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Research Article

Association of miR-132 and miR-185 Genes Methylation and their Expression Profile with Risk of Congenital Factor XIII Deficiency

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Abstract

Congenital factor XIII deficiency is a very rare bleeding disorder, but because of the high rate of consanguineous marriages, it is common in Sistan and Baluchestan Province of Iran. The discovery of promoter hypermethylation of numerous miRNAs in human diseases has demonstrated an epigenetic mechanism for aberrant miRNA expression. The present study has analyzed methylation and expression status of miR-185 and miR-132 genes in patients with inherited factor XIII deficiency in a sample of South-Eastern Iranian population. Promoter methylation of miR-185 and miR-132 was investigated by Methylation Specific Polymerase Chain Reaction (MS PCR) in blood samples of 75 factor XIII deficient individuals and 74 healthy controls. Expression level of these genes was also assessed in 15 blood samples of patients and 15 healthy controls using real-time quantitative reverse transcription PCR. Analysis of miR-132 and miR-185 promoter hypermethylation did not show any significant difference between cases and controls. Relative gene expression analysis in cases (n=15) with congenital factor XIII deficiency and healthy controls (n=15) revealed no statistically significant relationship for miR-132 (p = 0.126) and miR-185 (p = 0.165) genes. Our findings indicated that promoter methylation as well as gene expression of miR-132 and miR-185 had no significant effect on etiology of factor XIII deficiency.

***Keywords*:** XIII Deficiency, MicroRNAs, MiR-185, MiR-132, DNA Methylation

Introduction

Congenital factor XIII (FXIII) deficiency, which was first recognized by Duckert in 1960, is a rare bleeding disorder inherited as an autosomal recessive manner with an incidence of one per 2 million people in the general population. It is characterized by hemorrhagic diathesis frequently connected with spontaneous miscarriage and defective wound healing (Board et al., 1993; Muszbek et al., 2011; Ivaskevicius et al., 2007).

FXIII is expressed during compartmentalization of precursors of monocyte/macrophage and megakaryocyte/platelet cell lines in the bone marrow (Adany and Bardos, 2003). Plasma FXIII is a protransglutaminase, activated by thrombin and converted to transglutaminase in presence of calcium ions. It is a tetrameric molecule composed of 2 A-subunits and 2 B-subunits, which are noncovalently kept together in a heterologous tetramer. Subunit A with catalytic function is synthesized by hepatocytes, monocytes and megakaryocytes. Subunit B, which has no enzymatic activity and may serves as a plasma carrier molecule, is synthesized in the liver (Hsieh and Nugent, 2008; Ashcroft et al., 2000). In the cellular form and in tissues, the protein is only a homodimer of A subunits (A2). FXIII was first noted for its involvement in the coagulation cascade, where it covalently cross-links fibrin monomers and converts soft fibrin clots into stable hard clots. Factor XIIIa is able to cross-link α2-antiplasmin as well as extracellular matrix proteins such as vitronectin, fibronectin and collagen to fibrin, rendering the clot more resistant to lysis and anchoring the clot to the blood vessel wall, respectively (Takagi and Doolittle, 1974). MicroRNAs (MiRNAs) are considered as a regulator of platelet function. MiRNAs are small non-coding RNA molecules (22–25 nucleotides) regulating a range of biological processes by inducing RNA degradation and translation inhibition of targeted mRNAs by binding to complementary sites in the 3′-UTR of target transcripts via Watson-Crick base-pairing (Esquela-Kerscher and Slack, 2006). These molecules play

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pivotal roles in diverse gene regulatory pathways including timing control of developmental processes, hematopoietic cell differentiation, apoptosis, cell proliferation and organ development (Morita and Han, 2006; Robert et al., 2007; Amelia et al., 2005; Zhao, 2007). MiRNA deregulation can take place at both transcription and processing levels, and aberrant DNA methylation patterns have been implicated in altered expression of 30 or more miRNA genes in different diseases (Erik et al., 2011). Recently, they have also discovered that DNA methylation has emerged as an important mechanism in the regulation of MiRNA expression. Relatively little information is available on epigenetic regulation of miRNAs genes in coagulation disorders (Kunej et al., 2011). Recently, a role for miRNAs has been proposed in the regulation of innate immune responses in monocytes and macrophages. activation of the innate immune response has been associated with changes in the expression of some miRNAs including miR-132 (Taganov et al., 2006). Platelet-derived miRNAs could act as paracrine regulators of endothelial cell gene expression. activated platelets shed miRNAs including miR-185 that can regulate endothelial cell gene expression and a recent study demonstrated that platelet miRNA content of ST-elevation myocardial infarction (STEMI) patients is distinctly different to that of healthy individuals (Gidlöf et al., 2013).

