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Whole exome sequencing identifies a novel FANCD2 gene splice site mutation associated with disease progression in chronic myeloid leukemia: Implication in targeted therapy of advanced phase CML

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Abstract: Tyrosine Kinase Inhibitors (TKIs) have significantly improved the clinical outcome of BCR-ABL+ Chronic Phase-Chronic Myeloid Leukemia (CP-CML). Nonetheless, approximately one-third of the CP-CML patient's progress to advanced phases of CML (accelerated and blast phase). Impaired DNA repair including mutations in Fanconi anemia (FA) pathway genes are responsible for progression of many cancers. Nevertheless, FA-pathways genes have never been reported in myeloid cancers. Hence, this study was aimed to discover DNA repair genes associated with CML progression. AP-CML patients were subjected to whole exome sequencing along with appropriate controls. A novel splice site FANCD2 mutation was detected. FANCD2 is a well-known FA-pathway gene with established role in DNA repair. This is first report of FA-pathway DNA repair genes in myeloid cancers that can serve as a novel marker of CML progression to clinically intervene CML progression. Further studies are needed to establish the functional role of FANCD2 in CML progression that can provide novel insights into CML pathogenesis. This study also indicates that a combination TKIs and Poly (ADP-ribose) polymerase (PARP) inhibitors like Olaparib (FDA approved anti-cancer drug for FA-pathway gene mutations) could improve the clinical outcome CML patients in accelerated and blast-crisis phases of the disease.

Keywords: CML, progression, DNA repair genes, FANCD2, PARP inhibitors, combination therapy.

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal disorder arising due to translocation between chromosomes 9 and 22, t (9;22). This translocation results in a fusion oncogene, BCR-ABL, that encodes Bcr-Abl proteins directly involved in leukemogenesis. This Bcr-Abl target drugs for CML (Togasaki *et al.,* 2017). TKIs are very effective and even curative in chronic phase CML (CP-CML). Nevertheless, the effectiveness of TKI is highly compromised in the accelerated phase (AP-CML) and blast crisis phase CML (BC-CML). Approximately, one-third of the CML patients receiving TKIs becomes resistant and progress to advanced stages of the disease. The pathogenesis of chronic phase of CML is mostly dependent on BCR-ABL1. Mechanism of CML progression is poorly understood, and common molecular biomarkers indicating CML transformation to AP- & BC-CML are unknown (Kim *et al.,* 2017).

Human cells accumulate dozens of mutations in order to trigger the cancer onset and progression. In the case of CML, although BCR-ABL is considered the key molecular aberration involved, several other molecular abnormalities are accumulated during disease transformation and progression of CML from chronic to accelerated and blast crisis phases of the disease (Daley *et al.,* 1990; Quintás-Cardama and Cortes, 2009). BCR-ABL itself causes genomic instability in CML cells, leading to the accumulation of more mutations in itself as well as other genes involved in vital cellular functions. It has recently been postulated that unfaithful DNA repair could be responsible for the progression of CML by acquiring mutations in DNA repair genes (Branford *et al.,* 2019). Historically, mutations in DNA repair genes have been found to be associated with genomic instability and

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disease progression in cancers. Moreover, next-generation technologies like whole exome and whole genome sequencing have helped a lot in the last few decades to unravel the molecular basis of the disease pathogenesis in CML. Therefore, we carried out this study to find out mutations in DNA repair genes associated with CML progression by whole exome sequencing. Moreover, we confined our studies to DNA repair genes mutated in all advance phase CML samples in order to find out a common biomarkers leading to disease progression in CML.

MATERIALS AND METHODS

Patients and Setting

Our clinical studies included 141 CML patients from Hayatabad Medical Complex (HMC) Peshawar, Khyber Pakhtunkhawa (KP) Pakistan, from January 2012 to May 2018. For WES-based molecular biological studies, peripheral blood samples of 18 progressed CML patients (AP-CML, n=6, BC-CML, n=12) were collected. An equal number of CP-CML was included as gender/age-matched control. All patients received imatinib mesylate (IM) as first-line therapy and nilotinib (NI) as second-generation tyrosine kinase (TKIs) in the case of IM non-responders. All response criteria were per European Leukemia Net guidelines 2013 (Baccarani *et al.,* 2013).

The study protocols were approved by the institutional ethical review committees of Hayatabad Medical Complex (HMC) Peshawar and University of the Punjab, Lahore, Pakistan. A written informed consent was obtained from all participants in the present study. It was conducted according to the codes of the Declaration of Helsinki.

