Journal of Medical and Scientific Research

JMSR
www.jmsronline.com

Beevi SS et al. J Med Sci Res. 2023; 11(3):186-193
http://dx.doi.org/10.17727/JMSR.2023/11-35

ORIGINAL RESEARCH OPEN ACCESS

Impact of promoter region mutations of *Pim-1* on c-Myc expression as well as survival in patients with breast cancer

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Abstract

Purpose: Breast cancer accounts for most cancer-related deaths in women with over 2.3 million new breast cancer cases diagnosed every year worldwide. Pim kinases are oncogenic proteins that play important role in various cancers. *Pim-1* contains 1.7 kb promoter region without any transcriptional regulation, which is a characteristic feature of a housekeeping or constitutive promoter. This study was carried out to ascertain potential mutations in the promoter region of *Pim-1* in breast cancer and also to comprehend the effect of mutations on c-Myc expression as well as on patients' survival.

Methods: Ninety-six Indian subjects with a first diagnosis of breast cancer who underwent surgery at our hospital were recruited in this study. Genomic DNA was isolated from whole blood, amplified and sequenced to identify potential mutations at the promoter region of *Pim-1*. FFPE sections were used to determine the c-Mycexpression in all the studied groups.

Results: We observed a transversion (C>A at –502) and a deletion (–754Cdel) mutation in the promoter region with negligible association with tumor biology and c-Myc expression. Positive c-Myc expression was noted in almost 50% of the studied population with absolute nuclear staining or both cytoplasmic/nuclear staining. As per survival analysis, promoter region mutations did not modulate the patients' survival, but patients with negative c-Myc expression had better disease-free survival compared with positive c-Myc expression.

Conclusion: Our findings identified the presence of mutation in the promoter region of *Pim-1*with negligible impact on c-Myc expression in patients with breast cancer.

Keywords: *Pim-1* promoter; c-Myc; survival rate; transversion mutation; constitutive gene

Introduction

Breast cancer accounts for most cancer-related deaths in women with over 2.3 million new breast cancer cases diagnosed every year worldwide [1]. Current trends point out that breast cancer is on the rise, both in rural and urban India [2]. It is more common in the younger age group as compared to developed countries. Furthermore, the survival rate of patients with breast cancer is poor as compared to western countries owing to younger age at onset, disease presentation at late stage and delayed or inadequate treatment modalities [3]. Pressing priority is holistic multidimensional approaches including prevention, screening, early detection, appropriate treatment and care.

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Received 14 April 2023; *Revised* 14 June 2023; *Accepted* 21 June 2023; *Published* 30 June 2023

Citation: Beevi SS, Anbrasu K, Kannepalli KK, Verma VK, Pillarisetti RR, Madigubba S, Darapuneni RC, Bollineni BR. Impact of promoter region mutations of *Pim-1* on c-Myc expression as well as survival in patients with breast cancer. J Med Sci Res. 2023; 11(3):186-193. DOI: http://dx.doi.org/10.17727/JMSR.2023/11-35

The genomic backdrop of breast cancer is multifaceted and understanding mutation profiles and gene expression offer a new perception into tumor heterogeneity that eventually strategize the future development of clinical management of patients [4]. Gene promoter is usually located around the transcriptional start site, which through interaction with transcriptional factors controls transcriptional initiation. Mutations in the promoter region of gene could impede the interaction with transcriptional factors, thus altering transcriptional initiation and thereby leading to abnormal gene expression and cancer development.

Proviral integration site for Moloney murine leukemia virus (Pim) kinases are oncogenic proteins that play important role in various cancers [5]. To date, three Pim kinases such as *Pim-1*, 2 and 3 have been identified. *Pim* kinases are highly conserved throughout evolution and display early response to growth factors and cytokines [6]. Of the three kinases, *Pim-1* kinase has been found to be highly expressed mostly in hematological cancers [7]. Of late, several studies have also reported the overexpression of *Pim-1* kinase in various solid tumors including breast cancer [8].

