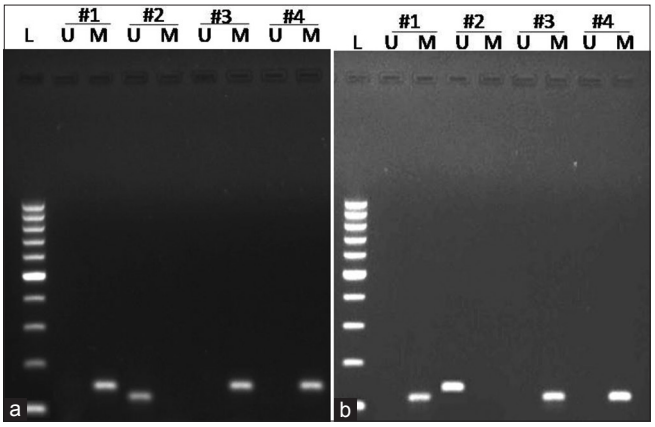

Figure 1: Representative images of methylation-specific polymerase chain reaction analysis for (a) secreted frizzled-related protein 1 methylation and (b) Dickkopf 3 methylation in breast carcinoma tissues. Bisulfite modified DNA was amplified using methylation-specific polymerase chain reaction primers specific to a CpG rich region of each gene promoter. Polymerase chain reaction-amplified products were resolved by 2% of agarose gel electrophoresis. DNA bands in lanes labeled with U indicate polymerase chain reaction products amplified with primers recognizing unmethylated promoter sequences. DNA bands in lanes labeled with M represent amplified products with methylation-specific primers

Association of secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation with clinicopathological parameters

As depicted in Table 4, correlation with clinical parameters revealed that the incidence of methylated *DKK3* promoter was significantly higher in premenopausal women as compared to postmenopausal women ($P = 0.016$) while it showed a trend of association with the younger age group as compared to older age group of breast cancer

Table 4: Correlation of secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation with clinicopathological parameters

Variables	*n*	*sFRP1* methylation		*DKK3* methylation	
		Unmethylated, *n* (%)	Methylated, *n* (%)	Unmethylated, *n* (%)	Methylated, *n* (%)
Age					
≤50	81	19 (24)	62 (76)	21 (28)	53 (72)
>50	79	19 (24)	60 (76)	34 (43)	45 (57)
χ^2, *r*, *P*		0.008, −0.007, 0.930		3.739, −0.153, 0.054	
Menopausal status					
Premenopausal	56	45 (80)	11 (20)	13 (23)	43 (77)
Postmenopausal	104	77 (74)	27 (26)	44 (42)	60 (58)
χ^2, *r*, *P*		0.803, −0.071, 0.374		5.786, −0.190, 0.016	
Tumor size					
T1	21	9 (43)	12 (57)	9 (43)	12 (57)
T2	113	25 (22)	88 (78)	43 (38)	70 (62)
T3	17	2 (12)	15 (88)	4 (24)	13 (76)
T4	9	2 (22)	7 (78)	1 (11)	8 (89)
χ^2, *r*, *P*		5.759, 0.163, 0.039		4.212, 0.143, 0.070	
Nodal status					
Negative	65	21 (32)	44 (68)	29 (45)	36 (55)
Positive	95	17 (18)	78 (82)	28 (30)	67 (70)
χ^2, *r*, *P*		4.427, 0.166, 0.036		3.858, 0.155, 0.050	
TNM stage					
Early (I + II)	99	29 (29)	70 (71)	43 (44)	56 (56)
Advanced (III + IV)	61	9 (15)	52 (85)	14 (23)	47 (77)
χ^2, *r*, *P*		4.406, 0.166, 0.036		6.905, 0.208, 0.008	
Differentiation grade					
Grade 1	13	5 (38)	8 (62)	9 (69)	4 (31)
Grade 2	103	24 (23)	79 (77)	40 (39)	63 (61)
Grade 3	44	9 (20)	35 (80)	8 (18)	36 (82)
χ^2, *r*, *P*		1.829, 0.082, 0.303		12.702, 0.274, <0.001	
Grade 1 + 2	116	29 (25)	87 (75)	49 (42)	67 (58)
Grade 3	44	09 (20)	35 (80)	8 (18)	36 (82)
χ^2, *r*, *P*		0.364, 0.048, 0.549		8.052, 0.224, 0.004	
Lymphatic permeation					
Absent	84	25 (30)	59 (70)	30 (36)	54 (64)
Present	76	13 (17)	63 (83)	27 (36)	49 (64)
χ^2, *r*, *P*		3.529, 0.149, 0.061		0.001, 0.002, 0.980	
Perinodal extension					
Absent	94	28 (30)	66 (70)	40 (43)	54 (57)
Present	66	10 (15)	56 (85)	17 (26)	49 (74)
χ^2, *r*, *P*		4.586, 0.169, 0.032		4.769, 0.173, 0.029	
Her2 status					
Negative	91	28 (31)	63 (69)	32 (35)	59 (65)
Positive	69	10 (14)	59 (85)	25 (36)	44 (64)
χ^2, *r*, *P*		5.741, 0.189, 0.016		0.019, −0.011, 0.890	
Molecular subtypes					
Luminal A	52	19 (36)	33 (64)	23 (44)	29 (56)
Luminal B	36	6 (17)	30 (83)	13 (36)	23 (64)
Her2 positive	35	4 (11)	31 (89)	12 (34)	23 (66)
TNBC	37	9 (24)	28 (76)	9 (24)	28 (76)
χ^2, *r*, *P*		8.634, 0.142, 0.074		3.771, 0.150, 0.058	

