SCREENING OF ENVIRONMENTAL RESPONSE GENES RELATED TO DENTAL FLUOROSIS

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SUMMARY: To explore the susceptibility and resistance or tolerance genes related to dental fluorosis, 30 children were selected at random from surveyed populations in two residential areas in China with different levels of fluoride (F) in their drinking water. Elbow venous blood samples were analyzed for leukocyte gene expression profiles using the cDNA chip for the control group, the high-loaded F group, and the dental fluorosis group. The results indicated that, compared with the control group, a total of 1057 genes were differentially expressed in the high-loaded F group. Of these, 148 were robustly up-regulated, and 61 were robustly down-regulated. In contrast, a total of 964 genes were differentially expressed in the dental fluorosis group as compared with the control group. These included 71 robustly up-regulated genes and 60 robustly down-regulated genes. In comparison with the high-loaded F group, 633 genes were differentially expressed in the dental fluorosis group. Of these, the number of robustly up-regulated genes and robustly down-regulated genes were 15 and 67, respectively. These findings suggest that differences in the occurrence of dental fluorosis are related to differences in gene susceptibility and resistance or tolerance.

Keywords: Dental fluorosis; Environmental response genes; Gene chip; High-loaded fluoride children; Susceptibility to fluorosis.

INTRODUCTION

Although endemic fluorosis is a systemic disease which greatly affects human health,[1-3] environmental epidemiological studies have shown that not all the residents living in areas with elevated levels of fluoride (F) intake as from their drinking water are afflicted with fluorosis. Similarly, in occupational settings, only a portion of workers develop fluorosis when exposed to high levels of fluoride in their working environment. These facts suggest that differences in individual susceptibility or tolerance related to F may exist. Obviously, therefore, if they exist, it is important to identify differences in susceptibility or tolerance genes associated with fluorosis. So far as we are aware, no data have been reported for such susceptibility or tolerance genes.

To the present, several studies have demonstrated that several critical oncogenes or tumor surpressor genes such as c-fos, c-jun, bax, and p53 are related to fluorosis.[4-7] However, conventional methods such as RT-PCR and hybridization *in*

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situ to search for those genes are not only time-consuming and labor-intensive, but also limited for obtaining a significant amount of information on gene expression profiles. Microarray technologies facilitate rapid measurement of the expression levels of thousands of transcripts in a single experiment and thus allow comparison of expression patterns across many samples.

In this study, in order to perform a rapid systemic screening of environmental response genes related to dental fluorosis, we used the gene chip HG-U133A available from the Affymetrix Company which contains 18,000 human genes. In this manner, we determined the gene expression profiles of leukocytes from representative control children, high-loaded F children, and dental fluorosis children.

MATERIALS AND METHODS

Study population: Over two hundred children from two villages in Anyang County (Henan Province, China) underwent initial dental examination. The first village was Renhegan, where F concentrations in the drinking water range from 1.1 to 2.0 mg/L. The second village was Xichangshan, where the drinking water contains 0.76 mg F/L. A questionnaire was used to collect information on the name, address, age, sex, school grade, drinking water source, duration of stay in the village, nutritional status, and dental fluorosis status of each child. According to dental examination results, the following three groups of children were chosen: First, 10 children in the 10-12 year age group with dental fluorosis, residing in Renhegan were chosen randomly as the dental fluorosis group. Then, in the same village, 10 healthy children without dental fluorosis, matched for age, sex, and nutritional status, were selected as the high-loaded F group. Finally, 10 healthy children without fluorosis, also matched for age, sex, and nutritional status were selected from Xichangshan as the control group.

