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Prenatal Diagnosis of Preeclampsia by Molecular of Circulating Corticotrophin-Releasing Hormone and Ep mRNA of Placental in Maternal Plasma

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Abstract

Objective: Preeclampsia (PE) is a common disorder in pregnancy. Corticotrophin-releasing hormone (CRH)ats through vasodilatory effects. The aim of this study is diagnosis of PE by molecular of circulating CRH and Ep mRNA of placental in maternal plasma. Material and methods: The study was performed in early pregnant women with PE (n= 28) and normal pregnant women (n=30) as control group, blood samples was taken from each mother. Total RNA was isolated from harvested plasma synthesized from RNA samples using reverse transcription kit (Gen net bio) by the instructions of the manufacturer. Results: The Results indicated that expression of CRH was significantly different amongwomen suffered from preeclamptic compare of healthy pregnant women. Conclusion: This study demonstrated that thosemRNA concentrations were increased in the plasma of PE patients compared with those of normalsubjects and suggest the opportunity to gain furtherinsight into pathological pregnancy through mRNA measurementsin maternal plasma.

Keywords: Preechlampsia, Circulating Corticotrophin-Releasing Hormone, Diagnosis, mRNA.

Introduction

Preechlamsia (PE), is a common multi system disorder in human pregnancy, (Stark et al., 2006) and is leading cause of maternal and perinatal morbidity and mortality (Purwosunu et al., 2007; Lian et al., 2013). Incidence of PE is 2-5% of pregnancies in the western developed countries, and around 10% of all pregnancies in the developing countries (Sanderson et al., 2000). The most prominent features of the PE syndrome isHemodynamic exaggeration (Hibbard, Shroff and Lang, 2004). Abnormal placenta formation is occurredfollowing incomplete or absent spiral arteries transformation that is a result of endothelial and vascular smooth muscle cells replacement that imposes endothelial damage by alterations in vasoactive mediators and maternal circulating levels of cytokines. The molecular pathways induce endothelial dysfunction, and abnormal placentation in PE remainsunclear (Redman and Sargent, 2005). Studies have shows that factors which release from placenta can change response of endothelial cell and vascular wall to constricting and relaxing hormones and proteins (Goulopoulou and Davidge, 2015). Up to now different aspects of normal and pathological pregnancy, such as EP is poorly understood.Investigation into fetal DNA in the maternal plasma led to new an approache for noninvasive prenatal diagnosis (Sekizawa et al., 2001; Sekizawa et al., 2002; Sekizawa et al., 2004). Analogous to these findings, fetal-specific RNA also has been identified in maternal plasma (Poon et al., 2000) and suggested a novel perspective for noninvasive methods in prenatal diagnosis. After the stability of endogenous RNAs in healthy individuals` plasma confirmed, this method introduced to scientists (Tsui, Ng and Lo, 2002). Evaluation of mRNAs expression in maternal plasma to monitor placental function could be a promising method that could be preventive, predictive or therapeutic (Zhong, Tuuli and Odibo, 2010).

One of the most important regulatory of hypothalamic-pituitary-adrenal (HPA) axis and modulates systemic response to stress is corticotrophin-releasing hormone (CRH). (Chrousos and Gold, 1992). CRH acts through vasodilatory effects (Clifton et al., 1995). Because of low concentration of CRH in normal circumstances, CRH is not detectable (Florio et al., 2007), but in at mid-pregnancy, the

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placenta secretes CRH which progressively rises, reaching levelsequivalent to the levels present in the hypothalamic portal blood during stress (Hobel, Arora and Korst, 1999). Compare of normal pregnant women CRH mRNA concentrations in plasma are higher in pregnant women with PE (Ng et al., 2003). Level of plasma CRH mRNA is associated with clinical severity of PE (Farina et al., 2004). According to these findings, plasma CRH mRNA could be considered as a new molecular marker for PE.

