Arch. Biol. Sci., Belgrade, 64 (2), 401-407, 2012 DOI:10.2298/ABS1202401M

EVALUATION OF THE ASSOCIATION BETWEEN $AT_1R1166C$ POLYMORPHISM AND THE INCIDENCE OF CAD AND CAC SCORE IN THE IRANIAN POPULATION

AMIR HOOSHANG MOHAMMADPOUR[1,2], SAEED NAZEMI[3], MARYAM FOROUGHI[1], MARYAM[1] GHORBANI[1], JAMAL SHAMSARA[4] and JAVAD BEHRAVAN[4*]

[1] *Department of Pharmacodynamy and Toxicology, School of Pharmacy, Mashhad University of Medical Science,* Mashhad, Iran
[2] *Pharmaceutical Research Center, Mashhad University of Medical Sciences,* Mashhad, Iran
[3] *Department of Cardiovascular Diseases, Razavi Hospital,* Mashhad, Iran
[4] *Department of Biotechnology, School of Pharmacy, Mashhad University of Medical Science,* Mashhad, Iran

Abstract - Most of the physiological effects of Ag II are mediated by the angiotensin II type 1 receptor. Polymorphisms of the AT_1R gene can affect the function of this receptor and subsequent atherogenic activity. In this study we investigated the correlation between AT_1R A1166C polymorphism and coronary artery calcification (CAC), a marker of the coronary artery burden. Fifty CAD patients and fifty healthy individuals fulfilled the inclusion and exclusion criteria entered this study. CAC was determined in the left main coronary artery (LMCA), left coronary artery (LCA), right coronary artery (RCA) and CX by CT-angiography and a blood sample was taken at this time. DNA extracted from whole blood leukocytes was analyzed by the polymerase chain reaction – restriction fragment-length polymorphism (PCR-RFLP) assay. There were no significant differences in genotype and allele frequencies between the CAD and control groups. The mean calcium score was compared in genotypes and alleles and no significant difference was seen. In addition, the frequency of genotypes and alleles was not significantly different in the calcium score groups (low<100, medium= 100-400, high >400). An analysis was performed separately in males and females and no significant correlation was found. According to our results, no association was found between $AT_1R1166C$ polymorphism and the incidence of CAD and CAC score in our study population.

Key words: Coronary artery calcification (CAC), coronary artery disease (CAD), Angiotensin II type 1 receptor (AT_1R)

INTRODUCTION

Coronary artery disease (CAD) continues to be the main cause of death and a major cause of morbidity and decline in quality of life. Atherosclerosis, the main cause of coronary artery disease (CAD), is influenced by a complex interaction among various environmental and genetic factors (Peyser, 1997). Familial incidence of CAD has been known for almost 100 years, and shows the genetic basis of many recognized CAD risk factors (Lusis, 2000). Even after managing CAD risk factors, there is a great risk of CAD associated with a family history of the disease. This suggests that numerous genetic factors underlying disease susceptibility are yet to be identified (Devereux and Alderman, 1993). One of the markers of coronary artery atherosclerosis is coronary artery calcification (CAC). Coronary calcium is a degenerative process with active rule in atherosclerotic plaque development (Rumberger et al. 1999). The degree of calcification in atherosclerotic plaques in coronary arteries, after

