Indian J Med Res 143, February 2016, pp 184-196
DOI:10.4103/0971-5916.180206

Investigation of *FANCA* gene in Fanconi anaemia patients in Iran

Ali Akbar Saffar Moghadam[1], Frouzandeh Mahjoubi[1], Nahid Reisi[2] & Parvaneh Vosough[3,4]

[1]Medical Biotechnology Institute, National Institute of Genetic Engineering & Biotechnology (NIGEB), Tehran, [2]Department of Pediatric Hematology & Oncology, Sayyd-al-Shohada Hospital, Isfahan University of Medical Science, Isfahan, [3]Division of Hematology & Oncology, Department of Pediatrics, Tehran University of Medical Science, Tehran & [4]Pediatric Hematology & Oncology, MAHAK Children's Hospital, Tehran, Iran

Received June 5, 2013

***Background & objectives*: Fanconi anaemia (FA) is a syndrome with a predisposition to bone marrow failure, congenital anomalies and malignancies. It is characterized by cellular hypersensitivity to cross-linking agents such as mitomycin C (MMC). In the present study, a new approach was selected to investigate *FANCA* (Fanconi anaemia complementation group A) gene in patients clinically diagnosed with cellular hypersensitivity to DNA cross-linking agent MMC.**

***Methods*: Chromosomal breakage analysis was performed to prove the diagnosis of Fanconi anaemia in 318 families. Of these, 70 families had a positive result. Forty families agreed to molecular genetic testing. In total, there were 27 patients with unknown complementary types. Genomic DNA was extracted and total RNA was isolated from fresh whole blood of the patients. The first-strand cDNA was synthesized and the cDNA of each patient was then tested with 21 pairs of overlapping primers. High resolution melting curve analysis was used to screen *FANCA,* and LinReg software version 1.7 was utilized for analysis of expression.**

***Results*: In total, six sequence alterations were identified, which included two stop codons, two frames-shift mutations, one large deletion and one amino acid exchange. *FANCA* expression was downregulated in patients who had sequence alterations.**

***Interpretation & conclusions*: The results of the present study show that high resolution melting (HRM) curve analysis may be useful in the detection of sequence alteration. It is simpler and more cost-effective than the multiplex ligation-dependent probe amplification (MLPA) procedure.**

Key words Fanconi anaemia - *FANCA* expression - High resolution melting curve

Fanconi anaemia (FA) is an inherited progressive bone marrow failure and cancer susceptibility syndrome, with both autosomal-recessive and X-linked patterns of inheritance[1,2]. Fanconi anaemia is characterized by progressive pancytopenia, congenital anomalies (thumb and skeletal deformities), growth retardation, renal malformations, abnormal skin pigmentation and high predisposition to acute myeloid leukaemia (AML) and other malignancies[3]. However, the phenotypes of FA patients vary from severe symptoms to moderate,

or even none at birth. Cellular hypersensitivity to DNA cross-linking agent such as mitomycin C (MMC) or diepoxybutane (DEB) is a gold standard for diagnosis of FA[4]. Clinical diagnosis of FA is further supported by a delay in the G2 phase of the cell cycle[5].

Seventeen genes belonging to the family of Fanconi anaemia complementation groups (*FANCA, FANCB, FANCC, FANCD1 or BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ or BRIP1, FANCL, FANCM, FANCN or PALB2, FANCI, RAD51C, FANCP or SLX4* and *FANCQ*) have been identified[6]. Furthermore, at least 13 distinct complementation groups of FA (genetic subtypes) are recognized[7]. The relative prevalence of each FA subtype varies widely according to the ethnic origin of the studied populations. Fanconi anaemia complementation group A (FANCA) is predominant in North America, South Africa (the Afrikaner population), Italy, Japan, and Turkey[8,9]. The largest complementation group is *FANCA* (60-65%) and together with *FANCB*, *C*, *E*, *F*, and *G* account for over 90 per cent of all FA cases[10,11]. The *FANCA* gene has 43 exons, and is on chromosome 16 (16q24.3), spanning about 80 kb of genomic DNA. It has an open reading frame of 4,365 bp and encodes 1,455 amino acids, with 32 variants[12].

The proteins encoded by the FA genes are assumed to act in a common pathway, termed the FA/BRCA pathway[13,14]. A nuclear multi-protein complex composed of the products of the *FANCA*, *B*, *C*, *E*, *F*, *G*, *L* and *M* genes is required for conversion of the small form of the *FANCD2* product in the monoubiquitinated larger form, *FANCD2-L*. These modifications cause this protein (a monoubiquitinated form of *FANCD2* product) directed to the site of the damaged chromatin where it interacts with other proteins, resulting in the repair of the damaged complex[15,16].

Knowledge of the mutation spectra of the FA genes is necessary to understand the pathogenesis of FA, and this spectra vary in different ethnic groups[17-19].

Complementation study or sub-typing of the patients in the FANCA group is the first step in the mutational analysis of the *FANCA* gene. However, mutation screening methods such as single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), multiplex ligation-dependent probe amplification (MLPA) and DNA sequencing are time-consuming and expensive. Hence, in this study, a novel method was developed which was not only cost-effective, reliable and sensitive, but covered all types of mutations, and included PCR, high resolution melting (HRM) curve analysis, real-time reverse transcription (RT)-PCR and sequencing.

Material & Methods

Patients and sample collection: Patients selected from 318 families were supposed to have Fanconi anaemia (FA). These families were referred to the Iranian Blood Transfusion Organization (IBTO) for confirmation of FA based on the standard test involving MMC-induced chromosomal breakage analysis[20]. Most of the patients and their cytogenetic status have been described by Tootain and colleagues[21]. The present study was carried out between 2010 and 2012 and most of experimental work was done in the division of Medical Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

Of the 318 families sent for MMC testing, 70 families had FA-affected children (positive test). From these 70 families, 40 families agreed to molecular genetic testing. In total, there were 27 live patients (Table I). The whole blood sample (10 ml) was obtained in EDTA from these patients and their family members. All subjects enrolled in this study gave written informed consent. The study protocol was approved by the Ethics Committee of the NIGEB, Iran.

The general manifestations of the 40 families with diagnosed FA patients are summarized in Table I. Clinical data were obtained based on history, physical examinations and reviewing medical records in the archives of the Iranian Blood Transfusion Organization and MAHAK hospital. These data are shown in Table II.

Genomic DNA extraction: Genomic DNA was extracted from fresh whole blood samples of patients using the Tadbir Tissue and blood DNA Extraction Kit (Tadbir DNA Extraction Kit, Tadbir Fan Azma Company, Iran). The genomic DNA samples were utilized to create a DNA bank for further studies.