Lipoprotein lipase (LPL) is considered as one of the enzymes classified in components of the lipase gene Family (Procopciuc et al., 2014) that manages metabolism of adipocytes store triglyceride from plasma lipoproteins (Huda et al., 2014). LPL gene correlation with a higher risk for dyslipidemia is suggested (Procopciuc et al., 2014) that involved in the development of cardiovascular diseases (Kalmar et al., 2005). In PE, most studies approvedexcessive and so early detection of maternal gestational hyperlipidemia (Barrett et al., 2015) which is thought to contribute to endothelial dysfunction (Bayhan et al., 2005). Furthermore, women with history of PE in their pregnancy often have persistent abnormalities in lipids postpartum (Magnussen et al., 2009)

Currently, there is no single reliable test for PE prediction before PE clinical onset (Paiva et al., 2011). Therefore, it is necessary to design novel an applicable non-invasive tests which can be able to identify high-risk women atearly stepsof PE and monitor them throughoutpregnancy period, and as a result, provide the best prenatal care. Undatisfied outcomes of pregnancies following to PE are affected by sex of fetus. Well designed epidemiologic study showed that male neonates remarkably in higher level suffered from mortality and morbidity ,

Wehave previously indicated that fetus sex is associated withaltered maternal physiology. These data suggest that factorsderived from the fetoplacental unit may influence maternalphysiology (Stark et al., 2006).

Evaluation of molecular-level of cell-free mRNAs in maternal plasma during both normal pregnancy and pregnancy complicated by PE would extremely interesting and useful results. Therefore, we evaluated CRH and LPL mRNAs expression level in the placenta as circulating biomarkers of PE cases. We compared the expression of these molecules in plasma of maternal during both normal pregnancy and pregnancy complicated by PE.

Method and Materials

Study population:

The study was a case control study.Out of the 1000 women who were enrolled, 30 singleton pregnancies women who experienced preeclampsia were matched as a group with 30 control women with a normal course of pregnancy.

Participants in the case group were early pregnant women (gestational weeks <20) with hypertension (≥160/110 milimeter Hg BP) and considerable proteinuria (higher than 5 gram/dliter). Normal pregnant women were consideredas control group(n=28), who visited at out patients Obstetrics clinic in the Al-Zahra and Taleghani Hospitals. Both of hospitals are affiliated to Tabriz University of Medical Sciences The study was conducted between December 2013 and February 2014. All women provided informed consent to participate. The study that was approved by the Institutional Research Ethics Committee of Tabriz University of Medical Science (ethic cod: 5.4.10366) in 2016.

Processing of Blood Samples:

Maternal peripheral blood samples (5–10 mL) was taken for the isolation of serum. In brief, 7-mL peripheral blood samples were collected in ethylene diamine tetraacetic acid (EDTA) containing tubes and centrifuged at 1600g for 10 minutes at 4°C twice. The samples were stored at -80 °C until subsequent Molecular processing.

RNA Extraction and complementary DNA (cDNA) synthesis:

Total RNA was isolated from harvested plasma with the Invitrogen Trizol LS reagent (Carlsbad, Calif. USA) according to protocols as recommended by the manufacturer. In brief, the plasma was mixed with 2 ml of Trizol LS reagent and 0.4 ml of chloroform. The mixture was centrifuged at 12,000 g for 15 min at 4 ° C; then the aqueous layer was transferred into new tubes. Subsequently, one volume of 70% ethanol was added to one volume of the aqueous layer; the mixture was then applied to an RNeasy mini column QIAGEN (China) and processed according to the manufacturer's recommendations. Finally, total RNA was eluted with 30 µl of RNase-free water and was quantified by Nanodrop which all samples had 260/280 ratios > 1.8. Directly cDNA was synthesized from RNA samples using Gen net bio reverse transcription kit (Belgium) by the instructions of the manufacturer. After stored at -80 °C for further analysis.

Real-Time Quantitative RT-PCR

One-step real-time quantitative reverse transcription-PCR (RT-PCR) was carried out using a PCR Master Mix Kit (QIAGEN, West Sussex, UK). Primers unique for the target gene and covering exon-exon junctions. The primer sequences for CRH were as follows: 5´-GCTGCTCTTATGCCATTTGT-3´ (Forwards) and 5´-TAAACACCTGGAAACGGAAA-3´ (reverse), generating a fragment of 164 bp. The LPL primer sequences were 5´-TCTCTTGGGATACAGCCTTG-3´ (Forwards) and 5´-CTGGTTTCTGGATTCCAATG-3´ (reverse), generating a fragment of 218 bp. The variability in RNA recovery was normalized by reference to a housekeeping gene, human B2M, using a primer set designed to amplify a 572-bp fragment with sequences 5´-TCTCTTGGGATACAGCCTTG-3´ (Forwards) and 5´-CTGGTTTCTGGATTCCAATG-3´ (reverse)(Ng et al., 2002). Housekeeping gene is a control gene which using for normalization of mRNA level. B2M is one of humane genes which candidate for housekeeping gene in related studies (Silver et al., 2006).

