Aberrant promoter methylation status is associated with upregulation of the E2F4 gene in breast cancer

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Abstract. E2F4 is an important basal transcription factor with the potential to promote tumor growth. Its upregulation in various types of cancer has been linked to numerous genetic factors; however, the nature of the involvement of epigenetic mechanisms, including DNA methylation, remains elusive. In the present study, E2F4 expression profiles were determined in 100 paired breast tumor and control samples, through RT-qPCR using the SYBR® green method. Furthermore, the E2F4 promoter methylation status in each of these samples was assessed using methylation specific PCR, in order to evaluate its impact on gene expression. A two-fold increase in E2F4 gene expression was observed in the breast tumors compared with in their respective controls (P=0.022); of these tumors, ~72% were under-methylated. The change in methylation status was also significantly higher (P<0.001) in the tumor samples. Methylation status was negatively correlated (r=-30) with E2F4 expression profiles, indicating that a decrease in methylation may promote higher expression of E2F4. The two study cohorts (>45 and ≤45 years) had comparable methylation profiles, though they had significantly decreased methylation status compared with controls. Various histo-pathological types also have different methylation profiles, indicating the presence of a tissue specific methylation signature. The results of the present study demonstrated that E2F4 methylation status

can have a notable influence on its expression, and that it may have prognostic value in breast carcinogenesis.

Introduction

Cell cycle regulation is critical to normal growth and division. Any deregulation of activity in the cell cycle regulatory proteins may proceed to uncontrolled division and tumor formation. Cell cycle checkpoints, particularly the G1-to-S checkpoint, serve crucial roles in the initiation and progression of cancer, due to the availability of checkpoint regulatory proteins, including E2F basal transcription factors and retinoblastoma (Rb) proteins (1).

The E2F transcription factors constitute a superfamily of basal elongation factors, which is comprised of eight members (2). Based on the regulatory roles that these proteins have, they are broadly categorized as activators (E2F1-E2F3) and repressors (E2F4-E2F8) (3-5). The association of pocket proteins (pRb, Rbl1/p107, Rbl2/p130) with E2F proteins abrogates their access to target promoters, hence hindering entry to the G1-to-S (6,7). The release of E2Fs from these pocket proteins is necessary for the transcription of genes that are required to cross this checkpoint (8,9). It has been previously reported that the regulatory roles of E2F1-E2F3 in differentiating cells may switch those of repressor proteins (10). The expression profiles of E2F1-E2F3 are strictly regulated in a cell cycle stage-specific manner; however, E2F4 and E2F5 have been demonstrated to maintain constant expression levels at all stages of the cell cycle (3), which indicates that their functions are not limited to any particular stage (11).

Studies researching the involvement of E2F4 in tumorigenesis first reported that a mutated form of this gene was identifiable in several types of cancer, including gastrointestinal and prostate cancer (12-14). Elevated expression levels of E2F4 have been associated with gastric cancer (1), solid osteosarcoma (15) and breast tumor progression (16); whereas E2F4-deficient mice exhibited severe defects in hematopoietic development (17). The E2F4 deficient cells have also been identified to easily undergo apoptosis upon DNA damage, possibly due to the absence of E2F4/DP1/p130 complexes (18). Therefore, it can be concluded that the elevated E2F4 gene levels can be attributed to frequent genetic alterations (13) and abnormal splicing (19). The

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epigenetic mechanisms, including promoter DNA methylation and histone modifications influencing E2F4 expression, remain elusive; although, the involvement of epigenetic mechanisms in cancer and metastasis, specifically in regulating the expression patterns of various genes, is well known (20,21).

Promoter DNA methylation can produce effects similar to gene mutation in order to achieve gain or loss of function of certain genes, leading to carcinogenesis (22). Furthermore, aberrant promoter methylation has been identified in various types of tumor cells, and thus has been theorized to be a biomarker for the early prognosis of cancer (23). In previous studies, aberrant methylation was identified at the pRb (24), Rbl2/p130 (25) and E2F5 promoters (26), and was significantly associated with their expression profiles in breast as well as head and neck cancer.

The present study was designed to investigate the expression levels of the E2F4 mRNA transcript and its promoter methylation status in breast tumor tissues, as well as in their adjacent normal control (ANCT) samples. This allowed an evaluation of whether promoter methylation is a decisive factor for E2F4 gene transcription. Associations between promoter methylation status and with various clinico-pathological outcomes of breast tumors were also established to evaluate the prognostic value of E2F4 promoter methylation in breast carcinogenesis within a clinical setting.

