RELATIONSHIP BETWEEN POLYMORPHISMS IN THE RENIN-ANGIOTENSIN SYSTEM AND NEPHROPATHY IN TYPE 2 DIABETIC PATIENTS

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SUMMARY - Background: Renin Angiotensin system is involved in renal function and its polymorphisms may influence diabetic nephropathy. ID ACE polymorphism modulates ACE level whereas M235T AGT polymorphism is involved in arterial hypertension. The A1166C AT1R polymorphism is involved in arterial hypertension and in diabetic retinopathy.

Methods: Two hundred thirty five type 2 diabetic patients were enrolled in this transversal study. Data were documented for clinical characteristics of the population, HbA1c, urinary albumin excretion, presence of retinopathy or antihypertensive treatment. Polymorphisms were analyzed by PCR techniques. The patients were divided into 3 groups: group 1, without nephropathy (n = 118), group 2, microalbuminuria (n = 78), group 3, macroalbuminuria (n=39).

Results: Diabetes duration was longer (p < 0.001), retinopathy (p < 0.001) and antihypertensive treatment (p < 0.02) were more frequent in group 3 compared to group 1 and 2. The I/D ACE and M235T AGT polymorphisms were not differently distributed between the three groups. In contrast, the CC genotype of the AT1R polymorphism was overrepresented in group 2 (p = 0.021). The presence of the CC AT1R genotype considerably increased the incidence of albuminuria after 10 years of diabetes (AA vs CC p = 0.01), particularly in men. No effect was seen with I/D ACE and M235T AGT polymorphisms.

Conclusion: In conclusion, we observed an interaction of A1166C AT1R polymorphism with diabetes in men but not of I/D ACE and M235T AGT polymorphisms.

Key-words: renin-angiotensin system, genetic polymorphism, diabetic nephropathy, type 2 diabetes.
Diabetic nephropathy is the first cause of chronic renal failure and comes along with cardiovascular morbidity and mortality. In France, 15% of the dialyses are caused by diabetes. Diabetic nephropathy affects 10 to 20% of type 2 diabetic patients. After twenty years of evolution, the prevalence becomes weaker. Genetic susceptibility has been found in type 2 diabetic patients for nephropathy development, particularly in PIMA Indians and in Caucasian subjects [1, 2].

Renin-angiotensin system plays a central role in blood pressure regulation and in renal homeostasis. Angiotensin II is cleaved from angiotensinogen (AGT) by the sequential action of renin and angiotensin converting enzyme (ACE). Angiotensin II interacts with two subtypes of the angiotensin II receptor: the angiotensin II type 1 receptor (AT1R) and the angiotensin II type 2 receptor (AT2R).

Genetic polymorphisms have been described for the different components of the renin-angiotensin system. Numerous studies have been conducted on the insertion/deletion polymorphism (ID) of an ALU sequence of 287 bp in the angiotensin converting enzyme gene. ACE polymorphism partly determined the level of circulating ACE. In a metaanalysis about I/D of ACE published in 1998 [3], which studied this polymorphism in type 1 and type 2 diabetic patients, a significant association between D allele and nephropathy was found but not with retinopathy. This insertion/deletion polymorphism was implicated in cardiovascular disease [4, 5] but not validated by all authors [6]. High angiotensinogen levels were linked with mutation M 235 T and should be associated with arterial hypertension [7] in the general population; in diabetic nephropathy, such association is still debated [8, 9].

Vascular actions of angiotensin are mediated by the type 1 angiotensin II receptor in which a polymorphism A 1166 C has been described which is involved in arterial hypertension [10] but it is still debated in diabetic microangiopathy [11, 12].

Interactions between these polymorphisms were described in type 1 diabetic patients by Marre et al. [13] who shown renal function deterioration with ID and DD ACE polymorphism associated to T allele of AGT. This association has been found in type II diabetic patients by Young et al. [14].

The purpose of this study was to determine whether diabetic nephropathy progression is dependent on one of these polymorphisms in type 2 diabetic patients.

Materials and Methods

Patients and clinical investigations

Patients for the study were recruited in the department of Endocrinology in Caen Hospital. The protocol of the study was approved by the ethical committee of the hospital and informed consent for genetic studies was obtained from all participants. Classification of type 2 diabetes was based on ALFEDIAM recommendations (Association de Langue Française pour l’Etude du Diabète et des Maladies Métaboliques). Patients were divided as follow into 3 groups according to their urinary albumin excretion (UAЕ) which was measured by nephelometry in urine samples collected on two consecutive visits:

- Group 1: no nephropathy (albumin excretion rate < 30 mg/24 h, < 20 µg/min or < 20 mg/l);
- Group 2: incipient nephropathy (albumin excretion rate between 30 and 300 mg/24 h, 20 µg and 200 µg/min or 20 and 200 mg/l);
- Group 3: established nephropathy (albumin excretion rate > 300 mg/24 h, > 200 µg/min or >200 mg/l).

