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Alteration of Splicing Pattern on Angiotensin-Converting Enzyme Gene Due To The Insertion of Alu elements

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ABSTRACT

Angiotensin Converting Enzyme (*ACE*) is a zinc metallopeptidase that has a significant role in blood pressure regulation and the pathophysiology of hypertension. ACE has two protein domains, the N-domain and the C-domain, which each has a single active site that functions independently of each other. There is insertion/deletion by 288 bp *Alu elements* in the intron 16 of *ACE* gene. The *Alu elements* potentially alter splicing process. The effect of the insertion of *Alu elements* in the splicing pattern of the *ACE* gene has not been reported. Here, we report on the results of splicing pattern analysis of the *ACE* gene due to the insertion of *Alu elements*. Using an in-silico approach, we found the presence of *Alu elements* insertion in intron 16 of *ACE* caused alternative splicing and experienced exonization. Further analysis showed that the exonization lead to a premature termination codon (PTC), which is raised protein shortening and lost one of its two protein domains. The loss of one protein domain may affect the catalytic activity of ACE. These findings suggest that the *Alu elements* I/D polymorphism is related to the differences in the catalytic activity of ACE that may influence blood pressure regulation and hypertension.

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1. INTRODUCTION

Hypertension is a cardiovascular disease suffered by 30% of the global population[1]. This disease is caused by multiple factors including the level of salt intake, kidney conditions, stress, genetics, obesity, and endothelial circumstances[2]. In general, blood pressure is regulated by the renin-angiotensin system (RAS) and kallikrein-kinin system (KKS)[3]. Angiotensin Converting Enzyme (ACE) is an enzyme that serves a crucial role in the RAS and KKS. In the RAS, ACE assists the conversion of the inactive decapeptide Angiotensin I (Ang I) into the active octapeptide Angiotensin II (Ang II), a vasoconstrictor. In KKS, ACE plays a role in the inactivation of bradykinin (BK), a vasodilator[3]–[8].

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ACE gene is 21 kb long, is located on the chromosome 17q23. *ACE* gene is consisting of 26 exons and 25 introns[6]. There is an insertion/deletion polymorphism in the intron 16 of *ACE*, characterised by the presence (insertion, I) or absence (deletion, D) of 288 bp *Alu elements*[7]. *Alu elements* are DNA repeat sequences, the group of *Short Interspersed elements* (*SINE*)[9]. In some genes, the insertion of *Alu elements* causes alternative splicing and results in exonization (the creation of cryptic exons)[10], [11]. Exonization can produce the mRNA transcripts to become longer, but such effects are not always applicable to the protein product. Exonization can cause a premature termination codon (PTC), resulting in protein shortening.

ACE with *Alu elements* insertion (ACE I) is believed to undergo protein shortening so that its molecular weight is lower than that of ACE Deletion (ACE D), which affects its activity. This phenomenon is corresponding with findings of Fuch *et al.* And Agerholm *et al.*[12], [13], ACE I activity is lower than that of ACE D.

ACE consists of two protein domains, the C-domain and the N-domain. Each domain has one active site that works independently of each other[12]. The *Alu elements* insertion may lead to protein shortening that causes loss of one active site in C-domain. For this reason, ACE I catalytic activity is not as high as that of ACE D. The low catalytic activity of ACE I in the conversion of Ang I to Ang II impacts the level activity of vasoconstriction. Therefore, this polymorphism is believed to have a huge effect on the structure and activity of the ACE protein. This study was conducted to determine the impact of *Alu elements* insertion to the splicing pattern of *ACE* and the 3D structure of its protein using the *in-silico* approach.

2. MATERIALS AND METHODS

2.1. Searching for the exonization of *Alu elements* in the GenBank database

According to data from GenBank, there are only three variant transcripts of ACE mRNA. Therefore, we have search transcript of any genes that contain *Alu elements* sequence. We used part of the *Alu elements* sequence (CGGGATGGTCTCGATCTCCTGACCTCGTGATCCGCCCGCCTCGGCCTC) to conduct a blast search in the ref_seq RNA NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All of these transcripts of cDNA sequences were saved in FASTA format. Individually of all variant were aligned with *Alu elements*, its genomic DNA and all variant of the transcript for each gene by ClustalW (Bioedit7.1.9).

We found over than five thousand transcripts that contain *Alu elements*. However, only 297 genes have inserted by *Alu elements* in the intron. Further, we focused on nine genes that inserted by *Alu elements* in the intron and have the definite motif of exonization (Table 1). This was done to identify the exonization of *Alu elements* in the cDNA transcripts was occurred in nature (GenBank).