Materials and methods

Recruitment of patients with breast cancer, and specimen collection. A total of 100 pairs of tumor and ANCT tissue samples were collected and stored in RNA Later™ stabilization solution (cat. no. AM7024; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the time of surgery from patients with breast cancer at Lady Reading Hospital (Khyber Pakhtunkhwa, Pakistan) between June 2012 and August 2015. All the patients were female. The ANCT sections were taken from an area ~2 cm from the cancer lesion site, as selected by a histo-pathologist. The patients with a history of other infectious, metabolic or familial diseases were excluded from the present study. Furthermore, prior approval was obtained from the Ethical Review Committee (approval no. CIIT-09-10-14) of the COMSTAS Institute of Information Technology (Islamabad, Pakistan) and the collaborating hospital aforementioned for the initiation of this project. In addition, informed and written consent was received from all patients enrolled in the present study, prior to sample collection.

mRNA extraction and real-time qPCR analysis. RNA extraction from all tissue samples (tumor and ANCT) was performed using the standard TRIzol® method (27). The extracted mRNA was converted to cDNA using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The primers used for mRNA expression analysis were as follows: E2F4 forward, 5'-TCAGAAATCTTT GATCCCAC-3' and reverse, 5'-AGATATAATCGTGGTCT CCC-3'; β-actin (internal control) forward, 5'-CACTCT TCCAGCCTTCCTTC-3' and reverse, 5'-TGATCTCCTTCT GCATCGTG-3'. PCR amplification was performed using SYBR®-Green method (28) through Step One Plus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycler conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C

for 15 sec, 56°C for 60 sec and 72°C for 20 sec; and a final extension at 72°C for 1 min. mRNA expression of the E2F4 gene was calculated using the $2^{-\Delta\Delta Cq}$ method (29).

Extraction and bisulfite modification of DNA. DNA from tumor and ANCT tissues was extracted using the standard phenol-chloroform method (30), and confirmed by 2% agarose gel electrophoresis. Genomic DNA (~2-5 μg) was bisulfite modified using an EpiJET™ Bisulfite Conversion kit (Thermo Fisher Scientific, Inc.), as per the manufacturer's instructions. Bisulfite treatment was used to convert non-methylated cytosine 'C' bases to thymine 'T' bases; however, the methylated cytosine 'C' was left unchanged.

Primer designing for methylation analysis. The methylation status of the E2F4 promoter was analyzed in the tumor and ANCT tissues from all recruited patients with breast cancer. The target region is located at a site 84 bp upstream and 117 bp downstream from the transcription start site (TSS). Furthermore, methylation-specific primers were designed for the target site, according to National Center for Biotechnology Information Nucleotide database (https://www.ncbi.nlm.nih. gov/nucleotide?cmd=search) and scanned 29 CpG sites within the targeted sequence. The two sets of methylated primers were as follows: Forward, 5'-CGTTACGTTTTTTGGAAG GC-3' and reverse, 5'-CGTACCGACTTAAAATACCCG-3'. Un-methylated primers were as follows: Forward, 5'-TAGTGT TATGTTTTTTGGAAGGTGT-3' and reverse, 5'-TTCATA CCAACTTAAAATACCCAAA-3'. Promoter methylation status was analyzed using the MethPrimer™ software (version 1.0; http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; Peking Union Medical Colege Hospital, Beijing, China) available online (31).

Methylation-specific PCR (MSP). The targeted promoter regions around the TSS of E2F4, amplified from bisulfite converted genomic DNA, was performed using MSP reactions. Bisulfite converted DNA was amplified in a one-step MSP reaction, using two independent sets of primers aforementioned (methylated and un-methylated) and Maxima® Hot Start Taq DNA polymerase (Thermo Fisher Scientific, Inc.). Briefly, bisulfite converted DNA (~50 ng) was amplified with 0.2 μM of each deoxynucleotide triphosphate and primer (both methylated and un-methylated forward and reverse primers) using 1 U Maxima® Hot Start Taq DNA polymerase.