RNA isolation and cDNA synthesis: Total RNA was isolated from the whole blood samples of patients using the High Pure RNA Isolation Kit (Roche, Germany), according to the manufacturer's instructions. The first-strand cDNA was synthesized by using the RevertAid First Strand cDNA Synthesis Kit (K1622, Fermentas, USA), as specified in the manufacturer's instructions.

RT-PCR analysis: The entire cDNA of *FANCA* was amplified via 21 pairs of specific primers which had an

Table I. General manifestations of Fanconi anaemia (FA) families (n=40)

Families	FA sibs		Consanguineous marriage of parents	BMT	Androgen therapy	Blood transfusion	Place of birth
	alive/dead	sex /age (yr)					
Fa1	+	F/28	No	No	Yes	Yes	Tehran
Fa2	+	F/13	No	No	Yes	No	Ardebil
Fa3	+	F/11	Yes	Yes	No	No	Esfahan
	+	M/24	-	No	No	No	Esfahan
Fa4	_	F/51	Yes	No	No	Yes	Tehran
Fa5	_	M/33	No	No	Yes	Yes	Gaem Shahr
	+	F/34	-	No	No	Yes	Gaem Shahr
Fa6	+	M/8	Yes	No	Yes	No	Tehran
Fa7	+	F/8	Yes	No	Yes	No	Tehran
Fa8	+	M/16	Yes	No	No	No	Hamadan
Fa9	_	M/9	Yes	No	Yes	Yes	Yazd
	+	F/6	-	No	No	No	Yazd
Fa10	+	F/10	Yes	No	No	Yes	Ardebil
Fa11	+	F/11	Yes	No	Yes	Yes	Esfahan
Fa12	+	M/8	Yes	No	Yes	Yes	Mashhad
Fa13	+	F/11	No	No	Yes	No	Esfahan
Fa14	+	M/8	Yes	No	No	Yes	Karaj
Fa15	+	M/21	Yes	No	No	No	Malayer
Fa16	+	F/8	Yes	Yes	No	No	Bandar Abbas
Fa17	+	M/8	Yes	Yes	No	No	Tehran
Fa18	_	M/7	Yes	Yes	No	Yes	Yazd
Fa19*	+	M/5	No	Yes	No	No	Kerman
Fa20	+	F/14	Yes	Yes	No	No	Tehran
Fa21	+	M/23	Yes	Yes	No	No	Tehran
Fa22	+	M/6	Yes	Yes	No	No	Karaj
Fa23	+	M/6	Yes	Yes	No	No	Tehran
Fa24	+	M/12	Yes	Yes	No	No	Tehran
Fa25	+	M/13	Yes	Yes	Yes	Yes	Tehran
Fa26	+	M/11	Yes	Yes	Yes	Yes	Tehran
Fa27	+	M/8	Yes	Yes	Yes	No	Ahwaz
Fa28	_	F/29	Yes	No	Yes	Yes	Rasht

Contd...

Families	FA sibs		Consanguineous marriage of parents	BMT	Androgen therapy	Blood transfusion	Place of birth
	alive/dead	sex /age (yr)					
Fa29	_	F/20	Yes	Yes	No	No	Ehar - Tabriz
Fa30	_	F/25	No	Yes	Yes	Yes	Shahre Kord
	_	M/21	-	No	Yes	Yes	Shahre Kord
Fa31	_	M/7	Yes	No	No	Yes	Ghom
Fa32	_	M/10	Yes	No	Yes	Yes	Tehran
Fa33	_	F/40	Yes	Yes	No	No	Tehran
Fa34	_	M/10	Yes	No	Yes	Yes	Qazvin
Fa35	_	F/18	Yes	Yes	Yes	Yes	Tehran
Fa36	_	F/6	Yes	No	Yes	No	Tehran
Fa37	_	F/10	Yes	No	Yes	Yes	Rodbar-Rasht
Fa38	_	M/15	Yes	No	No	No	Babolsar
Fa39	_	F/13	Yes	No	No	Yes	Mashhad
Fa40	+	M/6	Yes	Yes	No	No	Esfahan

Fa41 to Fa70 did not cooperate for this study. Information of these families available in archives of the Iranian Blood Transfusion Organization and MAHAK hospital.
M, male; F, female; BMT, bone marrow transplantation; +, alive; -, dead; *, twin pregnancy

overlap of 8 to 48 nucleotides with each other (cDNA walking). The primer sequences are shown in Table III. The PCR primers were designed according to the published nucleotide sequence of the *FANCA* cDNA available in the Ensembl Genome Browser (*http://www.ensembl.*org) and by using the Oligo primer analysis software version 5, (*http://www.oligo.net*).

The cDNA of patients samples were amplified by using the PCR Kit (CinnaGen, Iran) following the manufacturer's protocol. The PCR reactions were performed in a total volume about 25 µl containing 50 ng of cDNA, 0.5 µl of each primer (5 pmol), 1 µl of dNTP (2 mM), 5 µl of 10 x reaction buffer with $MgCl_2$ and 0.3 µl (1.5 U) of *Taq* DNA polymerase, (DNA free tested - mi-E8001L, Metabion, Germany). The PCR conditions were: 5 min at 95°C; then 35 cycles of 45 sec at 94°C, 10-60 sec at 56-68°C, and 45 sec at 72°C; and a final 5 min at 72°C. The smallest PCR product was 175 bp and the largest was 260 bp. The resulting PCR products were subjected to 2 per cent agarose gel electrophoresis, followed by ethidium bromide staining.

Table II. Clinical manifestations of the patients (n=27)

Abnormalities	No.	%
Low birth weight	20	45.45
Skin pigmentation	17	38.63
Delayed milestone	18	40.90
Short stature	21	47.72
Skeletal deformity (thumb)	13	29.54
Kidney	10	22.72
Bladder & urinary tract	5	11.36
Infertility	4	9.09
Gastrointestinal	3	6.81
Cardiac	0	0.0
Eyes abnormality	4	9.09
Hearing abnormality	4	9.09
Gonadal	5	11.36
Nervous	4	9.09
Cancers	3	6.81
Bone marrow transplant	18	40.90
Death	17	38.63

