Genetic polymorphisms in cardiovascular disease: focus on renin angiotensin system

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ABSTRACT

Cardiovascular Disease is an important cause of morbidity and mortality in Western Communities. The conditions of coronary artery disease, hypertension and heart failure predominate as the main types of cardiovascular disease. The renin angiotensin system (RAS) is one of the main systems responsible for cardiovascular homeostasis and the effect of genetic polymorphisms of components of this system may be important as it may affect the circulating or local concentrations of these components and thus predispose to the development of cardiovascular disease.

In addition, the possibility of these genetic polymorphisms affecting the response to treatment in patients with cardiovascular diseases needs further investigation, particularly in relation to pharmacological agents which target this system such as angiotensin converting enzyme inhibitors and more recently angiotensin II receptor antagonists.

This presentation describes the common RAS gene polymorphisms and discusses their associations with the common cardiovascular conditions as well as describes the rapid PCR based assays used in their detection. Information on the effect of these polymorphisms on treatment is presented. Examples of non RAS gene mutations are also highlighted.

Key words: angiotensin converting enzyme, hypertension, heart failure.

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INTRODUCTION

The Renin Angiotensin System is one of the major systems involved in maintaining cardiovascular homeostasis and as such polymorphisms in genes coding for components of this

system may have an impact on cardiovascular disease. Other systems that may also impact on cardiovascular disease include the fibrinolytic system and lipid metabolism.

THE RENIN ANGIOTENSIN SYSTEM

The renin angiotensin system (RAS) is one of the body's most powerful regulators of arterial pressure and body fluid volumes. Traditionally the RAS has been thought of as an endocrine system with circulating renin originating largely from the kidney where it is synthesised and stored in the juxtaglomerular cells of the kidney. The concept of the existence of tissue renin angiotensin systems proposes a paracrine mode of operation for the RAS with angiotensin II being produced in target organs via local RAS biochemical cascades. To support this RAS components have all been demonstrated at various tissue sites including the heart [1]. Enzymatic pathways however independent of renin and ACE also contribute to the development of angiotensin II in tissues, blood vessels and the heart. Chymase is an efficient angiotensin II forming enzyme and has been found in the human cardiac interstitium, endothelial cells and mast cells.

Renin

Renin was first discovered in 1898 [2]. It is synthesised as a pre-prorenin molecule within renin synthesizing cells. Human renin secreting cells have been identified in the juxtaglomerular apparatus (JGA) of the kidney with the aid of histochemical stains. The human renin gene is located on the short arm of chromosome 1 q32, [3] and contains ten exons (coding sequences) and nine introns (non-coding sequences) and spans 12.5 kilobases (kb) [4]. Various restriction fragment length polymorphisms are known to occur. These include the *Taq* I and *Bgl* I polymorphisms which are located in the 5' region, the *Hind*III in the 3' region and the *Hinf* I in the first intron. No significant associations have been found so far between human essential hypertension and the renin gene alleles [5].

Angiotensinogen

The angiotensinogen gene is located near the renin gene on chromosome 1q 42-3 [6]. It encompasses five exons and four introns and spans approximately 13 kilobases [7]. The first exon is short (37 nucleotides) and corresponds to the 5'untranslated sequence of the mRNA. The second exon codes for 59 percent of the protein and contains the nucleotide sequences coding for the signal peptide and ANG1. Exons 3 and 4 code for 48 and 62 amino acids of the protein and the last exon codes for the C terminal part of the protein and the 3' untranslated sequence of the mRNA. Mutations have been discovered within the 5' end of the gene as well as within exons and intron 3.

