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Studies on the Evaluation of Angiotensin-I Converting Enzyme Polymorphism under Fluorosis Mediated Renal Failures in Nellore District Andhra Pradesh, India

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Abstract: Excess levels of fluoride in drinking water leads to the development of fluorosis. Particularly in Andhra Pradesh state, next to Nalgonda, Nellore district seemed to be fluoride threaten area in India. The present study has been carried out at selective areas of Udayagiri mandal to evaluate the relation between renal failure and fluorosis in means of angiotensin-I converting enzyme (ACE) polymorphism. The contribution of insertion/ deletion (I/D) polymorphism of the gene encoding ACE has been investigated and the deletion type is documented to be a risk factor in the development of this disease. All the subjects, identified as DD, were reconfirmed with an insertionspecific primer. There was no significant difference in the distribution of DD, ID and II genotypes between renal failure and normal healthy subjects. The findings of the present study suggested that the ACE I/D polymorphism is not associated with advanced form of renal failures due to fluoride intake within the selected regional population.

Key words: ACE · polymorphism · Fluoride poisoning · Nellore District · Water fluoride

INTRODUCTION

Fluorine is the most electronegative element, distributed ubiquitously as fluorides in nature. Water is the major medium of fluoride intake by humans [1]. Fluoride can rapidly cross the cell membrane and is distributed in skeletal and cardiac muscle, liver, skin and erythrocytes [2, 3]. Fluorosis is a major public health problem resulting from long-term consumption of water with high fluoride levels.

In India, the states of Andhra Pradesh, Bihar, Chattisgarh, Haryana, Karnataka, Madhya Pradesh, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal are affected by fluoride contamination in water. This involves about 9000 villages affecting 30 million people [4]. It must be noted that the problem of excess fluoride in drinking water is of recent origin in most parts. Digging up of shallow aquifers for irrigation has resulted in declining levels of ground water. As a result, deeper aquifers are used and the water in these aquifers contains a higher level of fluoride [5-7]. Kidneys are among the most sensitive body organs in their histopathological and functional responses to excessive amounts of fluoride [8]. They are the primary organs concerned with excretion and retention of fluoride and thus are generally involved in chronic fluoride intoxication. In humans, only a few reports pertaining to kidney involvement in endemic fluorosis are available [9]. Kono *et al.* [10] reported impaired renal functions in fluoride-exposed workers. In contrast to cases of acute intoxication, the records of only a few autopsy reports of patients dying of chronic fluoride intoxication are traceable in the literature [9, 11].

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1), an exopeptidase , is a circulating enzyme that participates in the body's rennin-angiotensin system (RAS), which mediates extracellular volume (i.e. that of the blood plasma, lymph and interstitial fluid) and arterial vasoconstriction. It is secreted by pulmonary and renal endothelial cells and catalyzes the conversion of decapeptide angiotensin I to octapeptide angiotensin II. In recent years a vast amount of data has been published on the association between the insertion/deletion (I/D) polymorphism of the gene coding for angiotensin-

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converting enzyme and renal disease. It has become clear that the polymorphism does not affect the prevalence of renal disease. However, data on the association with progression of renal disease and therapy response are still contradictory. Moreover, sufficient data on the physiological significance of this polymorphism are still lacking. This contribution provides an overview of the available studies and the potential pitfalls in interpreting the data. We also discuss the putative mechanisms for the association between the DD genotype and progression of renal disease and suggest directions for the future that might be employed to further clarify the role in renal pathophysiology.

Studies related to exact evidence of fluoride involvement in the renal failures are no more. Most of the experiments were conducted in the renal failure patients under the supplementation of fluoride water. To know the specific mechanism of fluoride toxicity in the renal failures with reference to ACE polymorphism, we have designed the work in the patients who are not having hyper tension as well as diabetes. Since the diabetes patients are going to develop the renal problems due to hyperglycemic activity. From this background the study was started in the Nellore district region of Andhra Pradesh, which is geographically southern part of the India near to the Bay of Bengal.

MATERIALS AND METHODS

The study was conducted in the Nellore district region of Andhra Pradesh, which is geographically southern part of the India near to the Bay of Bengal. Nellore district is the coastal are of south India, which seems to be one of the most fluorosis threaten area of Andhra Pradesh state. From the data of water quality department as well as information from news papers, analysis has been initiated in the Udayagiri mandal of Nellore district. Among the mandal ten villages have been reported to be affected areas of fluorosis.

Selection of Samples: Five hundred individuals from 10 villages in Udayagiri mandal, Nellore district of Andhra Pradesh State were randomly chosen for survey work, which was highlighted by the local newspapers. The present study was constructed to analyze the samples that are having the renal disorders with the association of fluoride intake. Peoples suffering with regular renal failure with diabetes and hypertension were separated and omitted from the analysis.

Analysis of Water Quality and Fluoride Content: A total of 10 samples was collected from the selected locations of each village representing the water quality of the whole area. Fluoride concentration was spectrophotometrically determined using Alizarin red-S and SPADNS reagents [12]. Sodium fluoride was used to prepare the standard solution. The main sources of drinking water in these villages are open wells, hand pumps and municipal supply.