The PCR conditions for E2F4 were as follows: A single cycle at 98°C for 30 sec (initial denaturation); followed by 35 cycles at 98°C for 10 sec (denaturation), 62°C for 30 sec (annealing) and 72°C for 30 sec (extension); and a single cycle at 72°C for 7 min (final extension) using 1 U Maxima® Hot Start Taq DNA polymerase. MSP validity was checked by amplifying CpG-methylated human genomic DNA (Thermo Fisher Scientific, Inc.) as the positive control with PCR water (Solis BioDyne, Tartu, Estonia) used as the negative control; however, for the non-methylation-specific PCR, bisulfite-unconverted human genomic DNA was used as the positive control for all primers specific to the E2F4 gene promoter. All PCR reactions were carried out using a BIOER™ Thermal Cycler 9500 (Hangzhou Bioer Technology Co., Ltd., Binjiang, China).

Gel electrophoresis and quantification. The PCR products from the aforementioned reactions were resolved on a 2% agarose gel and visualized under an UV illuminator (BioDoc Analyzer, Biometra; Analytik Jena, Jena, Germany) using a 100 bp DNA size ladder. To establish the degree of methylation from qualitative MSP data, the change in methylation (Δ meth) value was calculated, as previously described (25,26). The Δ meth value was calculated by subtracting the un-methylation values of a particular sample from its methylation values, when considering un-methylation as the default condition.

Statistical analysis. Statistical analyses were performed using OriginPro 2015 software (OriginLab Corporation, Northampton, MA, USA). The results are expressed as the mean and (±) standard error of the mean to determine the descriptive statistics for qualitative data. For the analysis of transcript profiles, the expression data of the target gene (E2F4) were normalized against those of the internal control gene β-actin. The correlations between various factors were assessed using the Pearson's correlation coefficient. Depending on the experiment, the statistical significance was determined to 95% confidence intervals using Pearson's correlation coefficient, Kruskal-Wallis and Mann Whitney tests and one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis enabled an investigation of the correlations between the methylation frequency and expression levels of the E2F4 gene.
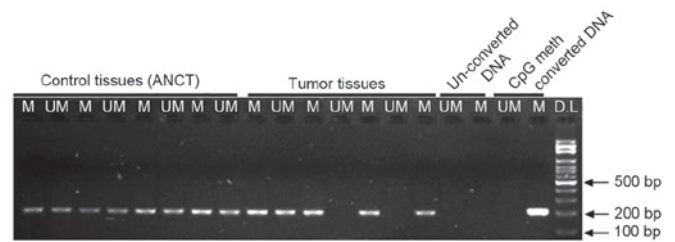

Figure 1. Agarose gel (2%) image demonstrating the promoter methylation status of the E2F4 gene in breast tumor and control tissue samples. Additionally, the methylation-specific PCR products are depicted for the ANCT and tumor tissues. Un-converted DNA is the genomic DNA that was not bisulfite-treated, while CpG meth-converted is bisulfite-treated and commercially available human methylated DNA. DL, DNA size ladder (100 bp); M, methylated; U, un-methylated.

