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Short Communication

Identification of novel target genes involved in Indian Fanconi anemia patients using microarray

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ABSTRACT

Fanconi anemia (FA) is a genetic disorder characterized by progressive bone marrow failure and a predisposition to cancers. Mutations have been documented in 15 FA genes that participate in the FA–BRCA DNA repair pathway, a fundamental pathway in the development of the disease and the presentation of its characteristic symptoms. Certain symptoms such as oxygen sensitivity, hematological abnormalities and impaired immunity suggest that FA proteins could participate in or independently control other pathways as well. In this study, we identified 9 DNA repair genes that were down regulated in a genome wide analysis of 6 Indian Fanconi anemia patients. Functional clustering of a total of 233 dysregulated genes identified key biological processes that included regulation of transcription, DNA repair, cell cycle and chromosomal organization. Microarray data revealed the down regulation of ATXN3, ARID4A and ETS-1, which were validated by RTPCR in a subsequent sample set of 9 Indian FA patients. Here we report for the first time a gene expression profile of Fanconi anemia patients from the Indian population and a pool of genes that might aid in the acquisition and progression of the FA phenotype.

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1. Introduction

Fanconi anemia (FA) is a rare autosomal or X-linked recessive (Taniguchi and D'Andrea, 2006) chromosomal instability syndrome characterized by multiple congenital abnormalities, bone marrow (BM) failure, aplastic anemia, cancer/leukemia susceptibility, and cellular hypersensitivity to clastogens such as cisplatin, mitomycin C (MMC), diepoxybutane (DEB), and melphalan. Treatment with these agents causes increased chromosome breakage in cells derived from patients with FA (D'Andrea and Grompe, 1997). FA patients are also susceptible to solid tumors, such as head and neck squamous cell carcinoma (HNSCC), gynecological squamous cell carcinoma (SCC), esophageal carcinoma, liver tumors, brain tumors, skin tumors and renal tumors.

The prevalence of FA is estimated to be 1 to 5 cases per million and heterozygous carrier frequency is estimated to be 1 in 300, although the actual frequency is probably higher (Orkin et al., 2009). The median age at diagnosis is 6.5 years for male patients, and 8 years for female patients, although the age at diagnosis ranges from 0 to 48 years. The male to female ratio is 1.24 (Taniguchi and D'Andrea, 2006). FA can be divided into fifteen and more complementation groups that include — A, B, C, D1, D2, E, F, G, I, J, K, L, M and N (Dokal, 2012). Proteins encoded by FA genes (FA proteins) include an ubiquitin ligase (FANCL/PHF9/POG), a monoubiquitinated protein (FANCD2), a helicase (FANCJ/BACH1/BRIP1), a protein with helicase motifs and a nuclease motif (FANCM) and a well-known breast/ovarian cancer susceptibility protein (FANCD1/BRCA2). FA proteins (including BRCA2) and another well-known breast/ovarian cancer susceptibility protein, BRCA1, cooperate in a common DNA repair process the “Fanconi Anemia–BRCA pathway” which is required for cellular resistance to DNA interstrand crosslinks (ICL). Furthermore, molecular and functional interaction of FA proteins with specific DNA repair proteins namely ATM (ataxia telangiectasia mutated), NBS (Nijmegen breakage syndrome), RAD 51 alongside many other DNA repair proteins ascertains the role of FA proteins in DNA damage (Kennedy and D'Andrea, 2005). However, symptoms like type 2 diabetes mellitus (Golubnitschaja et al., 2006; Pácal et al., 2011), café-au lait spots (Novellino et al., 1999) and other clinical manifestations are not entirely explained by DNA repair deficiency in FA (Pagano et al., 2005), suggesting a role for FA proteins in regulating functions beyond DNA repair. This also suggests the presence of other proteins that may interact with FA proteins or independently control certain pathways in FA. A well-accepted method of understanding the development and progression of a variety of diseases has been through the use of genome-wide expression array analysis. A genome wide expression array yields data about candidate genes that may be involved in disease acquisition and progression. The focus of this study was to elucidate the molecular basis of the phenotypic symptoms exhibited by FA patients using

Abbreviations: FA, Fanconi anemia; BM, Bone marrow; MMC, Mitomycin C; DEB, Diepoxybutane; HNSCC, Head and neck squamous cell carcinoma; ICL, Interstrand crosslinks; ATXN3, Ataxin-3; ETS1, v-ets avian erythroblastosis virus E26 oncogene homolog 1; ARID4A, AT rich interactive domain 4A (RBP1-like); ROS, Reactive oxygen species; SOD, Superoxide dismutase; AML, Acute myeloid leukemia; CMML, Chronic myelomonocytic leukemia; NK, Natural killer.

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microarray analysis. Previous studies with Indian FA patients have concentrated on chromosomal analysis. A study with 33 FA patients, revealed spontaneous chromosome breakage in 63.64% of patients, malignancies in 27.27% and chromosomal abnormalities in 55.5% (Korgaonkar et al., 2010a). Chromosomal breakage analysis in 195 pediatric patients revealed 17% of patients with classical FA, 4% with somatic mosaicism, 13% with high frequency of chromosomal breakage and without clinical features, and 66% with suspected FA but had no chromosomal breakage and clinical features of FA (Korgaonkar et al., 2010b). The current study reports the first ever genome wide expression study for Indian FA samples and details, for the first time, down regulation of three target genes ATXN3, ARID4A and ETS-1. The dysregulation of these genes can provide explanations for oxygen sensitivity (Pagano et al., 2011), certain aberrant hematological defects (Butturini et al., 1994) and impaired immunity (Myers et al., 2011) associated with FA.