2.2. Examination of HSF accuracy

The accuracy of Human Splicing Finder (HSF) 2.4.1 webserver software[14] for splicing pattern analysis was examined using known splicing data of the nine genes. The test was performed by inserting sequences that contain intron-exon-intron junctions of the genes with exonized *Alu elements* that have known splicing pattern and exonization locations. We also included 100 bases of the downstream intron and 100 bases of the upstream intron of an exon derived from *Alu elements*. HSF then scored the consensus value (CV) of every splice site it found. The splice site with the highest CV that is above the cut-off value is the most likely splice site. The cut off for the human 3' splice site is 75, and for the 5' splice site, it is 78[15]. Then we compare the HSF result (highest CV score) with the real data (transcripts) from the GenBank. The validity of HSF software was a test based on the outcome of the prediction is same with the real data.

2.3. Splicing pattern analysis of *Alu elements* inserted in intron 16 ACE

The splicing pattern due to *Alu elements* insertion in intron 16 *ACE* was analysed using HSF[14]. HSF identify and determine the potential splice sites through the method of the consensus value (CV) scoring. CV is the accuracy percentage of splice site base recognition by the spliceosome. CV values ranged from 0 (consensus with the worst recognition) to 100 (consensus with the best recognition). Splice sites are categorised according to whether their CV scores are above the cut-off. For human genes, the cut off is 78 for 5' ss, with a mean of 82, and 75 for 3' ss with an average of 80[15]. The potential splice sites were chosen based on the CV scores that were above the cut-off values. The splicing patterns of *ACE* intron 16 *Alu elements* that have been obtained by HSF software were then used to determine the cryptic exon sequence (exon derived from the *Alu elements*). These cryptic exons were then inserted between exon 16 and exon 17 in a somatic ACE CDS sequence (consisting of exon 1 to exon 25) using Bioedit 7.1.9 software. Further, the transcript sequence translated into amino acid sequences using the FR33 webserver (http://www.fr33.net/translator.php). Then we construct the three-dimensional structure of the ACE based on the splicing pattern.

2.4. Protein modelling and physicochemical analysis

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Protein structure of ACE I and ACE D were constructed using threading modelling using I-TASSER[16]–[18]. The physicochemical profiles of ACE proteins, including molecular weight, number and composition of amino acid sequences, were analysed by the Expasy Protparam tool webserver (web.expasy.org/protparam).

3. RESULTS AND DISCUSSION

Alu elements are the most abundant DNA repeat sequences from the *Short Interspersed Elements* (*SINEs*) group in primate genes. *Alu elements* make up not less than 10% of the mass of the human genome[19]. Cryptic splice site activation can result in alternative splicing and exonization so that a single gene can produce more than one mRNA transcript variant.

Approximately 400 of the 30,000 human genes that contain *Alu elements* are exonized in the coding region[20]. Through blast searching, we found that at least 5.000 cDNA transcripts of primates were indicated to have experienced exonization. Through ClustalW multiple alignments (Bioedit7.1.9), we aligned mRNA (cDNA) and DNA sequences of these 5.000 genes, individually, with *Alu elements* of *ACE* intron 16. We found no less than 407 cDNA transcripts of 297 primate genes having exonization. There are *Homo sapiens* (295 mRNA transcripts and 76 transcripts of non-coding RNA), *Pongoabelii* (27 mRNA transcripts), *Pantroglodytes* (3 mRNA transcripts), *Macacamulata* (5 mRNA transcripts), and *Callithrixjacchus* (1 mRNA transcript). Most of the exonisation on the transcript that we analysed occurred at the 3'and 5' UTR, and the whole sequence of *Alu elements* were exonized. For this reason, the splicing patterns were unclear. However, from these 407 cDNA transcripts, there were nine transcripts with partly exonised *Alu elements* with identical splicing pattern (Table 1), especially the acceptor splice site (5'ss). Thus, this is evidence that *Alu elements* were potentially becoming exonised.

To analyse the splicing pattern of *ACE* intron 16 with *Alu elements* insertion, we tested the Human Splicing Finder (HSF) webserver. Before using the HSF for *Alu* splicing pattern analysis, we checked its accuracy with the recognition and scoring of the splicing pattern of *Alu elements* in the genes with known splicing patterns. As a result, the HSF correctly assigned some cryptic splice sites, which is the highest CV score for potential splice sites has same with transcript sequence from GenBank.