management of the well-known CAD risk factors, is an indicator of plaque development and the calcium score of coronary arteries is related to atherosclerosis improvement and thus to CAD severity (Guerci et al., 1998; Bielak et al., 2000; Keelan et al., 2001). The renin-angiotensin system (RAS), which regulates blood pressure, has an important role in the pathogenesis of the different stages of atherosclerosis and accelerates the disease process (MacGregor et al., 1981; Cusi, 1997). Angiotensin II, its receptors and the angiotensin-converting enzyme (ACE), are the main components of the renin-angiotensin system. Angiotensin II is an effective regulator of cardiovascular homeostasis and acts through two different G-protein-coupled receptors. Most of the known effects of angiotensin II are mediated through the angiotensin II type 1 receptor (AT1R), which mediates the cardiovascular procedures of angiotensin II and it has been studied the most extensively (Takayanagi et al., 1992; Herzig et al., 1997). It is expressed in vascular smooth muscle cells and also in the myocardium, thus a possible relationship between the AT1R gene and myocardial infarction has been investigated (Berk and Corson, 1997). There are controversial reports regarding the role of the AT1 receptor gene A/C polymorphism as a risk factor for MI (Tiret et al., 1994; Ulgen et al., 2006). Also, ACE gene polymorphism may be associated with severe aortic valve calcification (AVC) and the risk of coronary artery disease, and development of the stenosis of coronary artery. The AT1 receptor CC genotype is also associated with coronary disease, hypertension, and ischemic heart disease in Caucasian populations (Gardemann et al., 1998; Jones et al., 2003; Pullareddy et al., 2009). The AT1R gene exhibits polymorphism, and an A/C transverse at the 3′-untranslated region has been related to essential hypertension (Bonnardeaux et al., 1994). The most widely studied polymorphism in the AT1R gene is the A1166C variant.

In different studies the correlation between AT1R gene polymorphism and CAD and atherosclerosis, which are linked to CAC, has been investigated, however, the probable relationship with coronary artery calcification has not been studied yet. We have examined this relationship in the present study.

PATIENTS AND METHODS

Patients

This study was carried out in the cardiovascular and pharmaceutical research center of the Mashhad University of Medical Science in Iran from November 2008 to September 2009. 50 CAD patients and 50 healthy volunteers entered the study. All of the patients fulfilled the following inclusion criteria: people aged 30-70 years (average age 43 years, range 26-50). The exclusion criteria were: patients using ACE inhibitors and ARBs, and patients with calcium and phosphor metabolism disorders, primary and secondary hyperparathyroidism, chronic renal failure, bone disorders and malignancies.

Blood sampling and procedure

CT angiography was performed for all patients and CAC was determined in the left main coronary artery (LMCA), left coronary artery (LCA), right coronary artery, (RCA) and CX. A peripheral venous blood sample was collected from each patient for biochemical measurement and the extraction of genomic DNA. Biochemical tests, including plasma lipid profiles, the presence of previous disease and administered drugs, FBS, ESR, PTH, hsCRP, Ca, phosphorus and Cr were determined. DNA was extracted from whole blood leukocytes by a salting-out method and samples were analyzed by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) assay.

Genetic analysis

Peripheral leukocytes, taken after centrifugation of 2 mL blood, were used to isolate genomic DNA using the proper nonenzymatic extraction kit (BioGene Ltd, UK). DNA concentrations and purification percentages were determined by gel electrophoresis and spectrophotometry.

The determination of the A/C-polymorphism at the 3′-untranslated region of the AT1R gene was based on the triple-primer polymerase chain reaction (PCR) method that used a primer including a mismatch, introducing a site for the Ded I restriction enzyme. A 540 bp fragment was amplified under the following conditions: 200 ng/ μL target DNA in a final volume of 20 μL containing 15pM (1μL) of each primer, 1.5 mM MgCl2 (1.2μL), 0.2 mM (0.4 μL) of each dNTP, and 1-2 U/mlTaq polymerase, 1X PCR buffer 10X (2 μL) and distilled water to 20 μL. The amplification profile included an initial denaturation at 95°C for 5 min and 30 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds with a final extension time of 5 min. The primers were as follows: 5′-GCACCATGTGAGGTTG-3′ (named AT1RM) and 5′-CGACTACTGCTTAGCATA-3′ (named AT1RH). The PCR yields of the expected size (540bp) were evaluated on 0.8% agarose gels. To illustrate the polymorphism, PCR products were processed overnight (24 h) with the restriction endonuclease Dde I at 37°C, which cuts the product into two pieces, 430 bp and 110 bp long. An additional Dde I detection site was created in the C-type variant at nucleotide 1166. Thus, the homozygote CC created two bands (430 and 110 bp long), the homozygote AA produced one band (540 bp long), and the heterozygote AC produced all three bands (540,110 and 430 bp long). Digested products were detected on 3% agarose gel and by staining with ethidium bromide.

