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Frequency Determination of c.1115_1118delTTGG and c.3788_3790delTCT FANCA Gene Mutation in North of Khyber Pakhtunkhwa (KPK) Pakistan Fanconi's Anemia Population

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Abstract

Background: This study aimed to assess the frequency determination of c.1115_1118delTTGG and c.3788_3790delTCT Fanconi's anemia A gene (FANCA) gene mutation in the North of Khyber Pakhtunkhwa (KPK) Pakistan Fanconi's Anemia Population.

Materials and Methods: A cross-sectional study was conducted at Khyber Medical University, Peshawar, Pakistan. For the Exon 13 mutation c.1115_1118delTTGG, the amplification-refractory mutation system polymerase chain reaction (ARMS PCR) was developed. Sanger sequencing confirmed the ARMS PCR results for Exon 13 and found an exon 38 mutation of the FANCA (Fanconi anemia complementation group A) gene (c.3788_3790delTCT). Sanger sequencing results analyzed on Bio edit sequence aligner software confirmed the results of PCR. The four incidental single nucleotide polymorphisms (SNPs) discovered were examined in several variation databases.

Results: The mean age for the patients was 9.68±3.02 years, with an age range of 5–16 years. Pedigree analysis of Fanconi Anemia patients revealed an autosomal recessive pattern of inheritance. Physical characteristics such as skeletal abnormalities, specifically thumb abnormalities and Fanconi's facies, are distinctive diagnostic features of FA. Pedigrees also showed bone marrow hypoplasia in 65% of patients and red cell aplasia in 6%. PCR results from all samples revealed tahat none of them had the Exon 13 mutation. In addition, none of the samples had the Exon 38 mutation. Four SNPs were found in the Sanger sequencing. Two of them were in the intron 12 region, one in each of Exons 13 and 38.

Conclusion: Results show that mutations in Exon 13 and Exon 38 of the FANCA gene are uncommon in our Pakistani FA population. SNPs established in the Pakistani population of Khyber Pakhtunkhwa (KPK) province have not been reported before.

Keywords: Aplastic anemia, Complementation group A protein, Fanconi anemia, , Polymerase chain reaction, Single nucleotide polymorphism

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Introduction

Fanconi's anemia (FA), an autosomal recessive inherited aplastic anemia (AA), is characterized by hypocellular bone marrow with various somatic abnormalities (1). The hallmark of the disease is the hypersensitivity of cells to DNA crosslinking agents, increasing vulnerability to acute myeloid leukemia (AML), myelodysplasia, and solid tumors (2). FA results from genetic mutations in genes responsible for DNA repair (3).

At least 19 genes have been identified linked with DNA repair, also known as the FA/BRCA pathway (4, 5). Fanconi's anemia A gene (FANCA) mutations account for 60%–70% of cases of FA. In the remaining 30% of cases, Fanconi's anemia C gene (FANCC) and Fanconi's anemia G gene (FANCG) are proven to be the most commonly mutated genes (5). Diagnosis of FA should be suspected in all patients presenting with AA,

myelodysplastic syndrome, AML, or any solid tumors (head, neck, liver, esophageal, vulvar cancers of squamous origin) before the age of 50 years (6). Prompt and well-timed diagnosis of FA in suspected cases of aplastic anemia is significant for better-directed treatment, ensuring optimal treatment results.

The FANCA gene is accompanied by the FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM genes, and their proteins participate in the monoubiquitination of FANCD. They are involved when cell division is in S-phase or during exposure to DNA crosslinking agents to help repair DNA (7, 8). The FANCA protein is a fairly large protein involving 1455 amino acids and a protein size of 163 kDa (9). The FANCA protein is a critical part of the FA core complex in the FA/DNA repair pathway (4).

The FANCA mutational variation is a very diverse, vastly polymorphic, and hypermutable gene with almost 200 different types of mutations described. These vary from insertions and frameshift mutations to deletions, missense, splicing, and nonsense mutations (10). The mutations in Exon 13 and Exon 38 of the FANCA gene are most commonly reported worldwide and can be used for diagnostic purposes (5, 11-13).

A well-timed and accurate diagnosis of FA among AA patients is essential to protect them from experiencing the chief toxicities associated with standard-dose conditioning regimens necessary for bone marrow transplant (BMT). This study aimed to determine the prevalence of the most commonly reported FA mutations in Pakistani FA patients to develop rapid diagnostic tests other than the chromosomal breakage test (CBT) for FA diagnosis. The amplification-refractory mutation system for polymerase chain reaction (ARMS PCR) was devised for common availability, cost-effectiveness, and rapid result generation.