The potential role of the AGT gene in human essential hypertension has been investigated in two large series of hypertensive sibships. Evidence was obtained of genetic linkage between the AGT gene and hypertension. In particular a strong linkage was found in patients characterised by a diastolic blood pressure greater than 100mmHg or the prescription of two or more antihypertensive drugs. One common variant has been shown to be linked to the determination of circulating angiotensinogen levels and hypertension. This variant is found within exon 2 and is the result of a single DNA base dimorphism (T or C). This produces a substitution of methionine (ATG) for threonine (ACG) at position 235 of the angiotensinogen amino acid sequence. Possession of alleles specifying threonine at this position is associated with high plasma angiotensinogen concentrations with 10 and 20 percent increases in heterozygotes (MT) and homozygotes (TT) respectively compared with wild type homozygotes. This AGT variant was found more frequently in hypertensive probands in some populations of White European ancestry [8] especially in the more severe index cases than in controls. The association of this variant with hypertension has been confirmed in Caucasians [9] and Japanese hypertensive patients [10] but not in African Caribbeans [11].

Angiotensin Converting Enzyme

ACE is a carboxypeptidase which hydrolyzes di- and tripeptides from the C terminus of peptides. The majority of the human ACE molecule projects into the extracellular space and is bound to the plasma membrane via a C-terminal region which spans across the membrane and is linked to a short cytoplasmic tail. ACE converts angiotensin I (Ang I) to angiotensin II (Ang II) by removal of the C terminal peptide His-Leu. ACE also cleaves the C-terminal dipeptide (Phe-Arg) from bradykinin, thus giving it the ability to activate a pressor agent and inactivate a vasodilator. ACE is homogenously distributed throughout the human heart with only low levels in the valves.

ACE GENE AND ACE I/D POLYMORPHISM

The Nancy study [12] showed a familial resemblance in plasma ACE levels between genetically related adult subjects. This was consistent with a major genetic contribution to the determination of plasma ACE levels. Two ACE alleles were discovered [13] which differed in size due to the insertion of a 287 base pair (bp) DNA sequence in intron 16 of the ACE gene. The larger allele was assigned the symbol I (insertion) and the shorter allele the symbol D (deletion).

Population Variations in ACE I/D genotypes

There are important population differences in the frequency of genetic markers including those of the renin angiotensin system. The ACE DD genotype shows a wide variation in frequency between populations. It is found in only 2% of Samoans but up to 42% in Caucasians [14]. Ishigami *et al.* [15] have reported significant differences in genotype frequences between Japanese population and Caucasians. DD genotype frequency in their normal control population was 18%. In Lee's [16] normal subjects, the D allele frequency was 0.3. The D allele frequency is higher in Black Nigerians [17] and the Afro Caribbean population compared to Europeans [18].

Effect of ACE gene I/D polymorphism on ACE levels

The effect of ACE genotype on tissue and plasma ACE levels is co-dominant with mean plasma ACE levels in DD subjects approximately twice those of II caucasian subjects. The ID genotype individuals have intermediate ACE levels [13,19]. The I allele is a marker for the major gene effect on serum ACE which is due to an as yet unidentified variant of the ACE gene. There is tight linkage of this ACE polymorphic marker site to a locus that regulates gene expression (site of major gene effect). This major gene has co-dominant effects and accounts for 44% of the total variability of ACE levels while the marker I/D polymorphism accounts for 28% of the variation [20].

Population variations in the effect of the ACE I/D genotype on serum ACE levels

Genetic studies in African-Caribbean families from Jamaica have shown that the ACE I/D polymorphism is in moderate linkage disequilibrium with an ACE linked quantitative trait locus. In this population two quantitative trait loci jointly influence serum ACE levels with one locus located close to or within the ACE locus and accounting for 27% of the total variability [21]. In a black healthy population no variation in serum ACE activity was found with ACE genotype [22]. The effect of ACE genotype on serum ACE levels also applies in the Caucasian hypertensive population [23]. No variation has been found in plasma angiotensin II levels and aldosterone with ACE genotype [24].