Statistical analysis

Statistical analysis was performed with SPSS 11.5 software. Differences in allele frequencies and genotype distribution between cases and controls and in the different groups according to their calcium scores were analyzed by chi-squared statistics. Comparison of the mean calcium scores in different genotypes and alleles in the CAD patients and control was performed by one-way ANOVA and Tukey post test. The results were recorded as mean ± SD, a p-value of 0.05 or less was considered as significant.

RESULTS

Population study

The main characteristics of the study population include demographic characteristics, biochemical parameters, CAC and population AT1R phenotype and genotype frequencies and are shown in Table 1.

CAC comparison between CAD patients and control

The study population (control and patients) was divided into 3 groups according to the calcium score (group 1 = <100; group 2 = 100-400, grades 2-4; group 3 = >400). The calcium score was significantly higher in the CAD patients than in the control ($p<0.001$) (Fig. 1).

Comparison of genotype and allele frequencies between patients and control

In the study population there was no difference in genotype and allele frequencies between the patients and controls with different calcium scores ($p>0.05$) (Fig. 2).

Comparison of genotype and allele frequencies in the study population

There was no significant difference in these frequencies in the whole study population, patients and controls with different calcium scores ($p>0.05$) (Figs. 3, 4, 5). This was investigated in men and women separately, and no correlation was found (data not shown).

DISCUSSION

In this study, the correlation between A1166 and C AT1R gene polymorphism with coronary artery calcification score and coronary artery diseases (CAD) in patients and healthy individuals was investigated. Genotype frequencies AA, AC and CC were similar to other studies (70%, 24% and 6%, respectively) and the CC genotype frequency was the lowest. There

Table 1. Study population characteristics

	patients	Control
number	50	50
Women percentage	26.1	36
age	56.52+_11.04	54.3+_9.1
BMI	28.21+_4.69	26.87+_4.23
Laboratory findings		
Total cholesterol(mg/dl)	170.13+_42.22	171.72+_40.62
HDL(mg/dl)	97.87+_35.39	95.86+_29.75
LDL(mg/dl)	43.95+_14.10	47.07+_10.44
triglyceride(mg/dl)	148.43+_57.86	144.65+_81.43
glucose(mg/dl)	103+_29.99	144.44+_41.66
CAC	356.256+_441.29	51.84+_51.54
With hypertension	60.9%	56%
Dislipidemic patients	78.3%	46%
Diabetic patients	17.4%	28%
Smoking people	28.3%	34%
Familiar history	45%	8%
Genetic findings		
Genotype AA	69.6%	70%
Genotype AC	26.1%	22%
Genotype CC	4.3%	8%
Allele A	82.6%	81%
Allele C	17.4%	19%

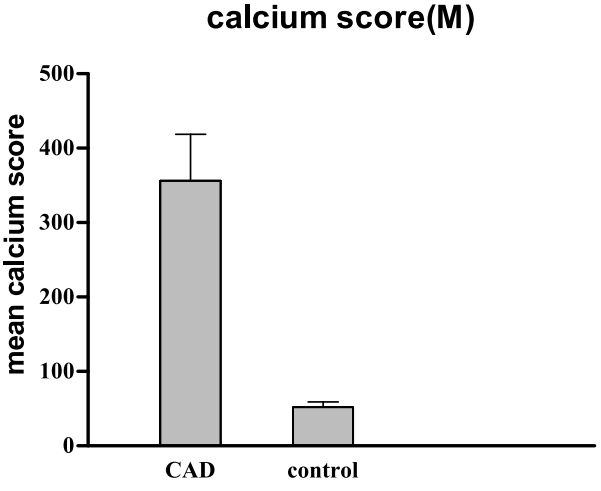

Fig. 1. Mean calcification score in patients (CAD) and control.