Table III. Specific primers used to amplify *FANCA* cDNA

Primers	Primer sequence (5′→3′)		Amplification of exon/exons	Melting temperature Tm (°C)	Product size (bp)
	Forward	Reverse			
61.1	CCAAGGCCATGTCCGACTC	TTTTTTACACAGTGGACCTTCTACCTC	1-2-3	57.5	221
61.2	GACCTGAATGCCCTTTTGCTTGA	AGGGTGACTGGTCTCCGCTG	2-3-4	59	237
61.3	CCAGCGGAGACCAGTCACCC	GAGCCACGATCCCACAGCATG	4-5-6-7	59.5	249
61.4	GTGGGATCGTGGCTCTTCAGG	TACTCCAGCAGCCAAAGCGTC	7-8-9-10	61	240
61.5	GCTGATTTTTGCACTTGACGC	GGTGAGCAGAGGGTGTGTCC	9-10-11-12	62.5	250
61.6	GCGGACACACCCTCTGCTCACC	AAACGCGCCACCCAGTCTTCA	12-13-14	62	195
61.7	GCGCGTTTGATGGCCCAG	ACCGGGGAGACTCAAAAGGCA	14-15	59.5	229
61.8	TCGTGCCTTTTGAGTCTCCCC	TTCCCCGTATGCTCAAACACC	15-16-17-18	57.5	250
61.9	TGGTGTTTGAGCATACGGGGA	AGGGCTGCATCTTCTGGCTTC	18-19-20-21-22	56	244
61.10	AGAAGCCAGAAGATGCAGCCC	AGTCTCGGCGTGTTGATGCTG	21-22-23	68	244
61.11	GCATCAACACGCCGAGACTGGA	GGTGACGGAGCAGCTGGCAGA	23-24-25	62	201
61.12	TCTGCCAGCTGCTCCGTC	AGAGAGAGTAAGAAATTGCTGCTG	25-26-27	61	243
61.13	TACAGCAGCAATTTCTTACTCTCTC	CTCTCGGAAGGTTCTGTGTGTC	27-28	56	244
61.14	CTGCCCTCTCTCTCTGGACAC	TGACAAGAATGGTACACGCAG	28-29-30	57	240
61.15	AGGCTGCGTGTACCATTCTTG	CGTCTGCGGAAAATCTCAAAG	30-31-32	56	232
61.16	CTTTGAGATTTTCCGCAGACG	GTGGAAGAACTGCTCGCATC	32-33	57	187
61.17	TCTTCCACTTGGTCAACTCTG	ATTAAGGGGCATTTCGTCTG	33-34-35	57	175
61.18	CCAGACGAAATGCCCCTTA	TCCCTGACTTGTTGAATCGC	35-36-37	56	240
61.19	GCGATTCAACAAGTCAGGGA	GAAAGAGTGCCAGCCAGGATA	37-38-39	57	223
61.20	TATCCTGGCTGGCACTCTTTC	CTGGAGGTGAAACTGTGCTTG	39-40-41	61	240
61.21	CAAGCACAGTTTCACCTCCAG	CAGGTCCCGTCAGAAGAGATG	41-42-43	61	260

1. Primers for the *FANCA* gene were designed by using the Oligo 5 software.
2. Primers made inTAG Copenhagen;
3. The minimum PCR product was 175 bp in 61.17 and maximum 260 bp in 61.21.
4. The overlapping fragments were minimum 8nts in primers 61.6 and 61.7 up to 48nts in primer 61.1 and 61.2

High resolution melting (HRM) curve analysis: The HRM analysis was carried out by using the Rotor-Gene 6000 Multiplexing Instrument (Corbett Research Pty. Ltd., Australia). All primers for real-time PCR and HRM analysis were the same as those mentioned in Table III.

To validate this assay, the normalized melt and HRM curves for each of the 21 fragments were determined, utilizing the samples (cDNA) from 10 normal individuals. When normalized melt and HRM curves were established for each fragment, their resulting products were sequenced with the same forward or reverse primers. The sequencing results were aligned with those in the National Center for Biotechnology Information (NCBI) database (*http://www.ncbi.nlm.nih.gov*). If the query sequence had 97 per cent or greater similarity, its melt and HRM curves were accepted as a reference. The patient samples were then compared with these. The entire coding regions (cDNA) of the *FANCA* gene belonging to both healthy individuals and FA patients were amplified in the 21 overlapping fragments.

Fig. 1 shows an example of the normalized melt and HRM curves regarding fragment 19. The profile of the programme for amplification of this fragment was: 10 min at 95°C; then 45 cycles of 45 sec at 94°C, 20 sec at 56°C, and 20 sec at 72°C. For the HRM curve: wait for 90 sec of pre-melt conditioning on the first step at 70°C, rising by 0.1°C in each step and wait for two seconds for each step afterwards. The temperature ramp was from 70 to 95°C. Reactions were performed in a total volume of 15 µl that included 5 ng of cDNA, 0.3 µl of each primer, 4 µl of Master Mix (SensiMix HRM Lot No: 908/SMH, Quantace, Bioline, USA), 0.5 µl of EvaGreen dye (Lot No: 908/EG, Quantace Bioline, USA) and water.

Nucleotide sequencing: Significant aberrant patterns in the HRM curves of patients samples were sequenced by the ABI 3730XL, capillary DNA sequencing system (Macrogen, Korea) in both directions. The primers used for sequencing were the same as those used for PCR (Table III). Sequence data were analyzed by using the *FANCA* cDNA sequence available in the Ensembl and NCBI databases as reference sequences.

PCR with genomic DNA: To confirm genomic deletion of the *FANCA* in patient 610011, for whom an HRM curve was not obtained after fragment 3, another pair of primers was designed for exon 13 and intron 13. The PCR primers were designed according to the published nucleotide sequence of *FANCA* in the Ensembl database, and by using the Oligo primer analysis software version 5.

The sequences of primers were: forward, 5´-GCGGACACACCCTCTGCTCACC-3´, and reverse, 5´- CGCAGCCCCCTCACTTCCCAC-3´. The PCR conditions were: 5 min at 95°C; then 40 cycles of 60 sec at 95°C, 30 sec at 61°C, and 40 sec at 72°C; and a final 5 min at 72°C. The PCR reactions were performed in a total volume of 25 µl. The resulting PCR product was

