

Some Molecular Abnormality Indicators of Prenatal Cell Free Fetus DNA CFF-DNA

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Abstract— The discovery of CFF-DNA originating from the fetus in the plasma of pregnant women has facilitated the development of noninvasive prenatal testing (NIPT) that compensate for surgical tests that are expensive and expose mother and her fetus to danger beside it enable the detection of fetal abnormalities and malformation , especially in pregnant women suffer from problems such as preeclampsia , hypertension, gestational diabetes , and other disorders that increase the chance of having children with defects or problems in fetal differentiation. In this research quantitative real time PCR technology had been used to determine the amplicon's copy number variations, of *GATA1* and *MDM2 genes* between normal and pregnant suffer problem groups that had an important role in the embryonic development and differentiation. .

Keywords— CFF-DNA, GATA1, MDM2, Real time PCR

1. Introduction

The discovery of circulating fetal DNA in the plasma of pregnant women has greatly promoted advances in noninvasive prenatal testing. Screening performance is enhanced with higher fetal fraction and analysis of samples whose fetal DNA fraction is lower than 4% are unreliable. Although current approaches to fetal fraction measurement are accurate, most of them are expensive and time consuming. Here we present a simple and cost-effective solution that provides a quick and reasonably accurate fetal fraction by directly evaluating the size distribution of circulating DNA fragments in the extracted maternal cell-free DNA [1]. The discovery of cell-free fetal DNA (CFF_DNA) in maternal plasma has opened up new promises for the development of non-invasive prenatal testing (NIPT). Application of CFF-DNA in NIPT of fetus diseases and abnormalities is restricted by the low amount of fetal DNA molecules in maternal plasma. Fetus-derived CFF-DNA in maternal plasma are shorter than maternal DNA, thus

leveraging the maternal and fetus-derived CFF-DNA molecules size difference has become a novel and more accurate method for NIPT [2]. CFF-DNA could be defined as small fragments passes through placenta and enter maternal blood it can be extracted from maternal plasma at tenth week of gestation as its percentage (4-15)% at the end of first trimester of gestation and increased with progress in pregnancy age [3]. Pregnants require to have safer and more accurate molecular diagnosis technology for prenatal disease screening there for Fetus-derived CFF-DNA already had a worldwide utilization in clinical tests as potential markers for early screening for some maternal diseases and fetal abnormalities, such as chromosomal aneuploidy screening [4].

MDM2 gene is one of the three discovered genes (*MDM1*, *MDM2*, *MDM3*).. encodes the protein E3 ubiquitin ligase that stimulates tumor formation by targeting tumor –suppressing proteins such as p53, and is located on chromosome 12q14.3-q15 and consists of 13 exons [5]. MDM2 protein is a negative regulator for tumor suppressor p53, contain ring finger domains at their C terminal and this ring domain of MDM2 protein harbors E3 ligase activity which giving it the ability to initiate P53 protein degradation. and many experiments have been conducted across different stages of development, to explore the role of these protein in mice, it was found that when *MDM2* deleted in specific tissues, various phenotypic defects are observed, some of which lead to embryonic death, which indicates that MDM proteins are essential for growth and can salvage the phenotypes with concomitant p53 loss. [6]. *MDM2* is also necessary for internal balance of smooth muscles and significantly affects fetal differentiation [7]. Muscle cell differentiation is controlled by a complex set of interaction between tissue –restricting transcription tumors and cell-regulating protein and the interaction of C- terminal domain of MDM2 protein with pRb promote premature differentiation of proliferation myoblast cells. [8]. Another important gene include in the fetal differentiation and development is human *GATA1* gene that located on the short arm P of X chromosome at site 11.23 with 6 exons coding for GATA1 protein that consists of 414 amino acids considered as phosphorylated transcription factor associated with DNA and this protein is highly expressed in all types of vertebrates [9].