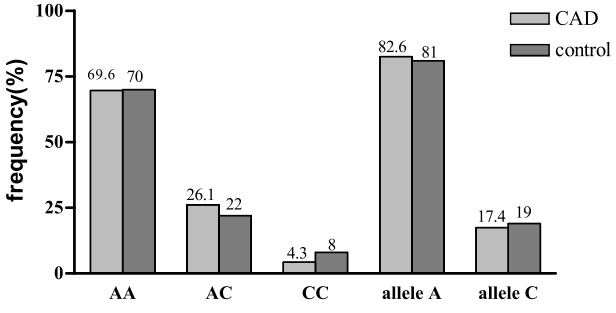

Fig. 2. Genotype and allele frequencies among patients (CAD) and control.

were no significant difference in genotype and allele frequencies in the patients and control group or in the different groups with different calcium scores. In other words, we did not find any significant correlation between the distribution of these genotypes and alleles and CAC and the incidence of CAD.

It is evident that both environmental risk factors and genetic factors could be the causes of CAD. Also, cardiovascular risk factors such as diabetes, dislipidemia, hypertension and obesity could have genetic

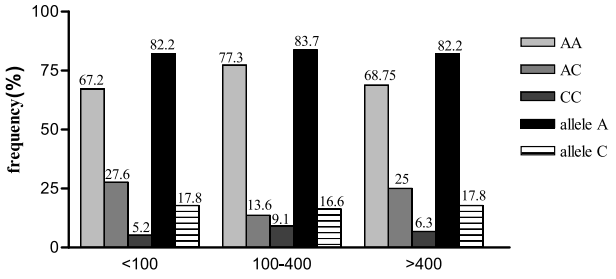

Fig. 3. Frequency of different genotypes and alleles in whole study group with different calcium scores.

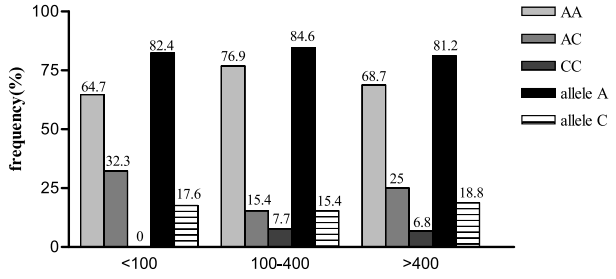

Fig. 4. Frequency of different genotypes and alleles in CAD group (patients) with different calcium scores.

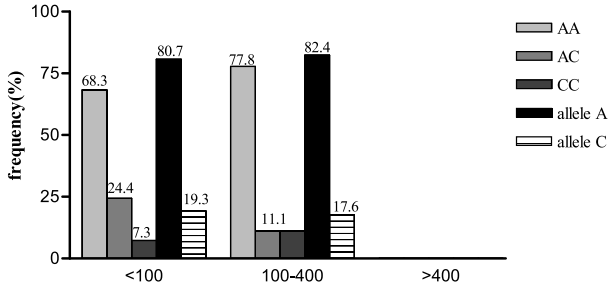

Fig. 5. Frequency of different genotypes and alleles in control group with different calcium scores.

and environmental causes. One of genetic causes is common polymorphisms at several genes in renin-angiotensin system (angiotensinogen, angiotensin converting enzyme and angiotensin receptor genes). For example, in several families the AT1R has been related to essential hypertension or diabetes, and its polymorphisms have been linked to an increased risk of hypertension and diabetes (Bonnardeaux et al., 1994).