Clinical and paraclinical data are summarized in Table I. Age, gender, diabetes duration, presence or absence of arterial hypertension (systolic pressure > 140 mm Hg and diastolic pressure > 80 mm Hg ) and of antihypertensive treatment, presence or absence of retinopathy (ocular fundus and retinal angiography), hemoglobin HbA1c measured by HPLC (N < 5.5%).

Genetic analyses

Genomic DNA from each patient was prepared from peripheral leucocytes by salts precipitation method [15]. The Met 235 Thr (MT) polymorphism of angiotensinogen localized in exon 2 was determined by the method of Russ et al. [16]. PCR products of 165 bp were digested by Asp I for 6 hours. Amplification of the Met allele is not digested by the enzymes whereas the digestion of Thr allele gives 2 products of 141 and 24 bp. Insertion/deletion polymorphism of the angiotensin converting enzyme gene located in intron 16 was determined by the method of Rigat et al. [17]. Amplification of the D allele resulted in a 190 bp fragment and amplification of the I allele resulted in a 490 bp fragment. Each sample which had the DD genotype was submitted to PCR amplification with a primer pair that recognizes an insertion specific sequence [18]. If the DNA was mistyped, a PCR product of 335 bp is present. Otherwise, no PCR product appeared. Polymorphism A 1166 C in the untranslated sequence of the AT1 R gene was determined by a method developed in the laboratory. A 1166 C mutation creates a palindromic sequence in the gene; with the PCR software from Intellegenics Company, we found the specific enzyme for this mutated sequence: Dde 1 enzyme. This enzyme is specific and cleaves the PCR product if the mutation is present [19, 20]. PCR primers have been chosen to obtain after electrophoresis, if the mutation is present, two fragments of different sizes easily visualized on a metaphor gel. Amplification product measures 350 bp and after 4 hours of
digestion, 1166 A allele is not cleaved whereas 1166 C allele generates two fragments of 237 and 113 bp (Fig. 1).

**Statistical analyses**

Firstly, data were presented as mean ± standard deviation (SD) or as proportions. For continuous and categorical variables, t-test and chi-square test were respectively applied. Secondly, a multivariate analysis of variance were used. Each comparison has been checked with Levene’s test. Statistical significance was defined as two-tailed p-value < 0.05. Data were analysed with SPSS® software version 10.0. Interactions between the three polymorphisms were not studied considering the low population size of the polymorphism subgroups.

### RESULTS

**Patients characteristics**

Two hundred thirty five patients were involved in the study. Clinical characteristics of the 3 groups of patients are summarized in Table I. We did not observe any significant difference between the 3 groups for age, gender and body mass index (BMI). Diabetes duration was significantly longer in group 3 than in groups 2 and 1. HbA1c was significantly higher in group 2 compared to groups 1 and 3 (p = 0.003). Creatinine levels up to 120 µmol/l were found in 30% patients of group 3 and in none patients from groups 1 and 2. Diabetic retinopathy was more frequent in group 3 (48.7%) than in group 2 (28.2%) and 1 (19.5%) p < 0.001. Percentage of patients receiving an antihypertensive treatment was higher in group 3 (82.1%) compared to groups 2 (71.3%) and 1 (59.3%) p = 0.02.

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**Table I. Clinical characteristics of patients in relation with their proteinuria.**

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No nephropathy</td>
<td>Incipient nephropathy</td>
<td>Established nephropathy</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.2 ± 8.3</td>
<td>56.8 ± 9.4</td>
<td>57.9 ± 9.3</td>
<td>p = 0.21</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>64/54</td>
<td>39/39</td>
<td>23/16</td>
<td>p = 0.60</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>9.5 ± 8.1</td>
<td>9.8 ± 8.4</td>
<td>15.4 ± 9.3</td>
<td>p = 0.001*</td>
</tr>
<tr>
<td>BMI</td>
<td>31.2 ± 6.5</td>
<td>32.6 ± 6.7</td>
<td>29.3 ± 4.6</td>
<td>p = 0.082</td>
</tr>
<tr>
<td>HbA1c</td>
<td>8.2 ± 2</td>
<td>9.4 ± 2</td>
<td>8.8 ± 3</td>
<td>p = 0.003**</td>
</tr>
<tr>
<td>Retinopathy (+/-)</td>
<td>23/95</td>
<td>22/56</td>
<td>19/20</td>
<td>*</td>
</tr>
<tr>
<td>Antihypertensive treatment (+/-)</td>
<td>70/48</td>
<td>55/23</td>
<td>32/7</td>
<td>p = 0.02</td>
</tr>
</tbody>
</table>

* Group 3 versus group 1 and group 3 versus group 2.
** Group 2 versus group 1 and group 3.

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**Fig. 1. Ethidium bromide gel for PCR with digestion for AT1R polymorphism (lanes 1 & 6 100 pb DNA ladder, lane 5 PCR product before digestion, lane 2 C/C homozygote with 2 fragments of 237 and 113 bp, lane 3 A/C heterozygote with 3 fragments 350, 227 and 113 bp, lane 4 A/A homozygote with the PCR product non digested of 350 pb).**
ACE, AGT and AT1R polymorphisms frequency

All genotype frequencies were in Hardy-Weinberg equilibrium. There were no differences in the frequencies of the ACE (II, ID, DD) and AGT (MM, MT, TT) between the 3 groups. There was a significant difference in the frequency of AT1R (AA, AC and CC) between group 2 and group 1 (p = 0.021) (Table II).

