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ASSESSMENT OF SINGLE NUCLEOTIDE POLYMORPHISMS IN SCREENING 52 DNA REPAIR AND CELL CYCLE CONTROL GENES IN FANCONI ANEMIA PATIENTS

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Fanconi anemia (FA) is a rare genetically heterogeneous disorder associated with bone marrow failure, birth defects and cancer susceptibility. Apart from the disease-causing mutations in FANC genes, the identification of specific DNA variations, such as single nucleotide polymorphisms (SNPs), in other candidate genes may lead to a better clinical description of this condition enabling individualized treatment with improvement of the prognosis. In this study, we have assessed 95 SNPs located in 52 key genes involved in base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double strand break (DSB) repair and cell cycle control using a DNA repair chip (Asper Biotech, Estonia) which includes most of the common variants for the candidate genes. The SNP genotyping was performed in five FA-D2 patients and in one FA-A patient. The polymorphisms studied were synonymous (n=10), nonsynonymous (missense) (n=52) and in non-coding regions of the genome (introns and 5 'and 3' untranslated regions (UTR)) (n=33). Polymorphisms found at the homozygous state are selected for further analysis. Our results have shown a significant inter-individual variability among patients in the type and the frequency of SNPs and also elucidate the need for further studies of polymorphisms located in *ATM, APEX APE 1, XRCC1, ERCC2, MSH3, PARP4, NBS1, BARD1, CDKN1B, TP53* and *TP53BP1*

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which may be of great importance for better clinical description of FA. In addition, the present report recommends the use of SNPs as predictive and prognostic genetic markers to individualize therapy of FA patients.
Key words: DNA repair, Fanconi anemia, single nucleotide polymorphisms

List of abbreviations

APEX APE1- APEX nuclease (multifunctional DNA repair enzyme) 1; *ATM*- ataxia telangiectasia mutated serine/threonine kinase; *BRCA1*- breast cancer 1, early onset; *BARD1*- BRCA1 associated RING domain 1; *BRCA2*- breast cancer 2, early onset; BER- base excision repair; *CCND1*- cyclin D1; *CCNH*- cyclin H; *p21/CDKN1A*- cyclin-dependent kinase inhibitor 1A (p21, Cip1); *CDKN2A*- cyclin-dependent kinase inhibitor 2A ; *CDKN1B*- cyclin-dependent kinase inhibitor 1B (p27, Kip1); *CDK7*- cyclin-dependent kinase 7; *CHEK2*- checkpoint kinase 2; DSB- double strand break; *ERCC1*- excision repair cross-complementation group 1; *ERCC2*- excision repair cross-complementation group 2; *ERCC4*- excision repair cross-complementation group 4; *ERCC5*- excision repair cross-complementation group 5; *FANCD2*- Fanconi anemia, complementation group D2; *GADD45A*- growth arrest and DNA-damage-inducible, alpha; *GRTH/Ddx25*- DEAD (Asp-Glu-Ala-Asp) box helicase 25; *LIG1*- ligase I, DNA, ATP-dependent; *LIG3*- ligase III, DNA, ATP-dependent; *LIG4*- ligase IV, DNA, ATP-dependent; *MGMT AGT*- O-6-methylguanine-DNA methyltransferase; *MLH1*- mutL homolog 1; MMR- mismatch repair; *MSH2*- mutS homolog 2; *MSH3*- mutS homolog 3; *MSH6*- mutS homolog 6; *MYH*- mutY DNA glycosylase; NER- nucleotide excision repair; *NBS1*- nibrin; *OGG1*- 8-oxoguanine DNA glycosylase; *PARP1*- poly (ADP-ribose) polymerase 1; *PARP4*- poly (ADP-ribose) polymerase family, member 4; *PCNA*- proliferating cell nuclear antigen; *PMS2*- PMS2 postmeiotic segregation increased 2 (S. cerevisiae); *POLB*- polymerase (DNA directed), beta; *RAD9A*- RAD9 checkpoint clamp component A ; *RAD23B*- RAD23 homolog B (S. cerevisiae); *RAD51*- RAD51 recombinase; *RAD52*- RAD52 homolog (S. cerevisiae); *RAD54B*- RAD54 homolog B (S. cerevisiae); *RECQL*- RecQ helicase-like; *XPA*- xeroderma pigmentosum, complementation group A; *XPC*- xeroderma pigmentosum, complementation group C; *XRCC1*- X-ray repair complementing defective repair in Chinese hamster cells 1; *XRCC2*- X-ray repair complementing defective repair in Chinese hamster cells 2; *XRCC3*- X-ray repair complementing defective repair in Chinese hamster cells 3; *XRCC4*- X-ray repair complementing defective repair in Chinese hamster cells 4; *XRCC5*- X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining); *XRCC9/FANCG*- Fanconi anemia, complementation group G; SNP- single nucleotide polymorphism; *TP53*- tumor protein p53; *TP53BP1*- tumor protein p53 binding protein 1; *TP53BP2*- tumor protein p53 binding protein 2

INTRODUCTION

Fanconi anemia (FA) is a recessive disorder considered to encompass a defect in maintenance of genome integrity. It is associated with bone marrow failure, birth defects and predisposition to cancer. The disease is genetically heterogeneous with 16 complementation groups (FA -A to FA –Q) and associated genes having been characterized (KEE and D'ANDREA, 2012; BOGLIOLO *et al.*, 2013; CHEN *et al.*, 2014). All of the 16 gene products operate in a common process to maintain genome stability. Some of these proteins have extranuclear activities affecting redox balance, apoptosis and energy metabolism (CAPPELLI *et al.*, 2013). The most frequent complementation group is FA-A (~ 60% of all cases of the disease); FA-C and FA-G account for

~ 10-15% of the cases while the rest of the cases (~ 15%) is distributed over the remaining complementation groups (e.g. the prevalence of FA-D2 is 3.3%) (MAGDALENA *et al.*, 2005; MATHEW, 2006; AMEZIANE *et al.*, 2012). Regardless of the complementation group, FA is a condition involving high risk of leukemia and solid tumors (KUTLER *et al.,* 2003). However, FA patients exhibit a high degree of clinical variability and have poor tolerance for radiotherapy and chemotherapy (MATHEW, 2006). Thus, the knowledge of factors that might predict either the best drug response or adverse outcome would greatly facilitate the decision making concerning the choices of treatment.