It is well established that the renin-angiotensin system (mainly Angiotensin II) has a key role in the development of atherosclerosis processing. The pro-inflammatory effect of the renin-angiotensin system and following inflammatory mediators' synthesis causes an interaction between leucocytes and endothelial cells, which is an important stage in the pathogenesis of atherosclerosis (Hirata et al., 2011), and Angiotensin II is able to directly affect the endothelium. These are important factors in plaque instability and natural fibrinolytic pathway destruction. Recently, an essential role has been established for the rennin-angiotensin system in CAC promotion. Angiotensin II activates the expression of genes related to calcification such as parathyroid hormone receptors and the hepatorenal-osteocyte alkaline phosphatase gene in heart smooth muscles (Armstrong et al., 2011). Angiotensin II acts through its receptors, mainly AT1R, which changes in its expression and function and leads to changes in the RAS and atherogenic activation. Due to these mechanisms, a correlation between atherosclerosis and CAC with AT1R polymorphism is reasonable.

Several related studies in different populations have reported controversial results. Kretowski showed that in diabetic patients there was no significant correlation between AT1R and ACE gene polymorphism and CAC. In addition, it has been shown that there is no correlation between AT1R and ACE and AGT gene polymorphism with clinical signs in patients with or without cardiac arrest history (Jeunemaitre et al., 1997), which is in agreement with Joseph (Joseph et al., 1998) who demonstrated no significant differences in AT1R genotypes in patients and healthy Indians. Evidence suggests that a genetic constituent affects the frequency and the degree of arterial calcification in humans (O'Donnell et al., 2002; Peyser et al., 2002). Many studies have found an association between insertion/deletion polymorphism of the *ACE* gene in atherosclerosis and coronary artery calcification (Pfohl et al., 1998; Agerholm-Larsen et al., 2000; Doherty et al., 2004). The study of RAS gene interaction in CAD patients showed that ACE and AT1R gene interaction could be effective in both CAD incidence and development (Ye et al., 2003).

Results from a study on Turkish patients suggest that ACE gene polymorphism may be associated with severe aortic valve calcification (Ertas et al., 2007).

Different alleles in other sites that are inherited simultaneously, and also heterogeneity and epistasis (a gene's effects hidden by another gene) could be responsible for this controversy (Carlborg and Haley, 2004). A1166C gene polymorphism occurs in the 3'-untranslated region, without alteration in receptor amino acid sequences. Functional modifications could be a result of changes in mRNA stability, processing, receptor activation, binding and in expression regulation. Thus, this polymorphism could be affected by other genes that control these pathways. According to an investigation into renin-angiotensin system polymorphisms (ACE, AGT and AT1R), in separated analyses none of them had correlation with CAD or cardiac arrest in people with or without risk factors (Tsai et al. 2006). CAD has a multifactor genetic and environmental basis and factors such as race, sex, physiologic disturbances like salt and other SNPs imbalances, could alter the relation between genotype and phenotype existence.

To our knowledge, there has been no other similar study to the presented work, and this is the first time that the correlation between AT1R gene polymorphism and CAD has been investigated. According to our results, there is no correlation between AT1R gene polymorphism and CAD in Iranian patients.

Conflict of interest: We have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinion stated.

REFERENCES

Agerholm-Larsen, B., Nordestgaard, B. G., and *A. Tybjaerg-Hansen* (2000). ACE gene polymorphism in cardiovascular disease: meta-analyses of small and large studies in whites. *Arterioscler Thromb Vasc Biol* **20** (2), 484-492.

Armstrong, Z. B., Boughner, D. R., Drangova, M., and *K.A. Rogers* (2011). Angiotensin II type 1 receptor blocker inhibits arterial calcification in a pre-clinical model. *Cardiovasc Res* **90** (1), 165-170.

Berk, B. C. and *M. A. Corson* (1997). Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. *Circ Res* **80** (5), 607-616.

Bielak, L. F., Rumberger, J. A. Sheedy II, P. F., Schwartz, R. S. and *P. A. Peyser* (2000). Probabilistic model for prediction of angiographically defined obstructive coronary artery disease using electron beam computed tomography calcium score strata. *Circulation* **102** (4), 380-385.