Normalization method:

To limit art factual regulation of sample normalization, only Ct below a minimal threshold (≤36) were normalized. Relative expression of mRNAs was normalized to the expression of B2M as housekeeping and calculated according to the $2^{-\Delta Ct}$ method.

Statistical analysis:

Statistical analysis of CRH and LPL mRNAs expression in plasma between preeclamptic women and normal pregnant groups were estimated using Mann Whitney test (as a non-parametric test) because the abnormality of the distribution of the variables confirmed according to Kolmogorov–Smirnov/Shapiro-Wilk test. Furthermore, Paired sample t-test was performed to explore the CRH and LPL expression in each group.All analyses were performed using the SPSS software (v.16), and so DATA ASSIST software (v3.01) for clustering. Furthermore, the correlation between the mRNA level and the clinicopathological characteristics of PE patients were explored with Spearmen and chi-square rank correlation. Furthermore, for estimating of sensitivity and specificity the receiver-operator curve (ROC) drowned and the area under the curve (AUC) calculated the accuracy of mRNAs and $P \leq 0.05$ was considered statistically significant.

Results:

Analysis of CRH mRNA in the plasma of preeclamptic pregnant women.

To compare the Results indicated that expression of CRH was significantly different between preeclamptic pregnant women in comparison with the control pregnant women ($p<0.000$) (Fig1a). Also, based on the ΔC_T values distances between study subjects and assays were determined for hierarchical clustering/heat map. Fig2 shows the statistically significant results ($P \leq 0.05$). ROC analyses of CRH was carried out to estimate the capability of this to predicting PE pregnancies. As depicted in Fig2, CRH produced. AUC value ($p<0.000$). According to Youdens· Index for the best optimum cutoff, CRH with result sensitivity and specificity, held promise as a candidate mRNA of maternal plasma for PE early diagnosis.

Relative expression of CRH and LPLmRNAs in plasma samples of two study groups was measured by Quantitative real-time PCR and normalized to the expression of B2M as control gene (HK), then calculated according to the $2^{-\Delta Ct}$ method ($P \leq 0.05$).

Based on the ΔCT method by using Pearson's Correlation, distances between individuals and assays in two groups were calculated for hierarchical clustering. For each assay, the middle expression of CRH and LPLmRNAs are set as the median of all of the ΔCT values, and data points were compared relative to other points for that assay (rows: represent mRNAs relative expression, columns: represent study subjects). Red indicates an increase in ΔCT values below the middle expression level, and so green shows a decrease in a ΔCT value above the middle expression level ($P \leq 0.05$).

ROC curves analysis of a) CRH and b) LPL in BAL and maternal plasma of 30 PE patients and 28 normal pregnancies.

Discussion:

Identification of cell-free CRH mRNA in human plasma during pregnancy was conducted in 1984 (Sasaki et al., 1984), and then in 1987, expression of CRHgene in the human placenta which induced elevated plasma CRH mRNA concentrations during pregnancy reported (Grino, Chrousos and Margioris, 1987). Upregulation of CRH in preeclamptic women with probable functional alterations in PE suggested by other studies (Laatikainen et al., 1991) which acts as a potent dilator for placental resistance vessels (Berkowitz et al.,

1996) and so a predictor of preterm delivery (Inder et al., 2001). Cell-Free mRNA CRH in the plasma of patients with PE was ten fold higher than the levels in normal pregnancy (Ng et al., 2003). The other studies in concordant with previous reports revealed that CRH transcript is increased in pregnancies complicated with PE (Purwosunu et al., 2007; Lian et al., 2013; Zhong et al., 2005; Mayor-Lynn K et al., 2011). Enders et al. in theirs study showd that concentration of maternal CRH has been increased in preeclampsia cases. They concluded that plasma CRH mRNA could be used as a new marker for prediction of preeclampsia (Ng et al., 2003). Purwosuni and colluges in their study fiound out similar results (Purwosunu et al., 2007).