Single nucleotide polymorphisms (SNPs) represent one of the largest types of inherited genetic variations (BOND and LEVINE, 2007) and are considered as predictive and prognostic genetic markers. Polymorphisms in DNA repair and cell cycle control genes may alter protein function leading to genomic instability and increased cancer risk (SIGURDSON *et al.*, 2004).

As already known, Fanconi anemia is a disease associated with deficiencies in DNA repair pathway, which may be the cause of the overall increased cancer risk in patients. FA proteins constitute the FA pathway responsible for repair of inter - and intra - strand DNA cross - links either occurring spontaneously or induced by clastogens as mitomycin C or diepoxybutane (JOENJE and PATEL, 2001; KIM and D'ANDREA, 2012). However, apart from the disease-causing mutations in FANC genes, the identification of specific variations in other candidate genes in FA patients may lead to a better clinical description of this condition enabling individualized treatment with improvement of the prognosis. In light of the fact that Fanconi anemia is a highly penetrant cancer susceptibility disorder, the products of the identified genes could become targets for innovative therapies in patients. In addition, the genotyping of SNPs in key genes involved in DNA repair may provide insight into the interactions between FA mutation and specific variations in DNA repair genes in each of the FA patients.

The results presented here focus on assessment of the known variations located in genes involved in base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double strand break (DSB) repair and also in the several genes related to the cell cycle control and apoptosis. Investigation was performed in patients belonging to complementation groups FA-A and FA-D2; the last one present as the most prevalent complementation group in Serbia.

MATERIALS AND METHODS

Patients

Six patients aged 3 to 13 years who had previously been diagnosed with FA based on clinical features were enrolled in this study. All of the patients had several developmental malformations (skin, skeletal, genitourinary and neurological anomalies) and developed bone marrow failure at an early age. At the moment of sampling, no one of the patients has been diagnosed with cancer. A clinical diagnosis of FA was confirmed by a positive diepoxybutane (DEB) test (AUERBACH, 1993). Complementation group assignment was performed by immunoblotting at the Department of Genetics Microbiology, Universitat Autonoma de Barcelona, Spain, and at Universität Würzburg, Institut für Humangenetik, Germany. The FA complementation group analysis showed that five patients belong to the complementation group FA-D2 (Patients #1 - #5), whereas only one patient belongs to the complementation group FA-A (Patient #6). Informed consent for investigation was obtained from all families, and the study was approved by the Ethical Committee of the Mother and Child Health Care Institute of Serbia.

DNA isolation

DNA was isolated from patients-derived fibroblasts using the phenol-chloroform method. The cells were treated with STE buffer (10 mM TRIS-HCl, 100 mM NaCl, 1 mM EDTA), proteinase K (10 mg/mL) (Sigma-Aldrich) and 10% SDS (sodium dodecyl sulfate) (Sigma-Aldrich) and incubated for 2 hours at 55 ºC. Afterwards, the upper layer (250 - 300 µL) was transferred to a new tube, and dH_2O (50 µL) and phenol (Sigma-Aldrich) (300 µL) were added. The suspension was vigorously vortexed and then centrifuged for 10 minutes at 12000 rpm in a microcentrifuge. The supernatant (250 - 300 µL) was placed into a new tube and chloroform (~150 µL) (Sigma-Aldrich) was added. After centrifugation for 10 minutes at 12000 rpm, the supernatant (250 - 300 µL) was transferred to a new tube and treated with 7.5 M ammonium acetate (150 µL) and ice-cold 100% ethanol (1.5 mL). After centrifugation for 5 minutes at 12000 rpm, the supernatant was decanted and the pellet was air-dried for 1-2 minutes and re-suspended in appropriate volume dH_2O (30-50 µL). The quantification of the concentration and purity of the DNA was performed using a spectrophotometer (Nanodrop-1000, Thermo Fisher Scientific, USA).

Single nucleotide polymorphism analysis

For SNP analysis, DNA samples (concentration 1 µg/µL at least) were studied using DNA repair chip which includes most of the common variants for the DNA repair genes as well as for the several genes related to the cell cycle control and apoptosis. The SNP genotyping was performed at the Asper Biotech (Tartu, Estonia) (http://www.asperbio.com/DNArepairchip.pdf). The total number of SNPs analyzed in this cohort is 95, and the total number of genes is 52.

RESULTS

In this study, we have assessed 95 SNPs located in 52 key genes involved in BER, NER, MMR, DSB repair and cell cycle control using an Asper Biotech array. The investigation was performed in five FA-D2 patients and in one FA-A patient. The summary of genes involved in different repair and cell cycle control pathways and corresponding SNPs are presented in Tables 1 - 2. Some genes are involved in more than one pathway, but each is listed only once. All of the SNPs studied are accessible at the NCBI (*National Center for Biotechnology Information*) database (http://www.ncbi.nlm.nih.gov/snp).

The polymorphisms studied were synonymous (n=10), nonsynonymous (missense) (n=52) and in non-coding regions of the genome (introns and 5 'and 3' untranslated regions (UTR)) (n=33). Polymorphisms found at the homozygous state (genotypes in bold, see Tables 1-2) are selected for further analysis.