Materials and Methods

A cross-sectional study of consenting patients with pancytopenia and characteristic physical features of FA (microcephaly, absent thumbs or hypoplastic thumbs, hypo or hyperpigmentation, growth retardation, short stature, and congenital anomalies of different organs) was performed at the Pharmacogenomics and Haematology laboratory of the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar. The calculated sample size was 22, but FA being a very rare disease, the maximum possible samples collected for 3 months were included in the study, which was 17. Sample size is calculated using the following formula: n = z2 P (1-P)/d2 (z = 95% confidence interval (1.96), P = 65% anticipated proportion of FANCA gene mutation in FA patients. d = 20% absolute precision).Parents/guardians provided at least three generations of the patient's family tree/pedigree. Strict aseptic measures, 3cc of blood was taken through a butterfly cannula from any visible vein of the patient, stored in EDTA tubes at 4-8°C for one week to be used for DNA extraction salting-out method (14). ARMS PCR was established as a rapid and cost-effective diagnostic test for the Exon 13 mutation c.1115_1118delTTGG (14). Sanger sequencing was used to confirm the ARMS PCR results for Exon 13. An additional global common mutation in exon 38 of the FANCA gene c.3788_3790delTGT was also confirmed by Sanger sequencing. PCR amplified products were sent to Macrogen Company for Sanger sequencing. 35ul of PCR amplified products were sent for Sanger sequencing of Exon 13 and Exon 38 for c.1115_1118delTTGG and c.3788_3790delTCT mutations. Sequencing was used as a confirmatory test for PCR results.

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Ethical Consideration

This study was initiated after approval was obtained from the Advanced Studies & Research Board and ethical board of Khyber Medical University, Peshawar. The current Study was approved by Ethical Committee of KHYBER Medical University (No. DIR/KMU-EB/FD/000407).

Results

Out of 17 registered FA patients, 76% were males, and 23% were females. The mean age for the patients was 9.68±3.02 years, with an age range of 5–16 years. The majority of the patients came from the far north of Khyber Pakhtunkhwa (KPK), Pakistan. Two patients presented from the Federally Administered Tribal Areas (FATA) adjoining the KPK. Two patients came from the neighboring country, Afghanistan. The mean Hb concentration was 6.7 ±1.6 g/dl. 29% had moderate anemia, and 71% had severe anemia. Assessment of family history was done through detailed pedigree analysis, considering any previous family member, sibling, or other child affected by the same disease. It was positive for 65% of patients and insignificant for 35% of patients. Wholly, pedigrees revealed an autosomal recessive pattern of inheritance, as evident from the skipping of generations with the disease and normal parents, who seem to be carriers of the faulty gene. Bone marrow examination reports displayed bone marrow hypoplasia in 65% of patients, aplasia in 24% of patients, and pure red cell aplasia in 6%. Physical characteristics such as skeletal abnormalities, specifically thumb abnormalities and Fanconi's facies, are distinctive diagnostic features of FA. 13 (77%) patients had Fanconi's facies as shown by microphthalmia, microcephaly, and small eyes with epicanthal folds. Four patients (24%) had Fanconi's facies and thumb abnormalities, including absent, double, and/or hypoplastic thumbs. Results from ARMS PCR of all the 17 samples showed the absence of the c.1115_1118delTTGG mutation in Exon 13 of the FANCA gene. Figure 1. Sanger sequencing results were analyzed on Bio edit sequence aligner software, thus confirming PCR results. Moreover, Sanger sequencing results showed the absence of the Exon 38 mutation of the FANCA gene. Sanger sequencing showed four single nucleotide polymorphisms (SNPs) (figures 2, 3, 4, 5). Two in the intron 12 region, one in exon 13, and one in exon 38. rs1800287, rs6500452, rs1800331 and rs11649210 respectively. Multiple variation databases such as NCBI SNP, ClinVar database, Ensemble variation database, and 1000 Genome Browser were used to analyze these SNPs and were benign. rs1800287 and rs6500452 in the intron 12 region were stated in 47% and 53% of the patients. rs1800331 (exon 13) and rs11649210 (exon 38) in 12% of the patients each. This study shows that the two most commonly reported worldwide mutations in Exon 13 and Exon 38 of the FANCA gene are uncommon in our Pakistani FA population. Such a study has not been conducted before on the Pakistani population. SNPs found in the Pakistani population of Khyber Pakhtunkhwa (KPK) have not been reported before.