Sample Collection & DNA extraction

We collected peripheral blood samples (3-5 ml) in EDTA blood collection tubes (BD Vacutainer Systems, Franklin Lakes, and N.J.). Genomic DNA (gDNA) was extracted from the whole blood of advanced phases CML patients by using QIAamp DNA Blood Mini Kit. The DNA was quantified by using NanoDrop Spectrophotometer (NanoDrop Technologies, Inc., USA) and diluted into aliquots of 70-80ng/µl for whole exome sequencing (WES), For mutation confirmation, the rest of the DNA was diluted to 40ng/µl for later use on Sanger Sequencing. DNA was stored in -80°C freezer till further testing.

Whole Exome Sequencing (WES)

We used the SureSelectXT V6-Post Capture Exome kit for the preparation of libraries and exome enrichment (SureSelect, Agilent). Target Enrichment was performed for exonic and intron flanking regions by using Illumina Paired-End Multiplexed Sequencing (SureSelect^{XT2}) as per manufacturer protocol. Briefly, DNA, 50ng, was fragmented, and tagmentation was done. The DNA tagmentation was followed by purification and amplification step. The amplified DNA libraries were purified with magnetic beads and target regions were captured with oligos (whole exome), it was followed by enriched libraries amplification by PCR. Qubit fluorometer was used to quantify the enriched libraries, and the distribution of library size was measured by Agilent Bioanalyzer. Lastly, the quantified DNA libraries were loaded on flow cell for cluster generation and whole exome sequencing on an Illumina NextSeq500 instrument (AlAsiri *et al.,* 2015).

Approach for Exome sequencing Data Analysis

The WES output BCL files were converted to FASTQ files with the help of BCL2FASTQ software. By using BWA-MEM algorithm, BWA aligner was used to align the FASTQ files to the human genome (GRCh37/hg19). Genome analysis tool kit (GATK), was used for variants calling. For genomic variants annotation and filtration, Illumina Variant Studio was utilized (AlAsiri *et al.,* 2015).

Strategy for variant analysis

First of all, we targeted only DNA repair genes mutated in all advanced phase CML patients in order to find out a common biomarker of CML progression. WES annotated Excel file was filtered as filtered through an approach follows as, firstly, all synonymous and intron variants were removed. Secondly, "rare variants calling" was done, which was defined as a filtration strategy for all those variants for which ≥70% of their allele frequencies were missing. Thirdly, all known Tolerant and Benign Variants (with known prediction) were removed. For multiple B or T, we considered it B, if the frequency of B was ≥70% or T, if the frequency of T was ≥70% and also all TBB combinations were removed. Lastly, WES data were further analyzed for driver mutations, gene variants not present in CP-CML patients but present in AP-BC CML patients, and thus these variants possibly could be involved in progression of disease

Variants validation by Sanger Sequencing

Sanger sequencing (Tsiatis *et al.,* 2010) was performed to validate the WES identified variants. Specific Genomic primers of the variants in identified *genes* were retrieved from the University of California Santa Cruz genome database browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The samples were amplified and PCR amplified products were sequenced (forward/reverse) by using by Sanger sequencer, ABI Prism 3730 Genetic Analyzer (Applied Biosystems, California, USA) and ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kits were used for preparation of samples (V.3).

Statistical Analysis of patient data

We presented the absolute number and percentages for categorical variables and mean and median with an

appropriate measure of variation for continuous variables based on the normality test. Comparisons between two groups for categorical data were by Chi-Square or Fisher's exact test, as appropriate. Two sample independent test or Mann Whitney U test was used for continuous data, depending on the normality hypothesis. For ≥3 groups, we used analysis of variance (ANOVA) or Kruskal-Wallis test. For data analysis we used [SAS/STAT] software version 9.4 (SAS Institute Inc., Cary, NC, USA.) and R foundation was utilized for statistical computing (Vienna, Austria). The Sokal risk score was calculated as described in the literature (Sokal *et al.,* 1984).

