Saudi Journal of Biological Sciences 26 (2019) 1123–1128

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Molecular analysis of V617F mutation in Janus kinase 2 gene of breast cancer patients

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ARTICLE INFO

Article history:
Received 27 May 2019
Revised 31 July 2019
Accepted 1 August 2019
Available online 2 August 2019

Keywords:
Breast cancer
JAK2
V617F mutation
Allele-specific PCR
Sanger's sequencing

ABSTRACT

Background: Breast cancer is a multifactorial disease with the highest frequency in females. Genetic and environmental factors can cause mutation in several genes like tyrosine kinase, JAK2 gene which may initiate cancer. Molecular analysis of mutations in the *JAK2* gene along with determination of environmental, clinical and haematological risk factors associated with breast cancer patients is need of hour to improve patient's healthcare. Somatic JAK2 valine-to-phenylalanine (617 codon) mutation is one of the widely prevalent mutations.

Methods: Blood was collected from seventy breast cancer patients after their consent. The questionnaire included risk factors, age group, locality, number of children, tumor type, family history, time of initial diagnosis, no of cycles/month, water conditions and exposure to radiations. Molecular analysis were carried out from genomic DNA using Sanger sequencing and allele-specific PCR to check the V617F point mutation.

Results: The breast cancer risk factors includes unfiltered water (68.57%), urban (58.57%), menopause (55.71%), family history of cancer (18.57%), tumor grades (II, 37.14% and III, 35.71%), consanguineous marriages (44.28%) and having more than 3–4 children (45.71%). Prevalence of breast cancer was higher after the age of 35 and maximum at 35–50. In allele-specific PCR of 70 patients, 25 patients were wild type (229 bp), 25 patients were with partially deleted gene (200 bp), and 20 patient had shown no or less than 40 bp size fragments. In Sanger's sequencing of 70 BC cases, 18% were found to be positive for V617F point mutation, including 6 homozygous (T/T) and 7 heterozygous (G/T) mutations at nucleotide position 1849 in exon 14 of the *JAK2* gene.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.08.002

Conclusions: Environmental and clinical risk factors were associated with breast cancer which can be overcome by improving awareness of associated risks, health facilities and reducing stress.

1. Introduction

Cancer is basically abnormal growth of cells with high potential to divide and spread to all parts of the body to form tumors at new places (Jayasekara et al., 2016). Cancer is a leading cause of death all over the world and main cancers are that of the breast, stomach, lungs, liver and colorectal cancer with an estimated 9.6 million deaths reported in 2018 (WHO 2018). According to the World Health Organization, in Pakistan, a death rate of 30.08% is due to breast cancer (BC) and ranked as the major cause of death in the country (WHO 2014). There is involvement of certain molecular and environmental factors that may change the expression of gene, genetic functions of gene and function of cells that leads to mutation (Ahmed et al., 2016).

Janus associated kinase 2 (*JAK2*) gene is present at chromosome 9p24.1 in humans. Deregulation of JAK-STAT (Signal Transducer and Activator of Transcription) signalling pathway is implicated in the promotion of oncogenic phenotypes encompassing tumorigenesis, invasion, metastasis, proliferation, survival, angiogenesis, anti-apoptosis and immune evasion (Balko et al., 2016). Involvement of JAK2 in myeloproliferative neoplasms is well-established (Bader and Dreiling, 2019; Mata et al., 2007; Panani, 2009; Xu et al., 2014). It is also associated with other malignancies like leukaemia (Singh et al., 2018) and cancers of skin (Pritchard et al., 2018), lung (Li et al., 2017; Xu et al., 2017), and stomach (Judd et al., 2014).

Missense substitution mutation V617F of *JAK2* gene (COSMIC ID: COSM12600) causes malignant transformation and proliferation and has been associated with triple-negative BC cases exhibiting poor prognosis and short survival time (Stanek et al., 2017). Mutation (V617F) in the *JAK2* gene is the results of G to T substitution at 1849 nucleotide position resulting in GTC converting to TTC at codon 617. In the wild types normal sequence G/G is present on both loci while in the mutants sequences it could be either heterozygous with one wild and another mutant allele (G/T) or homozygous with both the mutant alleles (T/T) (Tabassum et al., 2014).