Pim-1 contains1.7 kb promoter region with 40% GC rich region from nucleotide –1703 to –874 and 71% GC rich region from nucleotide –873 to –1. Functional studies confirmed that *Pim-1* promoter carries no TATA or CAAT elements and does not come under any transcriptional regulation, that is characteristics of a housekeeping or constitutive promoter. *Pim-1* promoter possess a proximal (nucleotide –104 to –1) and distal (nucleotide –427 to –336) elements, that are responsible for transcription. It has been confirmed that even though *Pim-1* promoter lacks TATA and CAAT elements, distant control elements such as silencer or enhancer modulate the transcriptional activity of the *Pim-1* promoter [9].

Gene expression is a meticulously regulated process to safeguard spatial and temporal expression, in which transcriptional initiation is the access point. Mutations in promotor region can modulate transcriptional initiation, gene expression level and trigger pathogenic outcomes. *Pim-1* kinase phosphorylates several substrates including c-Myc that initiate the transcription process [10].

c-Myc is a basic-helix-loop-helix/leucine zipper transcription factor that is critical for cell proliferation and differentiation. The expression level of c-Myc is tightly regulated by several mechanisms involving transcriptional regulation of proximal promoter region [11]. It can modulate several growth-promoting and growth-inhibiting genes for maintaining normal cell growth and function. c-Myc protein has a half-life of less than 20 min [12] and hence its precise regulation is extremely important for normal cellular processes. Any process that enhances the half-life of c-Myc and stabilizes the protein would promote cell transformation. It has been reported that overexpression of *Pim-1* kinase markedly stabilizes c-Myc through phosphorylation of c-Myc at Ser62 and thereby enhancing the transcriptional activity of c-Myc [10].

With this background, we hypothesize that specific mutations in promotor region of *Pim-1* could modulate the expression level of c-Myc that eventually impact the disease-free survival (DFS) and overall survival (OS) in patients with breast cancer. In this study, we amplified and sequenced the entire promoter region of *Pim-1* and determined the expression level of c-Myc to understand the synergism between *Pim-1* and c-Myc in influencing patients' survival.

Patients and methods

Ninety-six Indian subjects with primary breast cancer who visited the Department of Surgical Oncology, Krishna Institute of Medical Sciences, Telangana, between July 2017 and June 2022, were enrolled for this study. Institutional Research Advisory Board and Ethics Committee approved the study process and protocol. All individuals who participated in the study signed the written informed consent. The study was conducted by following guidelines of 1964 Helsinki Declaration and its later amendments. Patients' medical history, clinical reports, surgery approach, and treatment schedule were obtained from each patient.Each participant provided approximately 5 ml venous blood sample at fasting state for consequent mutational analysis before starting any medical intervention.

Mutational analysis

Deoxyribonucleic acid (DNA) was extracted from the whole peripheral blood using QiagenFlexiGene DNA kit according to the manufacturers' instruction. DNAs were screened for potential mutations in the entire promoter region of the *Pim-1* gene, using primers (Table 1) designed through Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Polymerase chain reaction (PCR) amplification was carried out in a total volume of 25 µl containing 100 ng of genomic DNA asa template, 1X reaction buffer, 1.5–3 mM magnesium chloride, 200 µM dNTP, 20–50 pm each PCR primer and 0.25 U Taq Polymerase. After PCR

amplifications, 5µl of the reaction product was analyzed via gel electrophoresis and ethidium bromide staining. PCR products were then cleaned using ExoSAP-IT™ and sequenced by Sangers' sequencing. If a mutation was identified, a new DNA aliquot from the same participant was sequenced to confirmed the result.

Immunohistochemical analysis of c-Myc

The breast tissue was fixed in 10% buffered formalin, dehydrated with ethanol gradient, permeabilized with xylene, and paraffin-embedded. Then, serial 5µm thick sections were cut, deparaffinized with xylene, hydrated with ethanol gradient, and proceeded further for c-Myc detection. Endogenous peroxidase inactivation was carried out with 3% H_2O_2, and antigen retrieval was performed in a microwave. The slides were washed in PBS and nonspecific binding was blocking by applying 5% bovine serum albumin in a humidity chamber for 1 hour. The slides were blotted with primary antibodies (anti-c-Myc) at 1:100 dilution and incubated overnight at 4°C. The slides were washed 3X with PBS and incubated with biotin-labeled secondary antibodies for one hour at room temperature. The slides were then washed 3X with PBS and the final signals were developed using the 3,3'-diaminobenzidine substrates (DAB). The sections were analyzed by optical microscopy after counterstaining with hematoxylin.