In this study, we assessed methylation and expression profiles of miR-185 gene on chromosome 22 (22q11.21) and miR-132 gene on chromosome 17 (17p13.3) predicted to bind the 3' UTR of A-subunit (FXIII-A) protein in normal controls and patients with severe congenital factor XIII deficiency.

Materials and Methods

Diagnosis of and characteristics study population

In hemophilia center of Zahedan city all suspected to factor XIII deficiency were referred to coagulation laboratory of Iranian Blood Transfusion Organization (IBTO). In coagulation laboratory patients were assessed by clot solubility test in 5M urea or 1% monochloroacetic acid environments. Since three separated molecular studies on more than three hundred of patients in this center revealed that all of them were homozygote of FXIII-A Trp187Trp polymorphism, these patients were not again analyzed molecularly for this polymorphism (Naderi et al., 2014; Naderi et al., 2013). 75 patients as well as 74 age and sex matched healthy individuals were selected for assessment the promoter methylation status miR-132 and miR-185 genes. Out of these two groups, two 15 persons groups were selected for the evaluation of miR-132 and miR-185 genes expression.

Bioinformatics

For the assessment the effect of miRNAs on factor XIII-A subunit we used algorithms of miRNA (http://www.microrna. org). According to these algorithms, we predicted that the FXIII-A gene might be a direct target of miR-132 and miR-185. Therefore the effects of these two miRNAs on factor XIII were assessed in this study.

Sample and DNA preparation

75 blood samples of patient with FXIII deficiency were collected from hemophilia center of Zahedan city, and 74 randomly control blood samples were obtained between November 2012 and May 2013 and all of them were age and sex matched. Informed consent was obtained from all the cases and controls and the study was approved by ethical committee of Sistan and Baluchestan University. Total DNA was extracted from frozen EDTA blood by using salting out method as described previously (Hashemi et al., 2014). Concentration of DNA samples was determined by spectrophotometer, and their integrity was assessed by gel electrophoresis. To modify DNA, 2 µg of extracted genomic DNA was incubated with 0.3 M NaOH for 20 min at 37°C and diluted with 550 µl of a 3.5 M sodium bisulfite (pH 5.0)and1 mM hydroquinone solution (both Sigma Aldrich), and then incubated at 55°C for 16 hr. The modified DNAs were then purified using a Wizard DNA Clean up System (Promega, Madison, WI). Purified DNAs were incubated with 0.3 M NaOH for 10 min at 37°C. DNA was precipitated with ethanol, and the treated DNA was diluted in 20 ml water, and was kept in −20 °C for use in later experiments.

Methylation Specific PCR (MSP)

Each PCR sample contained 1 µL bisulfite-modified DNA in a final volume of 25 ml including 16 µL RNase free double distilled water,0.5 µL Hot Star *Taq*® (Parstus: 2.5 U/ml), 1 µL dNTP mix (10 mmol/L), 2.5 µL 10× buffer, 1 µL each of primers (10 mmol/L), and 2 µL $MgCl_2$ (25 mmol/L). MSP amplification was set as follows: 94 °C for 10 min, followed by 39 cycles (94 °C for 30 s, the annealing temperature for miR-185: M=48/2, U=51; miR-132: M=51.8, U= 48.8 for 30 s and extension at 69 °C for 30 s). Final incubation was completed at 72 °C for 10 min. MSP products were separated by electrophoresis on 2% agarose gel and visualized

after ethidium bromide staining. The sequences of primers used for the methylation study are listed in Table 1.

Table 1. Primer sequences

miR-185 M	F: 5_TTTTTTTGTAGAGTTGGTTAATTCG_3
	R: 5_ATCACCATATCTTAATCTAAACGCA_3
miR-185 U	F: 5_TTTTTTTGTAGAGTTGGTTAATTTGG_3
	R: 5_ATCACCATATCTTAATCTAAACACA_3
miR-132 M	F: 5_TTTTTTGGGATATTTTTGACGTTAC_3
	R: 5_CCGACTAAAAACTCTACTACTCCG_3
miR-132 U	F: 5_TTTTTGGGATATTTTTGATGTTATG_3
	R: 5_CCAACTAAAAACTCTACTACTCCAC_3

M; methylated, U; unmethylated, F: Forward, R: Reverse

Gene expression analysis

Total RNA was extracted from fresh blood samples using bioZOL ™-B Blood RNA Isolation Kit (USA, bioWORLD.Cat No: 10760125-1) according to the manufacturer's instructions.
MiRNA 1st-Strand cDNA Synthesis Kit (Agilent technologies, Cat. no. 600036) was used for elongating miRNAs in a polyadenylation reaction to reverse transcribe the polyadenylated RNA into QPCR-ready cDNA. 5SrRNA was used as an internal standard. ABI 5700 sequence detection system (Applied Biosystems) was used to estimate the quantity of cDNA using real-time quantitative PCR. The sequences of primers used for the expression study are listed in Table 2.