2. Material and methods

2.1. Sample collection

Blood sampling was carried out from INMOL hospital, Lahore in collaboration with University of Health Sciences, Lahore, Pakistan. Total seventy blood samples (n = 70) of BC patients were included in current research with their consent and with the approval of the local ethical committee. Blood samples were collected in 5 ml EDTA tubes and were stored at 4 °C till use. Questionnaire was designed to know the patient's clinical history including environmental and socio-economic risk factors.

2.2. Clinical history

The clinical history was taken directly from patients which included age group, locality, lactation period, number of children, tumor type, marital status, dietary factors, family history of cancer, time of initial diagnosis, number of cycles per month, water conditions and exposure to radiations.

2.3. Extraction of genomic DNA and allele-specific PCR

300 μL blood of patients were used for the extraction of genomic DNA by using commercially available kit (QIAGEN) which were then subjected to electrophoresis with 1% agarose gel to check the quantity/quality of DNA. For the mutational analysis, Polymerase Chain reaction (PCR) analysis were carried out using four different sets of the *JAK2* gene primers as reported (Jones et al., 2005) (Table 1) to check the mutation (V617F) point in which valine gets changed into phenylalanine (G → T mutation). We wanted to check both the wild and mutant type gene in BC patients represented by 229 bp fragment of PCR for the wild type allele and 479 bp fragment for the mutant allele.

2.4. Direct Sanger sequencing of the amplified exon 14 of JAK2 gene

For all BC patients, exon 14 of the *JAK2* was amplified in 20 μL reaction volume with 10pMol of primer pair and sequenced by Sanger's method after purification. Standard sequencing protocol were followed using BigDye® Terminator v3.1 and Cycle Sequencing Kit (Applied Biosystems, USA). BioEdit tool was applied to analyze and detect mutations in sequenced data using NCBI GeneBank accession number [NG_009904.1] as reference for alignment. To ensure the heterozygous mutation detection, experiment was repeated with forward and reverse primers whenever noisy background found.

3. Results

3.1. Environmental risk factors

Several environmental risk factors were found associated with BC patients. Results showed that the use of unfiltered water (68.57%), urban living conditions of most of the patients (58.57%), poor economic status of the BC patients, low standard and unhygienic conditions were the main cause of BC. The residency of most patients was near to the factory area and the adjoining land present from where they fulfill their dietary needs most likely had a high concentration of harmful chemicals present and this possibly causes mutations as reflected by changes in the amino acid sequence (Table 2).

3.2. Clinical factors risk factors

The clinical factors were found associated with the Pakistani female who had BC. Seventy BC cases were enrolled of age between 20 and 65 years. The percentage of BC patients were found maximum those who have initial diagnosis within six months after

Table 1
Primers for the amplification of human *JAK2* (exon 14, V617F) gene.

Sr. no	Primer name	Base length	Nucleotide sequence (5′–3′)
1	JAK2 (FO)	23	AATGCTTTCCTTTTTCACAAGAT
2	JAK2 (RO)	21	TCCTCAGAACGTTGATGGCAG
3	JAK2 (R-Mutant) G > T	27	GTTTTACTTACTCTCGTCTCCACAAAA
4	JAK2 (F-Wild)	29	GCATTTGGTTTTAAATTATGGAGTATATG

Table 2
Environmental risk factors involved in breast cancer patients (n = 70).

Serial no	Environmental conditions		No. of patients	Percentage prevalence
1	Water	Filtered	22	31.42%
		Unfiltered	48	68.57%
2	Locality	Urban	41	58.57%
		Rural	29	41.42%
3	Status	Poor	36	51.42%
		Normal	34	48.57%

symptoms (39.51%), menopause (55.71%), family history of cancer (18.57%), tumor grading of cancer patients were more at 2nd and 3rd stage (37.14 and 35.71%), cousin marriages (44.28%), and the number of children's or pregnancies more than 3–4 (45.71%) (Table 3).