Bonnardeaux, A., Davies, E., Jeunemaitre, X., Féry, I., Charru, A., Clauser, E., Tiret, L., Cambien, F., Corvol, P. and *F. Soubrier* (1994). Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension* **24** (1), 63-69.

Carlborg, O. and *C. S. Haley* (2004). Epistasis: too often neglected in complex trait studies? *Nat Rev Genet* **5** (8), 618-625.

Cusi, D. (1997). Genetic renal mechanisms of hypertension. *Curr Opin Nephrol Hypertens* **6** (2), 192-198.

Devereux, R. B. and *M. H. Alderman* (1993). Role of preclinical cardiovascular disease in the evolution from risk factor exposure to development of morbid events. *Circulation* **88** (4), 1444-1455.

Doherty, T. M., Fitzpatrick, L. A., Shaheen, A., Rajavashisth, T.B, and *R.C. Detrano* (2004). Genetic determinants of arterial calcification associated with atherosclerosis. *Mayo Clin Proc* **79** (2), 197-210.

Ertas, F. S., Hasan, T., Ozdol, C., Gulec, S., Atmaca, Y., Tulunay, C., Karabulut, H., Kocum, H.T., Dincer, I., Kose, K.S. and *C. Erol* (2007). Relationship between angiotensin-converting enzyme gene polymorphism and severity of aortic valve calcification. *Mayo Clin Proc* **82** (8), 944-950.

Gardemann, A., Fink, M., Stricker, J., Nguyen, Q.D., Humme, J., Katz, N., Tillmanns H., Hehrlein, F.W., Rau, M. and *W. Haberbosch* (1998). ACE I/D gene polymorphism: presence of the ACE D allele increases the risk of coronary artery disease in younger individuals. *Atherosclerosis* **139** (1), 153-159.

Guerci, A. D., and *Y. Arad* (1998). Predictive value of EBCT scanning. *Circulation* **97** (25), 2583-2584.

Herzig, T. C., Jobe, S. M., Aoki, H., Molkentin, J.D., Cowley Jr, A.W., Izumo, S. and *B.E. Markham* (1997). Angiotensin II type1a receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. Proceedings of the National Academy of Sciences of the United States of America **94** (14), 7543-7548.

Hirata, Y., Fukuda, D. and *M. Sata* (2011). Critical role of renin-angiotensin system in the pathogenesis of atherosclerosis. *Nippon Rinsh*o **69** (1), 55-59.

Jeunemaitre, X., Ledru, F., Battaglia, S., Guillanneuf, M.T., Courbon, D., Dumont, C., Darmon, O., Guize, L., Guermonprez,

J.L., Diebold, B. and *P. Ducimetière,* (1997). Genetic polymorphisms of the renin-angiotensin system and angiographic extent and severity of coronary artery disease: the CORGENE study. *Hum Genet* **99** (1), 66-73.

J*ones, A., Dhamrait, S. S., Payne, J.R., Hawe, E., Li, P., Toor, I.S., Luong, L., Wooton, P.T.E., Miller, G.J., Humphries, S.E.* and *H.E. Montogomery* (2003). Genetic Variants of Angiotensin II Receptors and Cardiovascular Risk in Hypertension. *Hypertension* **42** (4), 500-506.

Joseph, A., Nair, K. G. and *T.F. Ashavaid* (1998). Angiotensin converting enzyme gene polymorphism in coronary artery disease: the Indian scenario. *Clin Chem Lab Med* **36** (8), 621-624.

Keelan, P. C., Bielak, L. F., Ashaid, K., Jamjoum, L.S., Denktas, A.E., Rumberger, J.A., Sheedy, P.F., Peyser, P.A. and *R.S. Schwartz* (2001). Long-term prognostic value of coronary calcification detected by electron-beam computed tomography in patients undergoing coronary angiography. *Circulation* **104** (4), 412-417.

