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Functional genetic variation in aminopeptidase A (*ENPEP*): Lack of clear association with focal and segmental glomerulosclerosis (FSGS)

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Abstract

The aminopeptidase A (APA) ectopeptidase is an integral membrane-bound zinc metalloprotease that cleaves aspartic and glutamic acidic residues from the N-terminus of a number of protein substrates that includes angiotensin II. Angiotensin II, the most vasoactive component of the renin–angiotensin–aldosterone (RAAS) pathway, can contribute to renal disease by causing an increase in arterial blood pressure leading to glomerular injury and fibrosis. APA is expressed in many organs, including the kidney where it localizes mainly to the podocyte cell membrane and brush borders of the proximal tubule cells. Antibodies directed to the APA peptide can induce an acute massive albuminuria in wild-type BALB/c mice after intravenous injection.

We examined whether variants in the APA encoding gene (*ENPEP*) are more frequent in individuals with the proteinuric disease focal and segmental glomerulosclerosis (FSGS) compared to control individuals. The *ENPEP* coding sequence was re-sequenced in 188 FSGS patients and 48 controls. Genetic variants were further genotyped in 181 individuals without any known kidney disease. We then examined the effect of the non-synonymous coding variants identified on their cell surface APA activity after transfection in COS-1 cells.

Several of these *ENPEP* variants lead to reproducibly altered APA activity. However, we did not see a clear correlation between the presence of a functional *ENPEP* variant and FSGS. However, the existence of these variants with marked effect on APA activity suggests that both rare and common variation in *ENPEP* may contribute to the development of renal and hypertensive disorders and warrants further study.

Keywords: Private non-conservative allele; Single nucleotide polymorphisms; DNA sequence analysis; Mutation; Hypertension

1. Introduction

Damage to the glomerular podocytes can occur through a variety of genetic, environmental and physiological mechanisms. Genetic insults include mutations in genes that encode major proteins of the podocyte slit diaphragm and cytoskeleton such as the *NPHS1* (nephrin), *NPHS2* (podocin), *ACTN4* (α-actinin-4) and *TRPC6* (transient receptor potential-canonical 6 ion channel) genes (Boute et al., 2000; Kaplan et al., 2000; Kestila et al., 1998; Reiser et al., 2005; Winn et al., 2005). Environmental causes such as the human immunodeficiency virus-1 (HIV) (Barisoni et al.,

Abbreviations: ACE, angiotensin I-converting enzyme; APA, aminopeptidase A; $CaCl_2$, calcium chloride; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetraacetic acid; EGTA; ethylene glycol tetraacetic acid; EMBL, European molecular biology laboratory; FSGS, focal and segmental glomerulosclerosis; HEPES, (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid; HCl, hydrochloric acid; ng, nanogram; nm, nanometer; *Pfu*, pyrococcus furiosus; RAAS, renin–angiotensin–aldosterone pathway; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline Tween 80; μg, micro gram.

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1999; Kaufman et al., 2007; Ross et al., 2005; Schwartz et al., 2001; Zuo et al., 2006), and glucose-induced injury (via type 1 and type 2 diabetes) (Mogensen, 1976; Mogensen, 1984; Nosadini and Tonolo, 2003) have been well described, while physiological triggers include (but are not be limited to) elevated blood pressure (Forbes et al., 2002; Ichihara et al., 2006; Kretzler et al., 1994; Nagase et al., 2006; Szokol et al., 1979).

The renin–angiotensin–aldosterone system (RAAS) is a central effector in the control of blood pressure. The RAAS is involved in the regulation of blood volume and systemic vascular resistance, which together regulate blood pressure (Bogatzki, 1964; Conn et al., 1965; Mulrow and Ganong, 1962; Nahum, 1965a; Nahum, 1965b; Tigerstedt and Bergman, 1898). Drugs targeting the RAAS (angiotensin I-converting enzyme (ACE) inhibitors or angiotensinogen II receptor type 1 antagonists) have been shown to reduce hypertension and decrease proteinuria (Collier et al., 1973; Gavras et al., 1974; Pickering and Prinzmetal, 1940) with a concomitant reduction in rate of renal injury in chronic kidney disease. These drugs have been shown to be effective, with a range of effects, in a majority of patients (Corvol and Plouin, 2002; Croog et al., 1990).

The aminopeptidase A (APA) ectopeptidase is a integral membrane-bound member of the zinc metalloprotease family (Jongeneel et al., 1989; Wu et al., 1990) that cleaves aspartic or glutamic acidic residues from the N-terminus of a large variety of protein substrates, including angiotensin II (Wolf et al., 1997; Zini et al., 1996), cholecystokinin-8 (Migaud et al., 1996), neurokinin B and chromogranin A (Goto et al., 2006) as part of their respective metabolic pathways. The protein consists of 3 domains; a 17-amino acid cytosolic N-terminal domain, a 22 amino acid transmembrane hydrophobic domain and a 906 amino acid extracellular C-terminal domain (Li et al., 1993; Nanus et al., 1993; Wu et al., 1990). The protein is encoded by *ENPEP*, a 20 exon gene located at chromosome 4q25 (Li et al., 1997; Wang et al., 1996).