In the BER pathway, the SNPs located in *APEX, LIG1*, *LIG3*, *MYH, OGG1, PARP1, PCNA, POLB* and *XRCC1* were evaluated. As shown in Table 1, the most of the studied polymorphisms were found as heterozygous variants, while the homozygous variants were found in *APEX APE1, LIG1, LIG3* and *PARP1*. The missense variation was found in *APEX APE1* (rs3136820) (p.Asp148Glu) in patients #2 and #5. In *LIG1*, an intronic variation (rs3730849) was found in patients #3 and #5, while the intronic rs1805403 of *PARP1* was detected in patients #3, #4 and #6. The 3`-UTR rs1052536 of *LIG3* was found in patients #5 and #6.

In the NER pathway, we analyzed polymorphisms located in *ERCC1, ERCC2 (XPD), ERCC4 (FANCQ), ERCC5 (XPG), GADD45A, RAD23B, XPA* and *XPC* (Table 1). The homozygous missense variation rs13181 (p.Lys751Gln) of *ERCC2* was found in patient #3. The intronic rs3212948 of *ERCC1* was detected in patients #1, #2, #4 and #5. In *GADD45A,* an

intronic rs532446 was detected in patients #1 and #2, while the 5`-UTR variant rs1800975 of XPA was detected in patients #1, #2 and #5.

Table 1. Summary of DNA repair genes screened for polymorphisms

					Detected polymorphisms in FA patients					
Gene	**Gene location**	**dbSNP (rs#)**	**Position in gene**	**Nucl. chan**	**Pat # 1**	**Pat # 2**	**Pat # 3**	**Pat # 4**	**Pat # 5**	**Pat # 6**
Base excision repair (BER)										
APEX APE1	14q11.2-q12	rs10489	Exon 3	G/C	G/C	G/C	G/	G/C	G/C	G/C
		rs31368	Exon 5	T/G	T/A	**G/C**	T/	TG/AC	**G/C**	TG/AC
LIG1	19q13.2-q13.	rs20579	Exon 2	C/T	CT/GA	C/G	C/	C/G	C/G	C/G
		rs37308	Intron 2	C/T	CT/GA	CT/GA	**T/**	CT/GA	**T/A**	C/G
		rs49870	Exon 13	G/A	G/C	G/C	A	G/C	G/C	G/C
		rs37309	Intron 9	A/G	AG/TC	A/T	A/	A/T	A/T	A/T
LIG3	17q11.2-	rs10525	3`-UTR	C/T	C/G	CT/GA	CT	CT/GA	**T/A**	**T/A**
MYH	1p34.3-	rs32194	Exon 12	G/C	G/C	GC/CG	G/	G/C	GC/CG	G/C
OGG1	3p26.2	rs10521	Exon 7	C/G	CG/GC	C/G	C/	CG/GC	CG/GC	CG/GC
PARP 1	1q41-q42	rs11364	Exon 17	T/C	T/A	T/A	T/	T/A	T/A	T/A
		rs18054	Intron 4	A/G	GA/CT	GA/CT	**G/**	**G/C**	GA/CT	**G/C**
PCNA	20pter-p12	rs25406	Intron 2	C/T	C/G	CT/GA	CT	C/G	C/G	CT/GA
POLB	8p11.2	rs31367	Exon 12	C/G	C/G	C/G	C/	C/G	CG/GC	C/G
		rs23071	5`-UTR	A/G	A/T	A/T	A/	A/T	A/T	A/T
XRCC1	19q13.2	rs17997	Exon 6	C/T	CT/GA	CT/GA	CT	C/G	C/G	C/G
		rs25489	Exon 9	G/A	G/C	G/C	G/	G/C	G/C	G/C
		rs25487	Exon 10	G/A	G/C	GA/CT	G	GA/CT	G/C	GA/CT
Direct reversal of damage										
MGMT AGT	10q26	rs1803965	Exon 5	C/T	C/G	CT/GA	C/G	C/G	C/G	C/G
		rs12917	Exon 5	C/T	C/G	CT/GA	C/G	C/G	C/G	C/G
		rs2308321	Exon 7	A/G	AG/TC	A/T	A/T	A/T	AG/TC	A/T
Nucleotide excision repair (NER)										
ERCC1	19q13.2-q13.3	rs3212948	Intron 3	G/C	**C/**	**C/G**	GC/CG	**C/G**	**C/G**	GC/CG
		rs11615	Exon 4	T/C	T/	T/A	TC/AG	T/A	T/A	TC/AG
		rs3212961	Intron 5	C/A	C/	C/G	CA/GT	C/G	C/G	CA/GT
		rs3212986	3`-UTR	G/T	G/	G/C	G/C	G/C	G/C	GT/CA
ERCC2 (XPD)	19q13.3	rs13181	Exon 23	A/C	A/	A/T	**C/G**	A/T	AC/TG	AC/TG
		rs238406	Exon 6	C/A	CA	CA/GT	C/G	C/G	CA/GT	C/G
ERCC4/FA	16p13.12	rs1800067	Exon 8	G/A	G	G/C	G/C	GA/CT	G/C	GA/CT
ERCC5	13q33	rs1047768	Exon 2	T/C	TC	T/A	TC/AG	TC/AG	T/A	TC/AG
XPG		rs17655	Exon 15	G/C	G/	G/C	GC/CG	GC/CG	GC/CG	GC/CG
GADD45A	1p31.2-p31.1	rs532446	Intron 3	T/C	**C/**	**C/G**	T/A	T/A	TC/AG	T/A
RAD23B	9q31.2	rs1805329	Exon 7	C/T	CT	C/G	CT/GA	C/G	C/G	C/G
XPA	9q22.3	rs1800975	5`-UTR	A/G	**G/**	**G/C**	GA/CT	GA/CT	**G/C**	GA/CT
XPC	3p25	rs2228001	Exon 16	C/A	AC	C/G	C/G	C/G	AC/TG	C/G