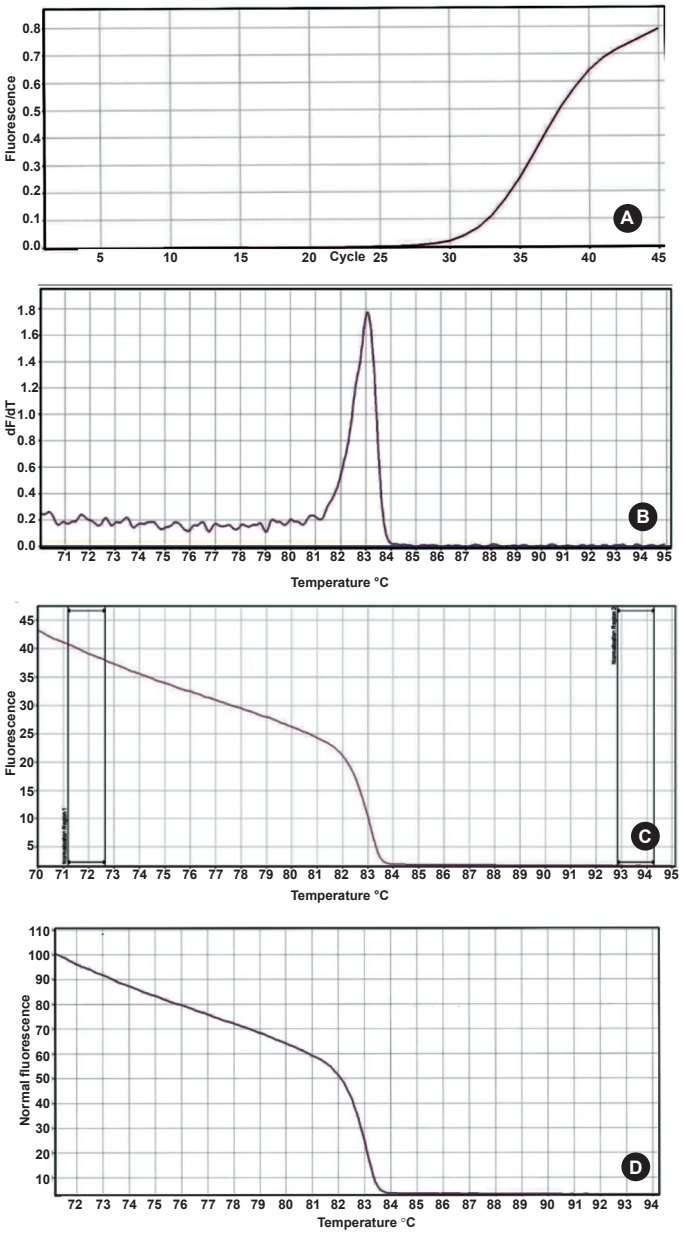


Fig. 1. An example of HRM normalized graph of fragment 19. **A:** Amplification curve, **B:** Melt curve, **C:** HRM analysis curve, **D:** HRM normalized graph.

351 bp, which was subjected to 2 per cent agarose gel electrophoresis, and visualized by ethidium bromide staining[19].

Real-time RT-PCR for expression study of FANCA: Real-time quantitative PCR is carried out by using the Rotor-Gene 6000 Multiplexing Real-Time Instrument (Corbett Research Pty. Ltd., Australia) with the newly released software version 1.7 (website: *http://www.corbettlifescience.com*).

Real-time RT-PCR was used to investigate the expression of patients samples relative to a housekeeping gene (*GAPDH*). The sequences of *GAPDH* primers were: forward, 5′-GCAGGGGGGAGCCAAAAGGGT-3′, and reverse, 5′-TGGGTGGCAGTGATGGCATGG-3′. Real-time reactions were carried out in a total volume of 10 µl containing 5 ng of cDNA, 0.3 µl of each primer, 2 µl of Master Mix, plus SYBR Green1 (LightCyler Fast Start DNA Masterplus SYBR Green1, 03515869001, Roche, Germany) and water. Real-time conditions were: 5 min at 95°C; then 50 cycles of 10 sec at 95°C, 10 sec at 59°C, and 25 sec at 72°C. The resulting 219 bp product was subjected to 2 per cent agarose gel electrophoresis, and visualized by ethidium bromide staining.

Data analysis was carried out by using the LinReg software (version 11.0), *http://LinRegPCR.nl*), according to the manufacturer's instructions.

Results

The patients' clinical, haematological and parental information was collected, and their past medical histories were recorded in individual forms. The lineage was constructed up to three generations. Although autosomal-recessive patterns were observed, no X-linked pattern of inheritance was detected.

In total, there were 24 males, and 20 female patients from 40 families. The male/female ratio (M/F) was 1.2: 1. In four families (designated; family 3, 5, 9 and 30), there were two FA-affected children (a boy and a girl) in each family. Families 3 and 9 had consanguineous marriages, but families 5 and 30 had non-consanguineous unions. The age of patients varied from 5 to 51 yr. More than 77 per cent of the FA patients were from families who had consanguineous marriages, and their degree of kinship was that of first cousins. Six families with non-consanguineous unions had ailing children. Eighteen patients (40.9%) received bone marrow transplants (BMT), four of whom died in less than seven months following BMT. Twenty patients (45.45%) received androgen therapy, and premature puberty was observed in one of them. Twenty one patients (47.72%) received blood transfusion (Table I).

One of the families without a consanguineous marriage (family 19) had a twin pregnancy. They had two children, one of whom was FA-affected and the other, healthy. The bone marrow of the healthy child was used to treat the FA-affected one (Table I).

Low birth weight was found in 20 (45.45%) patients. Eighteen (40.9%) patients had delayed milestones, and skin pigmentation was seen in 17 (38.63%). Skeletal malformations were present in 13 (30%) cases, which consisted of mainly thumb abnormalities (dual, hypoplasic). Short stature was prominent in 21 (47.72%) patients. The ultrasound study of the abdomen in 10 patients (22.72%) revealed a kidney abnormality (displacement or one kidney). In three patients lymphoma and acute myelogenous leukaemia (AML) had developed. Four (9.09%) patients developed abnormal ears. However, none of the patients had heart problems (Table II).

High resolution melting data analysis: Altogether, changes in the HRM curve were found in 14 patients samples (Table IV). Alterations in HRM and their melt curves in the 14 patients samples were investigated and compared with the corresponding HRM and melt curves of normal fragments. The shift in the HRM curve of fragment 20 (exon 39, 40 and 41) form patients 61004, 61006, 610010, 610012 and 610013 was conspicuous (Fig. 2a-f). In a patient (610011), with the exception of fragments 1, 2 and 3, the HRM curve of other fragments could not be detected. Therefore, this patient was designated as having a large genomic deletion, which was estimated to be 60 kb in size. The absence of an HRM curve was further confirmed by PCR of genomic DNA, as mentioned previously.

Sequencing analysis: After selecting the shift in the HRM curve of five patient samples (61004, 61006, 610010, 610012 and 610013), the HRM products of the corresponding fragments were sequenced.

Sequencing data of the patient 61004 showed an insertion of a single thymine (T) base at residue 4048 (c. 4048_4049insT) and change of the guanine (G) to the adenine (A) base at residue 4049 (c. 4049G>A) in exon 41. This alteration in the sequence was novel, leading to an altered stop codon (Fig. 3). In patient 61006, sequencing data showed the insertion of a single T base at residue 4046 (c. 4046_4047insT) in exon 41.