3.3. Biochemical parameters

Biochemical factors are included in the BC research and certain biochemical test like Creatinine, ALT (Alanine aminotransferase), bilirubin, AST (Aspartate aminotransferase), BUN and ALP (Alkaline Phosphatase) were studied. The normal range of ALP is 115–359, ALP values were high in 33 (47.14%) patients and 20 (28.57%) patients had low values. So, there was abnormal production of ALP in BC patients and the values of ALT and AST were also high in 20 and 23 patients respectively and low in 18 and 27 patients respectively. The values of the ALT and AST were disturbed due to malignancy and ongoing burden of chemotherapies because drug toxicities in the liver make these values high and when patient have malignant stage of BC then these values were disturbed as shown in Table 4. The values of BUN, creatinine, and bilirubin were not much disturbed as other biochemical values effected in BC patients.

3.4. Molecular analysis by allele-specific PCR

Allele Specific PCR was carried out to determine the mutation at position c.1849G > T in the *JAK2* gene of BC patients. Out of 70 patients, there were no mutation detected in 25 patients who have wild type gene (product size, 229 bp) (Fig. 1), 25 patients have partially deleted gene (fragment size, 200 bp) (Fig. 2) means that there were partial deletion at chromosome 9 at point 24.1 and 20 patients have shown no fragment size or less than 40 bp fragment size (Fig. 3) means their 24.1 part fully deleted at chromosome 9 (Table 5).

3.5. Sanger sequencing for V617F mutational screening of JAK2 gene

We screened whole exon 14 of the *JAK2* gene of all BC cases to detect possible G to T substitution at 1849 position resulting in V617F missense mutation at the protein level. We found the mutation in thirteen BC cases (18.56%) including six homozygous (G to T substitution, TT alleles) (Fig. 4) and seven heterozygous (G to T substitution, GT alleles) (Fig. 5).

4. Discussion

Presently, BC presents an awful scenario to doctors and scientists as the ratio of prevailed BC is quite greater after the lungs cancer worldwide including Pakistan. Approximately 1 million population are having BC and most of them are detected at advanced stages of malignancy (Ginsburg, 2013).

The most important factor that counts in BC risk factor analysis is the stress and literacy rate in Pakistan population. Most of the patients were familiar with the cancer symptoms and when they observed lump or any hard part near the breast or the nearby areas of breast (underarms) went for treatment and got diagnosed with BC. Awareness, literacy rate, and proper treatment can diagnose cancer at early stages (Di Lascio and Pagani, 2017). The most important thing is hesitation and sense of shame by females to express the symptoms of the disease to their families and in-laws, as a result, cancer develops from breast and gradually spreads to the whole body.

The other risk factor is the financial stress in the BC patients due to poor income. This financial stress is very harmful to the body because the stress related hormone cortisol has direct link with the carcinoma and it impairs the DNA activity and repair mechanism, and causes harmful impact on the body in general.

Table 3
Clinical parameters of breast cancer patients (n = 70).

Serial no	Clinical parameters of breast cancer patients		Total no of breast cancer patients (positive)	Percentage prevalence in breast cancer patients
1	Age	20–35 Years	16	22.85%
		36–50	32	45.71%
		>50	22	31.42%
2	Time of diagnosis about breast cancer	0.5 Year	16	22.85%
		0.6–1 Year	44	39.51%
		>1 Years	10	14.28%
3	Consanguinity	Yes	31	44.28%
		No	39	55.71%
4	No of children	0–2	17	24.28%
		3–4	32	45.71%
		>4	21	30%
5	Lactation period	0.6–1 Year	33	47.14%
		>1 Year	37	52.85%
6	Menstrual cycle per month	1/month	15	21.42%
		>1/month	16	22.85%
		Menopause	39	55.71%
7	Family history about breast cancer	Yes	13	18.57%
		No	57	81.42%
8	Tumor type	I	9	12.85%
		II	26	37.14%
		III	25	35.71%
		IV	10	14.28%

Table 4
Biochemical analysis parameters of blood of breast cancer patients (n = 70).

