# CHANGED MAPK1 POLYMORPHISM IN RESIDENTS LIVING IN THE COAL-BURNING TYPE OF ENDEMIC FLUOROSIS AREA IN GUIZHOU PROVINCE, PEOPLE'S REPUBLIC OF CHINA

Yan-Jie Liu,[a,#] Jie Deng,[a,b,#] Ke-Ren Shan,[b] Xiao-Lan Qi,[b] Na Wei,[a] Zhi-Zhong Guan,[a,b,*]

Guiyang, People's Republic of China

**ABSTRACT: Based on the results obtained in our group showing that chronic fluorosis can influence the expression of the extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) in the mitogen-activated protein kinase (MAPK) pathway, we further investigated whether the multiple single nucleotide polymorphisms (SNPs) of the MAPK1 gene (encoding ERK1/2) were connected to the injuries caused by long-term exposure to the fluoride ion. The presence of dental and skeletal fluorosis and the genotypes and imputed SNPs of the MAPK1 gene were determined in a total of 222 residents from the coal-burning type of endemic fluorosis area in Guizhou Province, People's Republic of China. The frequencies of the genotypes of the rs743409 and rs7286558 alleles were detected with the Hardy Weinberg equilibrium. The results showed that in the population living in the area of endemic fluorosis, the most frequently expressed genotype of the rs743409 allele was TT and that for the rs7286558 allele it was GG. A significant correlation was found between the female gender and presence of the CT and TT genotypes of rs743409. The incidence of fluorosis was: dental fluorosis: female 72.2%, male 71.9%; skeletal fluorosis: female 32.1%, male 28.8%. Interestingly, among these clinical manifestations, skeletal fluorosis was significantly connected with the GG genotype of rs7286558. The results suggested that sensitivity to skeletal fluorosis in the residents living in the coal-burning type of endemic fluorosis area might be associated with the modified gene polymorphism of the MAPK1 gene.**

Key words: Dental fluorosis; Endemic fluorosis; MAPK1; Single nucleotide polymorphism; Skeletal fluorosis

## INTRODUCTION

Endemic fluorosis occurs as a result of excess fluoride ion (F) intake due to environmental factors and induces damage to the human body characterized by a vast array of symptoms and pathological changes in addition to the typical skeletal or dental fluorosis.[1-2] Interestingly, a new type of endemic fluorosis has occurred as a result of consuming food contaminated with F in the regions of Southwest China, which is primarily the result of environmental pollution caused by smoke emitted during burning coal that contains a high concentration of F when the residents in these areas bake grain and warm themselves in winter.[3-5] In China, 34 million of the population live in the coal-burning type of endemic fluorosis area. Among them, are 18 million who suffer from dental fluorosis and 1.5 million afflicted by skeletal fluorosis.[6] The area with the most severe type of the coal-burning type of endemic fluorosis is located in Guizhou Province of the People's

[a]Department of Pathology in the Affiliated Hospital; [b]Key Laboratory of Endemic and Ethnic Diseases (Guizhou Medical University), Ministry of Education, Guiyang Medical University, Guiyang 550004, People's Republic of China; [#]Authors making equal contributions to the paper; *For correspondence: Professor Zhi-Zhong Guan, Department of Pathology in the Affiliated Hospital and Key Laboratory of Endemic and Ethnic Diseases (Guizhou Medical University), Ministry of Education, Guiyang Medical University, Guiyang 550004, People's Republic of China; E-mail: zhizhongguan@yahoo.com; 1457658298@qq.com

Republic of China. In addition to having high levels of F in the urine and blood, the patients with chronic fluorosis in this area often exhibit severe symptoms including headache, dental fluorosis, and skeletal fluorosis.[7]

Recently, awareness of the polymorphisms of the genotypes involved in endemic diseases has attracted more attention to the pathogenesis of chronic fluorosis. It has been found that the polymorphic status at the locus of GSTM1 may modulate the individual body burden of total arsenic in some ethnic groups in the areas with endemic arseniasis in Guizhou Province of the People's Republic of China.[8] A significantly higher presentation of the A/A35931 genotype was found in skin lesion patients, who lived in the villages with endemic arseniasis, in Guizhou Province.[9] Our previous study revealed that the mechanism for the elevated activity of myeloperoxidase (MPO) induced by endemic fluorosis may be connected to the stimulation of the expression of MPO mRNA as a result of gene changes from polymorphism.[10]