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Table I: Characteristics of the Patients. (n=17)

S.NO	Patient ID	Age	Sex	Consanguinity in parents	Physical & Clinical Characteristics SS	FF	SP	SK	BL	PA	RI	CP	UA	Family History
1	AH-5-17-FA-0970	9	Male	-	+	+	+	+	+	+	+			NS
2	AL-FA-0001-16	6	Female	+	+	+	+	+	+	+	+	+	+	+
3	AQ-FA-0004-16	7	Female	+	+	+	+			+		+		NS
4	FH-10-17-FA-1520	7	Male	-	+	+	+			+				NS
5	FR-AA-0063-16	7	Male	+	+	+	+		+	+	+			+
6	IH-AA-00910-16	13	Male	+	+	+	+		+	+	+			+
7	IJ-AA-0004-15	12	Male	+	+	+	+		+	+				NS
8	IA-5-17-FA-0899	10	Male	+		+	+			+	+			+
9	KA-FA-0007-16	13	Male	+	+	+	+		+	+				+
10	AS-5-17-FA-0896	9	Male	-		+	+			+	+			+
11	MA-FA-0010-16	5	Female	+	+	+	+			+	+			+
12	MU-FA-0003-16	9	Female	+	+	+	+			+	+			+
13	NA-FA-0008-16	9	Male	+		+				+				+
14	RE-5-17-FA-0898	12	Male	+	+	+	+	+		+	+			NS
15	RH-FA-0009-16	10	Male	+	+	+	+	+		+	+			+
16	SF-FA-0017	13	Male	+	+	+	+		+		+	+		NS
17	WU-5-17-FA-0938	16	Male	-	+	+	+			+	+			+

SS: Short stature, FF: Fanconi's Facies, SP; Skin Pigmentation, SK; Skeletal System, BL; Bleeding tendencies, PA; Pallor, RI; Repeated Infections, CP; Cardiopulmonary Involvement, UA; Urinary system involvement. NS; Not Significant

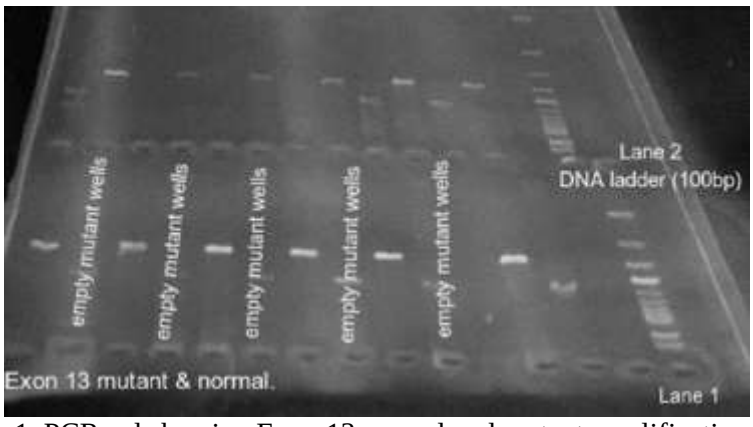

Figure 1. PCR gel showing Exon 13 normal and mutant amplification bands

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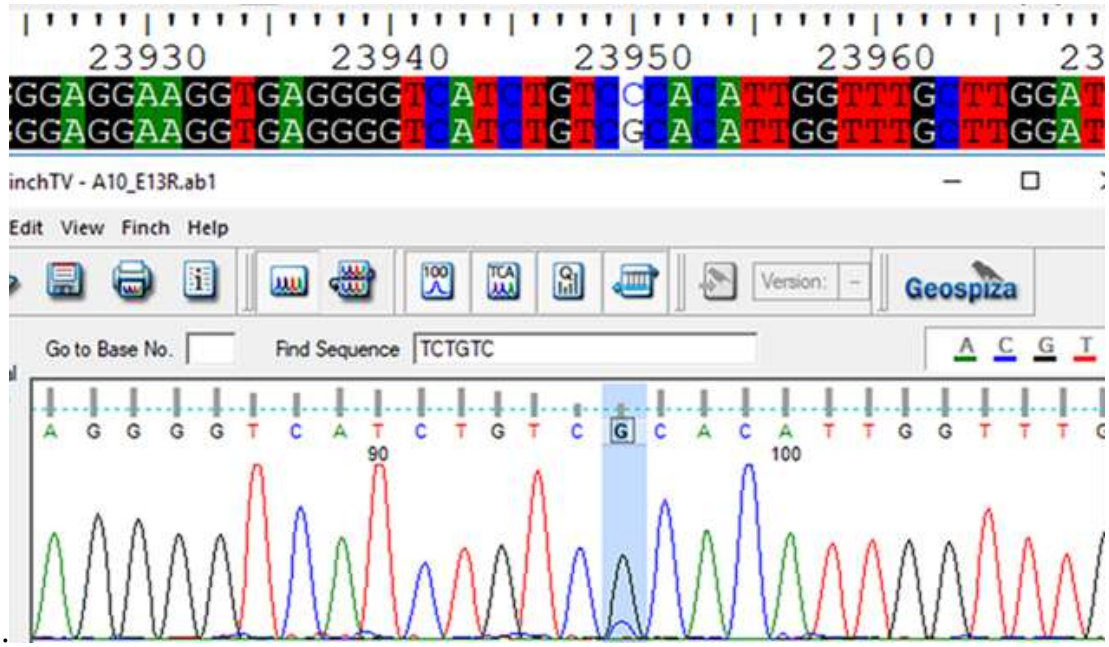