Table IV. HRM data of FA patients (n=14)

Primers and fragment correspondence to cDNA		61.3	61.4	61.5	61.6	61.7	61.9	61.10	61.11	61.16	61.19	61.20
		Frg.3	Frg.4	Frg.5	Frg.6	Frg.7	Frg.9	Frg.10	Frg.11	Frg.16	Frg.19	Frg.20
S. No.	Patients	Exon 4-7	Exon 7-10	Exon 10-12	Exon 12-14	Exon 14-15	Exon 18-21	Exon 21-23	Exon 23-25	Exon 32-33	Exon 37-39	Exon 39-41
1	61000											//
2	61001			//				//				
3	61003		//	//	//		//					
4	61004									//	//	//
5	61005							//				//
6	61006							//				//
7	61007							//				
8	61008	///		//	//							
9	61009	//						//				
10	610010							//				//
11	610012											//
12	610013	//		//								//
13	610014			//		//						//
14	610015	//							//			

Fig, fragment; //, shift of HRM curve

This sequence alteration was also novel, leading to a frame-shift mutation and thus an altered stop codon, 40 nucleotides afterward (Fig. 3). As for patient 610010, sequencing data showed the insertion of a single A base at residue 4021 (c. 4021_4022insA) in exon 41, resulting in a frame-shift mutation and then an altered stop codon, 12 nucleotides afterward, thus pointing to another novel sequence alteration (Fig. 3). Another new alteration, in the sequence leading to an altered stop codon, was also observed in patient 610012, whereby a change of the G base to the cytosine (C) base at residue 4046 (c. 4046G>C), and insertion of a single T base at residue 4048 (c. 4048_4049insT) in exon 41 were detected (Fig. 3). Sequence data of the patient 610013 indicated a change of the A to the G base at residue 3982 (c. 3982A>G) in exon 40 (Fig. 3). This sequence alteration led to substitution by an alanine (Ala) instead of a threonine (Thr) (p. Thr1328Ala).

In the case of patient 610011, following amplification of fragment 3, no such curves were detected in the remaining fragments, indicating the possible presence of a large genomic deletion (exon 8 to 43). In order to confirm this deletion, we chose randomly a position on the DNA between exon 12 to 14, and accordingly, designed a pair of primers for that region. The PCR was carried out by using normal, her parents and the patient's genomic DNA as template. The PCR of the corresponding region for the patient did not show an amplified product. Hence, deletion of almost the entire gene (nearly 60 kb) appeared to be the cause of FA in this patient (610011).

Real-time expression analysis: A study of the quantitative expression of *FANCA* as compared to the *GAPDH* gene (a housekeeping gene) was carried out. The raw real-time data were analyzed by the LinReg software (version 1.7), in accordance with the manufacturer's instructions.

The expression of six samples (61004 – 61006 – 610010 – 610011 – 610012 and 610013) relative to the *GAPDH* gene was investigated. All six samples showed a decrease in *FANCA* expression. The results are summarized in Fig. 4.

Discussion

In this study, clinical data from 40 families with 27 live patients were collected and investigated. Because complementation analysis was not studied in Iran, a strategy involving PCR, HRM, real-time RT-PCR and sequencing was designed to identify the spectrum of sequence alterations in the cDNA of the *FANCA* gene. Twenty one pairs of specific primers were designed