GATA1 protein has an important role in the development of red blood cells by regulating the transformation of fetal hemoglobin into adult hemoglobin. Mutation occur in this gene lead to defect in the formation of red blood cells in the bone marrow and placenta [10]. There is a close relationship between down syndrome and various blood malignancies. Fetus and children with down syndrome are highly predisposed to multiple disorders, including Transient Leukemia (TL), Transient Abnormal Myelopoiesis (TAM), Transient Myeloproliferative Disorder (TMD), and Acute Megakaryoblastic Leukemia (AMKL) [11][12].

The aim of this research is to shed light on the plasma prenatal CFF-DNA utilization as a molecular diagnostic indicator of prenatal diseases

And fetal abnormalities before the birth by utilization of qPCR Technique to determines the variation in amplicon copy numbers of certain genes such as *GATA1* and *MDM2* in the free fetal DNA between normal and pregnant suffer from problems

2. Material and Method

Sample collection: Blood sample were collected from 100 pregnant women healthy and pregnant suffer from pregnancy problems such as gestational diabetes, preeclampsia, previous recurrent miscarriages of unknown cause and pregnant aged over 40 year in the second and third trimester 18-40 week, with exclusion of pregnancy problems caused by a virus or parasite. Samples were collected, Venous blood sample 5ml was divided into two tubes the first, EDTA tube containing anticoagulant for plasma and the second gel tube for serum obtaining. Centrifugation was done to separate plasma and serum, then plasma and serum transferred to an eppendorf tube and frozen at -20 °C until use. Serum had been used for biochemical tests and plasma for DNA extraction and molecular tests.

Experimental design:

Blood samples had been classified into five groups as follow:

Control group: included 20 blood samples from healthy pregnant women in the second and third trimester of pregnancy (10 sample for each).

• **Malformation and miscarriage group:** included 20 samples from pregnant women who have previous and repeated births of deformed children and repeated miscarriages, 10 in the second trimester of pregnancy and 10 in the third.

• **Hypertension and preeclampsia group:** included 20 samples, 10 for each of the second and third trimester of pregnancy.

• **Gestational diabetes group:** 20 samples, 10 for each of the second and third trimester of pregnancy.

• **pregnant women group over 40 years old:** also included 20 samples 10 for each of the second and third trimester.

DNA extraction: Extraction of free fetal DNA from maternal plasma had been accomplished by Addprep genomic DNA extraction kit made in Korea, then DNA concentration and purity determined by Nano-drop instrument. DNA concentration ranged between 1.5-7.5 ng/μl and its purity (absorbance ratio of 280/260) ranged 1.7-2

Gene primer design: The primers of *GATA1 gene* and *MDM2 gene* designed by using primer-blast from National Center for Biotechnology Information (NCBI).

GATA1 primer:

F: 5'-CATGGCTGCAATGTGTGACC-3'

R: 5'-GATTCTGTGCCACCACGTA-3'

Forward start: from 48794392 to:48794411

Reverse start: from 48794735 to: 48794716

**Fig. 1.** primer design of *GATA1*

Primer	sequence	TM(C)	GC%	Product size
Forward	5'-CATGGCTGCAATGTGTGACC-3'	60.11	55	344
Reverse	5'-GATTCTGTGCCACCACGTA-3'	60.4	55	base pair

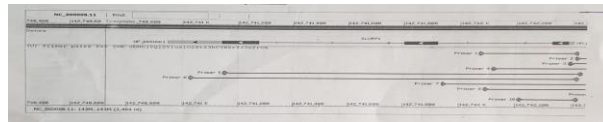
Fig. 2. primers of *GATA1***MDM2 Primers**

F:5'-TGAGCTGAGCCACAGACTTG-3'

R: 5'- AGGCTGGCTACCTCTCATCA-3'

forward Start: from 142742070 to :142742089

Reverse start : from 142742422 to: 142742403

**Fig. 3.** primer design of

Primer	sequence	TM(C)	GC%	product size
forward	5'-TGAGCTGAGCCACAGACTTG-3'	59.97	55	353
Reverse	5'- AGGCTGGCTACCTCTCATCA-3'	60.3	55	Base pair

Fig. 4. primers of *MDM2***GAPDH:**

F:5'-CGGGTCTTTGCAGTCGTATG-3'