Determination of ACE Genotypes: The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from intron 16 of the ACE gene and size fractionation and visualization by electrophoresis. DNA was extracted from peripheral leukocytes with standard techniques. PCR was performed with 20 pmoles of each primer in a final volume of 25 µl, containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2 mM of each dNTP and 1.25 unit of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). The DNA was amplified for 30 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min (DNA Thermal Cycler 480, Perkin Elmer-Cetus) [13, 14]. PCR products were electrophoresed in 2% agarose-gel with 5 µg ethidium bromide per milliliter. The amplification products of the D and I alleles were identified by 300-nm ultraviolet trans-illumination as distinct bands (D allele: 191 bp; I allele: 478 bp) Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence, with identical PCR conditions except for an annealing temperature of 67°C. The reaction yields a 335-bp amplicon only in the presence of an I allele and no product in samples homozygous for DD [15, 16].

Statistical Analysis: Statistical analysis was carried out using SPSS for windows 10.0 software (SPSS Inc.,Chicago, IL, USA) and Microsoft Excel. P value <0.001 was considered statistically significant.

RESULTS AND DISCUSSION

Udayagiri mandal of Nellore district andhra Pradesh, India seems to be the more threaten area by fluoride toxicity in drinking water. A sum of total ten fluoride

Table 1: Flouride contents in water samples of the selected ten villages in and around Udayagiri Taluk (Nellor edistrict, A.P., India)

Name of the village	Flouride content in water
Turkapalli	4.01 ± 0.83
Pakeerpalem	4.00 ± 0.66
Varikunta padu	6.74 ± 1.24
Bijjam palli	2.92 ± 1.02
Masi peta	2.37 ±0.98
Singa reddy palli	2.98 ±1.31
Boda banda	3.47 ± 0.88
Kolangadi palli	5.12 ± 1.56
Gangireddy palli	4.43 ± 1.98
Basine palli	3.12 ± 1.22

affected villages has been find out with the help of water control department and the water samples has been taken for the analysis of water fluoride content. Water samples from different bore wells of ten villages showed a maximum range of 2.37 to 6.74 ppm by SPADNS method (Table 1). Among the selected ten villages three are showing high levels of fluoride content in their drinking water (ranges 4-7 ppm). Particularly Varikunta padu showing a maximum fluoride content of 6.74 ppm. These three villages namely, Varikunta padu (6.74 ppm), Kolangadi palli (5.12 ppm) and Gangireddy palli (4.43 ppm) were take for the further entire study. Almost all the selected villages are higher than the permissible level of 1 ppm according to WHO [1].

ACE, a potent vasoconstrictor, catalyzes the conversion of angiotensin I to angiotensin II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis [17]. Hence present study was designed to study the role of ACE polymorphism of the fluoride affected people. The DNA samples from 90 fluoride mediated nephropathy and 60 normal healthy controls were amplified for I/D polymorphism in the ACE gene and analyzed. Figure 1 represents the PCR products of 190 and 490 bp indicating the presence of deletion (DD) and insertion (II) genotype, respectively. The preferential amplification of the D allele and inefficiency of the amplification of I allele may result in the mistyping of ID heterozygotes as DD homozygotes. Therefore, in order to increase the specificity of DD genotyping, all samples, identified as DD after initial amplification were reconfirmed with an insertion-specific primer pair, as mentioned in material and method section. The presence of insertion sequence was revealed by the amplification of a 275 bp fragment, while DD homozygotes failed to amplify due to the lack of annealing site (Fig. 2).

Table 2 shows the distribution of ACE genotypes in fluoride mediated nephropathy patients and normal controls. The frequency of D allele and DD genotype was only marginally higher in fluoride affected patients as compared to the normal controls. The observed and expected genotypic frequencies were in Hardy-Weinberg Equilibrium.

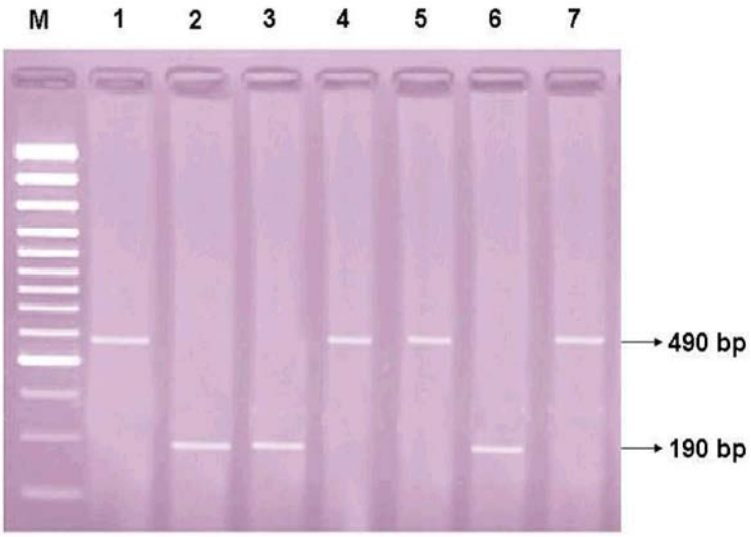

Fig. 1: Agarose gel electrophoresis stained with ethidium bromide, showing the initial amplification for ACE I/D polymorphism. Lane M represents the 100 bp ladder. The II genotype for I allele was identified by the presence of single 490 bp product (Lanes 1, 4, 5 and 7). The DD genotype for D allele was identified by the presence of a single 190 bp product (Lanes 2, 3 and 6). The DD homozygotes were reconfirmed with insertion specific primer pair to avoid mistyping as ID heterozygotes.