Results

Promoter methylation analysis of E2F4 for patients with breast cancer. The promoter methylation status of the E2F4 gene was analyzed through an MSP approach. Of the breast tumors that were observed, ~71.5% were un-methylated at the E2F4 promoter; whereas ~21.5% of breast tumors were determined to be exclusively methylated at this promoter (Fig. 1 and Table I). Conversely, ANCT control tissues were identified as differentially methylated (~51.5% methylated and ~48.5% un-methylated) at the E2F4 promoter. The Δ meth value also exhibited a remarkable difference between the breast tumor (mean=3,450.76) and the ANCT (mean=722.29) tissue samples (Fig. 2A; Table I). This indicates a statistically significant
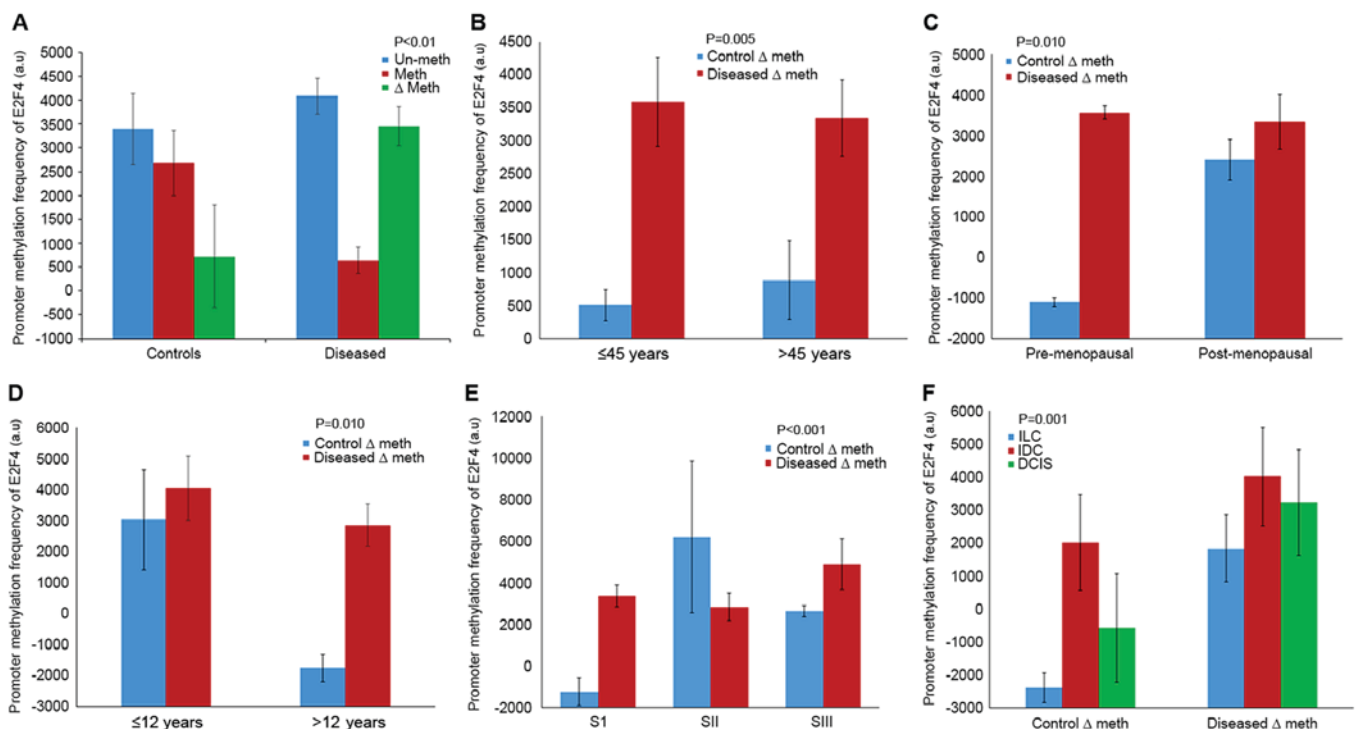

Figure 2. Promoter methylation status of the E2F4 gene, in particular the clinico-pathological factors for the controls and for patients with breast cancer. (A) The reduced methylation levels for tumor and control samples. (B) The age wise (≤45 and >45 years) promoter methylation status of the E2F4 gene in the study cohorts. (C) The promoter methylation status of the E2F4 gene in pre- and post-menopausal patients. (D) Variation in E2F4 gene promoter region methylation between patients with differing ages of menarche. (E) The promoter methylation status of the E2F4 gene at various disease stages. (F) The promoter methylation status of E2F4 in various histological cases of breast cancer. Un-meth, un-methylation; meth, methylation; Δ meth, change in methylation.

Table I. Statistical analysis of E2F4 mRNA transcript expression and its promoter methylation status in various breast cancer cohorts.

