RESEARCH ARTICLE

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Predisposition of an Intronic Duplication in *CHEK2* Gene in the Cases of Breast Cancer from Balochistan

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Abstract

Objective: After *BRCA 1&2, CHEK2* is the most frequently predisposing altered gene causing breast cancer in female. The prime objective of the current study was to analyze germline *CHEK2* variants and their association with breast cancer in Balochistani population. **Methods:** Breast cancer is among the most prevalent cancers worldwide and most common diagnosed cancer in women. Mutations in many proto-oncogenes and tumor suppressor genes lead to the development of cancer. Along with the highly penetrance genes *BRCA1* and *BRCA2* increase the risk of breast cancer, mutations in other genes including *CHEK2*, a tumor suppressor gene have also been reported to be associated with breast cancer. In current study *CHEK2* gene was analyzed for variation in breast cancer patients and controls of Balochistani population. Sequencing results of the DNA samples of the registered cases of breast cancer in CENAR were analyzed using Chromas software and bioinformatics tools including BLAT and RNAfold web server. **Results:** An intronic variant c.319+38-43dupA falling 38 nucleotide away at splice donor site of the *CHEK2* gene exon 2, in 9% breast cancer cases, was identified which has also been previously reported in non-Hodgkin Lymphoma cases. All the cases with identified variant, were affected with invasive ductal carcinoma. Higher tumor grade (III) was reported in >50% of the patients and > 70% of patients diagnosed with advanced stage of cancer. The RNA prediction results revealed the variant falling in the intronic region may code for miRNA that could play an important role in cancer progression. **Conclusion:** Our results suggest that the intronic variant identified in breast cancer cases as well as reported previously may act as a cancer marker and causing a splice site disruption or altering the posttranscriptional modification of mRNA encoded by *CHEK2* gene.

Keywords: Intron- Variant- Ethnic- Stage- Grade

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Introduction

Breast cancer, a global health concern, is a complex disease resulting from diverse lifestyle and genetic risk factors [1]. It is among the most highly prevalent cancers accounting ~1 in every 8 cancer cases diagnosed and is the most common cancer being diagnosed in women with a growing prevalence worldwide particularly in developing countries [2, 3]. It accounts 1 in every 6 cancer deaths in women globally [4, 5]. The occurrence of breast cancer is even worldwide but the rates of incidence, survival and mortality are considerably varying associated with the population structures, environmental, lifestyle and genetic factors.

The survival rate from breast cancer being reported has improved in past 2 -3 decades with even a high incidence rate whereas in various localities high mortality rate has also been reported that indicate a diverse pattern of breast cancer incidences worldwide [4, 6]. Various factors causing sporadic and hereditary mutations in many tumor-suppressor and proto-oncogenes influence the occurrence to breast cancer. The highly penetrance genes involved in increasing the risk of breast cancer are *BRCA1* and *BRCA2* with ~50% of the hereditary breast cancer cases whereas mutations in other genes including A*TM, TP53, BRIP1, CHEK2, CDH1, PALB2* and other low penetrance genes also increase the risk of breast cancer [7].

The *CHEK2* gene (ENST00000382580.6) located on 22q12.1 (Figure 1) coding for checkpoint kinase 2 (a human Cds-1 related kinase) that response to DNA damage, activated through phosphorylation by ataxia telangiectasia mutated (ATM) gene, resulting in modifications of the *p53, CDC25A,CDC25C, KAP1* and *BRCA1* genes substrates downstream, producing a

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signaling cascade to instruct cells to repair DNA damage and stop proliferating or induce apoptosis [8-10]. *CHEK2* gene encodes a tumor suppressor protein that regulate cell cycle by repairing DNA damage, mutations to *CHEK2* gene cause disruption to the normal function of this protein and leads to the development of cancer. Mutations to the *CHEK2* gene such as 1100delC has been reported as a very common variant associated with the risk of breast cancer. *CHEK2* gene variants (pathogenic and likely pathogenic) have been reported to be associated with breast cancer [11-13]. The association of variants in *CHEK2* gene with other cancers like Li-Fraumeni Syndrome, Kidney, Prostate, colorectal, stomach and thyroid have also been reported [14-16]. The prime goal of current study was to analyze germline *CHEK2* variants and their association to breast cancer in Balochistani population.