Table 1 Continued

Gene	Gene location	dbSNP (rs#)	Position in gene	Nucl. chan	Detected polymorphisms in FA patients					
					Pat # 1	Pat # 2	Pat # 3	Pat # 4	Pat # 5	Pat # 6
Mismatch repair (MMR)										
MLH1	3p21.3	rs1799977	Exon 8	A/G	AG/TC	AG/T	AG/TC	AG/TC	A/T	AG/TC
MSH2	2p22-p21	rs4987188	Exon 6	G/A	G/C	G/C	G/C	G/C	G/C	G/C
MSH3	5q11-q12	rs184967	Exon 21	A/G	GA/CT	**G/C**	GA/CT	GA/CT	**G/C**	GA/CT
		rs26279	Exon 23	G/A	AG/TC	AG/T	G/C	G/C	**A/T**	G/C
MSH6	2p16	rs1800935	Exon 3	T/C	T/A	CT/G	T/A	T/A	T/A	**C/G**
PMS2	7p22.2	rs1805324	Exon 11	G/A	G/C	G/C	G/C	G/C	G/C	GA/CT
RECQL	12p12	rs13035	3`-UTR	A/C	AC/TG	AC/T	AC/TG	A/T	A/T	A/T
Double strand break (DSB) repair										
BARD1	2q34-q35	rs2070094	Exon 6	G/A	**A/T**	G/C	GA/CT	GA/CT	G/C	**A/T**
		rs2070093	Exon 6	T/C	**C/G**	**C/G**	**C/G**	CT/GA	CT/GA	**C/G**
BRCA1	17q21	rs799917	Exon 10	C/T	CT/GA	C/G	CT/GA	CT/GA	CT/GA	C/G
		rs4986850		G/A	G/C	G/C	G/C	G/C	G/C	G/C
		rs16941		A/G	AG/TC	A/T	AG/TC	AG/TC	AG/TC	A/T
		rs4986852		G/A	G/C	GA/C	G/C	G/C	G/C	G/C
		rs1799950		A/G	A/T	A/T	A/T	A/T	AG/TC	A/T
BRCA2/ FANCD1	13q12.3	rs1799943	5`-UTR	G/A	GA/CT	**A/T**	GA/CT	G/C	G/C	GA/CT
		rs144848	Exon 10	A/C	A/T	A/T	A/T	A/T	A/T	A/T
		rs4987117	Exon 11	C/T	C/G	C/G	CT/GA	C/G	C/G	C/G
		rs15869	3`-UTR	A/C	A/T	A/T	AC/TG	AC/TG	A/T	AC/TG
FANCD2	3p26	rs3732974	5`-UTR	C/G	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**
LIG4	13q33-q34	rs1805388	Exon 2	C/T	CT/GA	C/G	C/G	CT/GA	CT/GA	CT/GA
		rs1805389	Exon 2	C/T	CT/GA	C/G	C/G	C/G	CT/GA	C/G
NBS1	8q21	rs1063045	Exon 2	G/A	G/C	G/C	GA/CT	**A/T**	G/C	G/C
		rs1805794	Exon 6	G/C	G/C	G/C	GC/CG	**C/G**	G/C	G/C
PARP 4	13q11	rs1050112	Exon 31	C/A	C/G	CA/G	C/G	C/G	**A/T**	C/G
		rs13428	Exon 31	G/C	G/C	CG/G	G/C	G/C	**C/G**	G/C
		rs4986817	Exon 21	A/T	A/T	A/T	A/T	A/T	A/T	A/T
		rs4986819	Intron 19	C/G	C/G	C/G	C/G	C/G	C/G	C/G
		rs7571	Exon 33	G/C	G/C	CG/G	G/C	G/C	**C/G**	G/C
RAD51	15q15.1	rs1801320	5`-UTR	G/C	G/C	G/C	GC/CG	GC/CG	G/C	G/C
RAD52	12p13-	rs11226	3`-UTR	C/T	C/G	C/G	CT/GA	**T/A**	CT/GA	CT/GA
RAD54B	8q21.3-	rs2291439	Exon 5	T/C	**C/G**	TC/A	TC/AG	TC/AG	T/A	TC/AG
XRCC2	7q36.1	rs3218536	Exon 3	G/A	GA/CT	G/C	G/C	G/C	G/C	G/C
		rs718282	3`-UTR	C/T	C/G	C/G	C/G	C/G	C/G	C/G
XRCC3	14q32.3	rs861539	Exon 8	C/T	CT/GA	CT/G	CT/GA	C/G	C/G	C/G
		rs1799796	Intron 7	A/G	AG/TC	AG/T	AG/TC	AG/TC	**G/C**	A/T
		rs1799794	5`-UTR	A/G	A/T	A/T	A/T	A/T	A/T	A/T

Table 1 Continued

					Detected polymorphisms in FA patients					
Gene	**Gen**	**dbSNP**	**Position**	**Nucl.**	**Pat # 1**	**Pat #**	**Pat # 3**	**Pat # 4**	**Pat # 5**	**Pat # 6**
XRCC4	5q13	rs180537	Intron 7	G/A	G/C	GA/C	**A/T**	G/C	G/C	G/C
XRCC5	2q35	rs105167	3`-UTR	T/C	TC/AG	T/A	T/A	T/A	T/A	T/A
		rs2440		G/A	G/C	GA/C	G/C	GA/CT	GA/CT	G/C
XRCC9/FANCG	9p13	rs223785	Exon 7	C/T	C/G	C/G	C/G	C/G	C/G	C/G