Serial no	Biochemical tests	Samples with high values	Samples with low values	Samples with normal values	Normal range
1	Alkaline Phosphatase (U/L)	33(47.14%)	20(28.57%)	17(24.28%)	115–359 U/L
2	Alanine aminotransferase (U/L)	20(28.57%)	18(25.71%)	32(45.71%)	Up to 40 U/L
3	Aspartate aminotransferase (U/L)	23(32.85%)	27(38.57%)	20(28.57%)	Up to 35 U/L
4	Bilirubin (mg/dL)	0(0%)	0(0%)	70(100%)	0.3–1.2 (mg/dL)
5	Blood Urea Nitrogen (mg/dL)	1(1.42%)	0(0%)	69(98.57%)	3–20 (mg/dL)
6	Serum Creatinine (mg/dL)	3(4.28%)	26(37.14%)	41(58.57%)	0.7–1.4 (mg/dL)

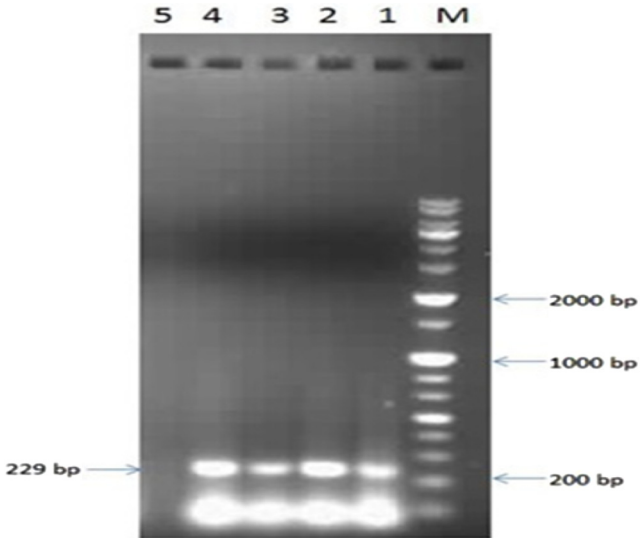

Fig. 1. Agarose gel photograph of PCR product showing wild type of JAK2 (exon 14) gene as detected by presence of 229 bp amplified fragments. **M** is 1 Kb DNA ladder and lane **1**–**4** are PCR product of breast cancer patients.

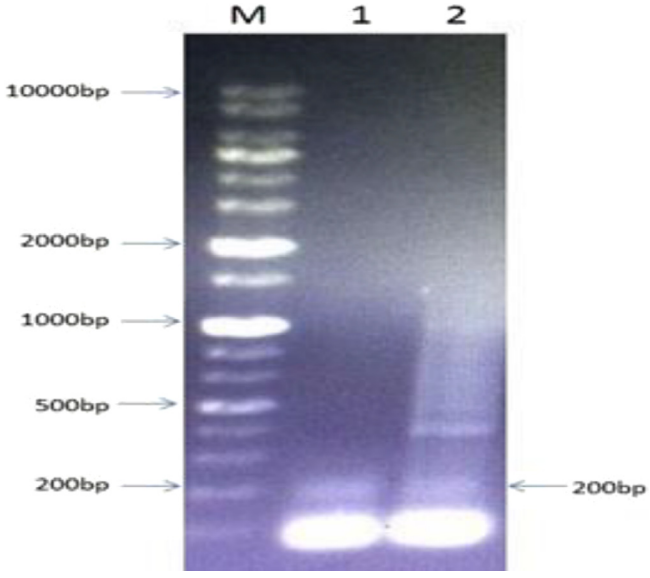

Fig. 2. Agarose gel photograph of amplified PCR product showing partial deletion of JAK2 gene as detected by the presence of 200 bp and absence of 400 bp fragment. **M** is 1 Kb DNA ladder and lane **1 and 2** are PCR product of amplified fragment of breast cancer patients.

