Polymorphisms of the Receptor of Advanced Glycation Endproducts (RAGE) and the development of nephropathy in type 1 diabetic patients

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SUMMARY

Objectives: We investigated the association of the RAGE (Receptor for Advanced Glycation End products) exon3 gene polymorphisms with stages of nephropathy in type 1 diabetes. Methods: The RAGE exon 3 genotype was assessed by Denaturing Gradient Gel Electrophoresis (DGGE) procedure in 487 type 1 diabetic patients with proliferative retinopathy subdivided into four groups according to their level of renal involvement and in 351 control subjects (GENEDIAB study).

Results: We reported here three main low frequency dimorphisms, previously submitted to data banks, Gly82Ser, Val89 CTC/CTG, and Arg77Cys. The genotype distribution of these polymorphisms was not statistically different in type 1 diabetic patients compared to healthy controls (p = 0.37). Among the three described polymorphisms, only the RAGE Gly82Ser genotype frequency was significantly increased in the group with advanced nephropathy (11%) defined by a chronic renal failure compared to the three others groups: no nephropathy, 5%; incipient (microalbuminuria) 5%; established (macroalbuminuria), 2% (P = 0.04). The 82 Ser allele was identified as an independent risk marker for the stage of advanced nephropathy: adjusted odds ratio 3.17(95% CI 1.32-7.85, p = 0.008).

Conclusion: These data suggest that the 82 Ser allele of the RAGE gene is a risk allele for developing advanced nephropathy. This suggests that some RAGE gene polymorphisms may be associated with progression to diabetic advanced nephropathy in Caucasian type 1 diabetic patients.

Key-words: Type 1 diabetes mellitus · Receptor for Advanced Glycation End products (RAGE) · Denaturing Gradient Gel Electrophoresis (DGGE) · Polymorphisms - Nephropathy.

RéSUMÉ

Polyorphismes du récepteur des produits de la glycation avancée (RAGE) et développement de la néphropathie chez des diabétiques de type 1

Objectif : Nous avons évalué l’association entre les polymorphismes de l’exon 3 du gène RAGE et les différents stades de néphropathie dans le diabète de type 1.

Méthodes : L’analyse du polymorphisme de l’exon 3 a été réalisée en « Denaturant Gradient Gel Electrophoresis » chez 487 patients diabétiques de type 1 porteurs d’une rétinopathie diabétique proliférative et divisés en 4 groupes en fonction de la sévérité de l’atteinte rénale ainsi que chez 351 sujets témoins (étude GENEDIAB).

Résultats : Nous rapportons 3 dimorphismes, peu fréquents, répertoriés dans les bases de données : Gly82Ser, Val 89 CTC/CTG et Arg77Cys. La distribution de ces polymorphismes n’est pas statistiquement différente dans le groupe des patients diabétiques type 1 et le groupe de sujets témoins (p=0.37). Parmi les trois polymorphismes décrits, seule la fréquence du gène Gly82Ser est significativement plus élevée dans le groupe des patients porteurs d’une néphropathie avancée définie par une insuffisance rénale (11%) comparé aux autres groupes : absence de néphropathie, 5% ; néphropathie incipiens (micro albuminurie), 5 %, néphropathie établie (macro albuminurie), 2% (p = 0.04). L’allèle 82Ser est un facteur de risque de néphropathie avancée : Odds ratio = 3,17 (95 % IC 1.32-7.85, p = 0.008).

Conclusion : Ces résultats suggèrent que l’allèle 82Ser est un allèle à risque de néphropathie avancée et par conséquent le polymorphisme du gène RAGE pourrait influencer la progression de la néphropathie chez les patients caucasiens diabétiques de type 1.

Mots-clés : Diabète de type 1 · Récepteur des produits de la glycation avancée (RAGE) · Électrophorèse en gel dénaturant (DGGE) · Polymorphismes - Néphropathie diabétique.

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Increasing evidence suggests that Advanced Glycation Endproducts (AGE) contribute to the development of diabetic complications including nephropathy [1-4]. The effects of AGE are partially mediated through their interactions with cell surface receptors [5]. Among these receptors, RAGE (receptor for the AGE) is the best characterised [2]. This 35 kDa polypeptide receptor is a member of the immunoglobulin family (a V type domain followed by two C type domains) [6]. It is present in a range of cell types like endothelium, mesangial cell, vascular smooth cell and monocytes, all cell types that are involved in diabetic nephropathy. The interaction of AGE with RAGE leads to the synthesis of the extracellular matrix proteins by mesangial cell, to activation of cytokines by monocytes and enhanced permeability of the endothelium [7-9].