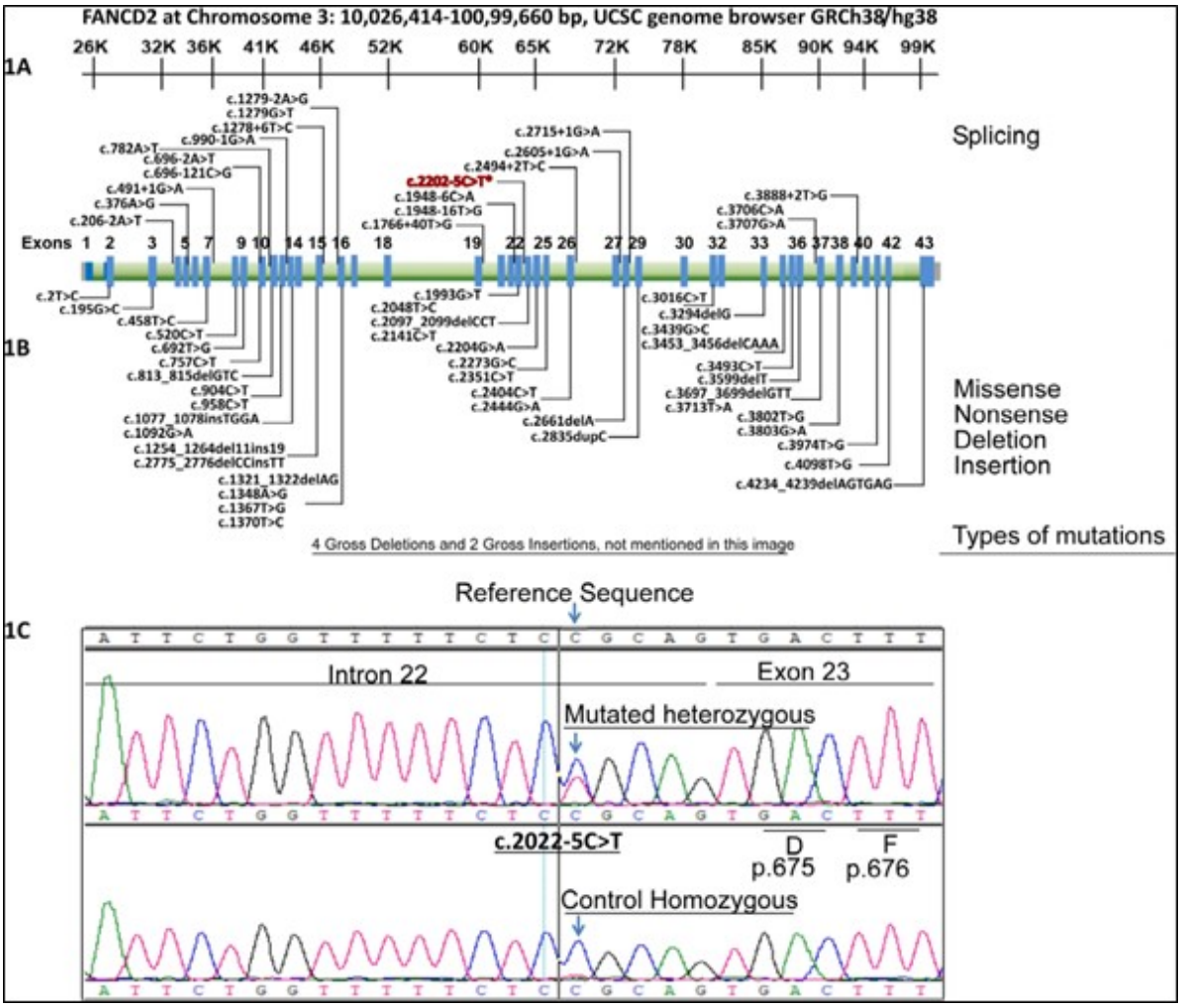

Fig. 1A to 1C: (1A) Showing location of *FANCD2 at* Chromosome 3: 10,026,414-100, 99,660 bp using UCSC genome browser GRCh38/hg38. (1B) Schematic representation of exon-intron structure of *FANCD2* gene; exons are designated as numbers Exons (1-43). Splice site location of Intron 22 where the mutation c.2022-5C>T resides is shown highlighted in red with asterisk (*). (1C): Showing sequencing chromatogram showing the normal control homozygous and mutated heterozygous, with mutation position marked by an arrow.

Table 1: Primers designed to amplify the regions around the detected variants in ATXN3, and FANCD2 by Sanger sequencing.