The intensity of c-Myc expression was scored relative to that in benign breast tissue. Mostly nuclear expression was detected in breast cancer tissues. Diffused cytoplasmic expression was observed in benign breast tissue and in certain patients with breast cancer. Nuclear expression was scored as being either strong (positive) or weak (negative) while cytoplasmic expression was determined as intense (positive) or diffused (negative).

Patients' review and follow-up

All the patients were followed-up at regular intervals till the end of study period (60 months). Patients alive or lost to follow-up were censored at the date of their last contact. Progression-free survival (PFS) was determined from date of initial diagnosis to first documented disease recurrence. Overall survival (OS) was calculated from date of recruitment into the study to the date of death or last follow-up.The median age of the patients inducted in the study was 48 (23–75) and the median follow-up period was 57 months (range 1 – 60). At the end of the follow-up period, 19 (19.79%) patients had expired of their disease. Patients who had deceased of causes other than breast cancer were censored from the survival analysis.

Statistical analysis

Patient outcomes were compared according to *Pim-1* promoter region mutational status. Baseline characteristics of patients were compared by Fisher's exact test for qualitative variables and Kruskal–Wallis test for quantitative variable. Survival curves were plotted by the non-parametric Kaplan–Meier method, and compared by the log-rank test. All statistical analyses were performed using an R Statistical Package (version 4.2.2 for Windows) with significance set at the 5% level.

Results

Entire promoter region (1.7 kb) of *Pim-1* gene is amplified using four different primers set (presented as Table 1). 13 out of 96 patients showed variations in the promoter sequence during analysis of Sanger's sequencing data. We identified two mutations in the promoter region namely, C>A transversion located – 502 C>A and deletion of C at – 754delC, upstream from the translational start site. All the patients carried single mutation, either – 502 C>A or – 754delC. We next analyzed the sequence of promoter region in order to identify the importance of these mutations in terms of gene functionality. FASTA sequence of promoter region is illustrated as Table 2 where transcription factors such as Sp1, AP2 and PPF-348 were marked exclusively [9]. None of the mutations identified in our study occurred at the site of marked transcription factors.

Table 1: Primers used for the detection of *Pim-1* promoter mutation.

Promoter	Primer sequence	PCR product (bp)
1.1	F – AGCTTTGAATACTCAGGAGGTGA R - CATGTAGAGTTGATAACGACGTG	460
1.2	F – ACTCTACATGAAGTTTAAAAGGACAAA R - AGGAGTCAGGCCTGGAACAG	480
1.3	F – CTGTTCCAGGCCTGACTCCT R – GGAAAAGGCGCCAAAATG	470
1.4	F – CATTTTGGCGCCTTTTCCT R - CCAGGACTAAAGGGGAGGAG	352

Patients' baseline characteristics harboring a *Pim-1* promoter region mutation was compared with wildtype *Pim-1* (Table 3). Only 13.5% of patients harbored promoter region mutations compared to 86.4% with wildtype or native sequences. *Pim-1* promoter mutations were not significantly associated with any of the pathological parameter such as menopause, tumor grade, receptor expression and c-Myc expression.

Table 2: Promoter sequence (1.7 kb) of *Pim-1 gene*. Sites of transcription factor binding are indicated as different colored boxes. Blue Box – AP2; Green Box – Sp1; Red Box – PPF-348. Mutations noted in the sequences indicated in Black Box; C>A at –502 and a deletion at –754Cdel.