Interestingly, RAGE engages distinct ligands including the members of the S100/calgranulins. S100/calgranulins are polypeptides released from inflammatory cells. The interaction of S100/calgranulins and RAGE mediates activation of endothelial cell, macrophages and lymphocytes, central cells to the inflammatory response [10].

Recent in vivo studies confirm the pathogenic effects of ligand-RAGE interactions. Glomeruli of patients with diabetic nephropathy demonstrated diffuse upregulation of RAGE expression in podocytes and staining for S100/calgranulins could be identified in the distribution of infiltrating mononuclear phagocyte in the glomeruli of diabetic nephropathy [11]. A role for RAGE in the pathogenesis of diabetic nephropathy has been most demonstrated in transgenic mice overexpressing RAGE in vascular cells. These animals develop glomerulosclerosis more rapidly compared to non transgenic mice, an effect blocked by an AGE inhibitor (OBP9195)[12]. In parallel, most structural and functional abnormalities associated with diabetic nephropathy were both prevented by pharmacologic blockade of RAGE by soluble RAGE and in the model of RAGE null mice [13].

The RAGE gene is located in chromosome 6 near the HLA class II region [14]. Hudson has identified four functional amino acid changes and he described a simple PCR-RFLP technique to screen the most common Gly82Ser mutation in the exon 3 [15].

The hypothesis that variants in the RAGE gene may influence the development of diabetic complications has previously been drawn. Up to now, most studies haven’t observed any association between RAGE polymorphisms and macrovascular or microvascular diabetic complications in type 2 diabetes [15-20]. By contrast, the C-1152A polymorphism located in the promoter region of the RAGE gene has been described as a potential protective marker for nephropathy in type 1 diabetic patients [21].

In a preliminary work (data non published), we have screened polymorphisms by Denaturing-Gradient gel Electrophoresis (DGGE) technique in the part of the RAGE gene that encodes the binding domain of AGE e.g. intron 2, exon 2, exon3. No polymorphism was found in intron 2 and exon 2 so we focused only on the exon 3 RAGE gene in the further studies.

The aims of our study were 1) to evaluate the RAGE exon 3 polymorphism by DGGE technique in a French previously published population of diabetic type 1 patients with nephropathy and in controls matched for ethnicity (GENEDIAB STUDY) [22], 2) to look for a relationship between RAGE polymorphisms and the level of renal involvement.

Material and methods

Population

A cross sectional multicentre study (GENEDIAB STUDY) was conducted in 17 diabetic clinics in France and in Belgium as detailed in Marre et al. [22]. Inclusion criteria were type 1 diabetes (ketosis prone diabetes requiring insulin treatment within one year of the diagnosis) before the age of 35 years and past or present proliferative diabetic retinopathy. Patients were collected as described earlier [22]. Briefly, 56% were men, the mean age was 44 years, and the mean diabetes duration was 29 years. The mean HbA1c was 8.6%. The 487 patients included in our study were not different from the initial cohort. They were further subdivided into four groups according to their level of renal involvement. 154 patients presented no nephropathy (normal urinary albumin excretion and plasma creatinine < 150 μmol/L), 103 patients had incipient nephropathy (microalbuminuria, plasma creatinine < 150 μmol/L), 124 patients had established nephropathy defined as past or present occurrence of macroalbuminuria and plasma creatinine < 150 μmol/L, 106 patients had advanced nephropathy (past or present occurrence of macroalbuminuria and plasma creatinine > 150 μmol/L).

The control subjects were 351, age and sex matched healthy persons participating in a prospective study on insulin resistance and cardiovascular disease.

Detection of the exon 3 RAGE gene mutation by DGGE technique

For the exon 3 amplification the forward primer was 5’-gTC ACT CTg CCT CAC AgT CCT TTC-3 and the reverse primer was 5’-CCg ggC ggg CgC ggC gAg ggC ggg Cgg ACg ggC Cgg gCA ggg ggC gcC gcAg gcC gGC gcC gcC gcC gcC gcC clamp TTC TTA ggT AAg Agg gAg gcC TTG-3’. Amplification was carried out as follows: 38 cycles of 30” at 94°C, 30” at 60°C, 30” at 72°C. Subsequently one extension cycle of 7” at 72°C. The specificity of PCR was monitored by polyacrylamide gels electrophoresis prior to DGGE.
Denaturing-gradient gel electrophoresis GC-clamped PCR products were analysed by DGGE based on the protocol of Myers et al. [23]. Bands were visualised under ultra-violet light after ethidium bromide staining. The computer programs MELT 87 and SQHTX were used to predict optimal conditions for the amplification and focusing of exon 3 [24]. The gels used were 6% acrylamide containing a denaturing gradient of 40-80%. Electrophoresis was carried out at 160 V for 4 h at 60°C in a circulating buffer. The samples that displayed different patterns of migration were identified by direct sequencing of the PCR product. These samples of which we knew the sequencing could be deposited as markers to verify the quality of PCR product migration for each electrophoresis.

