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Comparative expression profiles for KiSS-1 and REN genes in preeclamptic and healthy placental tissues

Fernando Vazquez-Alaniz [a,b], Carlos Galaviz-Hernandez [a,*], Laurence A. Marchat [c], José M. Salas-Pacheco [d], Isaías Chairez-Hernandez [e], José J. Guijarro-Bustillos [f], Alberto Mireles-Ordaz [f]

[a] Academia de Genómica, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, IPN Unidad Dgo., Durango, Zip Code 34220, Mexico
[b] Becario PIFI, Doctorado en Ciencias en Biotecnología, Nodo Durango, SIP-IPN, Mexico
[c] Escuela Nacional de Medicina y Homeopatía IPN, Mexico City, Zip Code 07320, Mexico
[d] Instituto de Investigación Científica, Universidad Juárez del Estado de Durango, Durango, Dgo., Zip Code 34000, Mexico
[e] Academia de Entomología, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, IPN Unidad Dgo., Durango, Zip Code 34220, Mexico
[f] Servicio de Ginecología y Obstetricia, Hospital General de Durango, Durango, Dgo., Zip Code 34000, Mexico

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ABSTRACT

Objective: The aim of the present work was to look at differences in the placental tissue expression of KiSS-1 and REN genes from preeclamptic and healthy pregnant women, that could account for a possible synergistic function for both genes in the pathogenesis of preeclampsia.
Study design: This case–control study involved 27 preeclamptic women and 27 normoevolutive pregnant women. cDNA was obtained from placental tissue to carry out qPCR for both KiSS-1 and REN genes in order to compare mRNA expression levels in the studied groups. Statistical analysis showed expression differences that correlate with clinical and/or biochemical variables.
Results: Higher expression for KiSS-1 in PEE vs. control woman ($p = 0.001$) was observed, whereas no difference was observed for REN expression ($p = 0.300$) when all the subjects were included. However, REN expression was significant higher when the samples were stratified according to preeclampsia severity. For 18 mild preeclamptic patients the p-value was $p = 0.001$ compared to their controls, while for the remaining nine with severe preeclampsia the expression became significant ($p = 0.001$).
Conclusion: Our results suggest that the high KiSS-1 expression seen in preeclamptic patients is in accordance with its role as an inhibitor of trophoblast invasiveness and maintained until the end of gestation. On the other hand, aggressive therapeutic management and/or severity status of patients have a direct effect on placental REN expression levels, masking the natural high expression of this gene on preeclamptic placental tissue. Therefore it was not possible to establish a real concordant expression profile for KiSS-1 and REN genes.

1. Introduction

Preeclampsia (PEE) is one the leading causes of fetal and maternal morbimortality in the world [1]. PEE is classified as mild [blood pressure (BP) ≥ 140/90 mm Hg and proteinury ≥ 30 mg/dL] and severe (BP ≥ 160/110 mm Hg and proteinury ≥ 2000 mg/dL). Many different theories [2] have been advanced to understand its basis. However, a clear deficiency of trophoblast invasiveness (TI) to maternal spiral arteries in placentas from early and term pregnancies [3–6] is central to all of them. A number of genes have been involved in trophoblast invasiveness (TI): LEP [7], MMP-9 [8], INSL4, KiSS1-R and KiSS-1 [9], being the last highly expressed in syncytiotrophoblast cells [6].

Kisspeptin 10, the protein product of KiSS-1 gene, reduces the normal invasiveness of trophoblast [4] through control of its migratory properties [9]. Decreased plasma levels of this peptide were reported during pregnancy [10]. Normal TI processes guide the normal transformation of spiral arteries in the myometrial segments and the impairment of this transformation [11] is noticed in preeclamptic patients [12]. It is suggested that TI of the spiral arteries is a continuous process [13,14] until the end of pregnancy.