ACE gene I/D polymorphism and myocardial infarction

The deletion polymorphism in the ACE gene (DD genotype) was reported to show an increased frequency in patients with myocardial infarction who were recruited between three and nine months after the event in a retrospective multicenter, case-control study [25]. The overall increase in risk was modest with an odds ratio of 1.34 for patients with the DD genotype (an increase in risk of MI with the DD genotype of 34%). This varied between 1.1 and 2.1 in the four populations studied. A sub-group analysis in patients who had sustained an MI despite being at low risk on established criteria (low body mass index and low apolipoprotein B) showed that the DD genotype assumed much greater importance with an odds ratio of 3.2. The DD genotype thus seemed to act independently and in a non-additive fashion to other known risk factors. At least 15 other studies have been published. They support or question this association. Samani et al. [26], performed a meta-analysis of the available studies and found support for the proposition that the D allele confers an increased risk of MI with a pooled estimate odds ratio of 1.26 (increased risk of 26%). The studies however were conducted in different ethnic groups and as recruitment was at variable intervals post MI, there is the possibility of selection by mortality affecting the results. The mechanism by which the D allele influences the risk of MI is unclear with published studies reporting different effects on coronary atheroma [27, 28]. Ludwig et al. [28] suggests that the polymorphism confer significant risk of infarction by influencing the conversion to MI with no apparent effect on the development of atherosclerotic coronary artery disease.

Effect of ACE I/D on Left Ventricular dilatation after Myocardial Infarction

There is evidence that the ACE genotype may affect ventricular remodelling presumably through its effects on circulating and tissue renin angiotensin systems. Ohmichi *et al.* [29] assessed 79 post myocardial infarction patients (4-7 months post MI) and found that patients with the ACE DD genotype had lower left ventricular ejection fractions and greater end systolic volume indices as determined by left ventriculography compared to II patients. Schunkert *et al.* [30], in a study of 96 patients, found DD patients to have significant LV dilatation at 1 year post MI compared to ID and II patients using echocardiographic assessment. Raynolds *et al.* [31] showed a higher frequency of the DD genotype in-patients with ischaemic cardiomyopathy. Sakuramoto *et al.* [32], showed that patients with idiopathic dilated cardiomyopathy and the DD genotype had a worse clinical response to ACE inhibitor therapy. These initial reports suggest a role for the ACE polymorphism in ischaemic heart failure and LV dilatation after myocardial infarction.

ACE gene I/D polymorphism and hypertension

The ACE gene has been shown not to be associated with a genetic predisposition to high blood pressure in a Caucasian population [24, 33, 34]. Other studies have shown associations with the I allele [35] and the D allele [36]. No significant differences have been found between the I/D allele frequency in the Japanese population with and without essential hypertension [37] or in the Chinese population [38]. In the African-American population however, Duru *et al.* [39] found a significant difference in genotype frequency with a higher prevalence of the D allele in patients with hypertension compared with normotensives. This finding was confirmed in this ethnic group by Rutledge *et al.* [40]. Also, in the Afric Caribbean population Barley *et al.* [41] found a positive association between the frequency of the D allele and increasing blood pressure.

ACE gene I/D polymorphism and Left Ventricular Mass and function

The DD genotype is known to be over represented in diseases characterised by left ventricular hypertrophy and dysfunction. Kupari *et al.* [42] have shown that in the absence of cardiac disease the ACE polymorphism has no effect on LV mass to body height ratio or indices of LV systolic or diastolic function detectable by echocardiography. The results of studies in which the study population is unselected are inconclusive. A large study on subjects from the Framingham Heart Study, Lindpaintner *et al.* [43] did not find an association between the D allele and LV mass using echocardiography while in the Japanese population, Iwai *et al.* [44] found an association between the D allele and LV mass.

ACE gene I/D polymorphism in Hypertensive Left Ventricular Hypertrophy

There is evidence that the ACE genotype is an independent predictor of the development of LVH in normotensive men [30] and hypertensives [44]. It has been suggested that the effect of blood pressure on left ventricular mass index (LVMI) in essential hypertension is expressed only in the presence of the ACE gene deletion allele [45]. Conversely, it can be hypothesised that for an equal reduction of blood pressure, DD patients may have less reduction in LV mass index. It is tempting to speculate that the cause of the association of the DD genotype with LVH is that subjects with the DD genotype have increased cardiac ACE activity and angiotensin II concentrations locally.