Her2: Human epidermal growth factor receptor 2, TNBC: Triple-negative breast cancer, TNM: Tumor, node, metastasis

patients (P = 0.077). On the other hand, incidence of methylated *sFRP1* promoter was not associated with the clinical parameters of breast cancer patients. Further, on correlating with pathological parameters, methylated *sFRP1*

showed significant association (P = 0.039) and methylated *DKK3* showed a trend of association (P = 0.070) with larger tumor size as compared to smaller size of breast tumors, respectively. The methylation frequency of *sFRP1* and *DKK3* gene promoter was also significantly higher in breast cancer patients with the presence of metastatic nodes (P = 0.036 and P = 0.05, respectively), advanced disease stage (P = 0.036 and P = 0.008 respectively), and perinodal extension (P = 0.032 and P = 0.029 respectively) as compared to their respective counterparts. Furthermore, methylated *DKK3* promoter was significantly higher in breast cancer patients with Grade 3 tumors (P = 0.004) and methylated *sFRP1* promoter showed a trend of association with the presence of lymphatic permeation (P = 0.061) as compared to their respective counterparts [Table 4].

Next, correlation with ER, PR and Her2 expression revealed that incidence of methylated *sFRP1* was significantly associated with Her2-positive tumors as compared to Her2-negative tumors (P = 0.016) while no significant association of either methylated *sFRP1* or *DKK3* was observed with ER or PR expression. In addition, on the basis of ER, PR, and Her2 expression, methylated *sFRP1* (P = 0.074) and *DKK3* (P = 0.058) genes showed a trend of association with different molecular subtypes of breast cancer patients. Further analysis of the molecular subtypes revealed that Luminal B and Her2-positive breast cancers exhibited increased *sFRP1* promoter methylation as compared to Luminal A (P = 0.043 and P = 0.009 respectively). However, increased *DKK3* promoter methylation was observed in triple-negative breast cancer (TNBC) patients when compared to Luminal A (P = 0.050) [Table 5].

Intercorrelation of secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation in breast cancer

The intercorrelation of *sFRP1* and *DKK3* promoter region did not show significant correlation between their methylation statuses.

Survival analysis

Univariate survival analysis revealed that breast cancer patients with methylated *sFRP1* promoter had poor OS as compared to patients with unmethylated *sFRP1* promoter (P = 0.082) while no such association was observed for methylated *DKK3* promoter [Figure 2 and Table 6].

