Angiotensin Converting Enzyme Insertion/Deletion Gene Polymorphisms in Leukemic Hematopoiesis

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ABSTRACT

Local bone marrow renin-angiotensin system (RAS) is an autocrine-paracrine system affecting normal and neoplastic hematopoiesis. Angiotensin converting enzyme (ACE) converts angiotensinogen-I to its physiologically active peptide angiotensin-II, which stimulates proliferation and differentiation of hematopoietic stem cells through angiotensin II type 1 receptors. We investigated the ACE insertion/deletion (I/D) gene polymorphisms in patients with hematological malignancies including acute and chronic leukemia, myelodysplastic syndrome and multiple myeloma. Our results showed that 80.4% of the patients represented ID/II genotype, whereas it was 55.9% of the control group and 3.2 fold increased disease risk in the existence of insertion allele (ID/II). This is the first study demonstrating possible effects of ACE I/D gene polymorphism of the local bone marrow RAS components on leukemic hematopoiesis.

Keywords: Angiotensin converting enzyme, Local renin-angiotensin system, ACE I/D gene polymorphism, Angiotensin II, Leukemic hematopoiesis

ÖZET

Lösemik Hematopoezde Kemik İliği Anjiotensin II Tip I Reseptör Gen ve Anjiotensin Dönüştürücü Enzim (ACE) İnsersiyon/Delesyon (I/D) Gen Polimorfizmleri

Lokal kemik iliği renin anjiotensin sistemi (RAS) normal ve neoplastik hematopoezi etkileyen otokrin ve parakrin bir sistemdir. Anjiotensin dönüştürücü enzim (ACE) anjiotensinojen-I'i kendisinin fizyolojik olarak aktif peptide olan anjiotensin II'ye dönüştürür ki, bu hemetopoetik kök hücrelerin çoğalmasını ve farklılaşmasını anjiotensin tip 1 reseptörleri üzerinden uyarır. Biz ACE insersyon/delesyon gen polimorfizmlerini akut ve kronik lösemi, myelodisplastik sendrom ve multiple myeloma gibi hematolojik malignasili hastalarda araştırdık. Çalışma sonuçlarımıza göre hastaların % 80.4'ü ID/II genotipi gösterirken bu oran kontrol grubunda % 55.9 olarak bulundu ve insersiyon alleli varlığında (ID/II) 3.2 kat artmış hastalık riski saptandı. Bu lokal kemik ilği RAS üyelerinden ACE'nin I/D gen polimorfizminin lösemik hematopoezdeki olası etkisini gösteren ilk çalışmadır.

Anahtar Kelimeler: Anjiotensin dönüştürücü enzim, Lokal renin-anjiotensin sistemi, ACE I/D gen polimorfizmi, Anjiotensin II, Lösemik hematopoez

INTRODUCTION

The new frontiers of the renin-angiotensin system (RAS) include organ-, tissue-, and even cell-based systems within the human body exhibiting autocrine, paracrine, endocrine and intracrine actions.[1] Presence of a local bone marrow RAS, affecting physiological and neoplastic blood cell production, has been evolved as a novel hypothesis in the past decade.[2-4] The forthcoming studies gave countenance to the potent stimulatory affect of angiotensin II in physiological hematopoiesis accordingly in an autocrine-paracrine manner by the findings of increased expression of RAS components such as renin, angiotensinogen and anjiotensin II it-self and its type-1 receptors (AT1R) on hematopoietic stem and precursor cells.[5,6] Angiotensin II stimulates the proliferation of bone marrow hematopoietic progenitors and umbilical cord blood cells.[7] Likewise, over-expression of RAS components were reported in leukemic hematopoiesis[6] and on acute myeloid leukemia cells.[8-10] Hence, the context of the local bone marrow RAS represents the clues for being a potent autocrin/paracrin system affecting both normal and neoplastic hematopoiesis.