Table 1. mRNA and non-coding RNA with identical *Alu elements* splicing patterns

No	RNA *accession number*	Description	Position of *Alu* exonization	*Acceptor splice site motif*	*Donor splice site motif*
1	NM_001131028.1	Homo sapiens autophagy-related 10 (ATG10), transcript variant 3, mRNA	Exon 2 (283...417)	ttgtatttttagTA (79,84)*	AAGgtaggc (88,53)*
2	NM_001257399.1	Homo sapiens ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1), transcript variant 11, mRNA	Exon 5 Var 11 (93...210)	ttgtatttttagTA (79,84)*	CAGgtatgt (91,2)*
3	NM_001146310.1	Homo sapiens chromosome 1 open reading frame 86 (C1orf86), transcript variant 1, mRNA	Exon - var 1 (179..331)	ttgtatttttagTA (79,84)*	ACGgtaggt (85,96)*
4	NR_026773.1	Homo sapiens chromosome 6 open reading frame 123 (C6orf123), non-coding RNA	Exon 4 (693..1652)	ttgtatttttagTA (79,84)*	Last exon
5	NM_145326.2	Homo sapiens zinc finger protein 493 (ZNF493), transcript variant 2, mRNA	Exon 4 var 2 (376..1702)	ttgtattttcagTA (87,52)*	Last exon
6	NM_005426.2	Homo Sapien's tumour protein p53 binding protein, 2 (TP53BP2), transcript variant 2, mRNA	Exon 3 var 2 (468..599)	ttgtatttttagTA (79,84)*	AAGgtaata (84,39)*
7	NM_031451.4	Homo sapiens testis expressed 101 (TEX101), transcript variant 1, mRNA	Exon 3 var 1 (186..279)	tgtatttttagTA (78,03)*	CAGgtatga (88,86)*
8	NR_024271.1	Homo sapiens septin 7 pseudogene 2 (SEPT7P2), non-coding RNA	Exon 10 (1160..1354)	ttttttttcagTA (92,59)*	TAGgtatgg (74,96)*
9	NM_001270393.1	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor beta (PKIB), transcript variant 4, mRNA	Exon - var 4 (215..334)	tatttttggtagAG (78,74)*	AAGgtaagt (99,05)*

* ConsensusValue(CV) score

HSF was then used to analyse the splicing pattern of *ACE* intron 16 *Alu elements*. Based on this method, it was found that *ACE* may potentially experience the alternative splicing, and we found six possible splicing patterns (Table 2). Alternative splicing is a mechanism of gene regulation in which the use of alternative splice sites in the splicing process generates multiple mRNA products from a single gene. This mechanism works to develop variations of the proteome. According to Desmet *et al.*[14], exon and intron existence is not static. Mutations in the gene can affect the spliceosomes in recognising the splice sites[14], resulting in alternative splicing. One type of alternative splicing is intron retention, called exonization. Exonization is the process by

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which genes acquire a new exon from the non-protein-coding sequence, mainly derived from introns. It is estimated that approximately 60% of human genes have alternative splicing[14].