Statistical analysis

Data were analysed using the Staview V program (Abacus Concepts, Inc, Berkley, CA, USA).

The $\chi^2$ for trend test was used for the comparison of qualitative variables, ANOVA and Mann-Whitney U test for quantitative variables.

Forward stepwise regression analysis were done to assess the influence of independent variables on the stage of advanced nephropathy.

Results

By DGGE technique and sequencing, three main dimorphisms, previously submitted to data banks, have been found: Gly82Ser (AF065211), Val89 CTC/CTG (AF065210), and Arg77Cys (AF065212). The genotype distributions of RAGE exon 3 polymorphisms were in Hardy Weinberg equilibrium in the entire type 1 diabetic patient population and in controls.

The Gly82Ser, CTC89CTG and Arg77Cys genotype distribution was not statistically different in type 1 diabetic patients and in healthy controls ($\chi^2 = 3.85$, $p = 0.37$) (Tab I).

We then investigated the relationship between RAGE exon 3 polymorphisms and the various stages of nephropathy (Tab I). Only the Gly82Ser dimorphism was weakly associated with the stages of nephropathy ($\chi^2$ for trend $= 8.9$, $p = 0.04$). This result was due to a significant increase of Gly/Ser genotype frequency in the advanced nephropathy group (11%) compared to the three others groups (no nephropathy, 5%; incipient 5%; established 2%). In contrast, no association was found between nephropathy and the two other CTC89CTG, Arg77Cys described polymorphisms (Tab I).

### Table I

Association analysis of RAGE exon 3 polymorphisms and nephropathy in type 1 diabetic patients.

<table>
<thead>
<tr>
<th>Gly82Ser</th>
<th>CTC89CTG</th>
<th>Arg77Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly/Gly</td>
<td>Gly/Ser, Ser/ Ser</td>
<td>CTC/CTC</td>
</tr>
<tr>
<td>Absent (n = 154)</td>
<td>146</td>
<td>8</td>
</tr>
<tr>
<td>Incipient (n = 103)</td>
<td>98</td>
<td>5</td>
</tr>
<tr>
<td>Established (n = 124)</td>
<td>121</td>
<td>3</td>
</tr>
<tr>
<td>Advanced (n = 106)</td>
<td>94</td>
<td>12</td>
</tr>
<tr>
<td>all diabetics (n = 487)</td>
<td>459</td>
<td>28</td>
</tr>
<tr>
<td>controls (n = 351)</td>
<td>326</td>
<td>25</td>
</tr>
</tbody>
</table>

Stages of nephropathy:

Gly82Ser polymorphism was X2 for trend $= 8.9$, $p = 0.04$
CTC89CTG polymorphism was X2 for trend $= 1.62$, $p = 0.89$
Arg77Cys polymorphism was X2 for trend $= 4.54$, $p = 0.02$

diabetics vs controls:

Gly82Ser polymorphism was X2 for trend $= 0.65$, $p = 0.42$
CTC89CTG polymorphism was X2 for trend $= 0.50$, $p = 0.82$
Arg77Cys polymorphism was X2 for trend $= 3.18$, $p = 0.07$.
We then evaluated the RAGE exon 3 distribution in patients with advanced nephropathy versus the three other group patients. There was an association between the Gly82Ser dimorphism and the advanced nephropathy (C2 = 6.5; p = 0.01).

This association was restricted to the Gly82ser dimorphism. The prevalence of the advanced nephropathy in the group of patients who carried the 82 Ser allele was 42% (12 patients) versus 21% in the group of patients who did not carry this allele (C2 = 7.53, Yates corrected p = 0.01).

There was no difference between patients carrying 82 Ser allele and those without regarding the diabetes duration, HbA1c, plasma creatinine and the blood pressure (Data not shown).