Materials and Methods

Current study was performed on100 breast cancer cases enrolled in Center for Nuclear Medicine and Radiotherapy (CNAR), Quetta for chemo and/or radiotherapy after surgical removal of the breast tumors and confirmatory diagnosis of the breast cancer. Control cases (100 number) with no breast or any other cancer history were also included in the cohort including the family members from the positive cases.

The study was approved by the Institutional Review Board of the Department of Biotechnology, Balochistan University of Information Technology, Engineering and Management Sciences (BUITEMS) Quetta. A written informed consent from all the volunteers was taken.

Venous blood samples (3-5ml) from each subject were obtained in heparinized vacutainers, transported into lab and stored at -20oC after being shifted into 15 ml falcon tubes. DNA was extracted in every weekend after sample collection, using" Thermo Scientific Genomic DNA Purification Kit" following the protocol of the company. Primers against all the coding exons of the *CHEK2* gene (Table 1) including adjacent intronic regions were designed using Amplifx primer designing software. DNA samples were amplified through thermal cycler (T-100 Bio-Rad) in a 25µl PCR reaction mixture containing 12.5µl Taq Master Mix Standard Buffer, 3.5µl forward and reverse working primers each, 3.5µl PCR graded RNase free H2O and 2µl DNA (25µg/µl). PCR products stained with ethidium bromide (6µl) were visualized under UV light following gel electrophoresis on 2% agarose gel and then cleaned with ExoSAP-IT PCR product cleanup reagent and incubated in thermal cycler at 37oC for 15 minutes and 80oC for 15 minutes. Sequencing PCR was performed in a 10µl reaction mixture containing 0.25µl sequencing buffer (5X), Big Dye (v 3.1) 0.25µl, Primer (forward) 1.0µl and 5.5µl PCR graded RNase free H2O. Sequencing PCR products were washed with 70% ethanol following precipitation using the precipitation mix and then dissolved in H_2O + HiDi 10µl each in ABI plate. The sequencing was performed on ABI PRISM 3100 Genetic Analyzer. Sequencing results were analyzed through Chromas (v.2.6.6) and BLAT (Ensembl genome browser 108). The DNA sequence of the region identified with the said variant was also analyzed for RNA secondary structure prediction by using RNAfold web server [17].

Results

The ethnic distribution of the breast cancer patients enrolled in current study were 32% Pashtun followed by 25% Afghani, 23% Baloch, 5% Hazara and 26% others including Panjabi, Sindhi and other ethnic groups. Sequencing of the PCR products (Figure 2) was performed to investigate variants in *CHEK2* gene. Sequencing results revealed a variant at intron 2, c.319+38-43dupA (Figure 3) in 9% of breast cancer cases whereas in normal randomly selected subjects the variant was not observed even in a single sample. 20% of the Afghani, 9.4% Pashtun and 4.3% Baloch patients were positive for c.319+38-43dupA variation and all the cases were affected with invasive

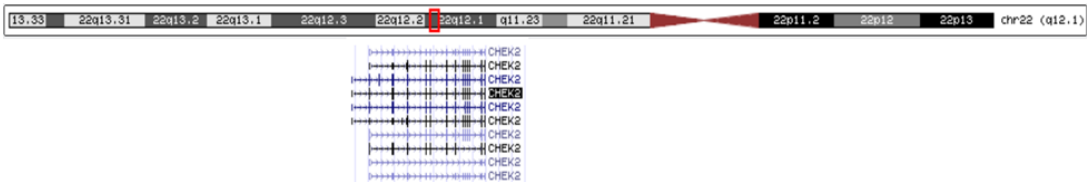

Figure 1. *CHEK2* Locus on Human Chromosome 22 (UCSC Genome Browser on Human (GRCh37/hg19)).

