Endothelial Nitric Oxide Synthase Gene Polymorphisms and Renal Survival

To the Editor:

Development of end stage renal disease (ESRD) and a blunted in vitro generation of nitric oxide (NO) have been recently associated with the 298Asp substitution in the endothelial NO synthase (eNOS) gene.1 Diabetic nephropathy was the culprit of ESRD in most patients (pts);1 however, the same association was recently reported in pts with ESRD due to polycystic kidney disease.2 Collectively these results would imply that a 298Asp allele–determined predisposition to generate less NO is mechanistically related with decreased renal survival. If proven, this appealing hypothesis could open new avenues to pharmacological prevention of ESRD.

However, some caveats suggest caution in jumping to this conclusion. First, criteria for patient enrollment were not given, and therefore a selection bias cannot be excluded.1 Second, both studies were small since each entailed less than 200 ESRD pts. Sample size calculation showed that even in the larger study1 a two-group $\chi^2$ test with a 0.05 two-sided significance level had only 60% power to detect a Glu298Asp genotype frequency difference between ESRD pts and controls. Thus, these were either lucky or serendipitous findings. Under both circumstances larger studies are mandatory. Furthermore, eNOS allele frequency differs markedly between Japanese and Caucasians; thus, these results must be replicated in populations with different ethnic backgrounds. Third, the restriction fragment length polymorphism analysis (RFLP) used for genotyping1 cannot be as accurate as it should be. We replaced this methodology with the melting curve analysis2 because of inconsistent amplicon cleavage with BanII resulting in misdiagnosis of GT and GG as TT and GT, respectively. According to the Hardy-Weinberg equilibrium, most pts would be heterozygous; therefore, the rate of misgenotyped pts could not be negligible. Sequencing of amplicons may circumvent this bias, but it was performed only in a minority, eg, on uncleaved fragments.

Noiri et al stated that the Glu298Asp polymorphism is functionally relevant by quoting a study that claimed the 298Asp variant to be more vulnerable to intracellular cleavage.4 However, this contention was thereafter disproved by the demonstration that the intracellular cleavage found in cells harboring the 298Asp eNOS substitution was an in vitro artifact.5 According to both studies, the 298Asp replacement does not affect eNOS biological activity.4,5 Thus, it remains controversial whether the 298Asp variant implies a blunted eNOS activity; accordingly, the contention that the latter accounts for decreased renal survival cannot be taken for granted.

Other functional variants of eNOS polymorphisms, such as the $T^{786}C$ variant in the eNOS promoter, exist. In the large cohort of African pts of the GENICA study6 and in another study,2 the Glu298Asp and $T^{786}C$ genotypes were in linkage disequilibrium, albeit not strongly. We proposed that this genetic variant can be the most important for NO generation in essential hypertensive pts;2,3 we have not associated with ESRD in another study.2 However, the $T^{786}C$ allele was associated with multivessel coronary atherosclerosis;6 and since coronary events are the major cause of death in ESRD pts, premature mortality of $T^{786}C$ allele carriers could result in underrepresentation of this allele in the ESRD population. Recent findings by 2 independent groups support our contention.7,8

Response: Multifactorial Disease: Glu298Asp of Endothelial Nitric Oxide Synthase

Type 2 diabetes mellitus (DM) is one of the multifactorial diseases—as are hypertension, hyperlipidemia, and atherosclerosis—that develop from both genetic and environmental factors. The risk of their onset derives from the accumulation and/or combination of polymorphisms of susceptible genes. In our study,1 the enrollment criteria for the chronic hemodialysis patient included all patients with end-stage renal disease (ESRD) receiving treatment at 2 suburban hemodialysis centers in Tokyo as of March 1996, and all blood samples were decided at a single time-point basis, there was no selection bias. While we report that a probability value of $<0.05$ was considered significant, the probability value of Table 1 was actually 0.002; the odds ratio of the ESRD group was 1.65 (95% confidence interval: 1.21 to 1.25). The probability value of Table 2 was actually 0.001; the odds ratio of the DM-derived ESRD group was 2.02 (95% confidence interval: 1.34 to 3.07).

The observed frequency of the endothelial nitric oxide synthase (eNOS) Glu298Asp allele was similar to that described in other East Asian studies.2–4 In contrast, the allele frequency is different from that described in studies conducted in Western countries.5,6 Therefore, we agree that ethnic background has to be considered carefully. Nevertheless, previous studies from Europe have demonstrated the statistically significant accumulation of Glu298Asp in coronary artery disease5 and carotid atherosclerosis.6

The restriction enzyme BanII is not so delicate as to cause inconsistent cleavage of amplicon if the right buffer and the appropriate conditions are applied. Like previous researchers,2,3 we performed overnight digestion, which worked perfectly with the particular protocol used.5 The intracellular cleavage found by Te-sauro et al was argued by Fairchild et al to be an artifact due to acidic hydrolysis under higher temperature control during electrophoresis, which we did not mention in our report.1 Both studies failed to demonstrate the blunt eNOS activity of the 298Asp (191TT) variant. Unlike these researchers, however, we used


Teresa M. Seccia
Department of Clinical Methodology and Medical-Surgical Technologies
University of Bari, Italy

Gian Paolo Rossi
Department of Clinical and Experimental Medicine
Clinica Medica 4
University of Padova, Italy
stably transfected Chinese hamster ovary cells for comparing NO activity. It is well known that a stable system is better suited for the physiological analysis of NO production than a transient system, since the proper expression of protein at high levels is difficult to achieve in the transient system. The intracellular signaling pathway is not well preserved or adjusted in a transient system, depending on the cell type and the cellular toxicity of the method used.

Therefore, we consider the observation derived from our stable system to be more reliable compared with observations derived from transient systems. It is of note that the difference we observed in NO production between 298Glu and 298Asp was significant but moderate. In other words, it was not an all-or-nothing event. The polymorphic site related to the susceptibility of the multifactorial diseases should produce moderate differences in protein expression instead of remarkable differences. Thus, we believe it should be studied with a stable transfection system.

We did not perform the typing of T-H11002786C in our study. It raises the delicate issue of linkage disequilibrium (LD) between Glu298Asp and T-H11002786C, since Nakayama et al\(^9\) reported no LD after the analysis of the frequencies of these 2 polymorphic sites in more than 1000 individuals. It is certainly of interest in T-H11002786C for the association with ESRD. At the same time, the accumulated observation related to Glu298Asp should also be considered as a predisposing factor for hypertension, ischemic heart disease, atherosclerosis, and diabetic nephropathy.

Eisei Noiri
Toshiro Fujita
Departments of Nephrology and Endocrinology
Katsushi Tokunaga
Department of Human Genetics
The University of Tokyo, Japan


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Teresa M. Seccia and Gian Paolo Rossi

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