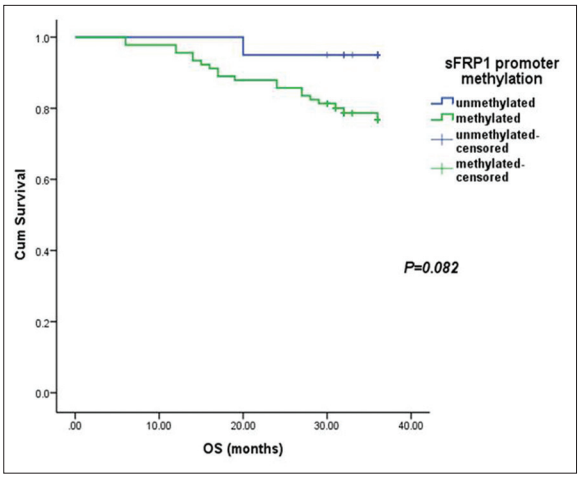

Figure 2: Kaplan–Meier survival curve for overall survival in relation to secreted frizzled-related protein 1 promoter methylation in breast cancer patients (n = 111). A trend of reduced overall survival was observed in patients with methylated secreted frizzled-related protein 1 promoter as compared to those with unmethylated secreted frizzled-related protein 1 promoter

Table 5: Correlation of secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation with molecular subtypes

Characteristics	*n*	*sFRP1* methylation		*DKK3* methylation	
		Unmethylated, *n* (%)	Methylated, *n* (%)	Unmethylated, *n* (%)	Methylated, *n* (%)
Luminal A	52	19 (36)	33 (64)	23 (44)	29 (56)
Luminal B	36	6 (17)	30 (83)	13 (36)	23 (64)
χ^2, *r*, *P*		4.130, 0.217,0.043		0.580, 0.081, 0.452	
Luminal A	52	19 (36)	33 (64)	23 (44)	29 (56)
Her2 positive	35	4 (11)	31 (89)	12 (34)	23 (66)
χ^2, *r*, *P*		6.782, 0.279, 0.009		0.860, 0.099, 0.359	
Luminal A	52	19 (36)	33 (64)	23 (44)	29 (56)
TNBC	37	9 (24)	28 (76)	9 (24)	28 (76)
χ^2, *r*, *P*		1.496, 0.13, 0.226		3.72, 0.204, 0.055	
Luminal B	36	6 (17)	30 (83)	13 (36)	23 (64)
Her2 positive	35	4 (11)	31 (89)	12 (34)	23 (66)
χ^2, *r*, *P*		0.402, 0.075, 0.533		0.026, 0.019, 0.874	
Luminal B	36	6 (17)	30 (83)	13 (36)	23 (64)
TNBC	37	9 (24)	28 (76)	9 (24)	28 (76)
χ^2, *r*, *P*		0.655, −0.095, 0.425		1.204, 0.128, 0.279	
Her2 positive	35	4 (11)	31 (89)	12 (34)	23 (66)
TNBC	37	9 (24)	28 (76)	9 (24)	28 (76)
χ^2, *r*, *P*		2.022, −0.168, 0.159		0.864, 0.11, 0.360	

Her2: Human epidermal growth factor receptor 2, TNBC: Triple-negative breast cancer

Table 6: Survival analysis of secreted frizzled-related protein 1 and Dickkopf 3 promoter methylation in breast cancer patients

Characteristics	RFS (*n*=109)			OS (*n*=111)		
	n	No recurrence, *n* (%)	Recurrence, *n* (%)	*n*	Alive, *n* (%)	Dead, *n* (%)
sFRP1 promoter						
Unmethylated	20	16 (80)	4 (20)	20	19 (95)	1 (15)
Methylated	89	68 (76)	21 (24)	91	71 (78)	20 (22)
Log rank, df, *P*		0.283, 1, 0.595			3.026, 1, 0.082	
DKK3 promoter						
Unmethylated	35	29 (83)	6 (17)	35	30 (86)	5 (14)
Methylated	74	55 (74)	19 (26)	76	60 (79)	16 (21)
Log rank, df, *P*		0.642, 1, 0.423			0.67, 1, 0.413	

RFS: Relapse-free survival, OS: Overall survival

Moreover, *sFRP1* and *DKK3* promoter methylation failed to predict the RFS in breast cancer patients.