					Detected polymorphisms in FA patients					
Gene	**Gene location**	**dbSNP (rs#)**	**Position in gene**	**Nucl. change**	**Pat # 1**	**Pat # 2**	**Pat # 3**	**Pat # 4**	**Pat # 5**	**Pat # 6**
ATM	11q22-q23	rs664677	Intron 20	T/C	TC/A	T/A	TC/A	TC/A	**C/G**	TC/A
		rs1800057	Exon 22	C/G	C/G	CG/G	C/G	C/G	C/G	C/G
		rs1801516	Exon 37	G/A	G/C	G/C	G/C	G/C	G/C	GA/C
		rs1801673	Exon 37	A/T	G/C	G/C	G/C	G/C	G/C	GA/C
		rs609429	Intron 46	C/G	CG/G	C/G	CG/G	CG/G	**G/C**	CG/G
CCND1	11q13	rs603965	Exon 4	G/A	GA/C	GA/C	**A/T**	GA/C	GA/C	GA/C
		rs678653	3`-UTR	C/G	**G/C**	GC/C	**G/C**	GC/C	GC/C	GC/C
CCNH	5q13.3-q14	rs2266690	Exon 7	T/C	TC/A	T/A	T/A	T/A	TC/A	TC/A
p21/CDKN1A	6p21.2	rs1801270	Exon 2	C/A	C/G	CA/G	C/G	CA/G	C/G	C/G
CDKN2A	9p21	rs3731249	Exon 2	G/A	G/C	G/C	G/C	G/C	G/C	G/C
		rs11515	3`-UTR	C/G	CG/G	C/G	C/G	C/G	C/G	C/G
		rs3088440	3`-UTR	C/T	C/G	CT/G	C/G	C/G	C/G	C/G
CDKN1B	12p13.1-p12	rs34330	Exon 1	T/C	CT/G	**C/G**	CT/G	CT/G	T/A	**C/G**
CDK7	5q12.1	- rs nr	Exon 10	C/T	C/G	C/G	C/G	C/G	C/G	C/G
CHEK2	22q12.1	- rs nr	Exon 13	C/del [1]	C/G	C/G	C/G	C/G	C/G	C/G
GRTH/Ddx25	11q24	rs551373	Intron 6	G/T	G/C	G/C	G/C	G/C	G/C	G/C
		rs683155	Exon 10	T/C	**C/G**	CT/G	CT/G	T/A	CT/G	**C/G**
RAD9A	11q13.1-	rs1064876	3`-UTR	G/A	G/C	G/C	G/C	G/C	G/C	G/C
TP53	17p13.1	rs1042522	Exon 4	C/G	GC/C	GC/C	GC/C	**G/C**	**G/C**	GC/C
TP53BP1	15q15-q21	rs560191	Exon 9	C/G	CG/G	CG/G	**G/C**	C/G	C/G	CG/G
		rs689647	Exon 11	G/A	GA/C	G/C	G/C	G/C	G/C	GA/C
TP53BP2	1q42.1	rs17739	3`-UTR	G/A	G/C	G/C	GA/C	GA/C	G/C	GA/C

In the MMR pathway, the SNPs located in *MLH1, MSH2, MSH3, MSH6, PMS2* and *RECQL* were evaluated. The homozygous missense variants rs184967 (p.Gln949Arg) and rs26279 (p.Ala1045Thr) of *MSH3* were found in patients #2 and #5, and patient #5, respectively. The synonymous rs1800935 of *MSH6* was detected in patient #6.

In the DSB repair pathway, which consists of both homologous recombination and non-homologous end-joining, SNPs located in *BRCA1, BRCA2 (FANCD1), FANCD2, LIG4, NBS1, PARP4, RAD51, RAD52, RAD54B, XRCC2, XRCC3, XRCC4, XRCC5* and *XRCC9 (FANCG)* were investigated (Table 1). Similar to the results obtained analyzing the BER, NER and MMR pathways explained above, the most of the nucleotide changes were at the heterozygous state. The

homozygous variants were found in *BARD 1, NBS1, PARP4, RAD54B, RAD52, FANCD2* and *XRCC4.* The missense rs2070094 (p.Val507Met) of *BARD 1* was detected in patients #1 and #6. The rs1805794 (p.Glu185Gln) of *NBS1* was detected in patient #4. The rs1050112 (p.Pro1328Thr) and rs13428 (p.Gly1280Arg) of *PARP4* were found in patient #5. The same patient also carried the rs7571 (p.Ala1656Pro) located in the same gene. Of the synonymous polymorphisms investigated, the rs2070093 of *BARD1* was found in patients #1, #2, #3 and #6. The rs1063045 of *NBS1* was found in patient #4, while the rs2291439 of *RAD54B* was found in patient #1. Considering the polymorphisms located in the non-coding regions, the 5`-UTR rs1799943 of *BRCA2* was found in patient #2. The 5`-UTR rs3732974 located in *FANCD2* was found in all of the patients. The 3`-UTR rs11226 located in *RAD52* was found in patient #4. The intronic rs1799796 of *XRCC3* was found in patient #5, while the intronic rs1805377 of *XRCC4* was found in patient #3.