ACE I/D Polymorphism and Antihypertensive Treatment

In a normal healthy population enalapril 10 mg reduced serum ACE activity in DD genotype subjects more than II subjects at 2, 4, and 6 hours post dose but the fall in ACE activity was greater in II subjects at 24 hours [46]. The expression of the AT_1 receptor in mononuclear cells of healthy subjects and patients with hypertension has been shown to vary with ACE genotype. The levels of expression decreased significantly with ACE inhibitor treatment in subjects with the DD genotype [47]. No association between the ACE I/D polymorphism and BP reduction has been documented in essential hypertensives treated with lisinopril 10 mg daily [48].

ANGIOTENSIN II RECEPTORS AND THE AT1 RECEPTOR POLYMORPHISM

Two subtypes (AT₁ and AT₂) of angiotensin binding receptors are known to exist in

humans. The AT1 subtype mediates the physiological actions of Ang II such as vasoconstriction and salt and water retention. Two structurally distinct AT_1 receptor isoforms however have been demonstrated in animal species (AT_{1a} and AT_{1b} . The AT_2 receptor is highly expressed in animal embryonic tissues such as the brain and kidney but is expressed at low levels in normal adult tissues [49]. There is evidence that the AT_2 receptor mediates an antigrowth effect and apoptosis and hence may exhibit functional antagonism to the AT_1 receptor. Both the AT receptors belong to the group of seven transmembrane receptors. The gene for the human angiotensin type 1 (AT_1) receptor is located on chromosome 3 while the X chromosome contains the gene for the angiotensin type 2 (AT_2) receptor. The angiotensin II type 1 receptor gene is polymorphic with the (A1166C) polymorphism located at the 5' end of the 3' un-translated region of the gene. This is the most relevant variant of this receptor's polymorphism to cardiovascular disease. It has been found to be associated with aortic stiffness in hypertensive but not normotensive subjects and it has been suggested that this may be due to a potentiation of angiotensin II effects in the presence of hypertension [50].

EXAMPLE OF RAPID METHODS FOR DETERMINING GENOTYPES

ACE

It is possible to genotype for the ACE gene polymorphism using molecular biological methods such as the polymerase chain reaction (PCR) based assay which was first described by Rigat *et al.* [51] and is an established rapid technique. This method requires the use of genomic DNA usually from blood samples. PCR is an *in vitro* method for selectively replicating a discreet DNA fragment using an enzyme. Two oligonucleotide primers (usually 18-25bp) which have complimentary sequences to the sense and antisense strands in the target DNA are used to define the fragment to be amplified. Amplification takes place via a repetitive series of thermocycles. The thermocycles correspond to the process of template denaturation (90-95°C), annealing of primers to the separated DNA strands (40-65°C), and extension of the annealed primers by a heat stable enzyme *Taq* DNA polymerase. The newly synthesised products can act as a template in the following cycles thus resulting in exponential increase in the desired product. The PCR based assay uses two primers which flank the insertion sequence and thus allows discrimination among three genotypes: II, ID, DD. Occasionally, amplification of the I allele is suppressed leading to mistyping of an ID heterozygote as DD [52].

Angiotensinogen M235T polymorphism

The T235 allele of the angiotensinogen gene can be detected by a modification of the method of Russ *et al.* [53]. PCR products are digested with 1U of the restriction enzyme Tth111 I at 65^oC for 6 hours.

AT1 Receptor Polymorphism

This can also be performed using a PCR based assay and by using a restriction enzyme to digest the PCR product. Genotyping for the A1166C AT₁R gene variant can thus be performed by using the sense primer: 5'ATAATGTAAGCTCATCCACC-3' and the antisense primer: 5'GAGATTGCATTTCTGTCAGT-3'.

The PCR reaction is cycled and the PCR product can be digested overnight with 5U of the

restriction enzyme DdeI at 37⁰C.