Isolation of leucocytes: A 3-mL sample of blood were collected from each child via the elbow vein into a sterile centrifugal tube containing sodium heparin. Then, 3 mL of ice-cold sterile phosphate-buffered saline (PBS) was added to the sample and mixed completely. The mixed liquids were gently transferred to a new sterile centrifugal tube containing 6 mL of lymphocyte separation medium, centrifuged at 4°C for 30 min at 3,000 *g*, and the resulting super white cell coats were transferred to a new RNase-free centrifugal tube, rinsed once with sterile PBS (centrifuging at 4°C for 5 min at 8,000 *g*) and the effluent discarded. Finally, the isolated leucocytes were suspended in Trizol (Invitrogen Life Technologies, Paisley, UK), diverted into a RNase-free tube in a refrigerator and preserved under liquid nitrogen.

RNA sample preparation and microarray processing: The sample preparation is described here in brief. Total RNA was extracted from the leucocyte samples by Trizol. RNA yields were measured by ultraviolet spectrophotometer UV-2401PC (Shimadzu Corporation, Japan), and RNA quality was assessed by agarose gel electrophoresis for visualization of ribosomal RNA band integrity. RNA from ten children of the same group was pooled for each microarray sample in order to acquire a sufficient quantity of RNA for the gene chip examination, eliminating individual variation, and reducing the cost for the gene chips.[8] Samples with 15 µg

of RNA were purified on RNeasy columns by Qiagen (Valencia) and then converted into double-stranded cDNA with a Superscript Double Stranded cDNA Synthesis Kit (Invitrogen Life Technologies, Paisley, UK). The cDNA was then expressed as biotin-labeled cRNA by *in vitro* transcription (IVT) with the Enzo RNA Transcript Labeling Kit (Affymetrix, Santa Clara, California, USA). Each sample was spiked with bioB, bioC, bioD, and cre (Affymetrix, Santa Clara, California). The biotin-labeled cRNA was fragmented non-enzymatically, and the fragmented cRNA was hybridized to the HG-133A GeneChip (Affymetrix, Santa Clara, California) in the Affymetrix hybridization buffer for 16 hr at 45°C. The hybridized arrays were washed and stained in the Affymetrix Fluidics Station 400 to attach fluorescent labels to the biotin, followed by biotin-labeled antibody, and then a second staining with fluorescent labeling of the biotin. Each array was scanned twice by the GeneArray Scanner G2500A.

Data analysis: The data were analyzed with Affymetrix Microarray Suite 5.0. The change in p-value was calculated by the Wilcoxon's signed-rank test. The signal log ratio (SLR) algorithm was used to estimate the magnitude and direction of change in a transcript, when two arrays were compared (experiment versus baseline). The robustly up-regulated or down-regulated genes were selected that conformed to all of the following criteria: present in the experimental sample, increase or decrease in expression, and SLR $\geqslant$ 1.0 or SLR $\leqslant$ -1.0.

RESULTS

The purity and integrity of the isolated total RNA: Table 1 shows that the RNA of the control, high-loaded F, and dental fluorosis group had an OD260/OD280 (optical density at 280 nm) ratio of 2.07, 2.05, and 2.07, respectively. Two clear bands at 18 s and 28 s were noted after electrophoresis on 1.2% agarose gel with a brightness ratio of 2 to 1 (shown in Figure 1). These results indicated that the purity and integrity of the isolated total RNA met the required standards.

Table 1. Ultraviolet absorbance of total RNA

Group	$OD_{260\ nm}$	$OD_{280\ nm}$	OD260/OD280
Control group	0.0310	0.0150	2.07
High-loaded F group	0.0273	0.0133	2.05
Dental fluorosis group	0.0309	0.0149	2.07

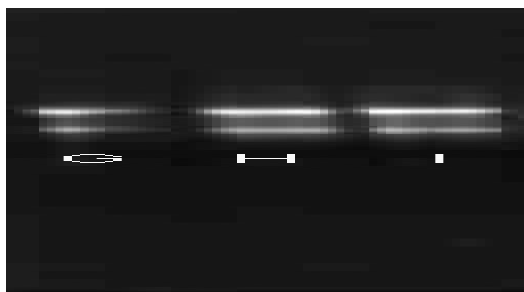

Figure 1. Agarose gel electrophoresis results of total RNA. From left to right are the total RNA band of the control, the high-loaded F, and the dental fluorosis group, respectively.