Table2. Real-time primer sequences

5Sr RNA (Real Time-PCR)	F: 5_CCAACGTCCATACCATGTTGA_3
	R: 5_GCGGTCACCCATCCAAGTA_3
miR-185 (Real Time-PCR)	F:5_GCGGCGGAGGGGGCGAGGGATTG_3
	R:5_ ATCCAGTGCAGGGTCCGAGG_3
miR-132 (Real Time-PCR)	F: 5_GCGGCGGCCGCCCCCGCGTCTC_3
	R: 5_ATCCAGTGCAGGGTCCGAGG_3

Statistical Analysis

Data were analyzed using SPSS software. The chi-square test was used for categorical variables, and the impact of methylation of miR-185 and miR-132 genes on the risk of congenital factor XIII deficiency was detected by estimating odds ratios (OR) and 95% confidence intervals (95% CI) using the binary logistic regression test. Analysis of relative gene expression ($2^{-\Delta\Delta CT}$) between patients and controls was done by independent t-test. The significance level was set at $p \leq 0.05$ for all the tests.

Results

Characteristics of study population

All study population had an abnormal clot solubility test and molecular analysis of two separated studies on 190 and 70 patients revealed that all of them were homozygous of factor XIII Trp187Arg polymorphism. These 260 patients were comprised more than two third of patients with factor XIII deficiency in this center. The mean age of patients was 14/65±11/35 years while in control group was 15/19±10/45 years (*p* value: 0.76) (Table 3).

Table3. Demographic characteristics of 74 patients with factor XIII deficiency as well as 75 healthy individuals

Characteristic		Controls N = 74 (%)	Cases N = 75 (%)	*p*-value
Mean age		15/19 ± 10/45	14/65 ± 11/35	0/76
Sex	male	36 ± 0/50	34 ± 0/50	0/57
	female	38 ± 0/06	41 ± 0/06	
Family history	Yes	-	47 (62/67)	-
	No	-	28 (37/33)	
Intracranial bleeding	Yes	-	17 (22/67)	-
	No		58 (77/33)	
The first symptoms	Umbilical cord bleeding	-	31 (41/33)	-
	Another symptoms	-	44 (58/67)	
Age at diagnosis	0-5	-	50 (66/67)	-
	5-10	-	10 (13/33)	
	10≤	-	15 (20/0)	

Promoter methylation of miR-132 and miR-185 genes

As shown in Table 4, promoter methylation frequency of miR-132 and miR-185 genes showed no significant difference between cases with congenital factor XIII deficiency and healthy controls. The analysis of OR detected no significantly increased risk of disease for homomethylated.

Table 4 . The socio-demographic characteristics of the case and control groups in miR-185 and miR-133.

Characteristic		Controls (N = 74)	MiR-185 methylation			MiR-132 methylation			Cases (N = 75) Present	MiR-185 methylation			MiR-132 methylation		
			M	U	MU	M	U	MU		M	U	MU	M	U	MU
Sex	male	36 ± 0/502	-	3 (8.3)	33 (91.6)	3 (8.33)	3 (8.33)	30 (83.33)	34 ± 0/503	-	1 (2.9)	33 (97.05)	1 (2.94)	0	33 (97.05)
	female	38 ± 0/058	2 (5.2)	5 (13.15)	31 (81.578)	1 (2.63)	-	37 (97.36)	41 ± 0/058	3 (7.31)	-	38 (92.68)	0	0	41 (100)
Family History	Yes	-	-	-	-	-	-	-	47	3 (6.38)	-	44 (93.61)	0	0	47 (100)
	No	-	-	-	-	-	-	-	28	-	1 (3.57)	27 (96.42)	1(3.57)	0	27 (96.4)
Intracranial bleeding	Yes	-	-	-	-	-	-	-	17	1 (5.88)	1 (5.88)	15 (88.23)	0	0	17 (100)
	No								58	2 (3.45)	-	56 (96.55)	1(1.7)	0	57 (98.27)

Table 5. Comparison of relative gene expression for Micro132 and Micro185 genes between in patients with congenital factor XIII deficiency and healthy controls.