In this study, which was conducted on the Pakistani population, the females were divided into three groups viz., 20–35 years: 16 females (22.85%), 36–50 years: 32 females (45.71%) and above 50: 22 females (31.71%) and BC rate was maximum between the

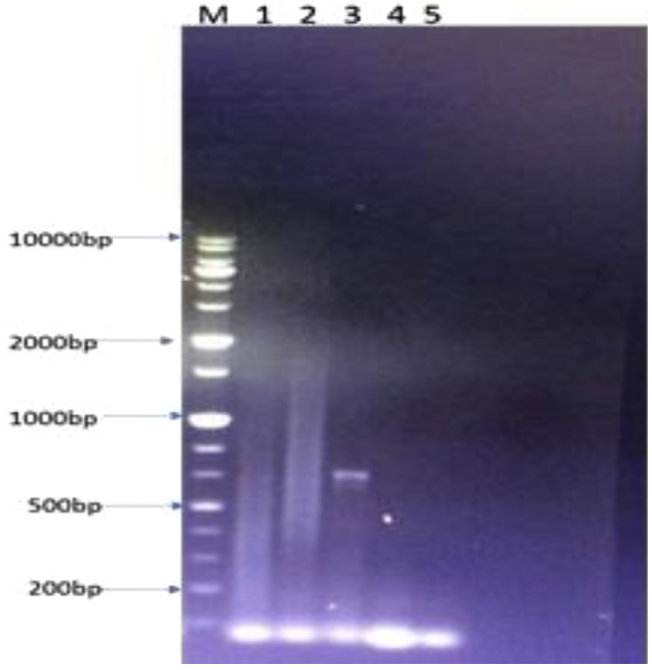

Fig. 3. Agarose gel photograph of PCR product showing full deletion of JAK2 gene at chromosome 9 as detected by absence of 229 bp amplicon. A 700 bp fragment size is a result of random amplification. Lane **M** is 1 Kb DNA ladder and lane **1**–**5** are PCR product of breast cancer samples without amplification.

age of 35–50 and it seems in this age BC risk is more. Whereas, contrastingly in a study conducted in Europe showed that females have BC at the age of 45 or above (Kelsey and Horn-Ross, 1993).

The other risk factor observed was late diagnosis and lack of treatment opportunities in Pakistan. Lack of annual mammography test results in late stage diagnosis of BC. In Pakistani population, diagnosis at stage II and III is more common due to delay in treatment and diagnosis. The percentage of type I tumor was (12.85%), type II (37.14%), type III (35.71%), and type IV (14.28%) which proves that type II and III are more frequent in patients due to lack of facilities for early diagnosis and treatment. According to the American Cancer Society, early screening, detection, and treatment may decrease BC risk successfully. Due to late diagnosis, usually metastasis occurred which spreads in the body after stage II. In our study, women who have more than one-year lactation period reported cancer which is contradictory with other studies in which lesser lactation period causes higher cancer frequency (Newcomb et al., 1994).

Further tests conducted at the molecular level may detect the tumor and location of tumor in the body followed by advanced techniques like RT-PCR, ELISA, and high throughput microarray technology (Slade et al., 1999). The results of these techniques can further guide for better targeted treatment options for BC (Osteen et al., 1994).

Allele Specific PCR was carried out to check the mutation in BC patients but half of the patient have no heredity link with the dis-

Table 5
Distribution of alleles in breast cancer patients determined by allele-specific PCR.