Gene expression analysis between two groups: As shown in Table 2, compared with control group, a total of 1057 genes were differentially expressed in the high-loaded F group. Of these, 148 were robustly up-regulated and 61 robustly down-regulated. In contrast, a total of 964 genes were differentially expressed in the dental fluorosis group as compared with control group, including 71 robustly up-regulated genes and 60 robustly down-regulated genes. Compared with the high-loaded F group, 633 genes were differentially expressed in the dental fluorosis group. Of these, the number of robustly up-regulated genes and robustly down-regulated genes were 15 and 67, respectively.

Table 2. Genes differentially expressed between two groups

	High-loaded F/control	Dental fluorosis/control	Dental fluorosis/ high-loaded F
Up-regulated genes	570	514	268
Down-regulated genes	487	450	365
Robustly up-regulated genes	148	71	15
Robustly down-regulated genes	61	60	67

Functional taxonomy of robustly differentially-expressed genes: According to their molecular function, the robustly differentially expressed genes were placed into different categories. The robustly differentially expressed genes included mainly transcription factors, genes related to signal transduction, structure proteins, transport proteins, cancer genes, genes related to immunity, and genes related to apoptosis in the high-loaded F group versus the control group. Some of these genes are listed in Table 3.

Table 3. A portion of robustly differentially-expressed genes in high-loaded F group versus control group

Category	UniGene ID	Gene Name	SLR
Transcription factors	Hs.23853	FLJ35036	1.2
	Hs.435949	ZNFN1A1	1.4
	Hs.460889	MAFF	1.5
Genes related to signal transduction	Hs.96	PMAIP1	1.5
	Hs.91662	KIAA888	1.4
	Hs.73793	VEGF	1.3
Structure proteins	Hs.434961	SCA1	1.0
	Hs.413045	CAP350	1.2
	Hs.412022	ABHD2	1.1
Transport proteins	Hs.434961	SCA1	1.0
	Hs.296323	SGK	1.4
	Hs.24485	CSPG6	1.3
Cancer genes	Hs.88297	STK17B	1.1
	Hs.73793	VEGF	1.3
	Hs.408528	RB1	1.7
Genes related to immunity	Hs.2050	PTX3	2.9
	Hs.179657	PLAUR	1.6
	Hs.89690	CXCL3	1.1
Genes related to apoptosis	Hs.2050	PTX	2.9
	Hs.242271	HHL	1.2
	Hs.20315	IFIT1	1.1

Again, the genes robustly differentially expressed included mainly genes related to immunity, transcription factors, genes related to signal transduction, structure proteins, and transport proteins in the dental fluorosis group versus the control group. Some of these genes are listed in Table 4.

Table 4. A portion of robustly differentially-expressed genes in dental fluorosis group versus control group

Category	UniGene ID	Gene Name	SLR
Genes related to immunity	Hs.449631	HBG1	-2.2
	Hs.198301	TBX6	-2.1
	Hs.87149	ITGB3	-3.3
Transcription factors	Hs.446532	LDC150759	-1.2
	Hs.449592	IGLJ3	-1.1
	Hs.500367	SPAG9	1.7
Genes related to signal transduction	Hs.310194	SNX16	4.6
	Hs.109438	KCTD12	1.3
	Hs.239176	IGF1R	1.1
Structure proteins	Hs.189829	EIF2C3	1.3
	Hs.306831	PTGDR	1.1
	Hs.436836	MX1	1.0
Transport proteins	Hs.276506	FYB	1.9
	Hs.73793	VEGF	1.4
	Hs.189829	EIF2C3	1.3

Likewise, the genes robustly differentially expressed mainly included genes related to immunity, transcription factors, genes related to signal transduction and structure proteins in the dental fluorosis group versus the high-loaded F group. Some of these genes are listed in Table 5.