One of the cornerstone enzymes in the RAS is angiotensin-l converting enzyme (ACE), which converts the angiotensinogen-I to its physiologically active peptide angiotensin-II. As a zinc metallopeptidase enzyme; ACE may play a major role in numerous physiological activities including cellular proliferation and metabolism of numerous other biologically active peptides owing to broad enzymatic specificity in the body. The encoding ACE gene is localized to long arm of the 17th chromosome (17q23.3) and responsible for the production of two isoenzymes- somatic and testicular form- according to alternative splicing of the single but duplicated ACE gene.[11,12] Additionally, it was identified that there were membrane-bound and circulating forms of the sACE enzyme. The membrane-bound form is present on various cell types, including vascular endothelial cells, epithelial cells, neuronal cells, macrophages and male germinal cells, in which C-domain of the sACE has found to be related to RAS by taking role in conversion of angiotensin-I to angiotensin-II and hydrolyses of bradykinin.[13] However, the circulating form of the ACE has found to be present in such biological fluids as plasma, amniotic and seminal fluids.

Differences in the circulating ACE levels among individuals have been determined in part by a polymorphic gene which has a major effect on the phenotype.[14] The gene responsible for this effect was ACE gene itself. An insertion/deletion (I/D) polymorphism located in the intron 16 of the ACE gene has found to be associated with differences of the plasma levels of ACE in the group of healthy subjects.[14] Moreover, I/D polymorphism of the ACE gene has been linked to coronary heart disease since subjects of the homogenous deletion genotype (DD) gave the impression of increasing myocardial infarction risk.[15] ACE has also been found in human monocytes/macrophages, and in the T-lymphocyte population. Thus, ACE likewise other ectopeptidases, may participate in the regulation of lymphocyte functions. The T-lymphocyte ACE levels of a given subject are highly reproducible when measured on two different occasions, but may vary widely between individuals in association with I/D polymorphism of the ACE gene.

Since plasma ACE has been thought to originate from endothelial cells and ACE was mostly a membrane-bound ecto-enzyme on those cells as on the other cell types, wherever it has been synthesized these observations indicated that the level of ACE expression was genetically determined. Therefore, in this study, we investigated ACE I/D gene polymorphisms, which might affect the behavior of the local RAS in hematological neoplastic disorders.

MATERIALS AND METHODS

Patients and DNA Isolation: In order to explore the ACE I/D gene polymorphisms, bone marrow samples of patients diagnosed with AML, CML, ALL, CLL, MM and MDS collected during their assessment and diagnosis at Hacettepe University Faculty of Medicine, Department of Internal Medicine, Hematology Unit in Ankara, Turkey and peripheral blood samples of healthy people were studied.

The genetic studies of the samples including isolation of Deoxyribonucleic acids (DNA), polymerase chain-reaction (PCR) and gel electrophoresis were done at Molecular Genetics Laboratory of Ankara University Faculty of Medicine, Department of Medical Genetics in Ankara, Turkey. DNAs of the patients were extracted from 3 ml bone marrow biopsy

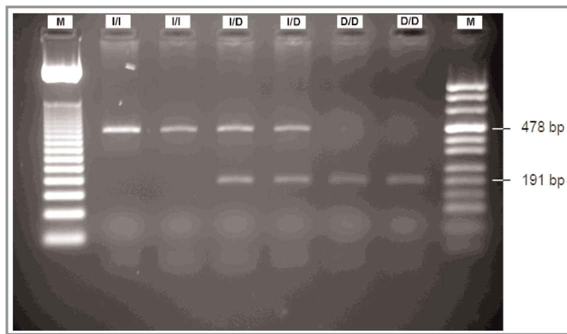

Figure 1. ACE I/D gene polymorphisms at 2% agarose gel visualized by Kodak Gel Logic 200.
I: Insertion, D: Deletion, M: Marker (Ladder)

samples which were obtained for routine cytogenetic examination either drawn by heparinized syringe or into EDTA containing tubes. Control group DNAs were extracted from peripheral blood samples drawn into 5-ml tubes containing EDTA. DNAs were isolated from all samples by proteinase K digestion, salt extraction, and ethanol precipitation method following standard protocols.