#	Gene Symbol	Sequence	Size (bp)	Tm
1	ATXN3-F	5` GCACAGCATCACTAAGCTGC 3`	324	59.8°C
	ATXN3-R	5` ACACCTGGCCCACATTTTAG 3`		59.9°C
2	FANCD2-F	5` TGCACCCTTAGCTCCAAAAT 3`	271	59.7°C
	FANCD2-R	5` CCCCATCTTTTGCAAAGTCCT 3`		62.5°C

Legend: F= forward primer, R= reverse primer, bp= base pair, Tm= melting temperature, °C= degree centigrade

Table 2: Whole Exome Sequencing of CML patient's demographics, clinical and laboratory parameters based on disease phase (CP-CML, n=123), (AP-CML, n=6) and (BC-CML, n= 12).

Characteristics	Patients Group		
	CP-CML, n (%)	AP-CML, n (%)	BC-CML, n (%)
# of Patients	123 (87.2)	6 (4.3)	12 (8.5)
Age, yrs			
Mean (range)	35.5 (9-67)	35.6 (27-43)	38.1 (29-50)
Gender			
Male	74 (60.2)	4 (60)	8 (66.7)
Female	49 (39.8)	2 (20)	4 (33.3)
Ratio: Male: Female	1.5-1	2: 1	2: 1
Hemoglobin (g/dL) Mean	10.1		
<12g/dl	69 (56.1)	5 (83.3)	9 (75)
>12g/dl	14 (11.4)	1 (16.7)	3 (25)
WBC count ($\times 10^9$/L) Mean	313.7	315	325
<50	20 (16.3)	1 (20)	2 (16.7)
>/=50	64 (52)	5 (80)	10 (83.3)
Platelets ($\times 10^9$/L) Mean	400.2		
<450	75 (61)	4 (66.7)	10(83.3)
>/=450	33 (26.8)	2 (33.3)	2 (16.7)
Imatinib			
Yes	82 (66.7)	4 (66.7)	7 (58.3)
Nilotinib as 2nd Line			
Yes	40.7 (33.3)	4 (66.7)	8 (66.7)
Hydroxyurea			
Yes	82 (66.7)	3 (50)	10 (83.3)
Interferon			
Yes	40.7 (33.3)	0 (0)	0 (0)
Chemotherapy			
Yes	10 (8.1)	4 (66.7)	9 (75)
Splenomegaly			
<5cm	4 (3.3)	0 (0)	0 (0)
5-8cm	9 (7.3)	1 (16.7)	3 (25)
>8cm	70 (56.9)	5 (83.3)	9 (75)
No splenomegaly	40 (32.5)	0 (0)	0
Hepatomegaly			
Yes	35 (28.5)	4 (66.7)	8 (66.7)
Anemia			
Yes	97 (78.9)	5 (83.3)	9 (75)
Pregnant			
Yes	4 (8.2)	0 (0)	0 (0)
Survival Status			
Confirmed deaths	10 (8.1)	0 (0)	9 (75)
Alive at last follow up	113(91.9)	6 (100)	3(25)

Legend: WES: Whole Exome Sequencing, WBC; White blood Cells, CP; Chronic Phase, AP; Accelerated Phase, BC; Blast Phase, CP-CML; Chronic Phase-Chronic Myeloid Leukemia, AP-CML; Accelerated Phase-Chronic Myeloid Leukemia, BC-CML; Blast Phase-Chronic Myeloid Leukemia,

Table 3: List of gene variants having role in DNA repair as found mutated only in advanced phase CML patients.

S#	#Ch.	Gene_Name	POS	REF	ALT	HGVS.c	HGVS.p	Effect
1	chr14	ATXN3	92,537,379	T	C	c.208A>G	p.Thr70Ala	Missense variant
2	chr3	FANCD2	10,106,408	C	T	c.2022-5C>T	NA	Splice region variant & intron variant

Legend, AP/BC-CML: accelerated phase/blast crisis-chronic myeloid leukemia, CP-CML: chronic phase- chronic myeloid leukemia, # Ch: Chromosome number, Pos: Position, Ref: reference, ALT: alternate, HGVS.c: Human Genome Variation Society.c, HGVS.p: Human Genome Variation Society.p

RESULTS

In our studies, only 18 out of 141 CML patients progressed to AP-/BC-CML from 2012-2018 (12.8%). Patient demographics, laboratory parameters and clinical outcome are tabulated in table 2. The mean age of CP, AP, and BC patients was 33.5, 35.6 and 38.1 years, respectively. In all phases of the disease, there was male patient's dominance; the ratio of male to female in CP, AP and BC patients was 1.5:1, 2:1 and 2:1, respectively. More than two-third of all patients were anaemic. Overall, 56% of CML patients (CML-CP, CML-AP & CML-BC) presented with a higher WBC count i.e. >/=50 × 109/L (n=79). Imatinib as first line drug was received by 66.7%, 66.7% and 5836% of CP, AP & BC CML patients, respectively. Chemotherapy was administered to 8.1%, 66.7% and 75% of by 40% and 71.4% CP, AP & BC CML patients, respectively. In the present study, overall 18 (12.7%; n=141) patients progressed to AP (n=6) or BC (n=12) (table 2).