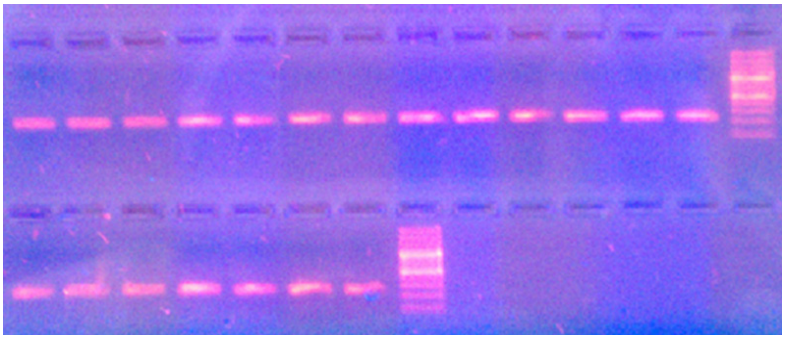

Figure 2. Amplified Exon 2 of Gene *CHEK2*

Table 1. List of Primers Used to Amplify *CHEK2* Gene Exon 2

Exon	Forward Primer	Tm	Reverse Primer	Tm
2	TTTGGAGAGCATGTTTGCCCT	57^{0}C	GAGTTCCTGAGTGGACACTGTCT	57^{o}C

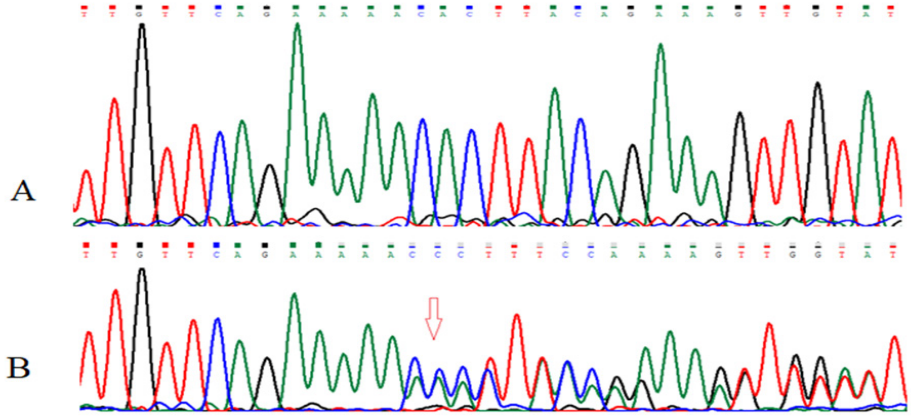

Figure 3. Chromatograph of the Variant c. 319+38-43dupA (A normal B variant)

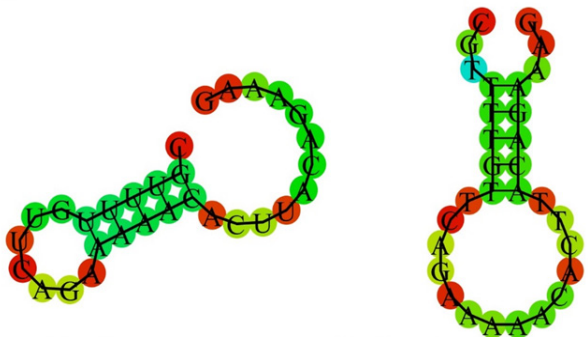

Figure 4. RNAfold Web Server Images Showing Hairpin Structure (A) MFE Secondary Structure (B) Centroid Secondary Structure

ductal carcinoma (IDC). The average age of the patients was 45.6 years, Tumor grade was reported to be 44.4% with grad II and 55.6% with grade III. All the patients with c.319+38-43dupA variation were diagnosed with advanced stage of cancer 77.78% with stage III and 22.22% stage IV (Table 2).