All patients provided written informed consent and study was approved by Hacettepe University Faculty of Medicine Research Ethical Committee (TBK 05/13-8).

Determination of ACE Genotypes: ACE I/D gene polymorphism has been investigated on the basis of PCR amplification of intron 16 of the ACE gene using forward and reverse primers, 5'-CCACTCC-CATCCTTTCTCC-3' and 5'-GGCCATCACATTC-GTCAGA-3', respectively, as previously described (16). After initial denaturation at 93^{0}C for 3 min; 30 cycles of 92^{0}C for 1 min, 57^{0}C for 1 min, and 72^{0}C for 1 min. were performed with a final extension at 72^{0}C for 7 min. PCR products were run on 2% agarose gel and visualized by Kodak Gel Logic 200 (Raytest GmbH, Straubenhardt, Germany) (Figure 1). All samples were studied twice. Because the D allele is preferentially amplified where the I allele is sometimes suppressed, samples found to have DD genotype were subjected to a second, independent PCR amplification with an insertion-specific primer as described previously.[17]

Table 1. Diagnostic features of patients

	Patients	
	(n)	(%)
MDS	7	15.2
AML	10	21.7
Minimal Changed	1	
Myelomonocytic	3	
Monoblastic	2	
t(8;21) +	1	
Multilineage Displasia	1	
Unclassified	1	
With Maturation	10	
ALL	8	17.4
T cell phenotype	1	
B cell phenotype	7	
CML	17	37
Chronic Phase	16	
Accelerated phase	1	
CLL	1	2.2
MM	3	6.5
TOTAL	46	100

Statistical Evaluation: In order to compare the frequency of genotypes within the groups, the Chi-square test and the Fisher's exact test for multiple groups were used within SPSS (Statistical Package for the Social Sciences Program) program for Windows version 11.5. Additionally, (Kolmogorov-Smirnov Test) was used for distribution of age and the likelihood ratio test (G statistic) was used to assess the agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium. The p value less than 0.05 has been considered as statistically significant ($p < 0.05$).

RESULTS

In order to evaluate the ACE I/D gene frequencies in 46 patients with leukemia and 59 healthy people were enrolled to the study between November 2005 and October 2007. Diagnostic features of patients are given in Table 1. 23 (59%) of the patients and 27 (46%) of the control group were male. Median age of patient group was greater than control group (49 years

Table 2. ACE I/D genotype frequencies in leukemic disorders.				
		ACE Polymorphism		**Total**
		DD	**ID/II**	
Control	(n)	26	33	59
	(%)	44.1	55.9	100
Patients	(n)	9	37	46
	(%)	19.6	80.4	100

old [range, 17-81] vs. 30 years old [range, 17-78], respectively) However, the distribution of age was homogenous in each group (Kolmogorov-Smirnov Test). The Hardy-Weinberg equilibrium was in agreement using likelihood ratio test (G statistic; G2= 0.039, freedom degree= 1 and p= 0.843).

ACE ID/II genotype frequency was significantly increased in patients with hematological disorders. Overall ID/II genotype frequencies was found in 80.4% of the patients while it was 55.9 % in the control group. There was significantly higher I allele (p= 0.008) in 46 patients compared to control group. Disease risk has found 3.2 times increased in patients who were carrying I allele (ID or II) (p= 0.008, OR: 3.2[95% C.I., 1.3-7.9]) (Table 2). Moreover, I allele (II or ID) carrying leukemia (CML, AML and ALL) patients were significantly higher compared to only D allele (DD) carriers (p< 0.001)(Table 3 and 4).

ACE ID/II genotype frequency was not different in patients with CML, AML or ALL. When we compared the patients with CML, AML and ALL in order to find out the possible ACE I/D polymorphism differences, there was no statistically significant difference between each diagnoses group (p= 1.0, multiple groups Fisher Test) (Table 3-4). Among them; 88.2% of CML, 90% of AML and 87.5% of ALL patients were carrying I allele.