The APA peptide is expressed in the many organs, but its expression profile in the kidney has been extensively studied, where it is expressed on the podocyte cell membrane and brush borders of the proximal tubule epithelial cells. Faint expression has also been observed in the juxtaglomerular cells, endothelial cells of the peritubular capillaries and in the pars media of the arteries (Assmann et al., 1992; Dijkman et al., 2006; Mentzel et al., 1996b).

The degradation of the angiotensin II peptide to angiotensin III is mediated directly by APA in both the brain and the kidney (Wolf et al., 1997; Zini et al., 1996), but other proteins can metabolize angiotensin II *in vitro* (neutral endopeptidase (Walter et al., 1980), prorylendopeptidase (Gafford et al., 1983) and angiotensinogen converting enzyme 2 (Tipnis et al., 2000)). Angiotensin II is thought to be the most vasoactive component of the RAAS (Wolf et al., 1997) and can contribute to renal insufficiency by causing an increase in arterial pressure and inducing glomerular injury and fibrosis in a variety of human kidney diseases (Brenner et al., 2001; Copelovitch et al., 2007). Interestingly, certain antibodies to the APA protein can induce an acute form of proteinuria in wild-type BALB/c mice that eventually resolves (Assmann et al., 1992; Mentzel et al., 1996a). A combination of two antibodies (ASD-37 and ASD-41) can induce a massive and acute albuminuria in BALB/c mice, that starts 6 h after administration and peaks to approximately 60 mg/ml at 8 h and subsides at day 7 (Dijkman et al., 2003; Mentzel et al., 1999). These antibodies either bind to the C-terminal 39 amino acids of the APA peptide (ASD-37), or very close to its active site (at amino acids 448–585 in the case of ASD-41) (Gerlofs-Nijland et al., 2003). Further evidence that the anti-APA antibodies cause albuminuria by affecting APA activity has been provided by studies that have administered them into APA homozygous null mice: these mice do not develop proteinuria (Lin et al., 1998).

The expression pattern of podocin, CD2AP, actin and nephrin in the podocyte of mice changes after injection of ASD-37 and ASD-41 (Dijkman et al., 2003). Both podocin and CD2AP have a more granular staining pattern 6 h after injection that changes to a more normal appearance after 7 days (during which time albumin excretion is low) (Dijkman et al., 2003). Twenty-four hours post-injection, podocyte-specific nephrin staining in the mice is decreased compared to non-treated mice, and actin aggregates into a more granular pattern (Dijkman et al., 2003).

The albuminuria and renal alterations induced by the injection of the ASD-37/41 anti-APA antibodies do not appear to be mediated directly by angiotensin II. This has been demonstrated using a combination of methods (Gerlofs-Nijland et al., 2001; Mentzel et al., 1999). Nevertheless, intrarenal angiotensinogen II levels are greatly elevated in the ASD37/41 induced proteinuria (Gerlofs-Nijland et al., 2001). Experiments showing either that ASD-37 (but not ASD-41) decreases APA activity *in vitro* (Mentzel et al., 1999), and mice injected with the ASD-37/41 antibodies respond to triple-therapy using enalapril (ACE inhibitor), losartan (angiotensin II type 1 receptor antagonist) and a β-blocker illustrates that defective APA enzyme activity per se does not contribute to albuminuria, and that the renal damage may be more related to systemic blood pressure (Mentzel et al., 1999). In addition, angiotensinogen (*AGT*) homozygous-gene knock out mice injected with ASD-37/41 also developed massive albuminuria, thus further demonstrating that albuminuria in the double-injected mice is not dependent on angiotensin II action (Gerlofs-Nijland et al., 2001). These results thus lead to the proposal that structural alteration of the APA peptide on the podocyte cell membrane may also directly cause renal disease.

We were therefore interested in investigating whether genetic mutations in the APA encoding gene (*ENPEP*) can either contribute to or cause the proteinuric podocyte disorder focal and segmental glomerulosclerosis (FSGS) in humans as it is possible that humans that carry alleles that alter the coding region of APA may have an increased susceptibility to FSGS. From the direct sequencing of the entire *ENPEP* coding sequence in 188 patients and 48 controls, we identified five private non-conservative alleles in the cases, and two of these in control samples. We also identified three known single nucleotide polymorphisms (SNPs) and four novel ones. The frequency of private non-conservative alleles did not differ significantly between cases and controls, but we did identify an increased frequency of both W413X(1239G>A) and Q213R(639A>G) single nucleotide polymorphism (SNP) alleles in cases compared to normal, while