-1704 ctttgaat actcaggagg tgaatttggg tcatttcatc tcctctctta gctgctgtat

-1646 gaccctgtgc catcctattt atgtttcatt ttcctatctg caaaaaggga gtaattcttg

-1586 ttcccttggg ttttctccta gtcataaaaa atgaatgagt tcggccacag tacacaaaca

-1526 aaaggaaaat atgtaatatt ttgtaggata aattcataaa agttgtggag gatctgggca

-1466 cttttataaa gcaagcactg tagaaaactt tcaaaggaaa catttaaatt cactaatgac

-1406 agctataggg atcctgattt ttcctttaaa tggcaggcac ttcaaaaaat taaaatagaa

-1346 tgtagttagc tttcaattac ttaggccact gacaattcaa tttatggatt atatagtatt

-1286 ttaatttact catttcacac gtcgttatca actctacatg aagtttaaaa ggacaaagga

-1226 tgcaagtgga attggtcagt gttccgggtt atttccaagg gaggcagagg gtgggggttt

-1166 tcctttgaga caagacttgg ggttggccaa taattgctgg tattgcctgc ctggtaataa

-1106 caggctgatg aaaaggtgac tacaacgtga aaactggtta aatcaagcgc accctcccac

-1046 cctcgtttta gatgaggaat tttccgccct cacagaaggg gctgaggcag catctggcat

-986 cacaacacta acatttgctt cgtgatttcc tctttacccg gccctttgac acacatccct

-926 tcccagaaat caggattcgc tggtgctttt gcatttctaa aatgggaatc ccgtggctga

-866 gcttttagcc ggccggaacg actgagggct gcatcccttt cccgcaggag cggggctccc

-806 gcctccagtt ctgttccagg cctgactcct ccactccctc cgtgactcat gtgctgcgga

-746 tccttcgccc ccgacgcgcc ccccaacaca caaaccccca gaatccgccc ccagcctaca

-686 gcgcgacgtc agcccgcccc agccgacttg gaggtctcgg gtctgagtca cacagaaaga

-626 ccaccctcgt cggcatcccc acacacagtc cgacacccgg cgcgccggcc tccccgcctg

-566 acacactaac gcccgtcgtc tccgcgcaac ttgttatgct ccggctcgag cccttgaccc

-506 aaaacctcta gcgaaacgga gagccgcaga gccggcctcg ggcggccttt gatggctttg

-446 ttattgtttg ggtttgaatc gatacgcccc tccccatcct tcctccctcg cggccctaca

-386 cccagctccc gcctcccctc acgccccgcg cccctccccc tccattttgg cgcctttcc

-326 ttcccgccac gtcgtggcgg cgtagagacc attctgaccg cgagagcttgg gcgggggcggc

-266 ggcgggggcc gccgagttat gcagatcaat cggcctctgg ttggctggag tagcgctggc

-206 agggggcgggg ccggggcgcg gccacagagc gcgcggggcg ggggccgagg ggagtcgccc

-146 agtcccgccg cttccccacc ccctctcctc cctcggccgg cccggcagcc ctgctccccg

-86 ccttggcctc ccggagaggc cccgccccgc ccccgcccgc cgcgcccctcc ccgcgcgccc

-26 tccccgccgc cgcgctcctc cccttt

Table 3: Characteristics of patients according to *Pim-1* promoter region mutational status.

		All n (%)	*Pim-1 promoter mutated n (%)*	*Pim-1 promoter WT n (%)*	*p value*
Patients		96	13 (13.54)	83 (86.46)	
Age Median (range)		48 (23 – 75)	48 (35 – 72)	48 (23 – 75)	
Pre-menopause		55 (57.29)	07 (12.78)	48 (87.22)	0.913
Post-menopause		41 (42.71)	06 (14.63)	35 (85.37)	
Grade					
	I	08 (8.33)	01 (12.50)	07 (87.50)	0.782
	II	54 (56.25)	08 (14.81)	46 (85.19)	
	III	34 (35.42)	04 (11.76)	30 (88.24)	
Estrogen receptor					
	Positive	61 (63.54)	05 (08.20)	56 (91.80)	0.085
	Negative	35 (36.46)	08 (22.86)	24 (77.14)	
Progesterone receptor					
	Positive	61 (63.54)	05 (08.20)	39 (91.80)	0.085
	Negative	35 (36.46)	08 (31.43)	24 (77.14)	
HER2 receptor					
	Positive	7 (7.29)	02 (28.57)	5 (71.43)	0.116
	Negative	89 (92.71)	11 (12.36)	78 (87.64)	
c-Myc expression					
	Positive	52 (54.16)	07 (13.46)	45 (86.54)	0.693
	Negative	44 (45.84)	06 (13.64)	38 (86.36)	
DFS (Median in months)		37.43	45.52	41.94	0.3021*
OS (Median in months)		48.27	45.84	47.26	0.7195*

*Log rank test.