Characteristics	ANCT					Tumors					Control vs. tumor
			95% CI					95% CI			
	Mean	SEM	Upper	Lower	Skewness	Mean	SEM	Upper	Lower	Skewness	P-value
mRNA expression											
Overall	1.00	0.56	2.15	-0.15	4.89	1.78	0.87	3.57	-0.01	4.09	0.022[a]
Methylation status overall											
Un-meth	3,398.61	745.61	4,937.47	1,859.75	4.59	4,087.59	375.31	4,862.19	3,312.98	0.11	<0.001[a]
Meth	2,676.32	685.99	4,092.12	1,260.51	1.02	636.82	275.64	1,205.72	67.93	2.67	
Δ meth	722.29	1,074.18	2,939.28	-1,494.70	1.93	3,450.76	407.76	4,292.34	2,609.19	-0.28	
Age (years) Δ meth											
≤45	509.88	235.73	5,754.31	-4,734.56	1.86	3,589.46	600.74	4,927.99	2,250.93	0.68	0.005[a]
>45	889.19	671.32	2,339.49	-561.12	-1.74	3,341.79	572.66	4,578.94	2,104.63	-0.85	
Age (years) of menarche Δ meth											
≤12 (early)	3,016.73	1,617.46	6,540.87	-507.42	2.45	4,025.39	440.89	4,986.02	3,064.76	0.51	0.010[a]
>12 (late)	-1,763.35	1,035.76	516.35	-4,043.05	-0.17	2,828.25	676.96	4,318.24	1,338.27	-0.09	
Menopausal stage Δ meth											
Pre-menopausal	-1,102.75	116.64	1,456.19	-3,661.69	-0.30	3,572.11	676.34	5,060.72	2,083.51	-0.06	0.010[a]
Post-menopausal	2,406.95	167.75	6,053.72	-1,239.83	2.49	3,338.75	500.26	4,428.71	2,248.79	-0.90	
Δ meth status of histopathological tumor type											
IDC	2,018.93	1,446.91	5,102.96	-1,065.09	2.21	4,010.89	1,484.53	5,043.65	2,978.13	-0.48	0.001[a]
ILC	-2,387.76	444.47	3,010.75	-7,786.27	-0.03	1,835.90	1,018.74	4,664.27	-992.47	0.73	
DCIS	-576.72	1,638.79	4,638.67	-5,792.13	-0.48	3,228.82	1,605.71	5,156.47	1,301.18	0.07	
Tumor stage Δmeth											
I	-1,227.12	855.83	587.15	-3,041.39	-0.43	3,372.69	519.79	4,474.62	2,270.78	-0.54	<0.001[a]
II	6,203.49	3,652.71	16,344.66	-3,937.69	2.23	2,841.46	667.11	4,693.58	989.34	1.34	
III	2,633.60	278.28	3,830.96	1,436.24	-1.73	4,908.64	1,226.92	10,187.65	-370.36	-0.19	

Δ meth, change in methylation; ANCT, adjacent normal control tissues; un-meth, un-methylation; meth, methylation; SEM, standard error of the mean (±); CI, confidence interval; [a]$P<0.05$; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*.

Table II. Correlation analysis of the E2F4 gene mRNA transcript expression and promoter methylation for various clinico-pathological factors in patients with breast cancer.

	ANCT				Tumors			
Characteristics	Pearson's r	Adj. R^2	F value	Prob>F	Pearson's r	Adj. R^2	F value	Prob>F
mRNA expression								
Overall	-0.07	-0.04	0.12	0.74	-0.24	0.02	1.41	0.25
Methylation status overall								
Un-meth	-0.05	-0.04	0.07	0.79	0.57	0.29	11.09	0.003
Meth	-0.10	-0.03	0.24	0.63	-0.06	-0.04	0.07	0.79
Δ Meth	0.03	-0.04	0.02	0.89	0.56	0.29	10.66	0.003
Age (years) Δ meth								
≤45	-0.07	-0.11	0.04	0.85	0.68	0.40	7.70	0.02
>45	0.26	-0.01	0.90	0.36	0.46	0.14	3.16	0.10
Age (years) of menarche Δ meth								
≤12 (early)	-0.25	-0.02	0.83	0.38	0.64	0.36	7.81	0.02
>12 (late)	0.40	0.08	1.94	0.19	0.61	0.31	5.93	0.04
Menopausal stage Δ meth								
Pre-menopausal	0.20	-0.06	0.41	0.54	0.78	0.58	16.59	0.01
Post-menopausal	-0.17	-0.06	0.36	0.56	0.32	0.02	1.23	0.29
Δ meth status of histopathological tumor types								
IDC	-0.11	-0.06	0.16	0.70	0.64	0.36	9.57	0.01
ILC	-0.13	-0.31	0.05	0.83	0.49	0.01	0.95	0.40
DCIS	0.22	-0.43	0.10	0.77	-0.22	0.43	0.10	0.77
Tumor stage Δ meth								
I	0.12	-0.05	0.22	0.64	0.60	0.31	8.04	0.01
II	-0.36	-0.16	0.46	0.54	0.35	-0.17	0.42	0.56
III	0.86	0.49	2.90	0.33	0.53	-0.43	0.39	0.64

Δ meth, change in methylation; ANCT, adjacent normal controls tissues; un-meth, un-methylation; meth, methylation; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*; prob, probability.

(P<0.001) decrease in E2F4 promoter methylation among patients with breast cancer, as compared with the controls. These findings highlight a possible role for E2F4 promoter methylation in breast cancer prognosis, via its elevated mRNA expression levels (Tables II and III; Fig. 2A).