Figure 2. SNP rs1800287 chromatogram, showing forward primers amplification Intron 12 region, against normal FANCA gene sequence

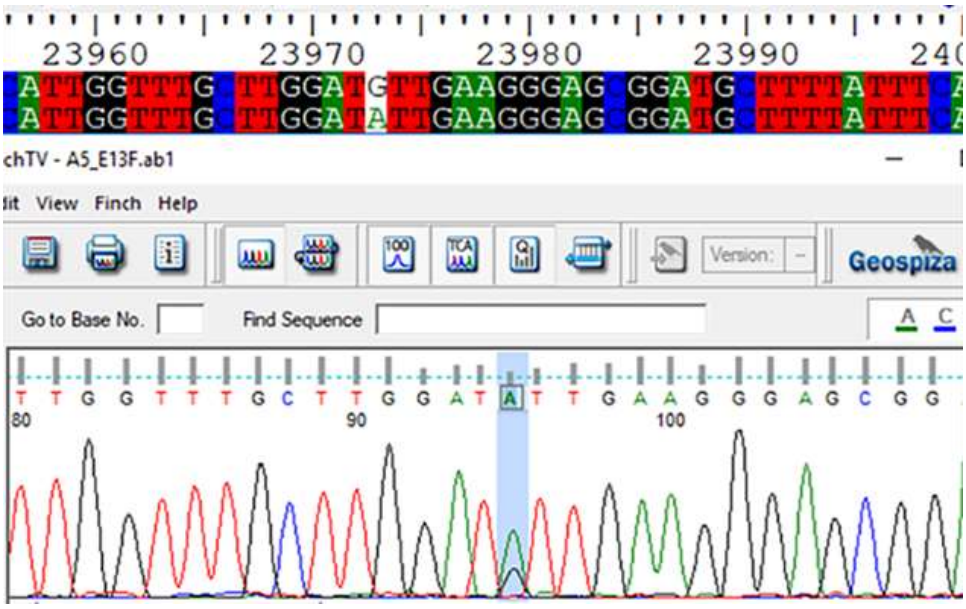

Figure 3: SNP rs6500452 chromatograms, showing forward primers amplification Intron 12 region, against normal FANCA gene sequence