PCR products or digested products are electrophoresed on 2.0% agarose gels and visualised via ethidium bromide mediated fluorescence using ultraviolet transillumination.

NON RAS POLYMORPHISMS

Lipoprotein lipase

Genetic variation helps to determine in part the levels of lipids in the blood. Lipoprotein lipase is a cofactor involved in the hydrolysis of triglycerides in chylomicrons and very low density lipoproteins. Over 50 deletions and point mutations have been reported in the gene for LPL. Two common mutations cause a substitution of aspartic acid at position 9 to asparagine and asparagine at position 291 to serine. These common mutations tend to cause an elevation in plasma triglyceride levels and or low high density lipoprotein levels. This effect is particularly associated with body mass index.

Fibrinogen genes

The three fibrinogen genes are together in approximately 50kb on the long arm of chromosome 4. Each chain is manufactured as a separate RNA with the levels of all three being coordinated. The promoter region for the beta fibrinogen gene is of the order of 150base pairs and sequence changes in this region of the gene may have a direct effect on the rate of transcription and plasma fibrinogen levels. One such change is a G/A variation at position -455 with the A allele in healthy individuals showing higher fibrinogen levels [54].

Homocysteine

Evidence is accumulating that an increased blood level of homocysteine is a risk factor for premature atherosclerosis and an independent risk factor for coronary artery disease. 5, 10 methylenetetrahydrofolate reductase (MTHFR) is a key enzyme catalyzing homocysteine. Recently an alanine/valine polymorphism of the MTHFR gene has been reported with the VV genotype correlating with increased plasma levels of homocysteine. Pharmacological doses of folate can reduce plasma homocysteine levels.

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<u>Discussion</u>: Genetic polymorphisms in cardiovascular disease: focus on renin angiotensin system

M.M. Reidenberg:

You mentioned that the ACE/ID polymorphism was in an intron. Is there any evidence that this is actually pathophysiologically important, or is it still possible that this could be a marker for something close to it on the chromosome?

G.K. Davis:

The current thinking is that it is possibly a marker for something close to it on the chromosome. It has not yet been identified exactly where the locus affecting ACE levels is located.

A. Breckenridge:

Does the level of ACE, whether or not determined by the polymorphism you have described, affect the expression of AII receptors? If so, this would be a very convenient homeostatic mechanism for the body and this might account for the diversity of the results of the studies that you have described. You said quite clearly that there is not a very strong or clear clinical correlate for the impressive genetic work you have described. Possibly, there is this kind of feed-back loop with respect to expression of AII receptor.

G.K. Davis:

I think there is some specific work in terms of either ACE and AT1 receptors, that we may have to look at. The expression of the AT1 receptor in mononuclear cells has been shown to vary with ACE genotype (Dieguez-Lucena *et al.*, Hypertension, 1996).

M. Pirmohamed:

Firstly, since tissue levels of ACE may be much more important pathophysiologically than serum levels of ACE, how closely do serum levels relate to tissue levels of ACE? And secondly, there was initially evidence that the ACE gene polymorphism could be associated with myocardial infarction, particularly in low-risk patients, although it was refuted through a prospective study.

G.K. Davis:

There is a relationship between the ACE I/D polymorphism and tissue levels of ACE for normal hearts, (Jan Danser *et al.*, Circulation, 1995). The overall result in the meta-analysis done by (Samani *et al.*, Circulation, 1996) was a positive effect of the D allele on the risk of MI. The large negative prospective study (by Lindpainter *et al.*, N Engl J Med, 1995) could be due to differences in genetic backgrounds.

P. du Souich:

A study about the effect of growth hormone on dilated cardiomyopathy was published two years ago (Fazio *et al.*, N Engl J Med, 1996) but it has not been reproduced. How could the polymorphism you have described affect the effect of growth hormone?