Promoter methylation status of the E2F4 gene among distinct clinicopathological breast cancer cohorts. The promoter methylation status of the E2F4 gene was analyzed in two age-based study cohorts, consisting of patient's ≤45 and >45 years. The study cohort comprising patients ≤45 years of age, exhibited hypo-methylation in ~66.67% of tumor; whereas, ~46.43% of their ANCT controls were hypo-methylated, indicating that non-cancerous tissues have higher methylation levels at the E2F4 promoter (Table I). Similarly, patients in the >45 years cohort exhibited hypo-methylation in ~75% of tumors, as compared with in ~50% of control samples, which again indicated higher methylation levels in controls. In addition, the Δ meth values between each study

cohort were identified to be significantly different (P=0.005). Furthermore, the Pearson's correlation coefficient values for Δ meth revealed a strong positive correlation (r=0.68 for >45 years and r=0.46 for ≤45 years) for each study cohort (Tables I-III and Fig. 2B). These findings indicated an age-independent trend in E2F4 promoter methylation status that may have implications for disease prognosis. Similar methylation profiles were also observed in pre- and post-menopausal study cohorts (Table I and Fig. 2C) with 45 years being the mean age for these cohorts. This indicated that the variable methylation signature in the cohorts may be a consequence of a hormonal imbalance in these patients, revealed a mechanistic insight into the whole process of DNA methylation. Patients with and early (≤12 years) or late (>12 years) age of menarche were observed to have statistically significant (P=0.010) variations in their promoter methylation status (Table I and Fig. 2D); the results demonstrated that there was a strong positive correlation for early (r=0.64) and late (r=0.61) age of menarche patients (Table II).

Table III. Promoter methylation frequencies of the E2F4 gene among various clinico-pathological parameters in breast cancer.

	ANCT		Tumors	
Characteristics	Meth no. (%)	Un-meth no. (%)	Meth no. (%)	Un-meth no. (%)
Overall	103 (51.5)	97 (48.5)	57 (28.5)	143 (71.5)
Age (years)				
≤45	45 (53.57)	39 (46.43)	28 (33.33)	56 (66.67)
>45	58 (50.0)	58 (50.0)	29 (25.0)	87 (75.0)
Age (years) of menarche				
≤12 (early)	53 (43.44)	69 (56.56)	20 (16.39)	102 (83.61)
>12 (late)	50 (64.10)	28 (35.6)	37 (47.44)	41 (52.56)
Menopausal status				
Pre-menopausal	80 (62.5)	48 (37.5)	46 (35.94)	82 (64.06)
Post-menopausal	23 (31.94)	49 (68.06)	11 (15.28)	61 (84.72)
Histo-pathological types				
IDC	63 (45.65)	75 (54.35)	35 (25.36)	103 (74.64)
ILC	31 (64.58)	17 (35.42)	19 (39.58)	29 (60.42)
DCIS	9 (64.29)	5 (35.71)	3 (21.43)	11 (78.57)
Stages				
I	61 (48.41)	65 (51.59)	40 (31.75)	86 (68.25)
II	23 (57.5)	17 (42.5)	10 (25.0)	30 (75.0)
III	19 (55.88)	15 (44.12)	7 (20.59)	27 (79.41)

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*; ANCT, adjacent normal control tissues; un-meth, un-methylation; meth, methylation.

This indicates that early age of menarche is a putative risk that may result in notable methylation variations.

Similarly, promoter methylation status among tumors at different disease stages revealed a gradual reduction in methylation frequency with advanced tumor stage, i.e. from SI to SIII (Table III; Fig. 2E). As the Δ meth was revealed to be statistically significant (P<0.001) among these tumors, it was suggested that promoter methylation had a notable implication for disease progression and prognosis. Furthermore, for the various histopathological types of breast cancer, including invasive ductal carcinoma, invasive lobular carcinoma and ductal carcinoma *in situ*, the Δ meth value was statistically significant (P=0.001; Tables I and III; Fig. 2F). This demonstrates that methylation may be controlled in a tissue specific manner.