An important finding was the discovery of a functional renin angiotensin system [RAS] in human [15,16] and rodent [17] placental tissue. The major extra-renal RAS producer during

* Corresponding author at: Academia de Genómica, CIIDIR-IPN Unidad Durango, Sigma #119 Fracc 20 de Noviembre II Durango, Dgo, Mexico c.p. 34220, Mexico. Tel.: +52 618 814 2091; fax: +52 618 814 4540.
E-mail addresses: carlosgalavizhernandez55@gmail.com, cgalaviz@ipn.mx (C. Galaviz-Hernandez).

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pregnancy is the placenta [18]. Renin, the protein product of REN gene, is a natural vasoconstrictor expressed during all pregnancy [19]. Increases in the expression of some components of RAS were noticed in serum of healthy pregnant women compared to those of preeclamptic patients [20]. Thus, poor TI and generalized endothelial dysfunction [21,22] are key players in the physiology of the disease.

On the other hand, Nistala et al. [23] isolated a PAC that includes the adjacent genes REN and KiSS1. Further studies confirmed the expression of those genes in placental tissue [24,25].

Some neighbor genes are co-expressed in common metabolic pathways [26] through shared promoters and transcription factors binding sites [27]. So it is tempting to think about the co-participation of KiSS1 and REN genes in PEE development. The aim of the present study was to look for differences in expression of KiSS-1 and REN genes in placental tissue between PEE and healthy pregnant women, assuming a possible concordant expression of both genes in the pathogenesis of PEE.

2. Materials and methods

2.1. Selection of patients

This case–control study was approved by an investigation ethical committee in the Hospital General de Durango México, in accordance with The Code of Ethics of the Declaration of Helsinki. Twenty-seven preeclamptic women under anti-hypertensive treatment and the same number of healthy pregnant women, agree to donate their placentas for the study after signing an informed consent letter.

Pairing was based on chronological age, gestational age and the number of pregnancies, as they are known to affect placental gene expression [28,29].

Inclusion criteria were those established by the Working Group Report on High Blood Pressure in Pregnancy and patients classified according to severity status in mild preeclampsia [blood pressure [BP] $\geq$ 140/90 mm Hg, measured twice within a 2 h interval and proteinury 300 mg/dL or 1+ inlab stick urine analysis)] or severe preeclampsia [BP $\geq$ 160/110 mm Hg, on two or more occasions 6 h apart and proteinury 2 g or more in 24 h or 2+ qualitatively) with new onset hypertension after 20 weeks gestation [30].

All patients under treatment with glucocorticoids, affected by gestational hypertension, gestational diabetes, diabetes mellitus types 1 and 2 and with hepatic or kidney diseases were excluded. A questionnaire with clinical relevant data was also obtained from all participants.

2.2. RNA extraction and cDNA synthesis

Within 30 min after delivery, placentas were washed with cold PBS at pH 7.4. Twelve different 5 × 5 mm biopsies were taken from maternal side of the placenta. Fifty to one hundred milligrams of the sample was kept in RNAlater® (Ambion™) at 4 °C until RNA extraction with TRIpure isolation reagent® kit (ROCHE™). RNA was treated with DNA-free® (Ambion™) and its integrity was verified by 1% agarose gel electrophoresis. Samples were stored at −72 °C until cDNA synthesis, which was carried out using the High Capacity cDNA Reverse Transcription® (Applied Biosystems™) kit, according to the manufacturer protocol; quantity and purity were evaluated through spectrophotometry. The synthesized cDNA was kept at −20 °C until further analysis.

2.3. qPCR

Gene expression was analyzed by semi-quantitative polymerase chain reaction (qPCR) in a SteptOne™ Applied Biosystems equipment, using FAM™ dye-labeled TaqMan® MGB probes (Applied Biosystems). KiSS-1 probe was based on RefSeq NM_002256.3; assay ID Hs00158486_m, which targets exons 2–3. REN probe was based on RefSeq NM_000537.3, assay ID Hs00166915_m1, targeting exons 1–2.