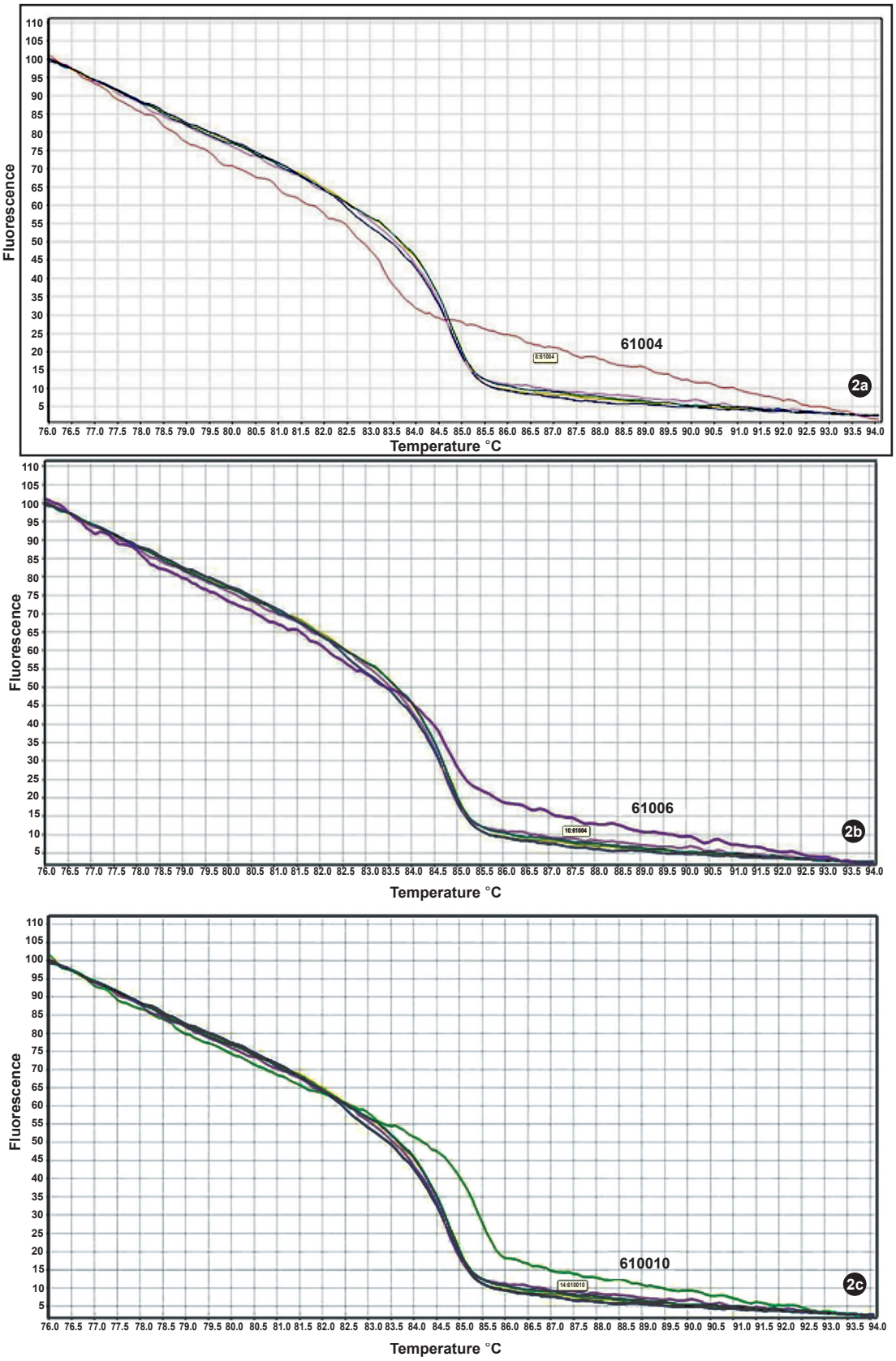
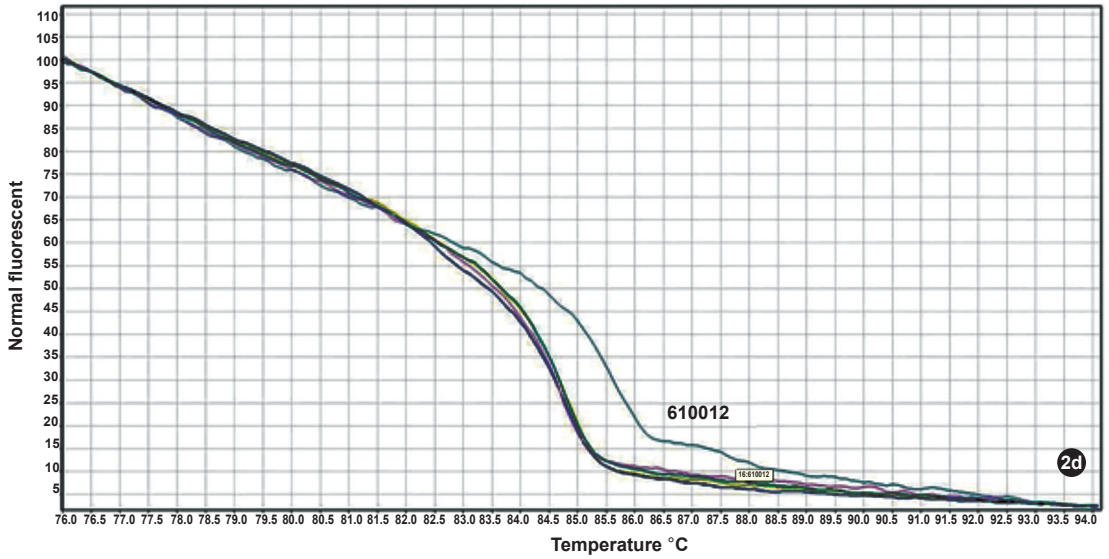
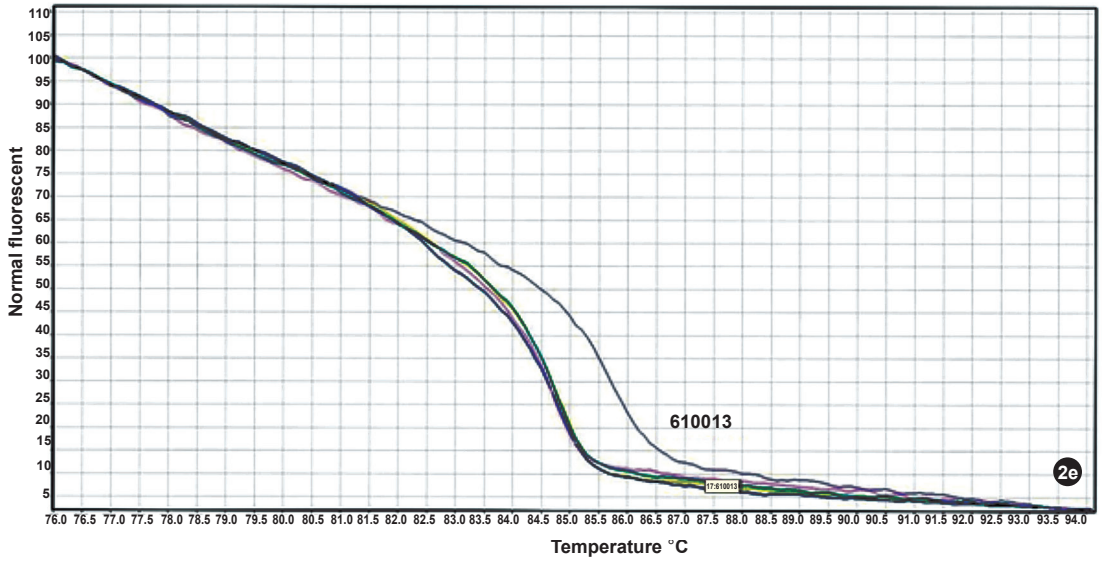
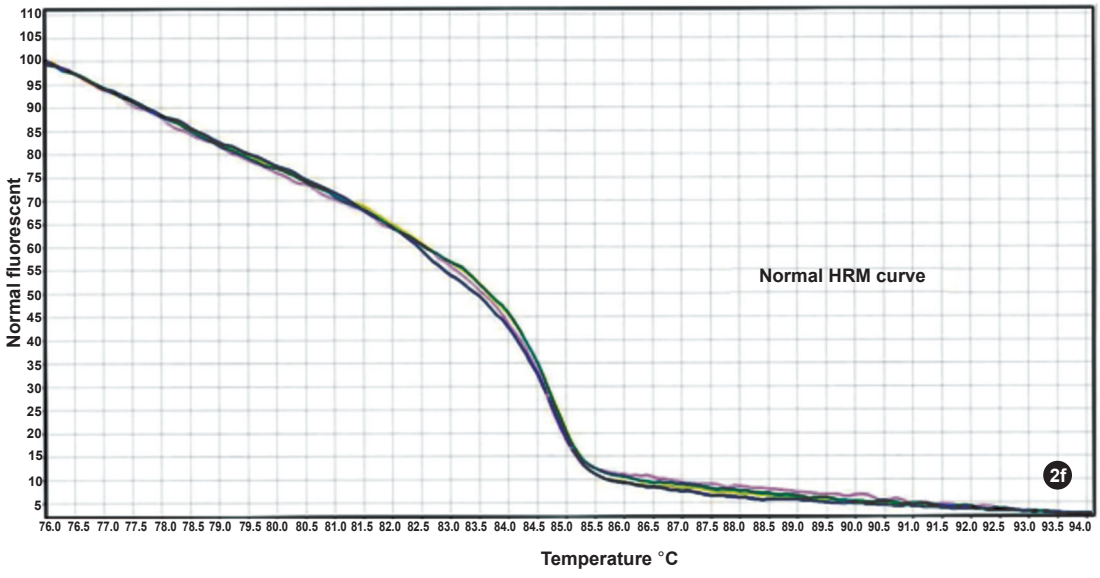


Fig. 2. Shift of HRM curve in five samples (a-f).

Contd...