MDS patients were carrying similar insertion allele frequency with control group unlike leukemia patients. MDS patients were carrying very similar frequency of I allele compared to control group (57.1 % to 55.9 %, respectively). The difference was not statistically significant (p> 0.05). However, insertion allele frequency in leukemia patients (88.9 %) was significantly increased compared to MDS (57.1 %) (p= 0.003). Additionally, presence of I allele had increased leukemia risk 6.3 times (p= 0.001 and [odds ratio: 6.3; 95% C.I., 1.98-20.1]) (Figure 2).

Insertion allele increased the leukemia risk in patients under 50 years old: I allele frequency under the

Table 3. ACE I/D gene polymorphism of CML, AML and ALL patients.				
		ACE Polymorphism		**Total**
		DD	**ID/II**	
ALL	(n)	1	7	8
	% within leukemias	12.5	87.5	100
AML	(n)	1	9	10
	% within leukemias	10	90	100
CML	(n)	2	15	17
	% within leukemias	11.8	88.2	100
Total	(n)	4	31	35
	% within leukemias	11.4	88.6	100

Table 4. ACE I/D polymorphism differences of leukemia subgroups

Diagnosis	I allel carrier (genotype ID/II) n (%)	Non-I allel carrier (genotype DD) n (%)	p value
Leukemia (ALL, AML, CML)	35 (85.4)	6 (14.6)	<0.001
ALL	7 (87.5)	1 (12.5)	0.034
AML	9 (90.0)	1 (10.0)	0.011
CML	15 (88.2)	2 (11.8)	0.002
Control	33 (55.9)	26 (44.1)	0.508

age of 50 were 83.3% in patients with hematological malignancies and 54.5% in the control group. I allele increased leukemia risk 4.2 times in this age group (p= 0.015) [OR: 4.2; 95% CI, 1.26-13.8]. I allele frequency was not different between patients and control group over 50 years old (77.3% and 75% respectively, p= 1.0) (Table 5).

DISCUSSION

In this study, we investigated I/D gene polymorphism of the ACE gene coding for ACE by polymerase chain reaction within leukemia (CML, AML, ALL, CLL,) patients including MDS and MM. To our knowledge, this is the first study to provide the evidence that ACE ID/II gene polymorphisms may be linked to the development of leukemia as a clue of activated local RAS in bone marrow during leukemogenesis.

Following the introduction of I/D gene polymorphism in the literature by Rigat et al. (14) , “D” allele has been reported to be related to increased serum enzyme levels compared to “I” allele (14; 18). The latter described as an insertional mutation, present in the intron 16 of the ACE gene, that caused by retrotransposition of 287 basepair of Alu repeat element.[19] Since, Suehiro et al.[20] reported that D allele had increased the ACE mRNA levels, it was also found to be a potent risk factor for cardiovascular diseases particularly on myocardial infarction in Turkish population.[20,21] Production of angiotension-II is increased as a result of increased serum ACE levels (15; 22-24). Reasonably thereafter increased Angiotensin-II causes vasoconstriction on renal vascular smooth muscle, increase fluid and electrolyte reuptake from renal tubule cells and finally augments the endocrine response by stimulating aldosteron and vasopressin release. Although, the latter has currently been ac-

Table 5. ACE I/D gene polymorphism and age distribution (cut off point is 50 years old).