The relative expression (RQ) of KiSS-1 and REN genes was normalized to the constitutive and endogenous control human gene beta-2-microglobulin (B2M) (FAM/MGB Probe, Non-Primer Limited RefSeq NM_ 004048.2), (Applied Biosystems™). This probe targets exons 2–3. Amplification efficiencies with values between 90 and 110% allowed establishing an optimal working concentration of 30 ng/μL. An expression test was also randomly run for B2M gene in five patients and five control samples in triplicate, to estimate significant differences between groups and determine the optimal PCR cycle at which fluorescence gave the better exponential cycle. The qPCR conditions were: 10 min of initial hold at 95 °C, followed by 48 cycles of 15 s at 95 °C for denaturing and 1 min at 60 °C for both annealing and extension. Cycle threshold values were obtained by triplicate and each sample was normalized with B2M endogenous control gene to calculate RQ values or ($2^{-\Delta\Delta Ct}$) for KiSS-1 and REN.

2.4. Statistical analysis

Media, range, median quartile and coefficient of variation were calculated for all clinical and biochemical features of the population. The expression of KiSS-1 and REN genes in placentas from both PEE and normoevolutive pregnancies (NEP) women was compared using the Student's *t*-test one way for independent data.

A cluster was created to stratify patients using the significant variables reported [31] in Mexican preeclamptic populations. A neural network analysis feed forward was done to find the most important variables. A new Student's *t*-test was performed for subgroups to compare REN expression between PEE patient's subgroups. A final correlation test was run out between relative expression gene and clinical and biochemical features of the patients. All statistical analysis was performed with Statistic® software V.7.0.

3. Results

Twenty-seven placental samples from PEE patients and the same number from healthy controls were studied. The media and coefficient range for chronological age was 20.0 ± 7.00 years. The media and coefficient variation value for gestational age was 38.8 ± 2.8 weeks. Nulliparity was found in 22 cases and 5 were

Table 1
Clinical characteristics for PEE and NEP patients.

Clinical characteristics	PEE group (n=27)	NEP group (n=27)	p-Value
Age (years)	22.2 ± 28.3[a]	22.2 ± 28.3[a]	NA
Gestational age (weeks)	38.3 ± 2.8[a]	38.3 ± 2.8[a]	NA
PEE antecedent (%)	22%	3%	0.000[b]
Primiparity/multiparity cases	19/8	17–10	0.300[b]
Delivery form (cesarean/vaginal) cases	22/5	3/24	0.001[b]
Mean arterial pressure in mm Hg	120.9 ± 9.9[a]	86.4 ± 4.6[a]	0.000[b]
PEE classification (mild/severe) cases	8/19	NEP	NA
Newborn weight (pounds)	5.88 ± 19.5[a]	6.77 ± 9.9[a]	0.001[b]
Serum uric acid (mmol/L)	354.5 ± 24[a]	154.9 ± 23.3[a]	0.000[b]
Treatment rate until delivery (h)	5.5 ± 39.6[a]	4.8 ± 24.5[a]	0.300[b]

Significant differences were appreciated for PEE antecedent, delivery form, MAP, newborn weight and serum uric acid.
[a] Media ± coefficient variation.
[b] Student's *t*-test.

multiparous. Preeclamptic (PEE) patients displayed higher values for mean arterial pressure (MAP) (120.9 ± 9.9 vs. 86.4 ± 4.6 mm Hg, $p = 0.000$), and higher values for uric acid blood (354.9 ± 24 vs. 154.9 ± 23.3 mmol/L ($p = 0.000$) than normoevolutive pregnant (NEP) women (Table 1).

The comparison of KiSS-1 and REN placental expression revealed higher KiSS-1 expression levels in preeclamptic (1.99 ± 0.80 and 0.99 ± 0.47; $p = 0.001$), than healthy women. In contrast, no significant differences ($p = 0.300$) between PEE (1.25 ± 0.51) and NEP (1.06 ± 0.46) for REN relative expression analysis were found (Fig. 1).