In one study from our group, we found increases of phospho- and total-extracellular signal regulated protein kinase 1 and 2 (ERK1/2), and decreased activation of phospho-ERK1/2 in brains damaged by chronic fluorosis.[11] ERK1/2 is a critical component of the biochemical cascades through which a variety of extracellular stimuli initiate and regulate processes of cellular transformation. The activation of ERK1/2 is connected to cell physiological metabolism, injury, and oncogenesis. Exposure of odontoblast-like cells to sodium fluoride (NaF) induced a biphasic phosphorylation of ERK and NaF-induced apoptosis was markedly suppressed by treatment with the JNK and MAPK/ERK inhibitors.[12] The function of ERK1/2 is reflected by the MAPK1 or MAPK3 genes. Many researchers believe that affecting the function of the MAPK genes by mutation would have lethal consequences.[13]

In the present study, we selected residents living in the coal-burning type of endemic fluorosis area in Guizhou Province, People's Republic of China, to investigate whether the clinical symptoms of endemic fluorosis were related to the molecular mechanism of MAPK1 gene polymorphism.

## MATERIALS AND METHODS

*Subjects:* A total of 222 cases, comprising 87 males and 135 females with an average age of 50.0±18.6 yr, with different symptoms of fluorosis were collected from Qi-Xing-Guan county, an area severely affected by the coal-burning type of endemic fluorosis in Guizhou Province, People's Republic of China, in which foods, such as corn and chilli, and air were polluted by F through burning coal with a high F content for food-drying and warming in winter. The subjects lived in similar social, environmental, and economic conditions, and had comparable life styles and food intakes. The health of all the participants was examined and approximately 2 mL of fasting venous blood was collected from each person for examination, including gene polymorphism analysis.

All of the subjects were examined for the presence of dental fluorosis. X-ray examinations for the presence of skeletal fluorosis were made of the pelvis, forearm, and leg by using digital radiography (DR) imaging systems.

*Ethical permission and informed consent:* Ethical permission for the study was authorized by the Regional Ethical Committee in Guizhou Province and all procedures were carried out in accordance with the principle of informed consent.

*DNA extraction:* The genomic DNA was extracted according to the standard phenol/chloroform method from whole blood collected in tubes containing the anticoagulant EDTA. The purity and concentration of the extracted DNA were determined by an UV–VIS Spectrophotometer. The extracted DNA was stored at 4ºC in TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0). Each DNA sample was subjected to ALM-ASA analysis.

*Detection of the genotypes of the MAPK1 gene (for ERK1/2) by the TaqMan® real-time quantitative PCR:* The single nucleotide polymorphisms (SNPs) of the ERK1 gene were investigated in the 222 subjects using a custom TaqMan® SNP Genotyping Assay (Applied Biosystems, USA) using specific PCR primers (sequence of the forward primer: 5'-TCTGTCCGTCCGTCCCT-3', and of the reverse primer: 5'-GCCAGATGTGCTTGTCAAAGAAG-3'), and probes (probe sequence to detect the wild type allele: TGGCCGAGCTGCAG labelled with VIC® fluorescent dye, and probe sequence to detect the mutant allele: TGGCCAAGCTGCAG labelled with FAM™ fluorescent dye). The final concentrations of 1×TaqMan® SNP Genotyping Assay Mix, 1×TaqMan® Genotyping Master Mix, and 10 ng canine gDNA were mixed in a 25 µL total volume on a 96-well plate, and the assay performed using a Stepone Real Time PCR System (Applied Biosystems, Foster City, USA). The results were analyzed using Stepone Software V2.1 (Applied Biosystems, Foster City, USA).

*Sequencing:* The sequence of primers is shown in Table 1. The genotyping of 5% of the samples was confirmed by sequencing. The amplified products were directly sequenced to identify the polymorphic site by the Automated ABI prism 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster city, Calif.) using the ABI prism Big Dye terminator kit (version 3.1).