Table 2. Summary of cell cycle control genes screened for polymorphisms

CCNH	**5q13.3-q14**	**rs2266690**	**Exon 7**	**T/C**	**TC/A**	**T/A**	**T/A**	**T/A**	**TC/A**	**TC/A**
p21/CDKN1A	**6p21.2**	**rs1801270**	**Exon 2**	**C/A**	**C/G**	**CA/G**	**C/G**	**CA/G**	**C/G**	**C/G**
CDKN2A	**9p21**	**rs3731249**	**Exon 2**	**G/A**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**
		rs11515	**3`-UTR**	**C/G**	**CG/G**	**C/G**	**C/G**	**C/G**	**C/G**	**C/G**
		rs3088440	**3`-UTR**	**C/T**	**C/G**	**CT/G**	**C/G**	**C/G**	**C/G**	**C/G**
CDKN1B	**12p13.1-p12**	**rs34330**	**Exon 1**	**T/C**	**CT/G**	**C/G**	**CT/G**	**CT/G**	**T/A**	**C/G**
CDK7	**5q12.1**	**- rs nr**	**Exon 10**	**C/T**	**C/G**	**C/G**	**C/G**	**C/G**	**C/G**	**C/G**
CHEK2	**22q12.1**	**- rs nr**	**Exon 13**	**C/del [1]**	**C/G**	**C/G**	**C/G**	**C/G**	**C/G**	**C/G**
GRTH/Ddx25	**11q24**	**rs551373**	**Intron 6**	**G/T**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**
		rs683155	**Exon 10**	**T/C**	**C/G**	**CT/G**	**CT/G**	**T/A**	**CT/G**	**C/G**
RAD9A	**11q13.1-**	**rs1064876**	**3`-UTR**	**G/A**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**	**G/C**
TP53	**17p13.1**	**rs1042522**	**Exon 4**	**C/G**	**GC/C**	**GC/C**	**GC/C**	**G/C**	**G/C**	**GC/C**
TP53BP1	**15q15-q21**	**rs560191**	**Exon 9**	**C/G**	**CG/G**	**CG/G**	**G/C**	**C/G**	**C/G**	**CG/G**
		rs689647	**Exon 11**	**G/A**	**GA/C**	**G/C**	**G/C**	**G/C**	**G/C**	**GA/C**
TP53BP2	**1q42.1**	**rs17739**	**3`-UTR**	**G/A**	**G/C**	**G/C**	**GA/C**	**GA/C**	**G/C**	**GA/C**

Results of the analysis of SNPs located in the genes involved in cell cycle control and apoptosis are presented in Table 2. We assessed polymorphisms located in *ATM*, *CCND1, CCNH, p21/CDKN1A, CDKN2A,* CDKN1B, CDK7, CHEK2*, GRTH/Ddx25, RAD9A, TP53, TP53BP1* and *TP53BP2.* The homozygous variants were found in *ATM, CDKN1B, TP53, TP53BP1, CCND1* and *GRTH/Ddx25.* The missense polymorphism rs34330 (p.Leu332Pro) of *CDKN1B* was detected in patients #2 and #6. The rs1042522 (p.Pro72Arg) of *TP53* was found in patients #4 and #5. The rs560191 (p.Asp353Glu) of *TP53BP1* was found in patient #3. The synonymous rs603965 located in *CCND*1 was detected in patient #3, while the rs683155 of *GRTH/Ddx25* was found in patients #1 and #6. In addition, the intronic variant rs664677 of *ATM* was identified in patient #5, while the 3`-UTR rs678653 located in *CCND1* was found in patients #1 and #3.

Considering the missense nucleotide substitutions in all of the studied genes, we have observed that among the 52 analyzed SNPs, the 29 (~56%) were identified in our patients as heterozygous variants, while the 12 of them (~23%) were detected as homozygous variants. Of the 10 synonymous SNPs, the six (60%) were found in our patients at the homozygous state. In

view of the 33 SNPs located in the non-coding regions of selected genes, the thirteen (~40%) of them were identified in some of our patients as homozygous variants.

Summarizing the results presented above it could be seen that SNPs identified at the homozygous state were not equally distributed among the patients. Patient #1 carried one missense, three synonymous and five polymorphisms in the non-coding region of the genes. Patient #2 carried three missense, one synonymous and five substitutions in the non-coding regions. Patient #3 carried two missense, two synonymous and five substitutions in the non-coding regions of the genes. Patient #4 carried two missense, one synonymous and four polymorphisms in the non-coding regions. Patient #5 carried seven missense variations, seven variations in the non-coding regions of the genes and no one homozygous synonymous substitution. Finally, patient #6 carried two missense variations, three synonymous and three variations in the non-coding regions of selected genes.

Taken together, the results of our investigation have shown a significant inter-individual variability among patients in the type and the frequency of SNPs.

Extensive analysis of the genotype–phenotype correlations in this cohort was not possible because of the small number of patients.

DISCUSSION

This study was aimed at defining the factors that might influence a DNA repair capacity in FA patients, which may allow for a more rational therapeutic approach. Accumulating evidence suggests that reduced repair capacity is a polymorphic phenotypic trait associated with an increased risk of developing tumors at several sites, including breast, lung, skin, liver, or head/neck (BERWICK and VINEIS, 2000; MOHRENWEISER *et al.*, 2002). Therefore, the data of the factors of the integrity of DNA repair pathways may be used not only in the development of novel therapeutic agents but also for the understanding of the high degree of phenotypic and clinical heterogeneity in Fanconi anemia which is in evident contrast with the uniform cellular phenotype (HODSON and WALDEN, 2013).