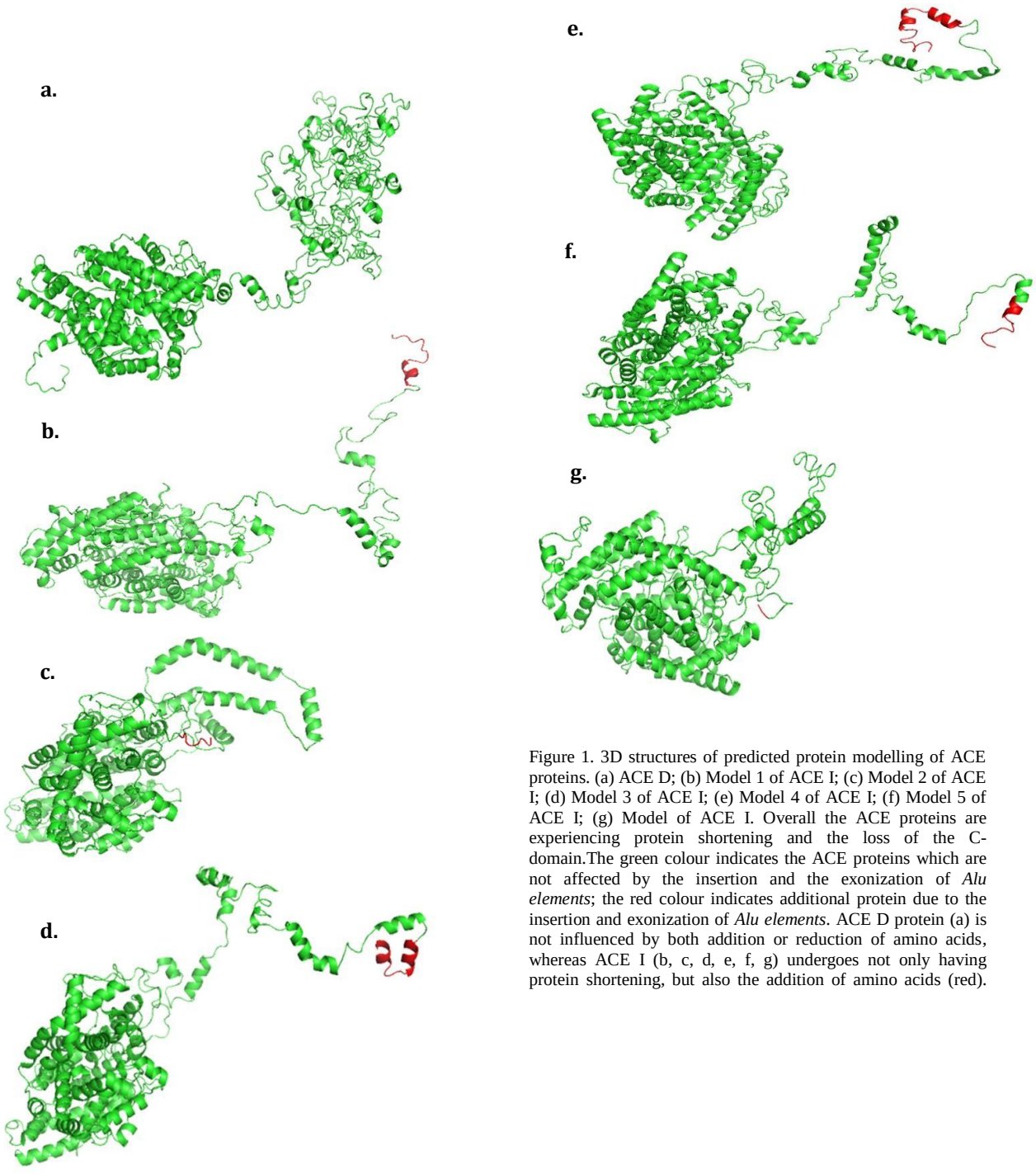

Figure 1. 3D structures of predicted protein modelling of ACE proteins. (a) ACE D; (b) Model 1 of ACE I; (c) Model 2 of ACE I; (d) Model 3 of ACE I; (e) Model 4 of ACE I; (f) Model 5 of ACE I; (g) Model of ACE I. Overall the ACE proteins are experiencing protein shortening and the loss of the C-domain.The green colour indicates the ACE proteins which are not affected by the insertion and the exonization of *Alu elements*; the red colour indicates additional protein due to the insertion and exonization of *Alu elements*. ACE D protein (a) is not influenced by both addition or reduction of amino acids, whereas ACE I (b, c, d, e, f, g) undergoes not only having protein shortening, but also the addition of amino acids (red).

Table 2. The six possible splicing patterns of *ACE*intron 16 *Alu elements*

Cons. Seq	ACE D	ACE I (1)	ACE I (2)	ACE I (3)	ACE I (4)	ACE I (5)	ACE I (6)
Upstream intron 5' ss (score)	CAGgtgaga (96,51)	CAGgtgaga (96,51)	CAGgtgaga (96,51)	CAGgtgaga (96,51)	CAGgtgaga (96,51)	CAGgtgaga (96,51)	CAGgtgaga (96,51)
Upstream intron BP (position)	-	ggctaAt (-17)	ggctaAt (-17)	ggctaAt (-17)	GgctaAt (-17)	ggctaAt (-17)	GgctaAt (-17)
Upstream intron 3' ss (score)	-	ctcctgcctcagCC (87,06)	ctgggaccacagCG (81,25)	TtgtatttttagTA (79,84)	ctgggattacagGC (85,4)	attttattccagCT (88,65)	CcttacaagcagAG (82,97)
Downstream intron 5' ss (score)	-	GAGgtgagc (94,91)	GAGgtgagc (94,91)	GAGgtgagc (94,91)	GAGgtgagc (94,91)	GAGgtgagc (94,91)	GAGgtgagc (94,91)
Downstream intron BP	agctcAa	agctcAa	agctcAa	agctcAa	AgctcAa	agctcAa	AgctcAa