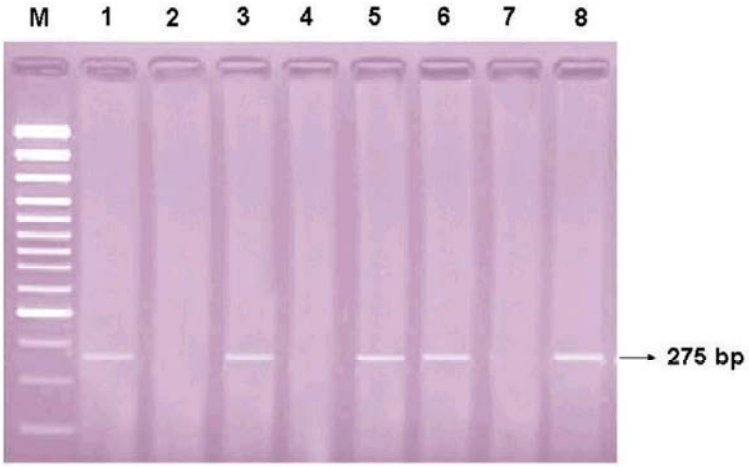

Fig. 2: Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of a product in the lanes 2, 4 and 7 confirms the presence of DD genotype. Heterozygous individuals (ID genotypes) were confirmed by the presence of a single 275 bp product (Lanes 1, 3, 5, 6 and 8). Lane M represents the 100 bp ladderFig. 2: Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of a product in the lanes 2, 4 and 7 confirms the presence of DD genotype. Heterozygous individuals (ID genotypes) were confirmed by the presence of a single 275 bp product (Lanes 1, 3, 5, 6 and 8). Lane M represents the 100 bp ladder.

Table 2: Distribution of the genotype and allele frequencies in the study groups for the angiotensin converting enzyme (ACE) I/D polymorphism

	Genotype frequencies (percentage)			Allele frequency	
Population (n)	DD	ID	II	D allele	I allele
Control (n=60)	10 (16.6%)	33 (55.0%)	17 (28.3%)	0.441	0.559
Fluoride affected (n= 90)	16 (17.7%)	48 (53.3%)	26 (28.8%)	0.44	0.56

÷2 based on allele frequency [degrees of freedom (df) = 1], (fluoride affected Vs Controls) = 0.00025

The study was started with a sum of total ten fluoride affected villages has been find out with the help of water control department and the water samples has been taken for the analysis of water fluoride content. Water samples from different bore wells of ten villages showed a maximum range of 2.37 to 6.74 ppm by SPADNS method (Table 1). Similar type analysis in the drinking water fluoridation has been earlier reported by several workers [18-21]. Among the selected ten villages three are showing high levels of fluoride content in their drinking water (ranges 4-7 ppm). Particularly Varikunta padu showing a maximum fluoride content of 6.74 ppm.

In recent years a vast amount of data has been published on the association between the insertion/deletion (I/D) polymorphism of the gene coding for angiotensin- converting enzyme and renal disease. It has become clear that the polymorphism does not affect the prevalence of renal disease. However, data on the association with progression of renal disease and therapy response are still contradictory. Moreover, sufficient data on the physiological significance of this polymorphism are still lacking. This contribution provides an overview of the available studies and the potential pitfalls in interpreting the data. We also discuss the putative mechanisms for the association between the DD genotype and progression of renal disease and suggest directions for the future that might be employed to further clarify the role in renal pathophysiology.

Renal failure is an outcome of complex pathophysiological process resulting from multiple etiologies with contribution from both genetic and environmental factors. A large variation abounds in the frequencies of ACE I/D polymorphism in different ethnic groups. It is evident from this table that the D allele frequency of our controls was intermediate to most reported Caucasian [22-26] and Asian [27-32] populations. However, two Caucasian [33, 34] and an Asian [29] population are reported to have comparable allele

frequencies. The failure to find statistically significant differences in the distribution of ACE gene I/D genotypes and their allele frequencies between the fluoride mediated nephropathy patients and the controls suggest that this polymorphism is not a risk factor for the development of renal failure in the studied population. These observations find support in the work of Tamaki *et al*. [29] and Ergen *et al*. [32].

In conclusion, our study suggests that the ACE I/D polymorphism is not associated with advanced form of renal failures due to fluoride intake within the selected regional population. From this it can be concluded that, we are in great need of more basic physiological studies that investigate the consequences of ACE I/D polymorphism in renal pathophysiology. Only then we can understand the impact of ACE I/D polymorphism on the onset and course of renal disease and eventually develop treatment strategies specifically adapted to certain genetic risk profile.

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