**Table 1.** Sequences of the primers employed for the genotyping

| Gene | Primer | Length of sequence (bp) |
|---|---|---|
| rs 743409 | Forward: 5'- AGTTGGCTTGGAGTGCCTGTC -3'<br>Reverse: 5'- ATCCCACTTTGCTTCCTGCTC -3' | 249 |
| rs 7286558 | Forward: 5'-ATGCTACCTAACAACTGTGAATGG-3'<br>Reverse: 5'-CTTAGGCTAGCTTTTAATAGTTCACTT-3' | 343 |
| β-actin | Forward: 5'- TGTCACATCCAGGGTCCTCACT -3'<br>Reverse: 5'- ACTCGTCATACTCCTGCTTGCTG -3' | 153 |

*Statistical analysis:* The allele frequency was calculated as the number of occurrences of the test allele in the residents divided by the total number of alleles. The Hardy-Weinberg equilibrium was applied to the allelic frequencies. $p<0.05$ was considered as significant for the data. The data from the study were analyzed using the SPSS 13.0 software. The LSD analysis, the t-test, and the Chi-square test were used to assess the associations of ERK1 gene polymorphism with gender, dental fluorosis, skeletal fluorosis with arthralgia, and blood F.

## RESULTS

Both the genotyped and imputed SNPs, of the 222 residents in the area of endemic fluorosis who participated in the study, were included in the analysis. The frequencies of the genotypes of rs743409 and rs7286558 following the Hardy-Weinberg equilibrium are indicated in Table 2.

**Table 2.** Genotype frequencies of the rs743409 (ERK197756T→C) and rs7286558 (ERK146788G→A) genes in the cases investigated

| Gene | n | Genotype frequency | | | Hardy-Weinberg equlibrium | |
|---|---|---|---|---|---|---|
| | | TT [% (n)] | CT [% (n)] | CC [% (n)] | $\chi^2$ values | p values |
| rs743409 | 222 | 47% (104) | 25% (56) | 28% (62) | $\chi^2$=50.46 | p=1.21 |
| | | GG [% (n)] | GA [% (n)] | AA [% (n)] | | |
| rs7286558 | 222 | 92% (204) | 8% (18) | 0% (0) | $\chi^2$=0.08 | p=0.78 |

Using the LSD analysis and the t-test, no significant difference was found between age and the genotypes of the rs743409 or rs7286558 alleles of the MAPK1 gene (p=0.54 and 0.56, respectively).

Using the Chi-square test, a significant difference (p=0.03) was found between gender and the genotypes of the rs743409 allele of the MAPK1 gene, but not with the genotypes of the rs7286558 allele of the MAPK1 gene (p=0.71) (Table 3).

**Table 3.** Association of age (years) and gender [female and male, % (n)] with the frequencies of the genotypes of the rs743409 and rs7286558 alleles of the MAPK1 gene

| Parameter | Allele of MAPK1 gene | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | rs743409 | | | | rs7286558 | | | |
| | Genotype | | | p* | Genotype | | | p* |
| | TT | CT | CC | | GG | GA | AA | |
| Mean age (yr) | 51.7 | 52.8 | 54.9 | 0.54 | 52.5 | 49.5 | 0 | 0.56 |
| Female [% (n)] | 15.8% (35) | 32.4% (72) | 12.6% (28) | 0.03 | 56.7% (126) | 4.1% (9) | 0% (0) | 0.71 |
| Male [% (n)] | 12.2% (27) | 14.9% (33) | 12.2% (27) | | 35.1% (78) | 4.1% (9) | 0% (0) | |

*The values were analyzed by the Chi-square test and the one way ANOVA.

The blood F levels were 1.21–4.03 μmol/L (0.023–0.077 mg/L or ppm) with no difference between the genders.

No gender difference was present in the incidence of dental fluorosis in the residents but skeletal fluorosis was significantly increased in females ($p<0.05$). The skeletal fluorosis usually presented with arthralgia and was mostly mild or moderate in severity (Table 4).

**Table 4.** Clinical manifestations of fluorosis in the residents living in the area of the coal-burning type of endemic fluorosis

| Gender | n | Dental fluorosis (%) | Skeletal fluorosis (%) |
|---|---|---|---|
| Female | 135 | 72.2% | 32.1%* |
| Male | 87 | 71.9% | 28.8% |

Compared to males by the Chi-square test: *$p<0.05$.

Comparison of the clinical manifestations of fluorosis in the residents with their genotypes of the MAPK1 gene showed skeletal fluorosis was significantly ($p=0.04$) connected to the rs7286558 allele of MAPK1 but not to the rs743409 allele ($p=0.79$). No significant association was found between dental fluorosis and the genotypes of the MAPK1 gene (Table 5).