To determine whether the association of the 82 Ser allele with advanced nephropathy was independent of the patients' characteristics, we used a forward stepwise regression analysis. The presence of advanced nephropathy was considered as the outcome variable and the following variables were used as covariates: HbA1c (divided for each 1% increase), diabetes duration, sex, and PAS (divided into three groups, < 135 mmHg, between 135 mmHg and 160 mmHg, and > 160 mmHg).

The 82 Ser allele was identified as an independent risk marker for the stage of advanced nephropathy: adjusted odds ratio 3.26 (95% CI 1.32-7.85, p = 0.008) (Tab II).

**Discussion**

In contrast to the PCR-RFLP method described by Hudson [18] for the analysis of the RAGE gene polymorphism which only detects the well-known Gly82Ser variant, the PCR-DGGE procedure we performed allowed us to screen two other variants of the exon 3 e.g. Val 89 CTG and Arg77 Cys. These dimorphisms were previously submitted to data banks but never reported in the prior studies. Hudson [18] for the analysis of the RAGE gene polymorphism where the D allele frequency increased with the nephropathy stages [22], the increase of the 82 Ser allele frequency was found only in the group with a chronic renal failure. This observation is questionable but we could speculate that the RAGE gene like others previously described could be an aggravating factor rather than an initiating factor for diabetic nephropathy [26], nevertheless these speculative assumption need to be proven in well designed future studies.

Though the association of the 82 Ser allele with advanced nephropathy should be confirmed in other large case control studies, a recent functional study makes this hypothesis relevant. Indeed, Hoffman et al. investigated the effects of the Gly82Ser polymorphism in the binding domain of RAGE on receptor function and on the inflammatory response. In transfected CHO cells, increased ligand affinity was found to be conferred by the 82 Ser allele which demonstrated up regulation of intracellular signaling pathway such an enhanced activation of NFkB upon interaction with S100/calgranulins. In parallel, mononuclear phagocytes retrieved from human subjects bearing the variant 82 serine allele displayed heightened activation of signal transduction molecule (MAP kinase pathway) in the presence of ligand [27]. Thus, these data suggest that the 82 Ser allele upregulates the inflammatory response upon engagement of S100/calgranulins. These findings could explain how the 82 Ser allele in the RAGE gene is a risk allele for previous study, where we had reported that the Gly82Ser variant despite a linkage disequilibrium with HLA class II DRB1 DR4 specificities was not associated with the susceptibility of type 1 diabetes [25].

Secondly, our major goal was to address the RAGE gene as a genetic determinant for diabetic kidney disease. Thus, we evaluated the RAGE polymorphism in the GENDIAB study population which includes a large population of well defined type 1 diabetic patients with a long diabetes duration and proliferative diabetic retinopathy. In this population, Marre et al. [22] described the implication of the Insertion/deletion angiotensin I converting enzyme polymorphism in the renal nephropathy. In our study, we have shown that only the Gly82Ser polymorphism by a significant increase of the 82 Ser allele in the group of patients with advanced nephropathy may play a role in the genetic of the diabetic nephropathy. The influence of the 82 Ser allele was independent from HbA1c, diabetes duration and PAS. Our data are quite different from Liu and Poirier's results [17, 21] who did not find any association between 82 Ser allele and the nephropathy stages neither in type 2 nor in type 1 diabetes. It could be explained by the size of the patient groups which was smaller in these prior studies and particularly, quite few patients with renal failure were analysed (24 patients with chronic renal failure in the Liu's study). As the prevalence of the 82 Ser allele in the control population is low (4%), we have chosen a large population for this case control association study.

In contrast to the ACE polymorphism where the D allele frequency increased with the nephropathy stages [22], the incidence of the 82 Ser allele frequency was found only in the group with a chronic renal failure. This observation is questionable but we could speculate that the RAGE gene like others previously described could be an aggravating factor rather than an initiating factor for diabetic nephropathy [26], nevertheless these speculative assumption need to be proven in well designed future studies.

**Table II**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted odds ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.95</td>
<td>0.58-1.58</td>
<td>0.86</td>
</tr>
<tr>
<td>PAS</td>
<td>8.29</td>
<td>3.77-19.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.77</td>
<td>0.36-1.65</td>
<td>0.49</td>
</tr>
<tr>
<td>Duration</td>
<td>0.98</td>
<td>0.3-3.26</td>
<td>0.97</td>
</tr>
<tr>
<td>Allele 82 Ser</td>
<td>3.26</td>
<td>1.32-7.85</td>
<td>0.008</td>
</tr>
</tbody>
</table>
developing advanced nephropathy. Nevertheless further investigations with other RAGE ligands are needed to confirm these results.

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References