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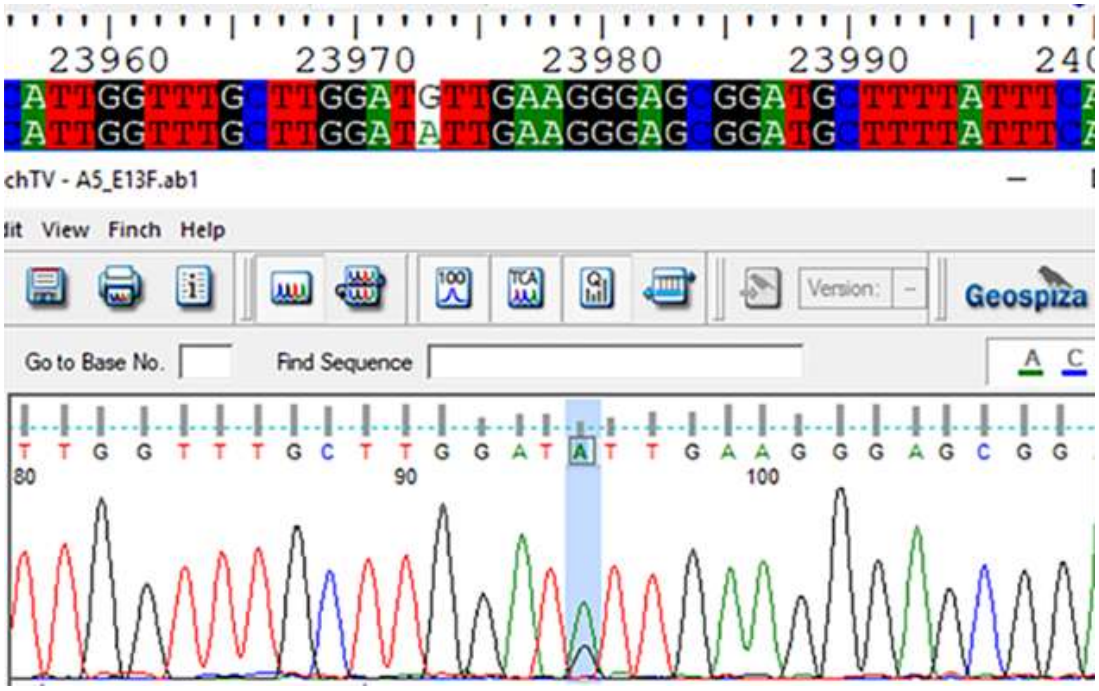

Figure 4: SNP rs1800331 chromatograms, showing forward primers amplification Exon 13 region, against normal FANCA gene sequence

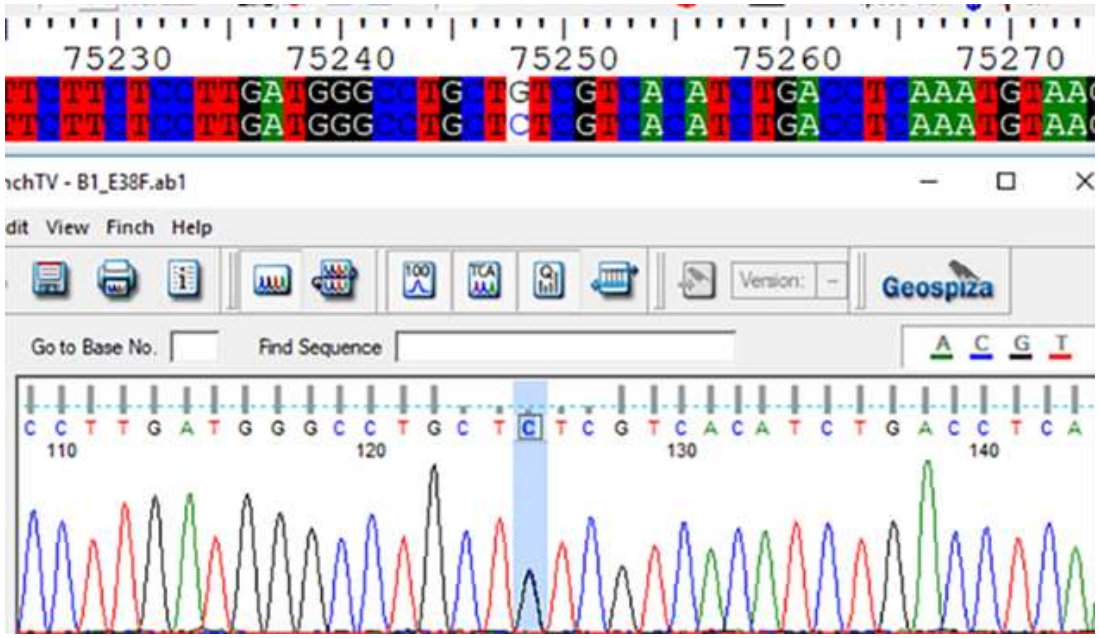

Figure 5: SNP rs11649210 chromatograms, showing forward primers amplification Exon 38 region, against normal FANCA gene sequence