Discussion

Wnt/β-catenin signaling is reportedly a crucial pathway in tumorigenesis and embryogenesis which is found to be inhibited by the antagonists, namely sFRP1 and DKK3.[7,8,13-16] Experimentally, it has been postulated that *sFRP1* and *DKK3* are putative tumor suppressor genes and frequent targets of epigenetic inactivation through promoter methylation in a variety of solid tumors such as colorectal cancer (CRC),[17] ovarian cancer,[18] mesotheliomas,[19] lung cancer,[20] and prostate cancer[21] including breast cancer.[8] Thus, identification of *sFRP1* and *DKK3* methylated genes could provide vital information specifically for breast cancer detection and targeted therapy. Hence, the present study examined the methylation status of *sFRP1* and *DKK3* in breast cancer patients. Dahl *et al*.[22] and Jeong *et al*.[23] reported a frequency of 75% and 83.3% for *sFRP1* promoter methylation in breast cancer cases which is in accordance to that observed in the current study (76%) of *sFRP1* in breast cancer patients. However, Lo *et al*.[24] and Veeck *et al*.[8] noted a slightly lower incidence (68% and 61%) of methylated *sFRP1* gene in patients with breast cancers. On the other hand, 64% of breast cancer patients exhibited methylated *DKK3* gene in the present study, which is similar to that observed by Veeck *et al*. in breast cancer patients.[25] In addition, methylated *DKK3* gene has been reported in other malignancies in prostate cancer (68%)[21] and in gastric cancer (67.6%).[13]

The correlation with clinicopathological parameters revealed that incidence of methylated *DKK3* was significantly higher in premenopausal breast cancer patients than postmenopausal patients. Similarly, Kloten *et al*. reported that sensitivity of *DKK3* methylation was more frequent in premenopausal women with breast cancer, indicating a pronounced benefit of *DKK3* for the early detection of breast cancer in premenopausal women.[26] Further, the present study observed a trend of higher incidence of methylated *DKK3* in patients of younger age group as compared to older age group. Contradictorily, Veeck *et al*. showed the association of methylated *DKK3* with advanced age of breast cancer patients.[25] On the other hand, a study by Yin *et al*. reported the association of methylated *DKK3* promoter with younger age group of papillary thyroid carcinoma patients, which is consistent with the current study.[27] Moreover, the current study observed a significant predominance of methylated *sFRP1* and *DKK3* in high-risk prognostic variables such as larger tumor size, positive lymph node status, advanced stage, high BR score, and presence of perinodal extension as compared to their respective counterparts. This signifies that aberrant *sFRP1* and *DKK3* promoter methylation plays a contributing role toward tumor aggressiveness leading to progressive breast cancer. Similar findings are being reported in literature by Xiang *et al*.[28] and Saied *et al*.,[29] demonstrating the association of methylated *DKK3* promoter with aggressive characteristics of breast cancer. Besides breast cancer, methylated *DKK3* promoter was significantly associated with advanced tumor stages in gastric cancer patients, larger tumor size in cervical cancer patients and with advanced stage, high-tumor grade and lymph node metastasis in papillary thyroid carcinoma patients; confirming the relation of methylated *DKK3* promoter with poor prognosticators.[13,27,30] Likewise, *sFRP1* methylation was reported to be more frequent in glioma patients with higher grade tumors, suggesting a direct correlation of *sFRP1* methylation with tumor aggressiveness.[31] However, Veeck *et al*.[8] and Kloten *et al*.[26] showed significant association of hypermethylated *sFRP1* and *DKK3* promoter, respectively, with smaller tumor size in breast cancer patients. Nevertheless, several other authors did not find any significant relationships between *sFRP1* and *DKK3* methylation and clinicopathological characteristics in breast cancer patients[25,32,33] and hepatocellular carcinoma (HCC) patients.[34,35]