We next continued to evaluate the expression and cellular localization of c-Myc in FFPE block of patients with breast cancer by immunohistochemistry, so as to comprehend any effect of *Pim-1* promoter mutations on c-Myc expression as *Pim-1* is known to phosphorylate and stabilize c-Myc. Positive expression of c-Myc was noted in 54.16 % of the patients which included 7 patients with promoter mutations. c-Myc expression was feebly cytoplasmic in adjacent normal breast epithelial cells (Figure 1a). Certain patients' population (46%) showed diffused cytoplasmic staining (Figure 1b) and considered as negative c-Myc expression. Patients who showed positive expression for c-Myc presented with either both nuclear and cytoplasmic staining (Figure 1c) or strong exclusive nuclear expression (Figure 1d).

Kaplan-Meier survival analyses were carried out to ascertain the impact of *Pim-1* promoter mutation (Figure 2a & b) and c-Myc expression (Figure 2c & 2d) on patients' disease-free survival (DFS) and overall survival (OS). *Pim-1* promoter mutations did not significantly influence both DFS and OS as patients with and without promoter mutations had almost similar survival rate.However, patients with negative c-Myc expression (p=0.0013) had distinctly improved median disease-free survival (DFS) as compared to patients with positive protein expression. Conversely, there was no considerable difference when computed for overall survival (OS) between the studied groups.

Discussion

The mutations in the promoter region of the oncogenes could influence the gene expression and play important role in the tumorigenesis, tumor progression and drug resistance [13]. Breast cancer is known to carry promoter sequence mutations in genes such as *TERT, AKT1, BRCA2, PRDM13, SLIT2, ELAVI2,* etc [14]. However, significance of *Pim-1* promoter sequence mutations in the breast cancer remains indistinct as it has not been investigated extensively. From our findings, we deduced that *Pim-1* promoter sequence mutations occur in a small percentage of breast cancer patients with