Quantitative PCR (qPCR) mRNA transcript analysis of E2F4. Quantitative mRNA transcript analysis of the E2F4 gene was performed using qPCR with all biopsy samples from the enrolled patients with breast cancer. β-actin was used as the internal standard for gene amplification. It was identified that there was an almost two-fold elevation in E2F4 gene expression in tumor samples when compared with ANCT tissues (Fig. 3); this difference was statistically significant (P=0.022; Table I) and indicated a potential involvement of E2F4 expression in tumor pathogenesis. The upregulation of E2F4 in breast tumor samples determined to be negatively correlated with its promoter methylation, as per the Pearson's correlation test. The value of Pearson's 'r' (Pearson's r=-0.24) indicated a
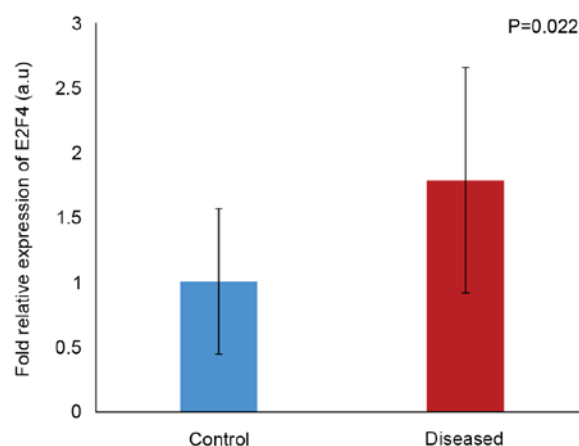

Figure 3. Relative mRNA expression of the E2F4 gene in breast tumor and control tissues. There was an almost two-fold increase in E2F4 expression detected in tumor tissues, as compared with in the control tissues.

negative correlation with disease outcome, which suggested that a higher E2F4 expression would lead to a poorer outcome. Notably, no correlation (Pearson's r=-0.07) was calculated for the control samples (Table II).

Expression of E2F4 correlates negatively with promoter methylation. The E2F4 promoter methylation status may correspond to its expression; the degree of E2F4 promoter methylation has a strong positive effect on its transcript

Table IV. Pearson's correlation between E2F4 gene expression and the promoter methylation status in patients with breast cancer.

	ANCT				Tumor			
Pearson's correlation	Relative expression	Un-meth	Meth	Δ Meth	Relative expression	Un-meth	Meth	Δ Meth
Control relative expression	1	-0.09	0.41[a]	-0.32	-0.08	0.33	0.81[a]	-0.25
Diseased relative expression	-0.08	0.89[a]	-0.09	0.67[a]	1	-0.34	-0.03	-0.30
Diseased un-meth	0.32	-0.16	0.12	-0.19	-0.34	1	0.24	0.76[a]
Diseased meth	0.81[a]	-0.13	0.67[a]	-0.52[a]	-0.03	0.24	1	-0.45[a]
Diseased Δ meth	-0.25	-0.06	-0.35	0.18	-0.30	0.76[a]	-0.45[a]	1
Control un-meth	-0.09	1	-0.12	0.77[a]	0.89[a]	-0.16	-0.13	-0.06
Control meth	0.41[a]	-0.12	1	-0.73[a]	-0.09	0.12	0.67[a]	-0.35
Control Δ meth	-0.32	0.77[a]	-0.73[a]	1	0.67[a]	-0.19	-0.52[a]	0.18

[a]Strong correlations. Un-meth, un-methylation; meth, methylation; Δ meth, change in methylation; ANCT, adjacent normal control tissues.

expression. Statistical analysis revealed a moderately negative correlation between promoter methylation (Δ meth) and gene expression (r=-0.30) in breast tumor tissues, as well as in the ANCT samples (r=-0.32; Table IV). This demonstrates that methylation in these samples may be the causative factor for their gene upregulation. Furthermore, there was a moderately positive association between the methylation status of controls and relative expression of E2F4 gene (r=0.41); whereas, there was no association observed between tumor methylation and their relative expression (r=-0.03), although, a moderately negative correlation (r=-0.34) between the un-methylation status of these samples and their expression was identified. This demonstrates an involvement of the hypo-methylated status of E2F4 in regulating its expression in breast tumor samples (Table IV).

Discussion

E2F4 being a basal transcription factor serves vital roles in cell cycle regulation, and its deregulated expression may lead to un-controlled growth and tumorigenesis (32). The transcriptional activity of E2F4 requires hetero-dimerization with its DP partner, which selectively binds to the CpG rich DNA sequence TTTC/GC/GCGC/G (33,34), regulating downstream sequences. Furthermore, the transcriptional domain of E2F4 is occupied by members of the RB family of proteins (pRB, p130/RBL2, p107/RBL1) (35,36); therefore, making a cyclin E repressor complex (CERC) (37), which causes the E2F4/DP complex to lose the ability to initiate transcription at the required promoters. As a result, the aberrant expression of E2F4 creates an unbalance in the normal composition of the cell cycle complex, causing uncontrolled cell division and tumor formation (26). High expression of E2F4 has previously been identified in various types of cancer, including gastric (1), prostate (5) and breast cancer (16); however, the key factors regulating E2F4 gene expression remain elusive.