Further, in relation to ER, PR, Her2 expression, the present study revealed that incidence of methylated *sFRP1* was significantly higher in Her2-positive tumors as compared to Her2 negative and was not associated with ER or PR status while methylated *DKK3* gene promoter had no influence on the expression of ER, PR, and Her2 status of breast cancer

patients. Likewise, several other studies also failed to find any significant correlation of methylated *sFRP1* and *DKK3* promoter with ER, PR, and Her2 expression in breast cancer patients.[8,25,26,29] However, Jeong *et al*.[23] has shown significant association of ER, PR, and Her2-negative tumors with low level of *sFRP1* gene methylation. Furthermore, Holm *et al*. has described the association of various gene methylation with the molecular subtypes of breast cancer, with a significant high frequency in Luminal B tumors and a low frequency in basal-like tumors.[36] Hence, it was important to explore whether there exists any difference in *sFRP1* and *DKK3* methylation pattern between the molecular subtypes. Indeed, *sFRP1* methylation displayed a trend toward higher frequency in Her2-positive subtype, followed by Luminal B, TNBC, and Luminal A subtypes. Jeong *et al*. indicated significantly low *sFRP1* gene methylation in basal-like subtype compared to the Luminal A, Luminal B, and Her2-positive subtypes.[23] Wang *et al*. reported that patients with TNBC have decreased *sFRP1* methylation as compared to other molecular subtypes.[37] The current study also observed decreased incidence of *sFRP1* methylation in patients with TNBC subtype than those with Luminal B or Her2-positive subtype. However, a trend of higher incidence of methylated *DKK3* gene in TNBC patients was noted as compared to those with Luminal A subtype. This could also be explained from a study by Lorsy *et al*. who observed loss of *DKK3* expression in aggressive TNBC subtype which was suggested to be due to increased promoter methylation of *DKK3* in this subtype.[38] Hence, the above findings suggest that biologically molecular subtypes not only display genetic aberrations but also harbor epigenetic aberrations.

In addition, the value of *sFRP1* and *DKK3* was elucidated as possible molecular markers of prognosis in breast cancer. Survival analysis revealed that methylated *sFRP1* promoter was associated with decreased OS in breast cancer patients, but with borderline significance. Consistent with present results, Veeck *et al*. observed significant association of *sFRP1* methylation with shorter OS in breast cancer and it emerged as an independent adverse prognostic factor.[8] Likewise, Majchrzak-Celińska *et al*. also observed a significant negative correlation of *sFRP1* methylation with survival time in glioma patients.[31] However, few other studies did not observe any significant associations of *sFRP1* methylation status with clinical outcome in CRC and acute myeloid leukemia patients.[7,39] Moreover, Vincent and Postovit performed pancancer analysis of different types of cancer and showed that *sFRP1* was associated with tumor suppressive functions but not with prognosis.[40] Furthermore, the current study did not observe significant difference in the survival of breast cancer patients with methylated *DKK3* promoter. However, several studies in literature reported significant association of *DKK3* methylation with poor survival in breast cancer, gastric cancer, and HCC patients.[13,25,41]

Hence, summarizing the present observations, high frequency of *sFRP1* and *DKK3* methylation was found in breast cancer patients, with significant associations with tumor aggressiveness contributing to the malignant behavior of the disease. Furthermore, methylation of both the genes was differentially present in various molecular subtypes of breast cancer patients that may suggest epigenetic aberrations and differences in the molecular pathogenesis in various subtypes. Moreover, not *DKK3* but *sFRP1* emerged as a prognosticator for breast cancer patients and thus might be useful as a potential prognostic marker in clinical oncology for breast cancer detection and therapy, thereby assisting to improve patient outcome. Moreover, as *sFRP1* and *DKK3* genes particularly regulate Wnt signaling pathway, it could be concluded that aberrant promoter methylation of these genes leads to dysregulation of the Wnt pathway in breast cancer

Acknowledgment

We would like to thank Gujarat Cancer Society and Gujarat Cancer and Research Institute.

Financial support and sponsorship

This study was financially supported by Gujarat Cancer Society, Gujarat Cancer and Research Institute.

Conflicts of interest

There are no conflicts of interest.