2. Materials and methods

2.1. Sample collection

Peripheral blood was collected from 15 Fanconi anemia Indian patients from Andhra Mahila Sabha Hospital, Chennai, according to the Indian Council of Medical Research (ICMR) New Delhi guidelines with the consent of the patients. All samples used for the study were characterized for the hypersensitivity to DNA damaging agent mitomycin C by the clinician prior to sample collection. The clinical features of the patients are provided in Supplementary information (Supplm data 1). Peripheral blood was also collected from 3 individuals with no symptoms of FA who served as control for the current study. Samples were collected in sterile PAXgene Blood RNA tubes (PreAnalytix GmbH, Switzerland) and stored at −20 °C until further processing.

2.2. RNA isolation

Total RNA was isolated from the samples using PAXgene Blood RNA isolation kit according to the manufacturer's instructions (PreAnalytix GmbH, Switzerland). Total RNA was quantified by measuring the absorbance at 260 nm using a PerkinElmer Lambda 650 UV/VIS spectrophotometer (PerkinElmer, USA).

2.3. Oligonucleotide array analysis of RNA samples

RNA was amplified using Express Art mRNA amplification kit micro version (Artus GmbH, Germany). Amplified RNA was labeled with Cy3 Post-Labeling Reactive Dye Pack (GE Healthcare UK limited, UK). Labeled RNA was fragmented and purified using Express Art Amino allyl mRNA amplification kit and YM10 columns (Millipore, USA). 10.0 mg of the labeled amplified RNA was used for hybridization with the Human 40K (A + B) OciChip array. Hybridization was performed using the automated hybstation HS 4800. The hybridized chips were scanned using an Affymetrix 428TM array scanner at three different PMT gains. The interest was to determine probes (genes) that were differentially expressed (DE) between the normal and FA samples. A threshold log fold change (LFC) of 3.0 was fixed to attain an FDR of less than 0.05 and accordingly the DE genes were obtained. The statistical analysis was carried out using the R package and the biological analysis was carried out using Genowiz™ software (Ocimum Biosolutions, USA).

2.4. Functional clustering and gene ontology analysis

The differentially expressed genes obtained through the aforementioned comparisons were functionally clustered under different gene ontology (GO) terms using the microarray analysis software — DAVID (The Database for Annotation, Visualization and Integrated Discovery) (Huang da et al., 2008). The terms were categorized into biological process, molecular function and cellular component. For each GO term, the p-value was calculated. A term with $p < 0.05$ was considered to be enriched with genes. The p-value was calculated as the hypergeometric probability to get so many probes/probesets/genes for a GO term, under the null hypothesis that they were picked out randomly from the microarray. The lower the p-value, the more significant the GO term.

2.5. Real time PCR amplification

Total RNA (1.6 μg) was reverse transcribed from oligo-dT primers (NEB, USA) and the resulting cDNA was amplified by PCR using gene-specific primers (MWG biotech, Germany). Real time PCR was carried out using a DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo, USA) on an Eppendorf mastercycler, ep realplex (Eppendorf, Germany). A two-step cycling protocol was performed consisting of a single 7 minute cycle at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 30 s at 60 °C. Melting curve analysis was performed after amplification to check for the presence of any spurious amplification or for the formation of primer dimers. Relative mRNA expression was determined by normalization against the expression of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences have been listed in Table 1.

3. Results

3.1. Identification of differentially expressed genes

Microarray analysis was carried out on 6 FA patient blood samples. Microarray analysis resulted in the identification of 233 commonly dysregulated genes. Out of the 233 genes, 52 were upregulated and 181 were downregulated. This list included 9 DNA repair genes, 54 genes involved in the regulation of transcription, 11 chromosome organizing genes and 14 genes regulating cell cycle. The entire dysregulated gene list is available in the supplementary data (Suplm data 2).

3.2. Gene ontology/functional enrichment analysis to identify dysregulated processes

Gene ontology/functional enrichment analysis was utilized to identify the involvement of differentially expressed genes in various biological processes and molecular functions using DAVID (Huang da et al., 2008). This analysis clustered genes into a variety of functional groups (Fig. 1, Table 2) with regulation of transcription, DNA repair, cell cycle and chromosomal organization being of utmost significance. Table 3 depicts the fold changes of genes functioning in regulation of transcription, DNA repair, cell cycle and chromosomal organization.

Table 1
Primer sequences.

Gene symbol	Sequence	Annealing tm
ATXN3		**60 °C**
FP	5′ AGCTGAGCACACACTGGATG 3′	
RP	5′ CTTGCACTGGCATCTTTTCA 3′	
ARID4A		**60 °C**
FP	5′ TGGTGGATTGTGGCAGTAAA 3′	
RP	5′ TCAGGTAGGCAGGCTCATCT 3′	
ETS-1		**60 °C**
FP	5′ TGGAGTCAACCCAGCCTATC 3′	
RP	5′ TCTGCAAGGTGTCTGTCTGG 3′	
GAPDH		**60 °C**
FP	5′ GAAGGTGAAGGTCGGAGT 3′	
RP	5′ GAAGATGGTGATGGGATTTC 3′	

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