As renin regulates blood pressure, it is expected that a treatment aimed to control it could modify REN expression. Therefore a more detailed cluster analysis for this gene was done. The considered variables for this analysis were PEE classification, MAP, uric acid, proteinuria, PEE history, creatinine, urea, edema, gestational age, drugs used for hypertension control and management time which revealed the presence of two clearly distinguishable subgroups $n = 18$ and $n = 9$ (circularized) (Fig. 2).

Resulting subgroups were analyzed again, revealing a high expression for REN in 18 mild preeclamptic women (1.44 ± 0.51) vs. their corresponding controls (0.86 ± 0.39) ($p = 0.001$) (Fig. 3). The high expression observed correlated with MAP, low proteins, urea and uric acid high concentrations in serum, newborn low weight, PEE history, increase of osteotendinous reflex (OTR) and the number of pregnancies ($p \leq 0.05$).

A decreased REN expression was found to be statistically significant ($p = 0.001$) in the remaining 9 severe preeclamptic patients (0.87 ± 0.51 and 1.46 ± 0.30,) compared to their controls (Fig. 3). These 9 affected patients were analyzed for all clinical and biochemical variables through neural network analysis feed forward, obtaining a classification of 100% for training data and 80% for test data. The analysis revealed that the 5 most important variables were: vomit, use of at least 3 hypertension control drugs, hepatalgy, alkaline phosphatase and treatment time before pregnancy resolution. All these data agree with those obtained in dendrogram.

The high KiSS-1 expression in PEE patients correlates with serum uric acid, newborn low weight, PEE history and number of pregnancies, meanwhile the high REN expression correlated with mean arterial pressure (MAP), used anti-hypertensive drugs and newborn weight (Table 2).

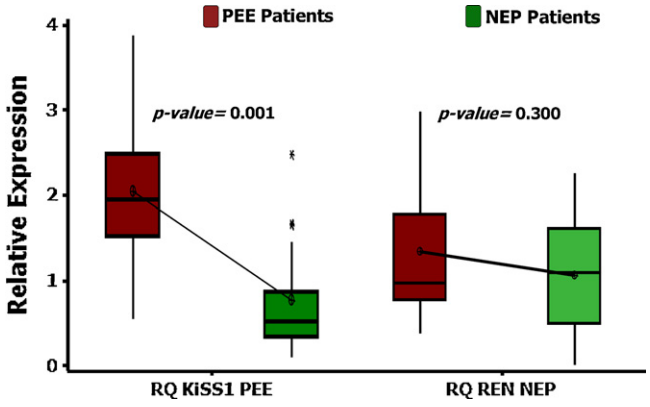

Fig. 1. Comparative expression analysis for KiSS1 and REN genes. Student's *t*-test showed differences for KiSS1 but not for REN gene placental expression between groups.

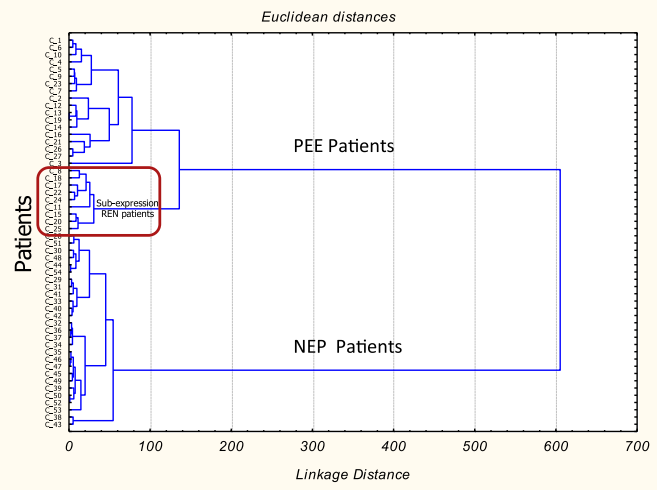

Fig. 2. Cluster diagram for PEE and NEP patients. Dendogram grouped the whole sample in two major groups. PEE sub-group was further divided, circle denotes the subgroup of PEE patients with MAP ≤126 mm Hg and the use of anti-hypertensive drugs as common variables.