G.K. Davis:

I cannot comment specifically on how ACE may interact with growth hormone. A point of interest I probably should mention is the fact that angiotensin II type 2 receptors exist in the heart as well as in other tissues. Whether or not the angiotensin II type 2 receptor is thought to have some form of anti-proliferative effect itself is another feature of the inter-relationship which has yet to be clarified.

A.J.J. Wood:

What should be our standard for reporting positive associations? Should we demand a pathophysiological hypothesis first, or should we view these as data-dredging exercises?

G.K. Davis:

I think the way cardiovascular medicine has developed is that we did the studies and found that some of the treatments were effective, for example, ACE inhibitors in heart failure and hypertension, although we did not actually have pathophysiological explanations at the time. We used the treatment based on the results we had at the time. Now, there is a lot of work on amongst different genes, and there is quite a list of different genes that may be related to coronary artery disease or hypertension. The information is forthcoming, but we should also look for some form of unifying mechanism, some pathophysiological reason to explain any effect.

A.J.J. Wood:

If you were a journal editor, for example, would you be more reluctant to accept an article that did not have a therapeutic basis to it? If you did not have a positive ACE inhibitor trial in post-myocardial infarction, should you not be less willing to accept a gene association?

G.K. Davis:

I would not say I would be less willing to accept a gene association, but I think that a positive trial result would give an association a lot more strength, at least in terms of the pathophysiology of the condition.

D.C. Brater:

There is an experiment of nature with high ACE levels in sarcoidosis where there is no increase in cardiovascular disease or hypertension. This seems to contradict hypotheses about the importance of these phenotypes?

G.K. Davis:

The fact is that the experiments done so far have shown that aside from hypertension, heart failure and hypertrophic cardiomyopathy, high ACE levels also seem to be a poor prognostic marker for quite a lot of independent cardiovascular conditions. To some extent, the question at the moment is whether or not it is all related to angiotensin-II, the main effector hormone and whether this is related to increased ACE levels. I think angiotensin-II is the effector hormone, and there is a lot of independent information as regards different cardiovascular conditions supporting this. Although I can not recall any increased cardiovascular events in patients with sarcoidosis, there is strong data with regard to a lot of the other conditions and increased ACE levels.

I.P. Hall:

Two issues should be considered. There is a high rate of polymorphic variation in the human genome, and therefore it is critical we do not do endless association studies. Therefore you need to have some functional evidence to indicate something is relevant. With respect to the ID polymorphism, I think it is problematic that it is intronic. One could argue that there is something else which is controlling ACE levels. This brings me to the second point: a lot of association studies have problems with drifting phenotypes. The difficulty is that, if you go after enough end-points, you will see associations, and looking at the ID story, some studies detect association with left ventricular change, some with risk of myocardial infarction and some have reported changes in blood pressure in some populations, but these are not the same pathophysiological processes. The danger is that one will assume that this is a meaningful polymorphism, because the body of evidence related to all the associations even if, on careful examination, the associations do not seem so valid.

E.M. Sellers:

In other fields, the explanation for varying reported results very often turns out to be inadequate control of ethno-racial origin. To what extent, can we be sure with these reports that in fact there is clear evidence that the ethno-racial groups have been homogeneous?

G.K. Davis:

From some of the study data actually presented, we can see that there is ethnic variation in the frequency of polymorphism. Therefore the interpretation has to be within the groups that are studied. There is certainly no firm explanation as to the actual pathophysiology, and studies have been carried out in different ethnic groups. We do have that sort of scepticism about intronic polymorphisms, and that has certainly been the feeling throughout the analysis of the data and accounts for the number of studies that have been performed looking at it in different ways.

M.M. Reidenberg:

I suspect this is an area where the risk of publication bias is very high, in that the positive studies get reported, and not the negative ones. At this stage, if there were some way to develop a registry of studies, such as has been attempted for clinical trials, it would be very fruitful in keeping people from making erroneous conclusions about the one positive study out of a series that looked at the same thing.

G.K. Davis:

Another area that we are studying is whether these polymorphisms themselves have any clinical relevance in terms of patient management and predicting patient response.