R:5'-CTGTTTCTGGGGACTAGGGG-3'

Primer	sequence	TM(C)	GC%
Forward	5'-CGGGTCTTTGCAGTCGTATG-3'	60.5	55
Reverse	5'-CTGTTTCTGGGGACTAGGGG-3'	62.5	60

Fig. 5. primer of *GAPDH*

Real time PCR: The reaction were performed with real - time quantitative PCR machine using Gotaq® qpcr master mix (72050) from USA promega company for the detection of amplification product. The system contains a fluorescent DNA-binding dye, the BRYT green ® DYE, that exhibits greater fluorescence

enhancement upon binding to double strand DNA than SYBR® green ϕ . and GoTag® qPCR master mix. Thermal cycling was started with a two - minute hot start polymerase activation at 95 °C, and this was followed by a first denaturation step of 15 - seconds at 95 °C and an annealing and extension in one minute at 60 °C.

Table 1. *Gata1* qPCR program amplification

No.	Step	Tm(°C)	Time	No. of cycle
1	Polymerase activation	95	2 min	1
2	Denaturation	95	15 sec	45
3	Annealing	60	1 min	
4	Extension	60	1 min	

Table 2. *Mdm2* qPCR program amplification

No.	Step	Tm(°C)	Time	No. of cycle
1	Polymerase activation	95	2 min	1
2	Denaturation	95	15 sec	40
3	Annealing	60	1 min	
4	Extension	60	1 min	

Statistical analysis:

The data have been analyzed by **IBM SPSS** statistics program for windows. all of the data in this study expressed likewise mean \pm standard deviation (SD), and the data have been analyzed by using T-test independent samples. Differences have been examined to be significant for values of P-value < 0.05 and p-value < 0.01

3. Discussion and the result:

Table 3. CFFDNA concentration in maternal plasma of healthy and pregnant suffer from pregnancy problem

DNA concentration ng/ μ l (Mean \pm standard deviation)	Control group (N=3)	Congenital anomalies and miscarriage group (N=3)	Hypertension and preeclampsia Group (N=3)	Gestational diabetes group (N=3)	Pregnant women over 40 years age group(N=3)
Second trimester	1.15 \pm 0.1*	2 \pm 0.73 ^{n.s}	4.22 \pm 1.26*	1.66 \pm 0.28 ^{n.s}	1.81 \pm 0.09

	(N=3)	(N=3)	(N=3)	(N=3))	(N=3)
Third trimester	6.60±1.08 *	1.38±0.12 *	7.40±0.55**	4.44±0.90 ⁿ .s	3.21±0.86

**significant difference at the level of probability $p < 0.01$

*significant difference at the level of probability $p < 0.05$

n.s the coefficients are not significantly different from each other.

Differences in prenatal plasma extracted CFF-DNA concentration between healthy pregnant group (control group) and pregnant suffer from problems groups in the second and third trimester of pregnancy had been shown in table (3), Elevated CFF-DNA concentration was observed in the pregnancy third trimester of all groups which was consistent with a previous study results of [13]. that revealed increases in the CFF-DNA concentration with the age of pregnancy due to gradual collapse of the placental septum especially at the last three months of gestation [13].

Beside the results showed that in congenital anomalies (malformation) and miscarriage group there was no significant difference in CFF-DNA concentration compared to control group in both second and third trimester of pregnancy. while a significant increase concentration was observed in the hypertension and preeclampsia groups at $p < 0.05$ and 0.01 probability level

in the pregnancy second and third trimester and this results was accepted with previous studies results that concluded the increase of free fetal DNA especially in the pregnancy third trimester of preeclampsia cases, due it is association with the placenta weakness, small size and the restriction of fetal growth. Placenta released factors circulate in the mother's blood vessel endothelial that increase in the lyses of the placenta trophoblast, thus increased CFF-DNA in maternal blood as a result of apoptosis. [14][15].