		ACE I/D gene polymorphism			Total
		DD	ID/DD		
≤ 50 years old	Control	(n)	25	30	55
		%	45.5	54.5	100
	Patients	(n)	4	20	24
		%	16.7	83.3	100
> 50 years old	Control	(n)	1	3	4
		%	25.0	75.0	100
	Patients	(n)	5	17	22
		%	22.7	77.3	100

cepted as a causative concept for D allele to be a cardiovascular risk factor, only few studies reported that there was no significant relationship between the ACE I/D gene polymorphism and serum angiotensin II levels or increased plasma ACE levels and angiotensin II levels.[25-29] These findings once more increase the importance of local ACE levels that could have much more potency on the Ang II levels within the tissues rather than in circulation.[30]

On the other hand, several investigators have been interested on the effects of ACE gene polymorphism on malignant diseases and have found the D allele to be the poor prognostic factor for several malignancies including breast cancer, intestinal type gastric cancer and prostate cancer.[31-34] However, our study showed that hematologic malignancies are more prone to the existence of I allele, as in endometrial and oral cancers in which I allele has found to be the poor prognostic factor (35; 36) that is consistent with our study in which presence of I allele increased the disease risk for 3.2 times compared to control group (p= 0.008) (Table 3).

Additionally, the report given by Haiman et al.[37] has shown that there was a positive correlation between ACE I/I genotype and the breast cancer, but stating with the discrepancy at each ethnic group. Moreover, if we took in consideration just only to AML, ALL, CML and CLL patients we achieved 6.3 times increased disease risk (p= 0.001) (Table 4). Hence, our data represented that insertion allele might be a potent risk factor for leukemia development in our population as a selected neutral genetic marker.[38]

Alternatively, in vitro incubation of AML cells with an ACE inhibitor decreased the growth and colony-forming ability of AML cells in a dose-dependent manner. Adding of angiotensin II peptide to AML cells partially rescued their colony-forming ability. In addition to these, over expression of a critical RAS component, ACE (CD 143) surface antigen has shown to be over-expressed in leukemic myeloid blast cells.[9] Moreover, a positive correlation has been found between the ACE and bone marrow blast count. Hence, local bone marrow RAS has been searched in acute and chronic myeloproliferative disorders and angiotensin has been suggested to act as an autocrine growth factor for AML cells. Those previous results could easily shifted us to expect a superior D allele frequency owing to higher serum ACE enzyme levels but surprisingly our result has shown that I allele has increased disease risk 3.2 times.

This may have several explanations. First of all one of the incompetence of our study was the small sample size that only 46 patients have been enrolled in the study. Larger sample sized studies would give most accurate polymorphism results. But, although we have had small sample size we have at most 4 diverse leukemia subgroups. So this deficiency might be negligible at all. Nevertheless, the local RAS might be an independent local mechanism than the circulating system. The clues for this are present in the literature that ACE is mostly a membrane-bound ectoenzyme and the cellular enzyme is known to be physiologically more important for peptide metabolism than the plasma enzyme. Abali et al. has found significant higher ACE concentrations in leukemic patient's bone marrow than in peripheral blood.[6] In addition to this, Beyazit et al. has found increased mRNA expressions of renin, angiotensinogen and ACE in local bone marrow of AML patients (10) as in erythroleukemic blast cells lines (K562).[39] These results are suggesting the importance and the possible underlying role of ACE I/D gene polymorphism in the malignant diseases in parallel and pertained to tissue level as if different genotypes would have effects on diverse tissues. Therefore, our result of significantly increased insertion allele frequency (p= 0.001) indicated that the level of expression of membrane-bound ACE in blastic cells was associated with the insertion/deletion polymorphism of the ACE gene (Figure 2). Thus, it could be attributed to insertion allele that increase in local ACE mRNA expression might lead to increase of effector peptide Ang-II which then could serve a potent proliferative growth affect inside leukemic bone marrow.[40]

Since the RAS peptides are involved in the control of cellular proliferation, Angiotensin II might have a role in the erythroid and myeloid differentiation of stem cells. Angiotensin II shows its effects in essence on AT-1 receptor, which is known as a G protein coupled 7-transmembrane receptor. At the molecular level AT-1 receptor is related to activation of lots of tyrosine kinase and non-tyrosine kinase signal transduction cascade activation such as mitogen activated protein kinase (MAPK), IP3-diachil glycerol (DAG), cAMP and JAK-STAT pathway. Additionally, Angiotensin II indirectly could activate the EGFR,