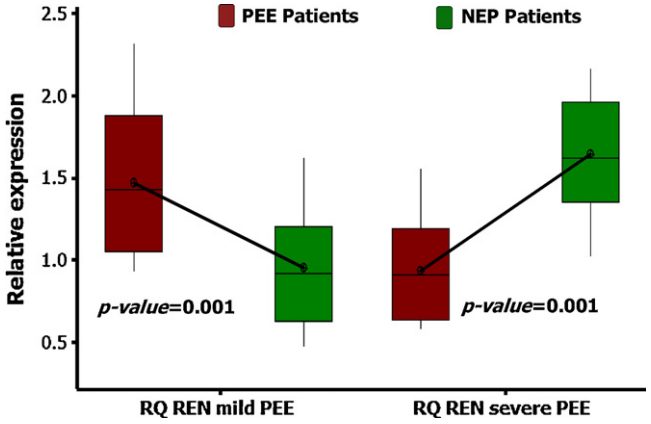

Fig. 3. REN gene expression level analysis for PEE subgroups. Over-expression was observed in 18 mild preeclampsia affected patients subgroup (left). Subexpression was advertised in the remaining 9 severe patients treated with as least three control blood pressure drugs in regard to their control (right). Significative Student's *t*-test, $p \leq 0.005$.

Table 2
Correlation test for distinct analyzed variables with gene expression.

Variable	Gene	Correlation coefficient, r^2	*p*-Value
Mean arterial pressure	KiSS1	−0.137	0.490
	REN	0.454	0.017
Serum uric acid	KiSS1	0.399	0.039
	REN	−0.067	0.730
PEE history	KiSS1	0.386	0.05
	REN	0.071	0.72
Pregnancies number	KiSS1	0.387	0.042
	REN	−0.230	0.574
Used anti-hypertensive drugs	KiSS1	−0.172	0.390
	REN	0.490	0.009
Newborn weight	KiSS1	0.412	0.035
	REN	0.421	0.030
OTR	KiSS1	0.217	0.25
	REN	0.402	0.03

Correlation test for clinical variables and gene expression in PEE patients. As can be appreciated, REN gene expression correlates tightly with the use of antihypertensive medications (significance *p*-value ≤ 0.05 and CI 95%).

Differences for gene expression were evaluated in the studied groups in regard to pregnancy way resolution (cesarean section vs. vaginal delivery). Observed p-values for KiSS-1 ($p = 0.062$) and REN ($p = 0.387$) in PEE patients and control subjects KiSS1 ($p = 0.276$) and REN ($p = 0.582$) did not reveal such differences.

4. Comment

Some examples are known of genes clustered [26,27] in the same chromosomal regions. Those genes used to be linked throughout evolution sharing expression and related functions in certain organs [24,25]. In this case–control study, gene expression profiles for two contiguous genes KiSS-1 and REN were compared in placentas of preeclamptic vs. healthy pregnant women.

The main clinical features in our population are in agreement with those observed in previous reports. However, in the present study most preeclamptic women delivered by cesarean section whereas controls did it through normal labor. No differences in KiSS-1 or REN gene expression were observed between PEE and NEP subjects in regard to delivering way, despite it is known to affect placental gene expression [32,33].

KiSS-1 is a metastatic inhibitor for many different types of cancer [34]. We observed a higher expression of KiSS-1 in 24 among 27 preeclamptic placental tissues vs. those of normoevolutive pregnant women. Matrigel analysis [35] demonstrated an altered migration pattern related to KiSS-1 and PlGF high expression [9] in normal trophoblast cells and choriocarcinoma cells. An increase of Kisspeptins in serum of patients with PEE [36] has also been reported. Thus, the observed KiSS-1 high expression could be related to impaired early trophoblast migration and maintained throughout pregnancy.