The results of the present study support those of prior studies, which reported upregulation of the E2F4 gene in breast tumor tissues (mean=1.78) compared with in the controls (mean=1.00). Furthermore, the present study demonstrated that promoter methylation status is a primary cause of

upregulation. The findings of the present study indicated that the frequency of E2F4 promoter methylation was notably low in the breast tumor tissues (28.5% of all cases), as compared with in the respective ANCT samples (51.5%). Furthermore, the Δ meth value was identified to be significantly different (P<0.001) between the control and tumor tissues. Promoter methylation is an epigenetic phenomenon that is crucial to activating or deactivating the regulatory sequence of a gene, and, therefore, to controlling the gene expression and effecting the normal growth pattern (21,22). This epigenetic phenomenon has already been demonstrated to serve an important role in influencing the expression profiles in a number of growth associated genes, including members of the RB family (pRB, Rbl2) (24,25), as well as other E2Fs (25,26). The CpG rich regions can be modified through methylation, which creates a competitive environment for E2F4 binding in these regions, even for the regulatory elements of E2F4 (38).

The elevated mRNA expression of E2F4 has a moderately negative association with its aberrant methylation level (r=-0.30) in cancerous tissues, and may serve a prognostic role. This indicates that un-methylation is associated with transcribing promoters; however, this must be further evaluated in other cancer types. The hypo-methylated promoters were repeatedly identified in all study cohorts, based on various factors, including patient age, the age of menarche, menopausal status, tumor histo-pathological types, and tumor stages. The decrease in promoter methylation highlights the prognostic involvement of E2F4 in tumor progression, and may be useful as a predictor for the early diagnosis of breast cancer. The hypothesis is supported by observations made during the present study, specifically that well-differentiated (SI) tumors were more methylated (~31%) compared with the poorly differentiated (SIII) tumors (~20%; Table III). Notably, analysis demonstrated that tumors from various histological origins exhibited varying degrees of methylation at the E2F4 promoter; this indicated that the methylation patterns could be a consequence of the activities of various methyl transferases and demethylases present at the tumor origin.

Generally, It is considered that gene silencing through aberrant promoter methylation is a consequence of aging (39).

However, the present study identified an age-independent pattern to the E2F4 promoter methylation status. Δ meth values obtained for patients of >45 years and ≤45 of age, indicated that Δ meth values between the tumor and control groups are age-independent, and may have a causative role in tumor progression. However, these patients (≤45 and >45 years) exhibited significant Δ meth values (P=0.005) when compared with their respective controls (Table II). Age- and disease-dependent mutations in CpG-rich regions of E2F4 gene promoter can be a consequence of variable methylation levels (40) in these patients. Hence scanning these methylation hotspots (CpG islands) for single nucleotide polymorphisms may be an interesting avenue to explore. This will also highlight an interesting interplay of genetic and epigenetic factors in determining disease fate and other ageing phenotypes. The E2F4 gene has 10 reported C/T transitions at the targeted chromosomal location (31) flanking over ~205 bp around the E2F4 TSS. This supports the assumption that promoter methylation in this region may have a crucial role in promoting tumor formation. There is currently limited knowledge regarding the disease susceptibility of a specific population harboring these single-nucleotide polymorphisms. Based on these observations, analysis of the C>T transition in a population of patients with breast cancer and other associated diseases may be a notable domain to investigate.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

FUF performed data acquisition and specimen analysis. FH performed data analysis and interpretation. NM performed data analysis and manuscript preparation. NA performed data analysis. HR performed data acquisition and interpretation. MS carried out study design, data analysis, interpretation, manuscript preparation and final approval of the version to be published.

Ethics approval and consent to participate

This study was initiated with a prior approval from departmental Ethical Review Committee (approval no. CIIT-09-10-14) of the COMSTAS Institute of Information Technology (Islamabad, Pakistan) and the Lady Reading Hospital (LRH) Peshawar. Informed and written consent was received from all patients enrolled in the present study, prior to sample collection for only data publication.

Consent for publication

All the information regarding patient's detail, are confidential and confined to Cancer Genetics and Epigenetics Laboratory, Department of Biosciences, COMSATS Institute of Information Technology (CIIT), 45550, Islamabad and the collaborating hospital LRH, Peshawar. Informed and written consent was received from all patients enrolled in the present study, prior to sample collection for only data publication.

Competing interests

The authors declare that they have no competing interests.

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