Quantitative aberrations of mRNAs in maternal plasma of preeclamptic pregnancies could justify with some theoretical mechanisms. Apoptotic changes in placental villous trophoblasts may be associated with the release of placental mRNAs into maternal plasma (DiFederico, Genbacev and Fisher, 1999; Hasselmann et al., 2001). The clearance half-life of nucleic acids even affects mRNA concentration (Purwosunu et al., 2007). In human placenta, CRHmodulates vascular tone, and it has been found in significantly higher concentrations in those conditions correlated to vascular damage as well as PE (Farina et al., 2004). High CRH levels may be detectable as early as the second trimester of pregnancy in women who subsequently develop PE (Leung et al., 2000).

The present study in agreement with mentioned findings showed that cell-free mRNA of CRH is detectable in the plasma of pregnant women and preeclamptic pregnancies with a significant difference. These findings indicate that upregulated expressions are derived from the placenta and are correlated to the pathogenesis of preeclampsia.

The data presented herein demonstrates the feasibilityof noninvasive profiling of placental gene expression,just by analyzing maternal samples. This developmentmight open numerous avenues for noninvasive evaluationof the placental function. RNA markers of placenta could be a hallmark for detection maternal plasma represents. The last one has a critical role for development of molecular markers. Theses markers have usage for pregnantsa without any limitation including genetic polymorphism of fetus and gender.

Conclusion:

In conclusion, this study is demonstrated that those mRNA concentrations were increased in the plasma of preeclamptic patients compared with those of normalsubjects and suggest the opportunity to gain furtherinsight into pathological pregnancy through mRNA measurementsin maternal plasma. Prospective and systematicinvestigation of the numerous genes that are known to be expressed in the placenta might be useful in clarifyingthe mechanisms of pathological pregnancies.

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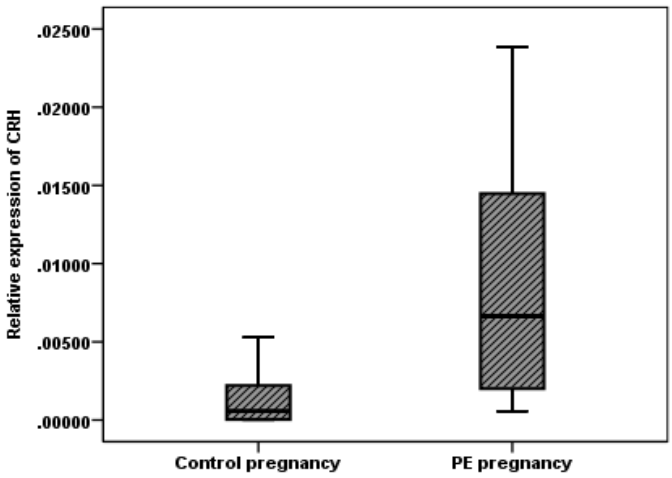

Fig. 1: Relative expression of CRH and LPLmRNAs in maternal plasma of PE patients and normal pregnancy

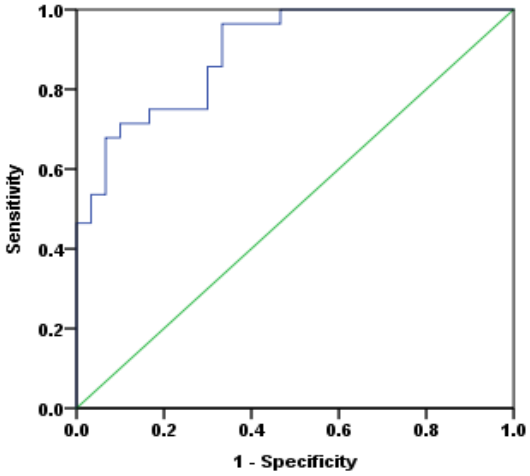

Fig. 2: Diagnostic value of CRH and LPLmRNAs quantifications by real-time PCR in maternal plasma of PE patients and normal pregnancy.