The DNA sequence of the region with variant c. 319+38-43dupA was analyzed for any possible non-coding RNA (ncRNA) by using RNAfold web server. The result showed a hairpin structure RNA (Figure 4) revealing a possible miRNA coding region.

Discussion

CHEK2 coding for checkpoint kinase 2, a tumor suppressor protein activated in p53 signaling pathway in response to DNA damage [18-19]. The *CHEK2* pathogenic variants have frequently been diagnosed in cancer panel testing and reported to be associated with breast, thyroid and kidney cancers and these data suggest informing for genetic counselling and care of the families and individuals positive for any *CHEK2* pathogenic variants [13, 20-21]. Molecular genetics research and techniques have mostly emphasized on the association of the non-synonymous variants with disease traits and their severity [22] but with advancements of modern techniques such as next generation sequencing [23], nanopore sequencing, HiC technology, Varfish data analysis including combined annotation dependent depletion (CADD) score [24] have invented the synonymous and non-coding region variants including intronic and intergenic variants and their association with disease traits [4, 25]. Intronic variants have been reported to dysregulate mRNA splicing and

Table 2. Clinical Presentation of the Cases with Variant, c.319+38-43dupA

S.No.	Case No.1	Ethnicity	Age	Type (Ca-Breast)	Tumor Grade	Stage of Cancer
1	20	Pashtun	46	IDC	III	IV
2	26	Pashtun	50	IDC	II	III
3	34	Baloch	50	IDC	III	IV
4	41	Afghani	38	IDC	III	III
5	44	Pashtun	40	IDC	III	III
6	51	Afghani	55	IDC	II	III
7	14	Afghani	40	IDC	II	III
8	55	Afghani	50	IDC	II	III
9	57	Afghani	46	IDC	II	III

thousands of intronic variants have been found pathogenic and documented to influence splicing [26-27]. Studies suggest that beside protein structural the RNA splicing and evolutionary conservation features also influence the characterization of intronic variants causing diseases [4]. In a study carried out by Wang et al., 2021 on Alport Syndrome (AS) caused by mutations in *COL4A3, COL4A4* and *COL4A5* genes revealed the 4 unrelated AS patients negative for next generation sequencing were analyzed by mRNA-based approach and reported deep intronic causative variants >100bp upstream/downstream in the probands [23]. In current study the intronic variant, c. 319+38-43dupA identified in breast cancer patients was also reported as the most frequent variant by Havranek et al 2015, in 22% of the patients of Non-Hodgkin Lymphoma and 31% control cases associated with decreased risk of non-Hodgkin Lymhpoma [28]. The RNA secondary structure prediction revealing a hairpin structure following the DNA sequence of the region with variant c. 319+38-43dupA suggests that this region may be coding for an miRNA. The function of miRNAs is reported to regulate a wide array of biological processes including oncogenesis and abnormal synthesis of miRNA in breast cell can lead to the occurrence of breast cancer by disrupting various biological processes [29-31].

In conclusion, we cannot conclude that c. 319+38-43dupA variant may be a cause or increase the risk of breast cancer as our study has limitations with that none of the patients have ever opt for any genetic analysis or any history of mutation in same or any other genes leading to breast cancer. This variant has also not been reported in any study carried out on breast cancer before but in line to the studies discussed above we suggest that this variant may be associated with breast cancer, and it may be further analyzed as it has been reported most frequent variant in the study carried out by Havranek as well as our study.

Author Contribution Statement

All the authors equally contributed to the production of data, processing of data, write up and editing of the study presented in this manuscript.

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Ethical Declaration

This study was approved by Institutional Review Board of the Department of Biotechnology, BUITEMS, Quetta, Pakistan.

Conflict of Interest

The authors have got no conflict of interest regarding the publication of this research article.

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