The presence of a placenta-specific RAS [37] showing differences of gene expression [20] between PEE women and NEP has been reported. We did not find statistically significant differences for REN expression between PEE and NEP groups as a whole. However, 18 mild affected patients showed a higher REN expression with regard to their respective controls. This high expression pattern has already been reported in preeclamptic uterine placental beds [38] and also in maternal decidua of PEE patients [39,40]. Our sampling technique removed all decidual tissue, implying that the higher REN expression can also be found in chorionic tissue.

Interestingly, the observed high expression coincides with a mild disease state and the use of one or two of the following drugs: alpha methyl dopamine and hydralazine. This suggests that neither alpha methyl dopamine nor hydralazine alone or in combination, affects the expression of REN at least in placenta.

All preeclamptic patients were subjected to different anti-hypertensive drugs regimes depending on severity status: mild/severe. KiSS-1 expression remains high in most preeclamptic women, independently of degree of severity and corresponding treatment. This could be expected as anti-hypertensive drugs are not aimed to affect TI driven by KiSS-1.

On the other side, a differential behavior for REN expression was advertised in preeclamptic patients depending on severity status and concomitant treatment. A higher expression of REN in 18 mild preeclamptic patients compared to the remaining 9 severe affected patients was observed. The last subgroup were treated with three (alpha methyl dopamine, hydralazine and magnesium sulfate) or 4 (alpha methyl dopamine, hydralazine, magnesium sulfate and nifedipine) antihypertensive drugs. These 9 severe preeclamptic patients presented a decreased REN expression. This lower expression pattern could be explained by two main possibilities: (1) the lowering of REN expression is due to the combined actions of the 3 or 4 antihypertensive drugs or (2) the severity of the disease explains such decrease in expression. To date, there is no report that supports the first assumption. However, nine differentially expressed genes were reported [41] between severe early onset vs. mild late onset preeclampsia.

The high KISS-1 and REN expression was also found associated [42] to high MAP values and newborn low weight, events commonly seen in preeclampsia. This is in direct relationship with a deficient uterus/placental blood perfusion, avoiding the proper transfer of nutrients from the mother to growing foetus [39,43].

One concern is that the observed changes in gene expression could be considered more a consequence than a cause of the disease. However this is difficult to prove due to ethical constraints imposed by the time of onset of illness and the taking of samples.

KiSS-1 and REN genes are involved in two of the main phenomena related to preeclampsia: trophoblast invasiveness impairment and hypertension respectively. So, it is possible to suggest the existence of a common regulatory mechanism for both genes based on (1) the physical contiguity for both genes on chromosome 1q32.1 [23] and (2) the presence of a couple of functional enhancers directing the expression of REN gene in kidney and placenta [44]. Then high expression levels for both genes would be expected in this pathology.

Here, we did not provide definitive evidence of a parallel expression for both KiSS-1 and REN genes in preeclamptic patients. However we cannot discard this phenomenon because of the presence of two variables which could mask the real expression profile for REN: severity status and concomitant treatment.

In this sense we found that the milder the presentation of the disease, the higher the expression of REN. Therefore a study including preeclamptic patients with the same severity status could support our hypothesis.

We also found that a less aggressive hypertension management is coincident with a higher expression of REN in placenta. Thus, it is possible that a treatment not affecting REN expression in placenta would leave them as high as those observed for KiSS-1.

Further studies are needed that focus on the molecular mechanism of transcriptional regulation of these genes. Our data also evidenced that REN could be an interesting therapeutic target to control PEE [45].

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Contributors

Vazquez-Alaniz F., first author, field and experimental work, analysis of results, manuscript writing. Galaviz-Hernandez C., corresponding author, design of the study, results analysis, manuscript writing. Marchat L.A., manuscript writing, results analysis. Salas-Pacheco J.M., guidance during experimental work development, results analysis. Chairez-Hernandez I., statistical analysis of data. Guijarro-Bustillos J.J., patient collection data. Mireles-Ordaz A., collecting placentas.

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