Gestational diabetes group, showed no significant difference in CFF-DNA concentration compared to control group in the second trimester of pregnancy but it was elevated significantly in the third trimester of pregnancy at $p < 0.05$ probability level compared with control group and this in agreement with a previous study that indicate significant differences of CFF-DNA concentration than control group [16]. The significant difference was also evident with the elevated concentration of CFFDNA in the plasma of pregnant women aged over 40 years at a probability level $p < 0.01$ compared to the control group in the second trimester of pregnancy, but in the third trimester it was elevated significantly at a probability level of $p < 0.05$, these result also was consistent with the result of previous studies that suggested the influence of CFF-DNA in maternal plasma with the characteristics of mother and fetus such as the age of mother and the mother's affliction with diabetes or obesity, hypertension and preeclampsia, that pointed indirectly to the nature and condition of the placenta, in

cases of restricted fetal growth in the uterus, the free fetal DNA concentration appears higher than what is expected in normal pregnancies and may increase the possibility of the fetus having aneuploidy chromosomal abnormality [17].

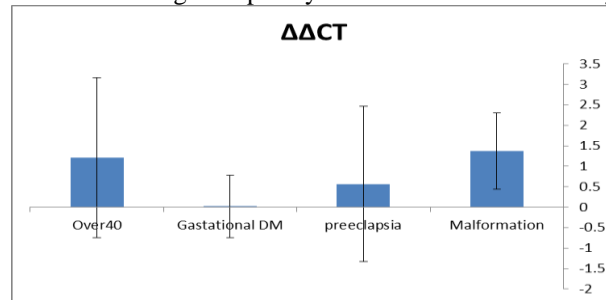


Fig. 6. Differences of GATA1 gene amplification between control and pregnant suffer problem groups in the third trimester of pregnancy

The result in fig (6) showed that the relative quantity of the *GATA1 gene* in the DNA from maternal plasma in pregnant groups suffering from malformation of previous births and miscarriage group is significantly higher than that of healthy pregnant at the probability level $P < 0.05$ and this revealed that any defect in the *GATA1 gene* had a role in the development of fetus red blood cells and its transformation to adult blood cells [10] or may indicate to the Down syndrome and leukemia suffering fetus [12].

While the results showed that there were no differences in the other pregnant groups compared with healthy pregnant group, thus *GATA1 gene* considered as a good indicator of fetus malformation presence which might suffered from down syndrome or other defect in the embryonic differentiation.

The results of *MDM2* gene relative quantity had been shown in fig (7) that indicate a significant difference at probability $P < 0.05$ in the CFF- DNA isolated from the maternal plasma of pregnant women compared with control group.

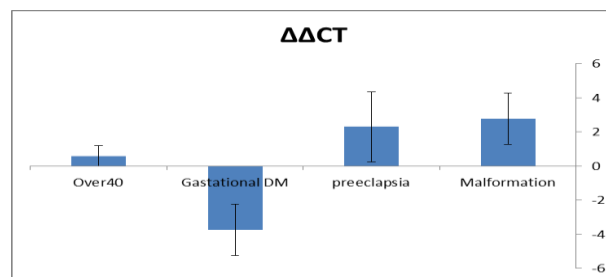


Fig. 7. Difference of MDM2 gene amplification between control and pregnant suffer problems groups in the third trimester of pregnancy

previous births and miscarriage group was higher than control but a significant decrease was observed in the group of pregnant women who suffer from gestational diabetes, and there was no significant difference of preeclampsia and pregnant women over 40 years groups as shown in fig (7).

4. Conclusion

Any defect in *MDM2* gene affects the balance of endogenous smooth muscle and had a clear effect on embryonic differentiation. thus we can rely on the up regulation of *GATA1 gene*, *MDM2 gene* as a good indicator of presence of abnormalities and malformation in the fetus. these results had been accepted with the results of [18] who concluded that the utilization of qPCR is the most sensitive method for the detection of any quantitative DNA abnormality and can be used for detection of fetal developmental disorders and could be monitored in the laboratory within few hours and [19] that revealed high efficacy of NIPT for copy number variations (CNVs) in over 42 000 pregnancies which indicated the potential significance not only for common aneuploidies but also for CNVs..

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