**Table 5.** Association of the genotypes of the rs743409 and rs7286558 alleles of the MAPK1 gene and the presence of dental and skeletal fluorosis

| Gene | Genotype | Dental fluorosis [% and (proportion affected)] | p* | Skeletal fluorosis [% and (proportion affected)] | p* |
|---|---|---|---|---|---|
| rs743409 | TT | 71.2 (37/62) | 0.44 | 24.2 (19/62) | 0.79 |
| | CT | 73.9 (65/105) | | 29.5 (31/105) | |
| | CC | 24.0 (32/55) | | 27.3 (15/55) | |
| rs7286558 | GG | 96.4 (102/204) | 0.56 | 30.9 (63/204) | 0.04 |
| | GA | 70.8 (7/18) | | 22.2 (4/18) | |
| | AA | 0.0 (0/0) | | 0.0 (0/0) | |

*The values were analyzed by the Chi-square test and the one way ANOVA.

## DISCUSSION

Changes in the ERK pathway have been implicated in the pathogenesis of many disorders, including brain, pulmonary, dental, and skeletal diseases, as well as in chronic fluorosis.[11,14,15] Recently, many researchers have focused on the correlation between mutations of the MAPK genes and symptoms or treatment because these encoding genes directly affect the expression and function of ERK1/2.[16,17] Some findings suggest that MAPK signals play important roles in incisor formation, differentiation of the dental epithelium, and tooth growth.[18] Interestingly, ERK phosphorylation, with triggering of mitogenic and osteogenic responses, was found to be active throughout the period of bone regeneration of skeletal defects induced by extracorporeal shock waves.[19]

In the present study, we detected two alleles (rs743409 and rs7286558) of the MAPK1 gene, three genotypes of rs743409 (TT, CT, and CC), and two genotypes of rs7286558 (GG and GA). To evaluate the correlation between the symptoms of chronic fluorosis and the genetic factors, we analyzed the association of characteristics of residents in the area of endemic fluorosis and the MAPK1 genotypes. The results showed a significant relationship between gender and the genotypes of rs743409, in which the women in the area of endemic fluorosis had more of the TC and TT genotypes.

Most of the residents in the investigation suffered from dental fluorosis and some of them also had skeletal fluorosis.[4,7] No significant relationship was found between the presence of dental fluorosis and the genotypes of the rs743409 and rs7286558 alleles of MAPK1.

In severe cases, skeletal fluorosis can cause serious joint deformity that limits a person's ability to work or his/her quality of life due to symptoms, including arthralgia, paresthesia, a limited range of motion, and even incontinence and paralysis. In the present study, we found a significant difference of skeletal fluorosis between the female and male residents, in which the women were more sensitive to the skeletal changes. From an epidemiological survey in the coal-burning type of endemic fluorosis area, the prevalence and the degree of skeletal change were higher and more severe in women than in men, which may be related to the metabolism of estrogen, pregnancy, birth, and breastfeeding, as well as more contact with F contamination from cooking.[20] In addition, the higher rates of skeletal changes in women may be related to the increased levels of the TC and TT genotypes of the rs743409.

Our results also showed, in the residents of coal-burning type of endemic fluorosis area, a significant interaction between the presence of skeletal fluorosis and the GG genotype of the rs7286558 allele of the MAPK1 gene. Changes in the gene polymorphism in the genotypes of the rs7286558 allele of the MAPK1 gene have been observed in some diseases.[21] In the present investigation, the exhibition of the GG genotype of the rs7286558 allele of the MAPK1 gene may be considered to be an indicator for an increased risk of suffering from skeletal fluorosis.

## CONCLUSIONS

In conclusion, in the residents living in the coal-burning type of endemic fluorosis area, the TT genotype was the most frequently expressed genotype of the rs743409 allele of the MAPK1 gene and the GG genotype was the most frequently expressed genotype of the rs7286558 allele. A significant correlation was found between the female gender and presence of the CT and TT genotypes of rs743409. A high rate of dental fluorosis (71.9% and 72.2% for males and females, respectively) was present in the residents and many had skeletal fluorosis (28.8% and 32.1% for males and females, respectively). Skeletal fluorosis was significantly connected with the GG genotype of rs7286558. The results suggested that increased sensitivity to the skeletal changes of chronic fluorosis in the residents living in the coal-burning type of endemic fluorosis area might be associated with gene polymorphism in the MAPK1 gene.

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