Gene	Wild type allele and percentage	Mutant type allele and percentage	Partial deleted gene and percentage	Fully deleted gene and percentage
JAK2 (V617F)	25 (35.71%)	0 (0%)	25 (35.71%)	20 (28.57%)

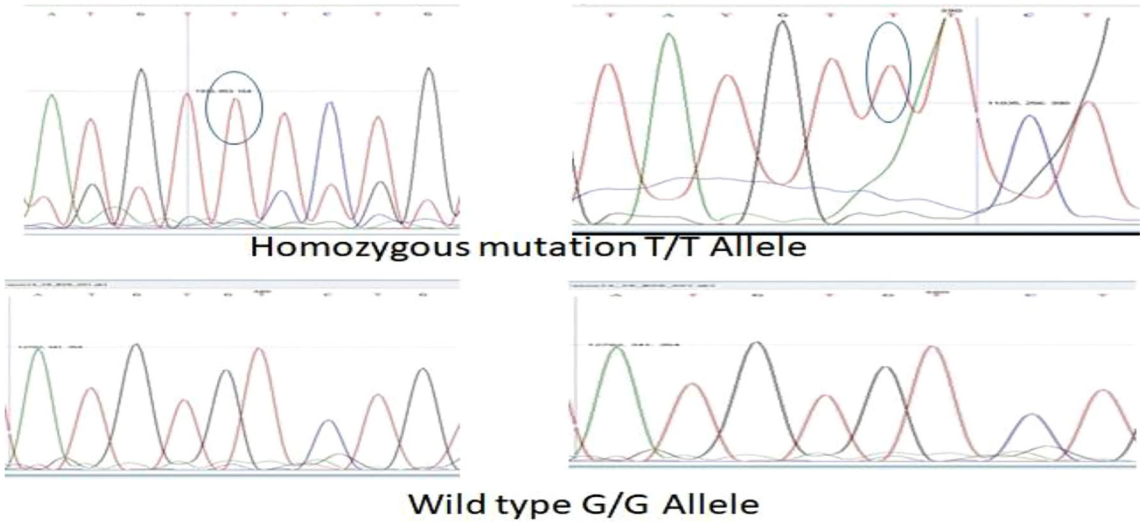

Fig. 4. Electropherogram showing homozygous mutations in JAK2 exon 14 [1849 G → T substitutions in both allele (T/T), resulting in Valine to Alanine (V617A) substitutions] of BC patient. For alignment and analysis NCBI gene accession number (NG_009904.1) was used as reference sequence.