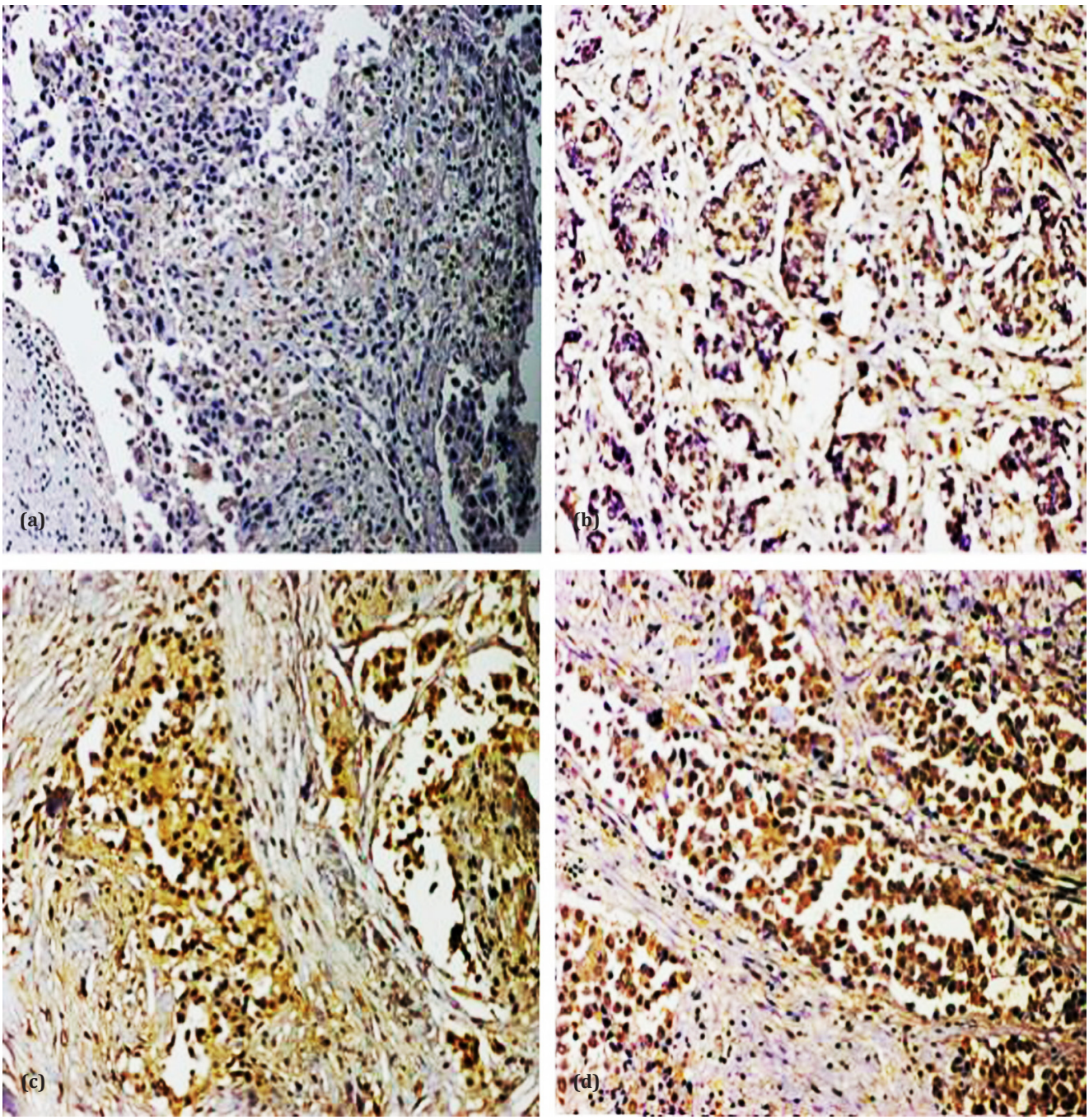

Figure 1: Immunohistochemistry of c-Myc in adjacent normal and breast tumor tissue. (Original magnification x 400), (a) Feeble cytoplasmic staining in adjacent normal breast tissue, (b) Diffused cytoplasmic staining in breast tumor tissue and regarded as Negative, (c) Both cytoplasmic and nuclear staining in breast tumor tissue and regarded as Positive, (d) Strong exclusive nuclear staining in breast tumor tissue and regarded as Positive.

negligible association with tumor pathology. However, these interpretations need further validation in large cohorts to evaluate the factual impact on breast tumor clinicopathology.

c-Myc is an essential signaling hub in several cellular processes that control cell proliferation, differentiation and death. Activation of c-Myc has been a common phenomenon in breast cancer progression [15, 16]. It has been reported that c-Myc is elevated in TNBC compared with other subtypes [17]. However, in our study, overexpression of c-Myc was observed in 50% of the studied population and distributed in all sub-types of breast cancers. Furthermore, there was no significant association between *Pim-1* promoter mutation and c-Myc expression.

Previous studies revealed the high prevalence of core promoter variation in breast cancer which can alter gene expression [14]. But, location of promoter mutation is the key to implicate in disease process as mutations at functional region of promoter may interrupt the