In this study, we have genotyped the known polymorphisms located in the DNA repair and cell cycle control genes in FA patients and observed that most of the polymorphisms are present at the heterozygous state. Considering the polymorphisms with functional consequences i.e., missense polymorphisms, it is possible that heterozygous nucleotide changes in the genes of interest could also have various biological and/or pathophysiological effects on FA patients. Recent report of Alsbeih and coworkers (ALSBEIH *et al.*, 2013) has shown that radiation toxicity in radiotherapy patients is associated with polymorphisms rs1801516 of *ATM* and rs25487 of *XRCC1*. Results of that study suggest that the presence of protective alleles (*ATM* rs1801516 A and *XRCC1* rs25487 A) at the heterozygous status would increase patients' follow-up after radiotherapy by 51 months, while homozygous status would raise this index by 77 months. In our cohort, all of the FA-D2 patients were homozygote carriers of the *ATM* G allele. These results are in concordance with our previous investigations reporting the marked radiosensitivity of FA patients (LESKOVAC *et al.*, 2010; JOKSIC *et al.*, 2012; LESKOVAC *et al.*, 2014) and support the use of SNPs in candidate genes as predictive markers of patients' radiosensitivity.

In the BER pathway, the homozygous missense nucleotide changes (rs3136820) were found in the *APEX*. This endonuclease recognizes and begins the process of removing abasic sites in DNA (WILSON and THOMPSON, 1997). It has been reported that 148Glu polymorphism does not reduce endonuclease activity but it is associated with increased mitotic delay after exposure to

ionizing radiation and an increased risk of colorectal cancer (HU *et al.*, 2001; KASAHARA *et al.*, 2008). Our previous investigations have shown that exposure to ionizing radiation *in vitro* significantly slowed down the proliferation of FANCD2 fibroblasts compared to control fibroblasts (JOKSIC *et al.*, 2012; LESKOVAC *et al.*, 2014).

In the NER pathway, we detected the homozygous missense polymorphism rs13181 (p.Lys751Gln) of *ERCC2* which is associated with a risk of acute myeloid leukemia, melanoma, chronic lymphocytic leukemia and squamous head and neck cancer (CHANG-CLAUDE *et al.*, 2009; GANSTER *et al.*, 2009; MITRA *et al.*, 2009; STROM *et al.*, 2010). Of the two homozygous missense polymorphisms located in MSH3 (MMR pathway), the rs26279 is associated with increased sensitivity to platinum-based chemotherapy in advanced non-small cell lung cancer patients (XU *et al.*, 2015).

In the DSB repair pathway, the homozygous missense variations were found in *BARD 1, NBS1,* and *PARP4.* The rs1805794 (p.Glu185Gln) of *NBS1* is associated with increased risk for urinary system cancer, especially for bladder cancer (ZHANG *et al.*, 2014), while the functional consequences of the rs1050112 (p.Pro1328Thr), rs13428 (p.Gly1280Arg) and rs7571 of *PARP4* as well as of the rs2070094 (p.Val507Met) of *BARD1* are still unknown. The significance of these findings is not known yet but may be important for future testing and improvement of the diagnosis and outcomes in patients with FA.

Considering the genes involved in the cell cycle control and apoptosis, the homozygous missense variations were found in *CDKN1B, TP53* and *TP53BP1*. The p53 protein is essential for regulating different cellular processes such as DNA repair, cell cycle arrest and apoptosis and therefore, has a crucial role in maintaining the genetic integrity of the cells (RILEY *et al.*, 2008). The functional SNPs located in *TP53* are confirmed to affect the p53 signaling pathway (GROCHOLA *et al.*, 2010). The rs1042522 (p.Pro72Arg) of *TP53* at homozygous state was found in two of our patients, while the rest of them were heterozygotes. It has been shown that 72Arg variant has a stronger ability to induce apoptosis then 72Pro variant. In other words, 72Arg is more efficiently translocated to the mitochondria, where it interacts with proapoptotic proteins such as GRP75 and Hsp60, consequently triggering apoptosis (DUMONT *et al.*, 2003). These results are in accordance with our previous investigation of FA patients describing the elevated level of leukocyte apoptosis either occurring spontaneously or induced by ionizing radiation (PETROVIC *et al.*, 2013). Additionally, the report of Litviakov and coworkers (LITVIAKOV *et al.*, 2010) has shown that 72Arg variant is coupled with higher frequency of aberrant cells and chromatid breaks in comparison to individuals with Pro/Pro genotypes. This polymorphism is also associated with high risk of diabetes II and acute lymphoblast leukemia (GAULTON *et al.*, 2008; DO *et al.*, 2009). As for the other two found polymorphisms, the rs34330 of *CDKN1B* is reported to have an association with breast cancer (DRIVER *et al.*, 2008), while the rs560191 (p.Asp353Glu) of *TP53BP1* is associated with a risk of lung cancer (TRUONG *et al.*, 2010).

As previously stated, the homozygous synonymous nucleotide changes were found in *BARD1, MSH6, NBS1, RAD54B, CCND1* and *GRTH/Ddx25.* The literature data provide the evidence that synonymous mutations are "silent" with respect to protein sequence but are not always "silent" regarding protein function (GINGOLD and PILPEL, 2011). Synonymous SNPs could influence protein function affecting mRNA secondary structure and stability consequently reducing protein expression (NACKLEY *et al.*, 2006). In other words, "silent" changes may possibly affect the mRNA translation (DRUMMOND and WILKE, 2008), folding (GOODMAN *et al.*, 2013) and splicing

(PARMLEY *et al.*, 2006), or, through translational pausing, a protein folding (ZHOU *et al.*, 2013). Silent mutations are proven to contribute to human cancer (SUPEK *et al.*, 2014).