Relationship between ACE, AGT, AT1R polymorphisms and nephropathy: effect of duration of diabetes and gender

Diabetes duration worsen nephropathy particularly in men (p = 0.01). There was no significant relationship between ID ACE polymorphism and M235T angiotensinogen polymorphism and the level of albuminuria whatever duration of diabetes or gender. The presence of the CC AT1R polymorphism considerably increased the albuminuria after 10 years of diabetes (AA versus CC, p = 0.01) but this correlation was restricted to men population (p = 0.001) (Fig. 2a and 2b).

DISCUSSION

Susceptibility factors to develop a diabetic nephropathy could be linked, via hyperglycemia, to an elevation of capillary pression, modifications of glomerular hemodynamics resulting in increased urinary excretion of albuminuria and eventually rearrangement of renal parenchyma. Angiotensin II level could modulate the development of diabetic glomeruloscle-

**Table II. Polymorphism distribution in the three groups of patients (* group 2 vs group 1).**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Group 1 n = 118</th>
<th>Group 2 n = 78</th>
<th>Group 3 n = 39</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>17.2</td>
<td>15</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>45.7</td>
<td>50</td>
<td>55.9</td>
<td>p = 0.87</td>
</tr>
<tr>
<td>DD</td>
<td>37.1</td>
<td>35</td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>33.9</td>
<td>40.5</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td>AGT (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>50</td>
<td>41.8</td>
<td>28.6</td>
<td>p = 0.19</td>
</tr>
<tr>
<td>TT</td>
<td>16.1</td>
<td>17.7</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>52.1</td>
<td>58.2</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>AT1R (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>43.7</td>
<td>30.4</td>
<td>17.6</td>
<td>p = 0.021*</td>
</tr>
<tr>
<td>CC</td>
<td>4.2</td>
<td>11.4</td>
<td>8.8</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.**

a) Relationship between proteinuria before and after 10 years of diabetes and AT1R polymorphism in men. (light grey diabetes < 10 years, dark grey diabetes > 10 years, p value: ns non significant).

b) Relationship between proteinuria before and after 10 years of diabetes and AT1R polymorphism in women. (light grey diabetes < 10 years, dark grey diabetes > 10 years, p value: ns non significant).
rosis [21]. A familial susceptibility was found for the development of nephropathy in type 1 [22] and type 2 [1] diabetes.

Renin-angiotensin system which acts at the peripheral level and at the tissue level on arterial pressure regulation, should modulate a risk for diabetic nephropathy.

The association between ID polymorphism of ACE and nephropathy is still debated but a metaanalysis published in 1998 [3] with a cohort of 4,778 patients concluded to a significant association between D allele and diabetic nephropathy according to a dominant model in type 1 and type 2 diabetes among two different ethnical populations (Asians and Caucasians). This association was not observed for the retinopathy. In our study, we did not find any difference between allelic distribution in the 3 groups of diabetic patients for ID polymorphism in univariate analysis. In a multivariate analysis including diabetes duration, D allele did not seem to have an influence on diabetic nephropathy in opposition with results from a recent Japanese study [23]. Patient number and duration of diabetes are the noticeable differences between the two studies, since the deleterious effect of D allele appeared only after 15 years of diabetes in the Japanese study.

Angiotensinogen is a candidate locus as M235T polymorphism is correlated with arterial hypertension [24] which is a strong predictor of nephropathy. We did not find any correlation between M235T polymorphism and nephropathy whatever its stage of progression in univariate or multivariate analyses. These results are similar to works of Schmidt [9], Rigal [25] and Gutierrez [26]. These studies, conducted with a control group or between groups with and without nephropathy failed to demonstrate a link between AGT polymorphism and nephropathy in type 2 diabetics.

We observed a significant difference in univariate analysis when comparing group 1 and group 2 for AT1R polymorphism since CC homozygotes were overrepresented in group 2. Similar studies in type 1 diabetic patients [11, 12, 27] and type 2 diabetic patients [28] failed to find such modification in the distribution of AT1R polymorphism according to the degree of albuminuria.

In multivariate analysis, we found a relationship between diabetes duration, albuminuria, and AT1R polymorphism. Albuminuria increased considerably after 10 years of diabetes in patients with CC polymorphism compared to AC polymorphism, with AA patients exhibiting an intermediate albuminuria. One might notice that this phenomenon was observed exclusively in men, which is not explained. The deleterious effect of the C allele was observed in coronary heart disease in non diabetic patients [29-31] in association with DD polymorphism. In type 2 diabetic patients, Tomino et al. [23] found a deterioration in renal albuminuria in women with the C allele but not in men. From our study, we found such effect of C allele to be restricted to homozygote men.

To conclude, AT1R polymorphism interacts with diabetes duration for the deterioration of nephropathy in type 2 diabetic patients but we did not find any influence of the ID ACE polymorphism or MT polymorphism of angiotensinogen.

REFERENCES

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