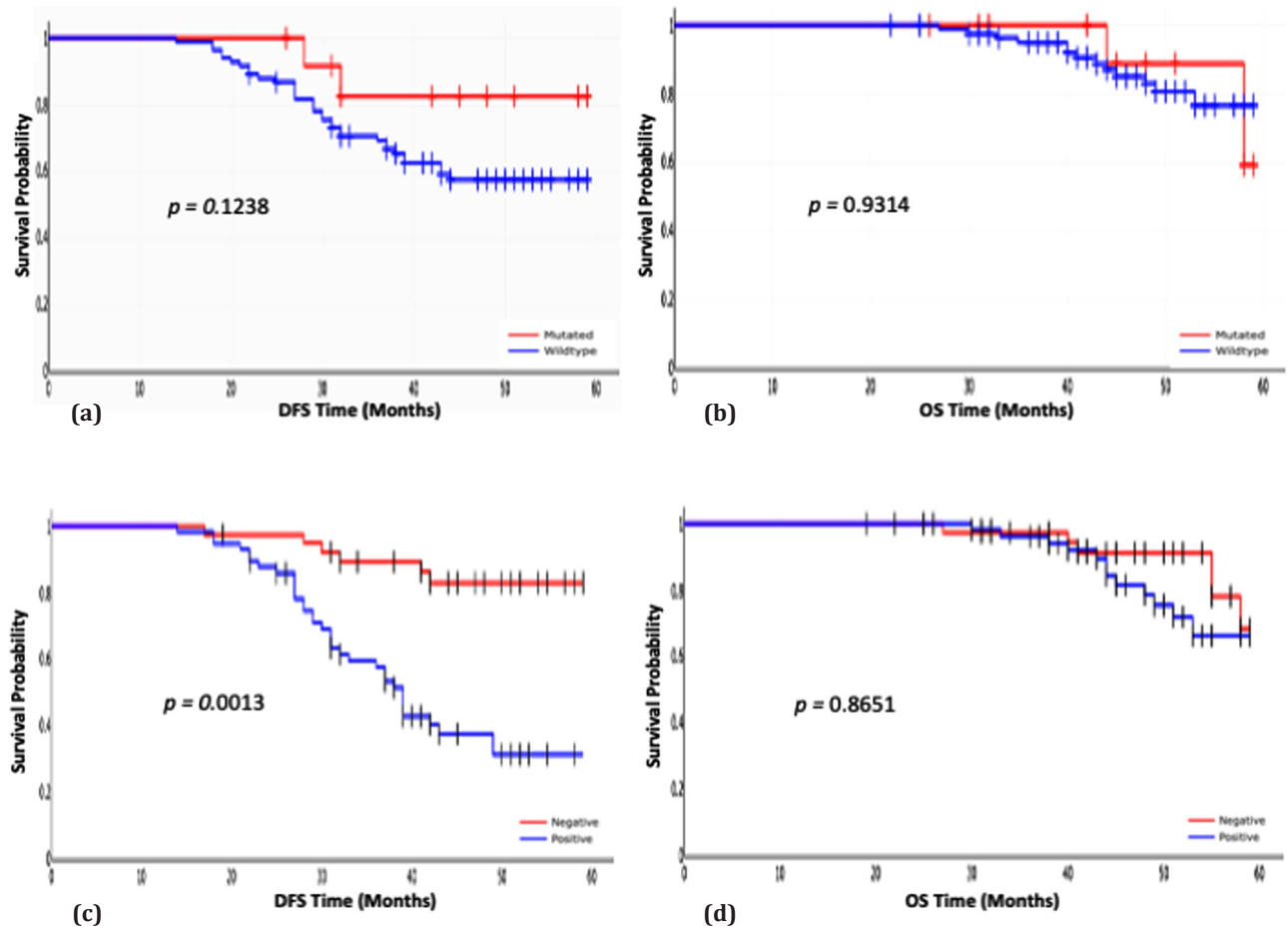

Figure 2: Kaplan–Meier survival curve showing the association of *Pim-1*Promoter mutation and c-Myc expression with disease-free survival (DFS) and Overall survival (OS). (a) Disease-free survival (DFS) of patients with mutant and wildtype *Pim-1*Promoter region, (b) Overall survival (OS) of patients with mutant and wildtype *Pim-1* Promoter region, (c) Disease-free survival (DFS) of patients with positive and negative c-Myc expression, (d) Overall survival (OS) of patients with positive and negative c-Myc expression.

normal processes of gene activation by disrupting the ordered deployment of transcription factors [18]. Since, promoter mutations observed in our study are present outside the supposed transcriptional factors [9], we presume that they may not have any potential impact in modulating the transcriptional activity of *Pim-1* gene, hence having negligible impact on patients' survival.

Several studies reported that a higher expression of c-Myc was observed in breast cancer tissue and associated with a rapid disease recurrence and progression [16, 17, 19], which is in line with our findings. Furthermore, Qu et al [20] have reported that c-Myc overexpression was associated with worse prognosis with reduced DFS and OS in a long-term follow-up study.

Conclusion

We are herein for the first time, report the presence of *Pim-1*promoter sequence mutations in breast cancer. We observed a transversion (C>A at –502) and a deletion (–754Cdel) mutation in the promoter region with negligible association with tumor biology and c-Myc expression. Likewise, mutations were not significantly associated with patients' survival rate. We further observed positive expression of c-Myc in almost 50% of the studied population and that patients with negative c-Myc expression showed improved median disease-free survival. Population based clinical study is warranted to ascertain the factual impact of *Pim-1* promoter mutations in breast cancer so as to assess its importance in disease prognosis.

Acknowledgements

We are grateful to KIMS Foundation and Research Centre for funding this project. We are thankful to all the patients who participated in the study. We would like to further acknowledge that there have been many other individuals who made a significant contribution to this study.

Conflict of Interest

All authors disclose no potential sources of conflict of interest.

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