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(position)	(-22)	(-22)	(-22)	(-22)	(-22)	(-22)	(-22)
Downstream intron 3'	aaccccctaccagAT	aaccccctaccagAT	aaccccctaccagAT	aaccccctaccagAT	aaccccctaccagAT	aaccccctaccagAT	AaccccctaccagAT
splice site (score)	(85,57)	(85,57)	(85,57)	(85,57)	(85,57)	(85,57)	(85,57)

BP = Branch Point; ss= splice site;

We got six exonization models of ACE based on the splicing pattern analysis described above (Table 3). At first glance, the presence of exonized *Alu elements* extended the CDS sequences. However, after being translated into amino acids, the sequences exhibited premature termination codons (PTC), which entailed enabling the protein shortening. ACEconsists of two protein domains, the C-domain and the N-domain; there is an active site that works independently in each domain[12]. Due to this protein shortening, the six models of ACE I proteins lost the large quantities of amino acids that constituted the C-domains. The phenomenon of protein shortening was discovered by Sorek *et al.* after they investigated the effects of *Alu elements* insertion in the protein coding region of several genes[20]. Their finding showed that 84% of exonized *Alu elements* that occupied protein-coding regions caused protein shortening through either frameshift or inframe stop codon mechanisms.

Table 3. Length of CDS Transcripts and protein profile of ACE D and ACE I models

Model	CDS sequence length without Alu exonization (base)	Alu exonization length (base)	CDS sequence length with Alu (base)	PTC Position	Amino Acid sequence (residues)	MW of ACE Proteins (kDa)
ACE D	3921	-	3921	1307	1306	149,7
ACE I (Model 1)	3921	237	4158	784	783	89,3
ACE I (Model 2)	3921	213	4134	776	775	88,6
ACE I (Model 3)	3921	174	4095	787	786	90,0
ACE I (Model 4)	3921	80	4001	797	796	90,9
ACE I (Model 5)	3921	35	3956	782	781	89,4
ACE I (Model 6)	3921	2	3923	771	770	88,2

PTC = Premature Termination Codon

Based on threading modelling results, protein shortening showed significant differences in the 3D structure and physicochemical profile of ACE D and the six models of the ACE I (Figure 1 and Table 3). Due to the loss of large number of amino acids, ACE I had a lower molecular weight than that of ACE D, thus affecting the level and concentration of plasma ACE. This is by the findings of Smithies *et al.,* in which the plasma concentration in subjects with the ACE II genotype was only 60% of the plasma concentration of the ACE DD genotype. Another effect of the protein shortening on ACE I was the loss of one active site. Consequently, ACE I activity to convert Ang I to Ang II is not as high as ACE D. This data explained the reason of subjects with the DD genotype has risk factors for hypertension higher than subjects with ID and II genotypes[21]. In addition, it has been reported that hypertensive patients with genotype II are hypersensitive to dry cough induced by the administration of ACE-inhibitors[22]. The low activity of ACE I in the Bradykinin inactivation leads to the accumulation of Bradykinin. The accumulation of Bradykinin in the lungs causes the activation of proinflammatory peptides (substance P, prostaglandins, neuropeptideY, and phospholipases C and A2) and the local liberation of histamine in the airway, with the consequence of stimulating of vagal afferent[23], [24]. A cough reflex follows the stimulation mechanism. Prostaglandins and histamine that are released as a result of Bradykinin accumulation also cause hypersensitivity to cough[24]. Thus, I /D polymorphism causes ACE gene splicing pattern variation. This impacts the protein profile of their products and subsequently affect ACE activity in the pathophysiology of hypertension and ACE inhibitor-induced cough. However, further research is required to collect the necessary data to investigate the effect of the *Alu elements* insertion in intron16 *ACE* by sequencing the cDNA, also the effect of other gene such as Bradykinin B2 Receptor[25].

4. CONCLUSION

Based on our research, *Alu elements* I/D polymorphism in intron 16 of *ACE* causes alternative splicing that impacts on the transcriptome variation. The *Alu element* insertion caused exonisation that implicated to protein shortening of ACE. The truncated ACE protein in one of the active site might reduce its activity.

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