Normal fluorescent
610012
2d
Temperature °C


Normal fluorescent
610013
2e
Temperature °C


Normal fluorescent
Normal HRM curve
2f
Temperature °C

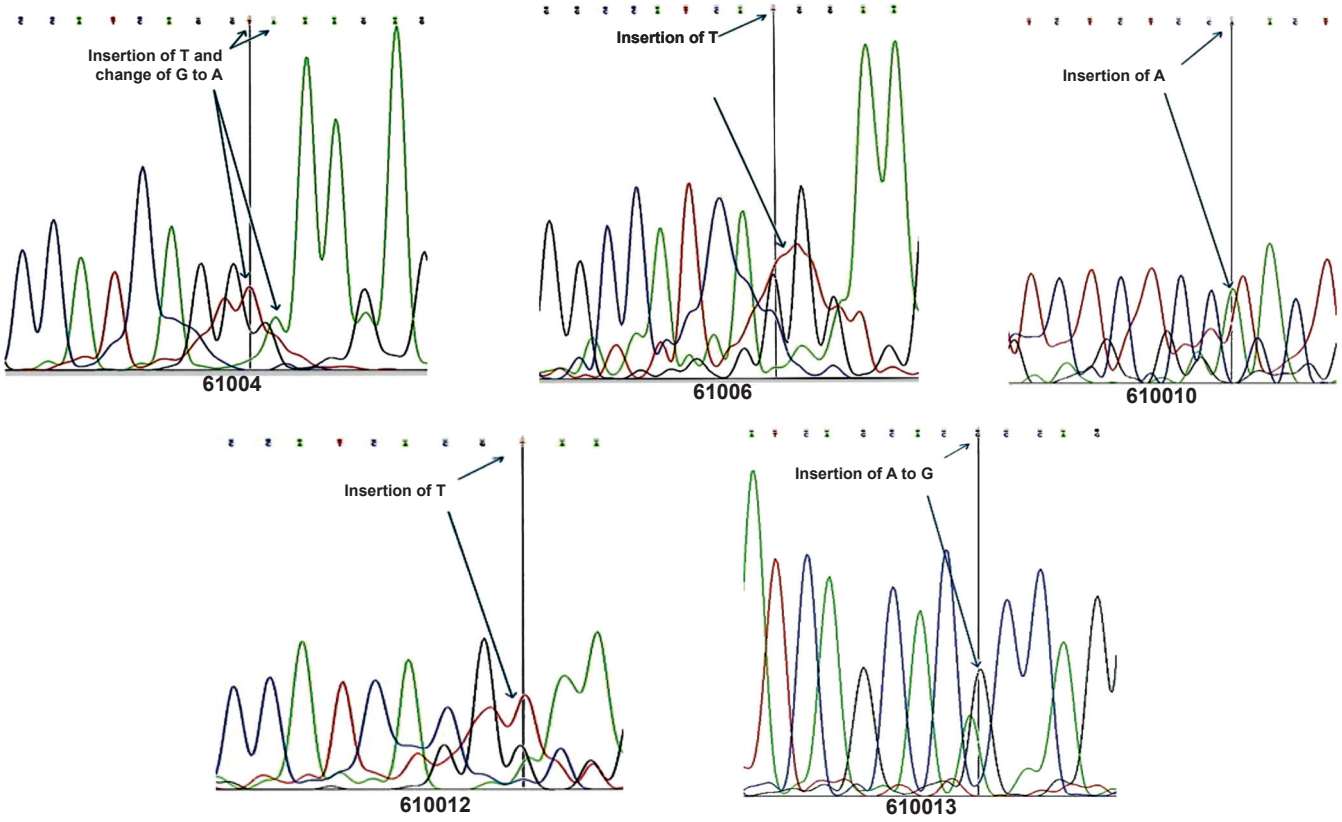


Fig. 3. Sequence alterations in five samples.

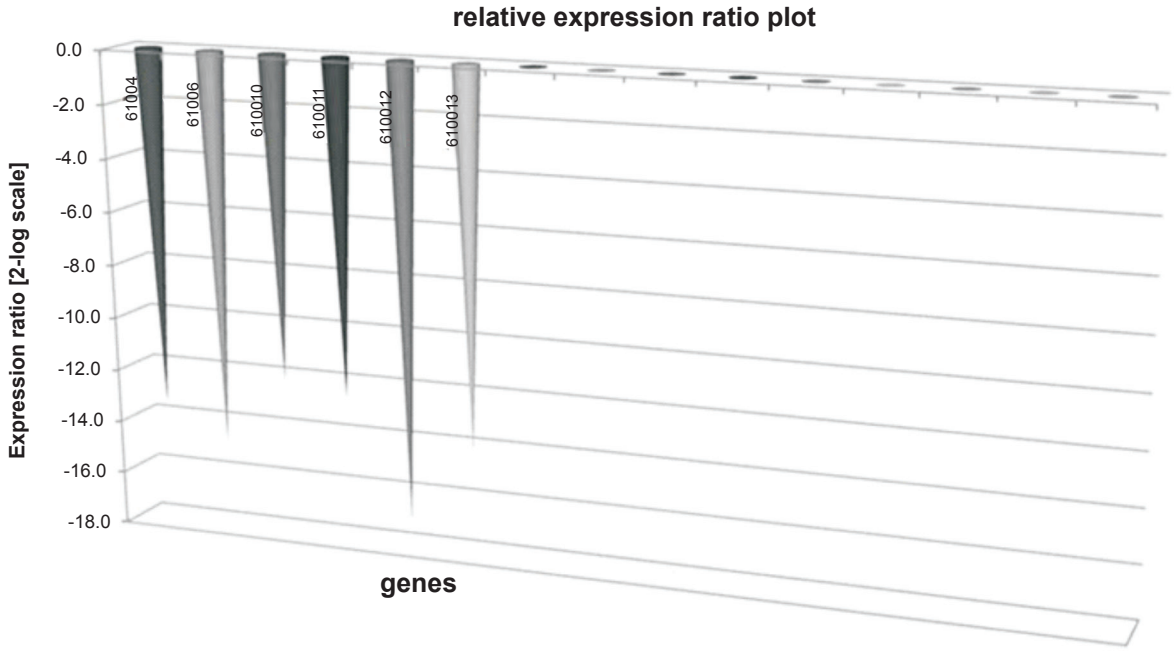


Fig. 4. Relative expression ratio of *FANCA* gene in six samples.

to cover the entire cDNA of *FANCA*. In the human genome, HRM has 64 per cent sensitivity with a T_M melt curve shift of > 0.5°C with regard to detection of C/T and G/A base changes. In general, the more base changes in the DNA sample, the easier these are to be detected by HRM[22].

There are certain limitations in the HRM procedure. DNA fragments should not be more than about 250 bp. Furthermore, formation of primer-dimmers or non-specific products can complicate the interpretation of HRM results. These complications can be eliminated by subjecting the HRM product on 2 per cent agarose gel electrophoresis, followed by visualization using ethidium bromide staining, and double-checking the HRM programme.

FANCA is a large gene (≈ 80 kb), and comprised 43 exons. Hence, screening of mutations with MLPA or the sequencing approach can be technically demanding, time-consuming and expensive. However, with the approach used in this study; five sequence alterations and one large deletion were identified.

In patients 61004 and 610012, the stop codons perhaps caused premature termination of translation, yielding a truncated protein, thus resulting in the disruption of gene function. In patients 61006 and 610010, frame-shift mutation and a subsequent altered stop codon could be a cause for premature termination of translation.

These sequence alterations have not been previously reported in the Fanconi Anemia Mutation Database (*http://www.rockefeller/fanconi/mutate*), and hence, can be regarded as novel.