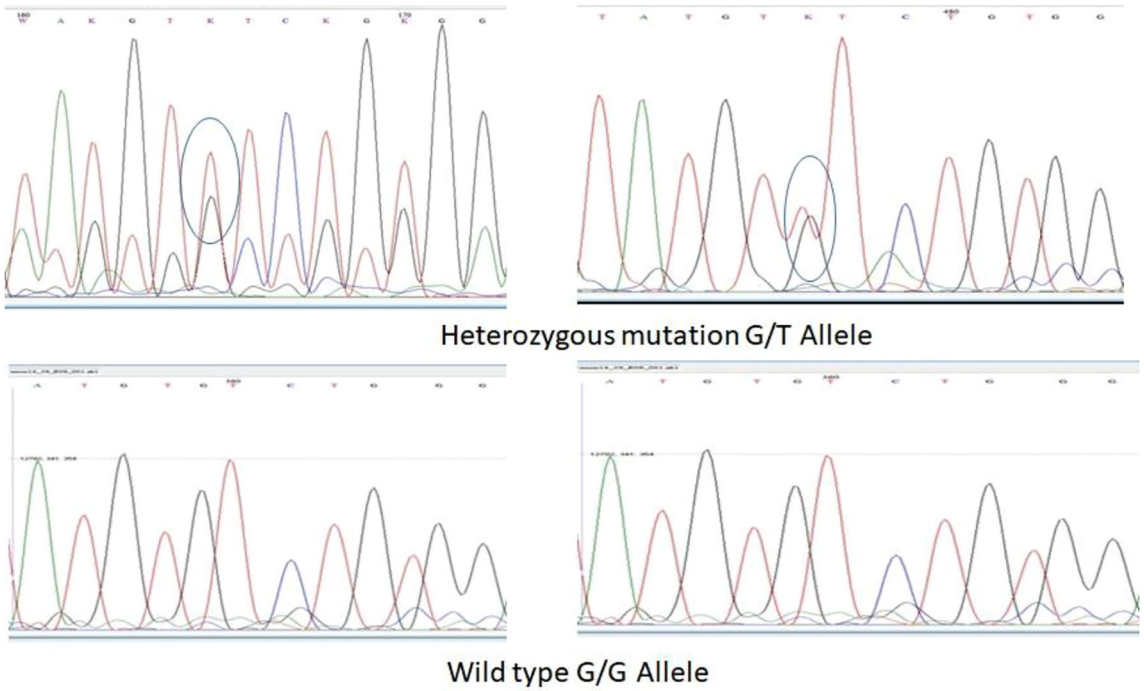

Fig. 5. Electropherogram showing heterozygous mutations in JAK2 exon 14 [1849 G → T substitutions in one allele (G/T), resulting in Valine to Alanine (V617A) substitutions] of BC patient. For alignment and analysis NCBI gene accession number (NG_009904.1) was used as reference sequence.

ease, the risk factors are implicated on BC. Most of the patients have wild type fragment size which means they have no mutation in the gene at the chromosome 9, and half of the patients have partial deletion have fragment size of 200 bp means they have some part of gene deleted on the chromosome 9. and on other hand some patients have no amplified fragment either on 200 or 229 that shows the gene is totally deleted in those patients because this gene is present at the ends of chromosome 9p at position 24.1. This is in accordance with a previous study on *JAK2* (V617F) in diagnostic labs from 2006 to 2016, where only 1.4% mutation in 13,411 patients and 0% mutation allele was present in the patients (Langabeer and Haslam, 2017). Telomere dysfunction are strongly associated with the BC and short arm telomere are considered as genetic effect in BC. Hormonal risk factors are playing major role in the BC revelation. So, after this it is reported that chromosomal instability is well involved in the BC having the chromosome arms at 17q and 20q

because of gain and due to loss 8p, 9p, 16a and 17p chromosomal dysfunctionality effects (Baudis, 2007). At the end, environmental and clinical risk factors were found associated with the BC in Pakistan which can be overcome by improving awareness, health facilities and reducing stress. In total seventy BC patients, 45 (64.28%) showed alterations (V6117F) in exon 14 of *the JAK2* gene.

Valine to phenylalanine is in itself a disfavoured substitution pathogenic mutation. Jak2-V617F lies in pseudokinase domain (JH2) and this mutation rigidifies α-helix C in the N lobe of JH2, facilitating trans-phosphorylation of tyrosine kinase domain (JH1) (Bandaranayake et al., 2012). This hotspot mutation is hyperkinetic and constitutive activation of Jak2-V617F is mediated by a π-π ring stacking mechanism involving F594 (αC), F595 (αC) and mutant F617 (Silvennoinen and Hubbard, 2015). Gene suppression via epigenetic modifications has been suggested as a possible reason for the resistance of Jak2 V617F against the cell signalling regulators (Gnanasambandan and Sayeski, 2011).

Notably, JAK2 (V617F) mutation is omnipresent in nearly all patients suffering from polycythemia vera and very commonly in patients suffering from thrombocythemia and primary myelofibrosis. Infact, potent inhibition of JAK2 by a novel substituted quinoxaline, NVP-BSK805, has been already reported This inhibitor has a good bioavailability and a long half-life, acts in an ATP-competitive manner, exhibits more than 20-fold selectivity towards JAK2 in vitro, and blunts constitutive STAT5 phosphorylation in JAK2 (V617F)-bearing cells, with concomitant suppression of cell proliferation and induction of apoptosis (Baffert et al., 2010). This particular mutation has also been implicated in non–small cell lung cancer (Lipson et al., 2012).

5. Conclusion

Present study establishes association of genetic and environmental factors with JAK2 gene in BC patients in Pakistan. Allelic variation of JAK2 V617F point mutation could provide a better understanding of BC progression and pathogenesis. In-depth structural analysis of JAK2-V617F has potential to pave way for targeted therapies using small rationally designed selective inhibitors.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgement

We are thankful to Dr. Ghulam Mujtaba, Institute of Nuclear Medicine and Oncology, Lahore, Pakistan for providing breast cancer samples for research work.

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