In this study, the thirteen homozygous variants were found in the non-coding regions of the genes tested. Several studies provided evidence that SNPs located in the non-coding DNA, especially in intronic gene regions near the exon/intron boundaries, could inactivate pre-mRNA splice sites consequently affecting gene expression (LOMELIN *et al.*, 2010; VOGELSTEIN *et al.*, 2013), or could activate cryptic splice sites leading to exonization (WANG and COOPER, 2007). Furthermore, the presence of SNPs in the 3`-UTR of selected genes could modify the binding with specific microRNAs (miRNAs) (NACCARATI *et al.*, 2012). It has been reported that one of the key mechanisms of gene expression control is a post-transcriptional gene regulation by miRNAs which interact with the 3`-UTR of target mRNAs inducing their degradation or inhibition of their translation, thus silencing gene expression (BARTEK and LUKAS, 2007; FILIPOWICZ *et al.*, 2008). Therefore, the SNPs could alter the miRNA–mRNA interaction consequently inducing the increase or decrease of protein translation (DUAN *et al.*, 2009). In addition, the sequence changes in the 5´ -UTR regions of mRNAs can influence translation regulation (WARD and KELLIS, 2012).

There are many association studies providing the linkage between SNPs in non-coding regions of candidate genes and cancer propensity. As for the homozygous polymorphisms detected in our patients, the intronic rs3730849 of *LIG1,* the 3`-UTR rs1052536 of *LIG3* and the intronic rs3212948 of *ERCC1* are associated with a risk of lung cancer (LANDI *et al.*, 2006; MA *et al.*, 2007). Similarly, the 5`-UTR variant rs1800975 of XPA is associated with lung cancer risk and also with squamous cell carcinoma (Lou et al, 2014). In *GADD45A,* an intronic rs532446 was reported to possess a functional role in acute lung injury (MITRA *et al.*, 2014). The 5`-UTR rs1799943 of *BRCA2* is associated with breast cancer susceptibility (SAPKOTA *et al.*, 2013). The 3`-UTR rs11226 located in *RAD52* is reported to has a significant association with myelodysplastic syndrome (BELICKOVA *et al.*, 2013). The intronic rs1799796 of *XRCC3* is associated with ovarian cancer risk (YUAN *et al.*, 2014), while the intronic rs1805377 of *XRCC4* is associated with an increased risk of glioma development (ZHAO *et al.*, 2013). In addition, the intronic variant rs664677 of *ATM* may play an important role in the development of thyroid cancer (SONG *et al.*, 2015) while the 3`-UTR rs678653 located in *CCND1* is associated with oral cancer (TSAI *et al.*, 2011). Regarding the intronic rs1805403 of *PARP1* as well as the 5`-UTR rs3732974 located in *FANCD2,* the clinical significance of these substitutions has not yet been elucidated.

Investigation of the relatively large number of SNPs, as in the current report, probably diminishes the significance of findings; therefore, many of the SNPs assessed (e.g. polymorphisms located in *ATM, APEX APE 1, XRCC1, ERCC2, MSH3, PARP4, NBS1, BARD1, CDKN1B, TP53, TP53BP1)* should be considered as promising candidates for further investigation in larger cohort. In addition, since Fanconi anemia is a genetically heterogeneous disease, the future investigation should also encompass the SNPs located in other genes that are proven to contribute to FA condition i.e., genes involved in bioenergetic pathways, antioxidant activities, response to stress and metal-chelating proteins, inflammation-related cytokines and also in some selected DNA repair genes such as type II DNA topoisomerase (PAGANO *et al.*, 2013).

The present report, although descriptive in its nature, may serve as a source for further studies, which may confirm the use of SNPs as predictive and prognostic biomarkers to individualize therapy of FA patients on genetic basis.

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ISPITIVANJE POJEDINAČNIH NUKLEOTIDNIH POLIMORFIZAMA U 52 GENA UKLJUČENA U DNK REPER I KONTROLU ĆELIJSKOG CIKLUSA KOD PACIJENATA OBOLELIH OD FANKONIJEVE ANEMIJE

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Izvod

Fankonijeva anemija (FA) je genetički heterogeno oboljenje koje se manifestuje progresivnom pancitopenijom, razvojnim abnormalnostima i predispozicijom ka razvoju kancera. Nezavisno od mutacija u FANC genima koje su direktni uzročnici bolesti, identifikacija varijacija u DNK, naročito pojedinačnih nukleotidnih polimorfizama u odabranim genima, može omogućiti individualizaciju pristupa svakom pacijentu i poboljšati prognozu bolesti. U ovom istraživanju, ispitali smo 95 pojedinačnih nukleotidnih izmena lociranih u 52 ključna gena uključena u bazni ekscizioni reper, nukleotidni ekscizioni reper," mismatch" reper, reper dvolančanih prekida DNK, kao i u nekoliko gena odgovornih za kontrolu ćelijskog ciklusa i apoptoze. Istraživanjem su obuhvaćena pet FA-D2 pacijenta i jedan FA-A pacijent, a genotipizacija polimorfizama je izvršena pomoću DNK reper čipa (Asper Biotech). Proučavani su sinonimni (n=10) i nesinonimni ("missense") polimorfizmi (n=52), kao i polimorfizmi u nekodirajućim regionima genoma (introni i 5' i 3' netranslatorni regioni) (n=33). Rezultati našeg istraživanja pokazuju da se pacijenti međusobno razlikuju po tipu i po broju nukleotidnih polimorfizama i istovremeno ukazuju na potrebu daljeg ispitivanja polimorfizama lociranih u genima *ATM, APEX APE 1, XRCC1, ERCC2, MSH3, PARP4, NBS1, BARD1, CDKN1B, TP53* and *TP53BP,1* na većem broju pacijenata, što bi omogućilo bolji uvid u kliničku sliku bolesti. Pored toga, pokazano je da pojedinačni nukleotidni polimorfizmi mogu imati značajnu ulogu u predikciji kancera kod FA pacijenata, kao i u proceni efikasnosti i toksičnosti različitih vidova terapije.

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