Sequence alterations in patient 610013 [a substitution of threonine (Thr) (p. Thr1328Ala) with alanine (Ala) amino acid] has been previously reported as an SNP, and described in the Fanconi Anemia Mutation Database. This observation has also been previously reported by Levran and colleagues[23]. In patient 610011, a surprisingly large genomic deletion was detected between exon 8 and 43, which has also not been previously reported. Hence, it can also be regarded as novel. None of these sequence alterations were detected in the 100 control alleles. In these six patients samples, *FANCA* expression was found to be reduced or downregulated when compared to that of *GAPDH*.

In the absence of functional data, it is difficult to determine conclusively whether an observed variation in sequences represents a pathogenic mutation. Although the sequence alteration is predicted to truncate the protein and change its conformation, such a variation in sequence is highly likely to be a pathogenic mutation. Additionally the results of this study (particularly that of patient 610011) have shown that HRM analysis may serve as an alternative to the MLPA.

Fanconi anaemia exhibits a high frequency of chromosomal damage and has the risk of malignancies due to genetic rearrangements. Various cancers have been reported in this regard, most notably acute myeloid leukaemia (AML)[24]. The patient 61004 died due to AML, during the course of this study. Although, phenotype–genotype correlation in FA would be more meaningful based on molecular studies, in actual fact, these are highly varied.

In conclusion, males were found to be more affected by FA than females and a high frequency of consanguinity was observed in the affected families. Other common abnormalities detected in FA-affected individuals included short stature and upper limb deformities, especially congenital thumb abnormality. However, congenital abnormality of the kidneys was ruled out as a symptom of FA. Finally, the clinical features of the patients were so varied that a genotype-phenotype relationship could not be found.

Conflicts of Interest: None.

Acknowledgment

Authors acknowledge Dr Thamasebi Enferadi, of the core facility lab in National Institute of Genetic Engineering and Biotechnology (NIGEB), and the Iranian Blood Transfusion Organization and MAHAK hospital. Authors thank the physicians who referred their patients and provided clinical data.

References

1. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. *Blood* 2006; *107* : 4223-33.
2. Levitus M, Joenje H, de Winter JP. The Fanconi anemia pathway of genomic maintenance. *Cell Oncol* 2006; *28* : 3-29.
3. Bouchlaka C, Abdelhak S, Amouri A, Ben Abid H, Hadiji S, Frikha M, *et al*. Fanconi anemia in Tunisia: high prevalence of group A and identification of new *FANCA* mutations. *J Hum Genet* 2003; *48* : 352-61.
4. Berger R, Le Coniat M, Gendron MC. Fanconi anemia. Chromosome breakage and cell cycle studies. *Cancer Genet Cytogenet* 1993; *69* : 13-6.
5. Sims AE, Spiteri E, Sims RJ 3rd, Arita AG, Lach FP, Landers T, *et al.* FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. *Nat Struct Mol Biol* 2007; *14* : 564-7.

6. Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. *Nature* 2013; *493* : 356-63.

7. Ameziane N, Errami A, Léveillé F, Fontaine C, de Vries Y, van Spaendonk RM, *et al*. Genetic subtyping of Fanconi anemia by comprehensive mutation screening. *Hum Mutat* 2008; *29* : 159-66.

8. Balta G, de Winter JP, Kayserili H, Pronk JC, Joenje H. Fanconi anemia A due to a novel frameshift mutation in hotspot motifs: lack of FANCA protein. *Hum Mutat* 2000; *15* : 578-83.

9. Tachibana A, Kato T, Ejima Y, Yamada T, Shimizu T, Yang L, *et al*. The *FANCA* gene in Japanese Fanconi anemia: reports of eight novel mutations and analysis of sequence variability. *Hum Mutat* 1999; *13* : 237-44.

10. Joenje H, Patel KJ. The emerging genetic and molecular basis of Fanconi anemia. *Nat Rev Genet* 2001; *2* : 446-57.

11. Joenje H, Lo ten Foe JR, Oostra AB, van Berkel CG, Rooimans MA, Schroeder-Kurth T, *et al*. Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. *Blood* 1995; *86* : 2156-60.

12. Centra M, Memeo E, d'Apolito M, Savino M, Ianzano L, Notarangelo A, *et al*. Fine exon-intron structure of the Fanconi anemia group A (FAA) gene and characterization of two genomic deletions. *Genomics* 1998; *51* : 463-7.

13. de Winter JP, van der Weel L, de Groot J, Stone S, Waisfisz Q, Arwert F, *et al*. The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. *Hum Mol Genet* 2000; *9* : 2665-74.

14. Garcia-Higuera I, Kuang Y, Naf D, Wasik J, D'Andrea AD. Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. *Mol Cell Biol* 1999; *19* : 4866-73.

15. Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, *et al*. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* 2001; *7* : 249-62.

16. Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER 3rd, Hurov KE, Luo J, *et al*. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* 2007; *129* : 289-301.

17. Nakamura A, Matsuura S, Tauchi H, Hanada R, Ohashi H, Hasegawa T, *et al*. Four novel mutations of the Fanconi anemia group A gene (*FAA*) in Japanese patients. *J Hum Genet* 1999; *44* : 48-51.

18. Savino M, Ianzano L, Strippoli P, Ramenghi U, Arslanian A, Bagnara GP, *et al*. Mutations of the Fanconi anemia group A gene (*FAA*) in Italian patients. *Am J Hum Genet* 1997; *61* : 1246-53.

19. Morgan NV, Tipping AJ, Joenje H, Mathew CG. High frequency of large intragenic deletions in the Fanconi anemia group A gene. *Am J Hum Genet* 1999; *65* : 1330-41.

20. Auerbach AD: Fanconi anemia diagnosis and the diepoxybutane (DEB) test. *Exp Hematol* 1993; *21* : 731-3.

21. Tootian S, Mahjoubi F, Rahnama M, Hormozian F, Mortezapour F, Razazian F, *et al*. Cytogenetic investigation in Iranian patients suspected with Fanconi anemia. *J Pediatr Hematol Oncol* 2006; *28* : 834-6.

22. Herrman MG, Durtschi JD, Bromley KL, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem* 2006; *52* : 494-503.

23. Levran O, Erlich T, Magdalena N, Gregory JJ, Batish SD, Verlander PC, *et al*. Sequence variation in the Fanconi anemia gene FAA. *Proc Natl Acad Sci USA* 1997; *94* : 13051-6.

24. Alter BP. Cancer in Fanconi anemia, 1927-2001. *Cancer* 2003; *97* : 425-40.

Reprint requests: Dr Frouzandeh Mahjoubi, Division of Medical Genetics, National Institute of Genetic Engineering & Biotechnology (NIGEB), Pajoohesh Blvd, Tehran - Karaj Highway, Tehran, Iran
e-mail: frouz@nigeb.ac.ir