Genes		Sex		p-Value	Age	p-Value	No.	Mean±SD	p-Value
		Male	Female						
Micro132	Cases	5	10	0.052	13.4		15	0.92±0.13	
	Controls	7	8		13.7	0.25	15	0.99±0.07	0.126
Micro185	Cases	5	10	0.052	13.4	0.25	15	0.93±0.1	
	Controls	7	8		13.7		15	0.98±0.1	0.165

Heteromethylated patterns of miR-132and miR-185 in comparison to the unmethylated pattern as reference [OR = 0, 95% confidential 0 - 65, p = 0.78 .OR = 4.41, 95% confidential 0.42 - 220.4, p = 0.33, OR = 0.08, 95% confidential 0.001 - 2.05, p = 0.19, OR = 0.74 , 95% confidential 0.06 - 6.68, p = 0.9 respectively].

MiR-132 and miR-185 genes expression

Because of limitation for obtaining fresh sample only 15 patients in case group as well as 15 cases in control group during the study time were eligible for sampling and obtaining fresh sample for RNA extraction and subsequently for gene expression. Among case group, five and ten and in control group seven and eight persons were male and female respectively and this difference was not statistically significant (p: 0.052).

Expression an analysis revealed no statistically significant difference between cases and healthy controls concerning the relative expression of miR-132 and miR-185 (Table 5).

Discussion

FXIII, a protransglutaminase distributed in blood plasma and platelets, is involved in clot preservation by converting loose fibrin polymers into an organized structure by cross linking the peptide-bound glutamyl and lysine residues of fibrinogen chains through an isopeptide bond. Therefore, it participates in physiologic processes including wound repair and healing (Fadoo et al., 2013). Unlike plasma FXIII, which is in the form of a tetrameric structure (A2B2), the cellular form of FXIII present in platelets, monocytes and tissue

macrophages is a homodimer of FXIII-A subunit (Hsieh and Nugent, 2008). FXIII-A is synthesized by megakaryocytes and packaged into budding platelets, and it is present in large amounts in circulating forms. It seems to play an important role in the cytoskeletal remodelling associated with activation stages of platelets. FXIII-A is also found in blood monocytes and in all subsets of monocyte-derived macrophages in every part of the body. FXIII-A is mainly localized in the cytoplasm in association with cytoskeletal filaments; however, in a relatively early stage of macrophage differentiation, it appears temporarily in the nucleus. Cytoplasmic expression has a very close relationship with phagocytic activities (Adany et al., 2003). mRNA 3´UTRs of FXIII-A has binding sites for multiple individual miRNAs, which acts as posttranscriptional regulators of gene expression. Srikanth Nagalla et al (2011) demonstrated that miRNAs are able to repress expression of platelet proteins (Nagalla, 2011). Emerging evidences now support the idea that DNA methylation is crucially involved in dysregulation of miRNAs in different type of disorders. Shilpa Jain et al (2013) showed that platelets from sickle cell anemia patients exhibit an altered miRNA expression profile (Jain et al., 2013). Michael Girardot et al (2010) found miR-28 overexpressed in platelets of a fraction of myeloproliferative neoplasm patients, while it was expressed at constantly low levels in platelets from healthy subjects (Girardot et al., 2010). Lu Qian Wang (2013) showed that in miR-9 family, miR-9-3 is a relatively frequently methylated tumor suppressor miRNA, and hence silenced in chronic lymphocytic leukemia (CLL), while miR-9-1 methylation is rare in CLL (Wang et al., 2013). Pinto R et al (2013) showed different methylation and microRNA expression patterns in male and female familial breast cancer patients (Pinto et al., 2013). Tao Huang et al (2012) detected dysfunctions associated with MiRNA expression in lung cancer (Huang et al., 2012). From a therapeutic standpoint, however, manipulating the expression level of a specific miRNA may have only a limited effect on expression of the desired target gene, but targeting a single 3′UTR by multiple miRNAs can result in a synergistic mode of regulation. Improved understanding of miRNA epigenetics might lead to the development of a new generation of diagnostic markers for congenital factor XIII deficiency (Thomson et al., 2011; Richardson and Patel, 2014) .A limitation related to the samples was that due to timing and problems associated with fresh sample collection, the same samples were not used in methylation and gene expression studies. The present study results suggest that further research is necessary in this field to find an exact epigenetic change during diagnosis and treatment of congenital factor XIII deficiency in the same blood sample, which can be used as a fast and simple marker to monitor the disease process.

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