References

1. Rivenbark AG, O'Connor SM, Coleman WB. Molecular and cellular heterogeneity in breast cancer: Challenges for personalized medicine. Am J Pathol 2013;183:1113-24.
2. Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, *et al.* Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer Res 2014;34:1071-7.
3. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225-9.
4. Mulero-Navarro S, Esteller M. Epigenetic biomarkers for human cancer: The time is now. Crit Rev Oncol Hematol 2008;68:1-11.
5. Howe LR, Brown AM. Wnt signaling and breast cancer. Cancer Biol Ther 2004;3:36-41.
6. Aguilera O, Muñoz A, Esteller M, Fraga MF. Epigenetic alterations of the wnt/beta-catenin pathway in human disease. Endocr Metab Immune Disord Drug Targets 2007;7:13-21.
7. Ghasemi A, Rostami S, Chahardouli B, Alizad Ghandforosh N, Ghotaslou A, Nadali F. Study of SFRP1 and SFRP2 methylation status in patients with *de novo* acute myeloblastic leukemia. Int J Hematol Oncol Stem Cell Res 2015;9:15-21.
8. Veeck J, Niederacher D, An H, Klopocki E, Herman JG, Graff JR, *et al*. Methylation-specific PCR: A novel PCR assay for methylation status of CpG Islands. Proc Natl Acad Sci 1996;93:9821-6.
9. Veeck J, Niederacher D, An H, Klopocki E, Wiesmann F, Betz B, *et al.* Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis. Oncogene 2006;25:3479-88.
10. Veeck J, Geisler C, Noetzel E, Alkaya S, Hartmann A, Knüchel R, *et al.* Epigenetic inactivation of the secreted

frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated with unfavorable prognosis. Carcinogenesis 2008;29:991-8.
11. Veeck J, Bektas N, Hartmann A, Kristiansen G, Heindrichs U, Knüchel R, *et al.* Wnt signalling in human breast cancer: Expression of the putative Wnt inhibitor dickkopf-3 (DKK3) is frequently suppressed by promoter hypermethylation in mammary tumours. Breast Cancer Res 2008;10:R82.
12. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: A novel PCR assay for methylation status of CpG Islands. Proc Natl Acad Sci U S A 1996;93:9821-6.
13. Yu J, Tao Q, Cheng YY, Lee KY, Ng SS, Cheung KF, *et al.* Promoter methylation of the wnt/beta-catenin signaling antagonist Dkk-3 is associated with poor survival in gastric cancer. Cancer 2009;115:49-60.
14. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017;169:985-99.
15. Su Z, Song J, Wang Z, Zhou L, Xia Y, Yu S, *et al.* Tumor promoter TPA activates wnt/β-catenin signaling in a casein kinase 1-dependent manner. Proc Natl Acad Sci U S A 2018;115:E7522-31.
16. Mohammed MK, Shao C, Wang J, Wei Q, Wang X, Collier Z, *et al.* Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. Genes Dis 2016;3:11-40.
17. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, *et al.* Epigenetic inactivation of SFRP genes allows constitutive Wnt signaling in colorectal cancer. Nat Genet 2004;36:417-22.
18. Takada T, Yagi Y, Maekita T, Imura M, Nakagawa S, Tsao SW, *et al.* Methylation-associated silencing of the Wnt antagonist SFRP1 gene in human ovarian cancers. Cancer Sci 2004;95:741-4.
19. Lee AY, He B, You L, Dadfarmay S, Xu Z, Mazieres J, *et al.* Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma. Oncogene 2004;23:6672-6.
20. Fukui T, Kondo M, Ito G, Maeda O, Sato N, Yoshioka H, *et al.* Transcriptional silencing of secreted frizzled related protein 1 (SFRP 1) by promoter hypermethylation in non-small-cell lung cancer. Oncogene 2005;24:6323-7.
21. Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 2005;65:4218-27.
22. Dahl E, Veeck J, An H, Wiesmann F, Klopocki E, Sauter G, *et al.* Epigenetic inactivation of the Wnt antagonist SFRP1 in breast cancer. Verh Dtsch Ges Pathol 2005;89:169-77.
23. Jeong YJ, Jeong HY, Bong JG, Park SH, Oh HK. Low methylation levels of the SFRP1 gene are associated with the basal-like subtype of breast cancer. Oncol Rep 2013;29:1946-54.
24. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, *et al.* Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in human breast cancer. Cancer Biol Ther 2006;5:281-6.
25. Veeck J, Wild PJ, Fuchs T, Schüffler PJ, Hartmann A, Knüchel R, *et al.* Prognostic relevance of wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. BMC Cancer 2009;9:217.
26. Kloten V, Becker B, Winner K, Schrauder MG, Fasching PA, Anzeneder T, *et al.* Promoter hypermethylation of the tumor-suppressor genes ITIH5, DKK3, and RASSF1A as novel biomarkers for blood-based breast cancer screening. Breast Cancer Res 2013;15:R4.
27. Yin DT, Wu W, Li M, Wang QE, Li H, Wang Y, *et al.* DKK3 is a potential tumor suppressor gene in papillary thyroid carcinoma. Endocr Relat Cancer 2013;20:507-14.
28. Xiang T, Li L, Yin X, Zhong L, Peng W, Qiu Z, *et al.* Epigenetic silencing of the Wnt antagonist Dickkopf 3 disrupts normal wnt/β-catenin signalling and apoptosis regulation in breast cancer cells. J Cell Mol Med 2013;17:1236-46.
29. Saied MH, Rady AS, El Naga GM, Sharaki OA. Clinical utility of promoter methylation of the tumor suppressor genes DKK3, and RASSF1A in breast cancer patients. Egypt J Med Hum Genet 2018;19:87-90.
30. Kang WS, Cho SB, Park JS, Lee MY, Myung SC, Kim WY, *et al.* Clinico-epigenetic combination including quantitative methylation value of DKK3 augments survival prediction of the patient with cervical cancer. J Cancer Res Clin Oncol 2013;139:97-106.
31. Majchrzak-Celińska A, Słocińska M, Barciszewska AM, Nowak S, Baer-Dubowska W. Wnt pathway antagonists, SFRP1, SFRP2, SOX17, and PPP2R2B, are methylated in gliomas and SFRP1 methylation predicts shorter survival. J Appl Genet 2016;57:189-97.
32. Suzuki H, Toyota M, Carraway H, Gabrielson E, Ohmura T, Fujikane T, *et al.* Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer 2008;98:1147-56.
33. Hayashi T, Asano H, Toyooka S, Tsukuda K, Soh J, Shien T, *et al.* DNA methylation status of REIC/Dkk-3 gene in human malignancies. J Cancer Res Clin Oncol 2012;138:799-809.
34. Shih YL, Shyu RY, Hsieh CB, Lai HC, Liu KY, Chu TY, *et al.* Promoter methylation of the secreted frizzled-related protein 1 gene SFRP1 is frequent in hepatocellular carcinoma. Cancer 2006;107:579-90.
35. Wu Y, Li J, Sun CY, Zhou Y, Zhao YF, Zhang SJ. Epigenetic inactivation of the canonical Wnt antagonist secreted frizzled-related protein 1 in hepatocellular carcinoma cells. Neoplasma 2012;59:326-32.
36. Holm K, Hegardt C, Staaf J, Vallon-Christersson J, Jönsson G, Olsson H, *et al.* Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. Breast Cancer Res 2010;12:R36.
37. Wang S, Dorsey TH, Terunuma A, Kittles RA, Ambs S, Kwabi-Addo B. Relationship between tumor DNA methylation status and patient characteristics in African-American and European-American women with breast cancer. PLoS One 2012;7:e37928.
38. Lorsy E, Topuz AS, Geisler C, Stahl S, Garczyk S, von Stillfried S, *et al.* Loss of Dickkopf 3 promotes the tumorigenesis of basal breast cancer. PLoS One 2016;11:e0160077.
39. Rawson JB, Manno M, Mrkonjic M, Daftary D, Dicks E, Buchanan DD, *et al.* Promoter methylation of Wnt antagonists DKK1 and SFRP1 is associated with opposing tumor subtypes in two large populations of colorectal cancer patients. Carcinogenesis 2011;32:741-7.
40. Vincent KM, Postovit LM. A pan-cancer analysis of secreted Frizzled-related proteins: Re-examining their proposed tumour suppressive function. Sci Rep 2017;7:42719.
41. Yang B, Du Z, Gao YT, Lou C, Zhang SG, Bai T, *et al.* Methylation of Dickkopf-3 as a prognostic factor in cirrhosis-related hepatocellular carcinoma. World J Gastroenterol 2010;16:755-63.