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Discussion

Our study was comprised of 17 patients. The diagnostic age is reported to be seven years of age in the literature (15), but in our study, the mean age at the time of diagnosis was nine years. Reasons can be remoteness from specialists, underprivileged socioeconomic status, and reduced treatment-seeking behavior, causing postponement in diagnosis. A case report from Italy stated a child of 8 years at the time of diagnosis of FA (16). A different study conducted on 550 FA cases from all over India described the mean age at diagnosis ranging from 1 to 30 years ago (17). In our study, most patients had pancytopenia; this was also supported by a study conducted on 283 suspected cases of FA (18). Patients with FA present with characteristic physical features and congenital abnormalities. Suspicion of FA rises following the onset of pancytopenia (17). A research study on the proband and parents and siblings revealed that the mutation was identified in the homozygous state in a FA patient. The same mutation was found in the heterozygote state in the parents. Such facts can be utilized to plan more pregnancies and prenatal diagnoses (19). We did a detailed pedigree analysis on all of our patients to discover positive family history. It was positive for 67% of patients. Positive was considered in the cases of having another sibling or first-degree kin having FA, a history of solid cell tumors, and congenital anomalies. Commonly, patients are born of consanguineous marriages. One of our patients was born in a consanguineous marriage, whose pedigree analysis showed another elder sibling having FA who died. Two first-degree cousins were also born in consanguineous marriages with FA, who is alive and undergoing treatment. The results are comparable to the study conducted in Turkey on four FA patients born in consanguineous marriages. Two patients were diagnosed as FA patients prenatally in that study, but the mother wanted to continue the pregnancy (20).As FA patients develop pancytopenia in the long run, bone marrow examination is a good indicator of pancytopenia. A prospective study on patients enrolled in the International Fanconi Anemia Registry (IFAR) showed that 75% of patients showed reduced bone marrow (BM) cellularity. The patients were followed up, and it was noted that after nine years of age, FA patients having BM failure progressed to developing AML or Myelodysplastic syndrome (MDS). It was also revealed that BM failure or hematological abnormalities triggered the maximum number of deaths in FA patients (21). In diverse populations globally (13, 14, 25), the two most frequent mutations are Exon 13 and Exon 38 of the FANCA gene (11, 12, 22). A study conducted on 67 Spanish FA patients revealed that 10 (9.4%) patients had the c.1115_1118delTTGG mutation in Exon 13 and 17 (20.7%) had the c.3788_3790delTCT mutation in Exon 38, which is mentioned to be present in Pakistani ethnic background patients as well (23). At IFAR, 300 patients from different ethnic backgrounds were screened, which yielded results as 3788_3790del was found in 33 patients (5% allele frequency), and c.1115_1118delTTGG was carried by 12 patients (2% allele frequency) (12). A study conducted on 80 Brazilian patients showed that the Exon 38 mutation c.3788_3790delTCT was positive in 30% of patients, suggesting a high frequency in Brazilian patients (12, 24). In our study, ARMS PCR was aimed at finding the most frequent worldwide mutation in Exon 13, reported as c.1115_1118delTTGG of the FANCA gene in our local FA patients, which came out to be negative in all samples. The results of the ARMS PCR were then confirmed by Sanger sequencing, which generated negative consequences as well. Sanger sequencing was performed directly for the Exon 38 mutation c.3788_3790delTCT.It was negative in all samples. The SNPs revealed

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due to Sanger sequencing have never been described in the Pakistani population before. SNPs c.1143G/T and c.3807G/C were described by Levran et al. (11) in his study, in which they were utilized to create haplotypes for FA patients and their families. Such data can be applied to explore the haplotype background of FA mutations to ascertain the initiator chromosomes. Another broad study done for mutation screening of the FANCA gene in French Canadian residents showed all these SNPs in many patients (25). A study on FANCA gene mutations and polymorphisms in 88 familial breast cancer patients showed that eight patients had SNP c.1143G/T in Exon 13 of the FANCA gene (26). A similar study on familial pancreatic cancer patients showed this SNP in Exon 13 (27). SNP in exon 13 has also been found in the case report of a four years old child from Korea (28). Making a correct diagnosis of FA is still challenging even after its first narrative many decades ago. The timely and accurate diagnosis of FA among AA patients is of utmost importance to protect them from suffering chief toxicities linked to standard dose conditioning regimens needed for BMT (29). FA is a rare inherited aplastic anemia. There are no studies on the Pakistani population that have targeted these patients. This study will further direct other researchers to haplotype analysis and look for other mutations besides these in the Pakistani population.

Conclusion

This study was carried out to study the pattern of FANCA gene mutations in Exon 13 (c.1115_1118delTTGG) and Exon 38 (c.3788_3790delTCT). These mutations were absent in our 17 FA patients. This might be due to a small cohort of patients, and it can be validated by carrying out a large-scale study. A large-scale genetic study of our FA population should be conducted to determine the common mutations in our population. The SNPs found in our research are all non-pathogenic and were incidental findings.

Conflict of interest

The authors declare no conflict of interest.

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