Lusis, A. J. (2000). Atherosclerosis. *Nature* **407** (6801), 233-241.

MacGregor, G. A., Markandu, N.D., Roulston, J.E., Jones, J.C. and *J.J. Morton* (1981). Maintenance of blood pressure by the renin-angiotensin system in normal man. *Nature* **291** (5813), 329-331.

O'Donnell, C. J., Chazaro, I., Wilson, P.W., Fox, C., Hannan, M.T., Kiel, D.P. and *L.A. Cupples* (2002). Evidence for heritability of abdominal aortic calcific deposits in the Framingham Heart Study. *Circulation* **106** (3), 337-341.

Peyser, P. A. (1997). Genetic epidemiology of coronary artery disease. *Epidemiol Rev* **19** (1), 80-90.

Peyser, P. A., Bielak, L. F., Chu, J.S., Turner, S.T., Ellsworth, D.L., Boerwinkle, E. and *P.F. Sheedy* (2002). Heritability of coronary artery calcium quantity measured by electron beam computed tomography in asymptomatic adults. *Circulation* **106** (3), 304-308.

Pfohl, M., Athanasiadis, A., Koch, M., Clemens, P., Benda, N., Häring, H.U. and *K.R. Karsch* (1998). Insertion/deletion polymorphism of the angiotensin I-converting enzyme gene is associated with coronary artery plaque calcification as assessed by intravascular ultrasound. *J Am Coll Cardiol* **31** (5), 987-991.

Pullareddy, B.R., Srikanth Babu, B.M.V., Karunakar, K.V., Yasovanthi, J., Kumar, P.S., Sharath, A. and *A. Jyothy* (2009). Angiotensin II type 1 receptor gene polymorphism in myocardial infarction patients. *Journal of Renin-Angiotensin-Aldosterone System* **10** (3), 174-178.

Rumberger, J. A., Brundage, B. H., Rader, D.J. and *G. Kondos* (1999). Electron beam computed tomographic coronary calcium scanning: a review and guidelines for use in asymptomatic persons. *Mayo Clin Proc* **74** (3), 243-252.

Takayanagi, R., Ohnaka, K., Sakai, Y., Nakao, R., Yanase, T., Haji, M., Inagami, T., Furuta, H., Gou, D.F., Nakamuta, M., et al. (1992). Molecular cloning, sequence analysis and expression of a cDNA encoding human type-1 angiotensin II receptor. *Biochem Biophys Res Commun* **183** (2), 910-916.

T*iret, L., Ducimetière, P., Bonnardeaux, A., Soubrier, F., Poirier, O., Ricard, S., Cambien, F., Marques-Vidal, P., Evans, A., Kee, F., Arveiler, D.* and *G. Luc* (1994). Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *The Lancet* **344** (8927), 910-913.

Tsai, I. J., Yang, Y. H., Lin, Y.H., Wu, V.C., Tsau, Y.K. and *F.J. Hsieh* (2006). Angiotensin-converting enzyme gene polymorphism in children with idiopathic nephrotic syndrome. *Am J Nephrol* **26** (2), 157-162.

Ulgen, M. S., Ozturk, O., Yazici, M., Kayrak, M., Alan, S., Koç, F. and *S. Tekes* (2006). Association Between A/C1166 Gene Polymorphism of the Angiotensin II Type 1 Receptor and Biventricular Functions in Patients With Acute Myocardial Infarction. *Circulation Journal* **70** (10), 1275-1279.

Ye, S., Dhillon, S., Seear, R., Dunleavey, L., Day, L.B., Bannister, W., Day, I.N.M. and *I. Simpson* (2003). Epistatic interaction between variations in the angiotensin I converting enzyme and angiotensin II type 1 receptor genes in relation to extent of coronary atherosclerosis. *Heart* **89** (10), 1195-1199.