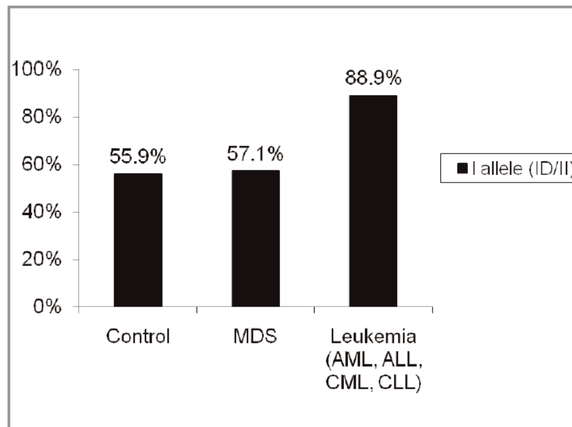

Figure 2. Comparison of ACE I/D gene polymorphism between leukemia and MDS

PDGFR by receptor transactivation.[41] One of the subtypes of PDGFR is ERB-B2 (HER-2/neu) that is known as an important target of breast cancer. By activating the tyrosine kinase receptors Angiotensin II could activate intracellular downstream effector molecules such as phospholipase-C and PI3-kinase, which are then result in increase of intracellular Ca^{2+} and Akt kinase formation, respectively.[42] The latter has a potent role in cellular proliferation and inhibition of apoptosis. Activation of signal transduction pathways in the cells result in activation of intranuclear transcription factor activation such as c-JUN, c-MYC and c-FOS. Heterodimerization of c-FOS and c-JUN as a posttranslational modification might result in production of transcription factor activator protein -1 (AP-1). Ang II has a role in inflammation by activation of AP-1 and NF-κβ activation over AT-1 receptor.[43] Additionally, it's shown that Angiotensin II could activate the JAK –STAT pathway via AT-1 receptor[44], that possible relationship between the local RAS, JAK-STAT pathway and leukomogenesis has been hypothesized before.[45] Consequently, increased production of Angiotensin II by membrane bound ACE could result in leukemic transformation of bone marrow hematopoietic cells by gathered activation of signal transduction pathways via AT-1 receptor and/or receptor transactivation.

Additionally, ACE hyperfunction under the effect of insertion allele may lead to the acceleration of the negative hematopoietic regulator peptide AcSDKP (goralatid) metabolism, which in turn lowers its level in the bone marrow microenvironment, and finally give rise to removal of anti-proliferative effect of the peptide on hematopoietic blast cells.[2,6,10,46] As a result, insertion allele might have a role on disease development and could be used as an aberrant marker of leukemia.

Another important result of the study was for the patients who were under 50 years old. Those patients also represented increased ACE ID/II frequency as a potent risk factor for leukemia under 50 years old. The disease risk has found to be increased 4.2 times compared to the control group (p= 0.015) [OR: 4.2, 95% CI= 1.26-13.8)] (Table 5). This effect was not seen in the patients over 50 years old (p= 1.0). Since the leukemia is a multifactorial diseases, important mutations occur on the patients genome during lifelong could change the effect of those polymorphisms in the patients[47], but its obvious that this effect, which might result in malign disease and poor prognosis, could be seen after 50 years old and before this polymorphic genes might have crucial role in the disease development.

In conclusion, ACE ID/II genotype frequency has increased in leukemic patients. Previous studies suggested that the genetic control of ACE synthesis is probably at the transcriptional level and the regulation of ACE synthesis in cells may also be genetically determined in leukemic patients. The pathobiological consequences of the genetic polymorphism of ACE levels for leukemic cell regulation and other ACE-mediated functions within the local bone marrow RAS remain to be investigated.

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finding patients, Mutlu Hayran has completed statistical analyses and interpreting the data, Ibrahim K. Onal has participate on organization of the study and grant application, Osman I. Ozcebe has participated on patient finding and Halil G. Karabulut and Ajlan Tukun has participated in designing and studying the molecular genetics and interpreting data.

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