Exome sequencing

Initial screening for DNA repair genes

Based on our screening strategy, we found 2 mutations in two DNA repair genes FANCD2 and ATXN3 exclusively mutated in advanced phase CML and none of the control samples (Table 3).

Mutation validation by Sanger Sequencing

We performed Sanger sequencing for confirmation of variants by WES. ATXN3 variant (c.208A>G) was not confirmed, however, a well-established DNA repair gene variant FANCD2 was found mutated (c.2022-5C>T) in all sequenced advanced phase CML patients that indicate a major role of impairment of DNA repair mechanism in CML progression. The location of the FANCD2 gene on chromosome 3, the exon-intron structure of the FANCD2 gene with splice site mutation, and Sanger validation image is depicted in figure 1A-1C.

DISCUSSION

CML disease progression leads to drug resistance, treatment failure, comorbidities, and mortality and hence finding genetic variants associated with AP-/BC-CML helps in early detection of fatal disease progression and timely clinical intervention to delay or prevent acute transformation in CML (Cortes *et al.,* 2012; Haznedaroglu, 2013). The eligible CML patients with advanced phase disease, i.e., accelerated and blast crisis (AP/BC) CML, were subjected to whole exome sequencing along with appropriate controls to find out genetic variants in novel/known genes associated with disease progression.

In the present study, the rate of progression of CML to advanced phase disease was 12.7% (n=141). In a study from Europe, the progression rate to accelerated or blast crisis was noted in 35 patients out of 553 (6.3%) (Druker *et al.,* 2006). This shows a significantly higher frequency of CML progression in our CML patients (p=0.02) that could be attributed to delayed diagnosis, availability of fewer TKIs, poor compliance, patients possibly wasting time with quacks, limited availability of molecular testing / MMR and shortage of trained hematologists.

The risk of developing cancer is high when the mutations happen in cancer susceptible genes or those normally looking after the integrity of our genome. These mutations can arise due to disruption in normal cell regulatory pathways, crucial for normal cell function and genome stability. One such a pathway is the Fanconi anemia (FA) protein family and its interaction with breast and ovarian cancer susceptibility genes (BRCA1/BRCA2). To date 22 FA genes have been described (Nalepa and Clapp, 2018) and mutation in any one of these genes leads to FA, which is associated with genetic defects in newborns, failure of bone marrow and predisposition of the genome to develop cancer (Timmers *et al.,* 2001; Nalepa and Clapp, 2018). Recently, the FA pathway has been linked with progression seen in many sporadic cancers (Lyakhovich and Surralles, 2006; Valeri *et al.,* 2010). In the present study, a novel splice site FANCD2 mutation (c.2202-5C>T) was detected in all advanced phase CML patients.

FANCD2: Possible role in CML

FANCD2 is a conserved FA gene that plays a crucial role in response to reactive oxygen species (ROS) related double strand breaks (DSBs) in DNA. FANCD2 has been reported up-regulated in CD34+ CML cells and BCR-ABL1 positive cells and associated with genome damage and leukemogenesis under the influence of BCR-ABL (Valeri *et al.,* 2010; Koptyra *et al.,* 2011). Valeri *et al.,* (2010) documented the disruption of the FA/BRCA pathway in hematopoietic BCR-ABL positive stem cells with altered FANCD2 foci, which is suggestive of instable genome and compromised efficiency of the DNA repair mechanisms. Role of genetic association in subjects with FA mutations versus without FA mutations has been investigated in CML and it was found that heterozygous mutations in the FA genes predispose to this cancer ((D'Andrea Grompe, 2003; Garcia Benitez, 2008; Garcia Benitez, 2008). Furthermore, a dual role of FANCD2 has been postulated in CML progression: Firstly the overexpression of FANCD2 is likely to promote the survival and propagation of CML cells (Koptyra *et al.,* 2011), while, the inhibition of FANCD2 foci under the influence of BCR-ABL fusion gene most likely contributes in genome instability (Valeri *et al.,* 2010). Nevertheless, most of the reported data in this regard is based on cell lines and mutations on FANCD2 in CML, specifically their association with CML progression have rarely been reported.

Association of FANCD2 mutations in cancer pathogenesis

FANCD2 mutations have been reported in different cancers like head and neck squamous cell carcinoma (Smith *et al.,* 2013; Chandrasekharappa *et al.,* 2017), breast cancer (Smith *et al.,* 2013) T-cell acute lymphoblastic leukemia (Borriello *et al.,* 2007), colorectal cancer (Smith *et al.,* 2013) and prostate cancer (Paulo *et al.,* 2018). To date, 19 splicing mutations have been reported in the FANCD2 gene in literature according to the human gene mutation database (HGMD), summarized in fig. 1B. Majority of splicing mutations are reported in Fanconi Anaemia phenotype (84.1%) (Timmers *et al.,* 2001; Kalb *et al.,* 2007; Knies *et al.,* 2012; Chandrasekharappa *et al.,* 2013; Cagnan *et al.,* 2018; Kanchi *et al.,* 2014) and 5.3% in each of prostate cancer (Paulo *et al.,* 2018), breast cancer (Maxwell *et al.,* 2016) and growth abnormality phenotypes (Retterer *et al.,* 2016).

FANCD2 splice-site mutations in pathogenesis

A splice site mutation is a result of alteration in DNA sequence at the junction of an exon/intron. The consequences of such changes include defects in RNA splicing, and the aftermaths of such mutation are usually loss of exons or gain of introns and a defective protein-coding sequence (Anna and Monika, 2018). Earlier studies reported heterozygous mutations in FA gene families like *FANCC* and *BLM* genes in breast cancer. There is a strong notion that heterozygous mutations genes involved in DNA repair like FANCD2, could contribute as an increased risk for genomic instability and cancer progression. This belief needs to be further explored as it has gained increased traction overtime. FA genes heterozygous mutations could have diverse biological and pathophysiological functions in the pathogenesis of the underlying cancers (Thompson *et al.,* 2012; Chang *et al.,* 2014).

FANCD2, FA Pathway, role of BRCA1/2 and targeted therapies

Various cancers with mutations in DNA repair genes have been successfully treated by poly (ADP-ribose) polymerase (PARP) inhibitors that activate impaired homologous DNA repair. Olaparib is an FDA approved PARP inhibitor for BRCA1/2 mutated breast and ovarian cancers (Liu *et al.,* 2019). As FANCD2 carries out DNA repair though BRCA1/2 proteins, PARP inhibitors could be very effective in CML patients with mutations in FANCD2 or other DNA repair genes. Specifically, in the case of AP-/BC-CML, ours and other studies indicate that Olaparib and other PARP inhibitors could help in the treatment of advanced phase CML patients with dismal response to TKIs and other available drugs for AP/BC-CML. In a recent study, a combination of PARP inhibitor (Olaparib) and PI3K inhibitor led to the attenuation of leukemogenic activity in Philadelphia chromosome (Ph+) leukemia cells in mice (Hiroki *et al.,* 2019). In another study, Faraoni *et al.* evaluated the anti-leukemic activity of Olaparib in primary acute myeloid leukemia blasts that showed Olaparib as a very promising monotherapy with anti-proliferative and apoptotic activities (Faraoni *et al.,* 2018). Liu et al have recently reported cytotoxicity of newly-approved PARP inhibitor (talazopraib) in cultured CML cells (Liu *et al.,* 2019). All of these recent studies indicate the great potential of PARP inhibitors in advanced phase CML, specifically in blast crisis. Therefore, we recommend further studies to find the role of DNA repair genes in CML progression and to carry out clinical trials in order to find out the efficacy of PARP inhibitors in advanced phase CML.

CONCLUSION

In conclusion, we found a novel splice site FANCD2 mutation in advanced phase CML. As FANCD2 carries out DNA repair through BRCA1/2 protein and drugs like PARP inhibitors are already in clinical practice for BRCA1/2 mutated cancers, we suggest initiating clinical trials to test efficacy of PARP inhibitors in advanced phase CML as monotherapy as well as in combination with other anti-leukemic drugs. This may provide novel therapeutic options for fatal disease progression in CML.

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