Table 5. A portion of robustly differentially-expressed genes in dental fluorosis group versus high-loaded F group

Category	UniGene ID	Gene Name	SLR
Genes related to immunity	Hs.624	IL8	-2.1
	Hs.82120	NR4A2	-2.1
	Hs.79197	CD83	-1.4
Transcription factors	Hs.282204	NSBP1	-3.9
	Hs.326035	EGR1	-1.5
	Hs.306802	HCRP1	-1.8
Genes related to signal transduction	Hs.368178	RHAG	-3.9
	Hs.126256	IL1B	-3.1
	Hs.82120	NR4A2	-1.6
Structure proteins	Hs.460	AIF3	-1.5
	Hs.153138	ORC5L	-1.8
	Hs.177486	APP	-1.1

DISCUSSION

Gene microarray is a technology by which a large number of target genes are sequence arrayed in high density onto a vector made from glass or silicon slides. Compared to conventional technology of nucleic acid blot hybridization, it has many virtues such as high effectiveness, high comparability, and ease for automated operation. But in practice, technical requirements for gene microarray are very strict. To obtain reliable results, rigorous and uniform standard procedures must be carried out to decrease false positive and false negative rates. Therefore,

to ensure reliability of the results, this study used the oligonucleotide gene chip manufactured by Affymetrix Company, which is considered internationally to be of excellent quality and uniformity of standardization. Directions for the standardized sample preparation, gene chip examination conditions, and data analysis systems were provided by Affymetrix Company to screen genes related to dental fluorosis.

Previous investigations of the molecular mechanisms involved in fluorosis showed that genes related to signal transduction (G protein, MEK1, MEK2, ERK1, p38, JNK), transcription factors (NF-kappaB, CREB), genes related to immunity (IL6, IL8), cancer genes, and genes related to apoptosis (c-fos, c-jun, bax, bcl-2, p53) may be associated with the occurrence and development of fluorosis.[7,9-16] The present study showed that differentially expressed genes also include structure and transport proteins in addition to those kinds of genes previously identified.

Lau et al.[17] reported that mitogenic concentrations (50–100 mmol/L) of AlF_4^- could increase mRNA and protein levels of IGF-2 and stimulatory IGFBP-5 but either reduced slightly or had no effect on the mRNA and protein levels of the inhibitory IGFBP-4. Conversely, similar mitogenic concentrations of NaF had no significant effects on the protein or mRNA levels of IGF2, IGFBP-4, or IGFBP-5.[17] However, the AlF_4^- complex can form in food, drinking water, and in the organism after administration of NaF.[18] Our gene chip examination revealed that the expression of IGF1R significantly increased in both the high-loaded F group and the dental fluorosis group compared with control group. Moreover, the IGFBP-7 expression was significantly up-regulated. These results further demonstrated that the insulin-like growth factor (IGF) regulatory system may play an important role in the process of fluorosis.

Many reports indicate that F can induce IL6 and IL8 expression.[15-16,19-20] In the present work, up-regulated expression was observed for IL6 and IL8 in the high-loaded F group. However, this expression was not up-regulated in the dental fluorosis group when compared to the control group. In addition, significant up-regulations were observed for cytokines such as IL1B, IL1RN, and TNF in the high-loaded F group compared with both the control and the dental fluorosis group. These results imply that these cytokines may be related to the observed tolerance or resistance to fluorosis.

The genes related to dental fluorosis as screened through gene chip technology only provide clues for further intensive investigation of the molecular mechanisms underlying fluorosis. The significance of differential expression of these genes also needs further investigation.

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REFERENCES

1 Chakma T, Vinay Rao P, Singh SB, Tiwary RS. Endemic genu valgum and other bone deformities in two villages of Mandla District in central India. Fluoride 2000;33:187-95.
2 Shivashankara AR, Shankara YMS, Rao SH, Bhat PG. A clinical and biochemical study of chronic fluoride toxicity in children of Kheru Thanda of Gulbarga District, Karnataka, India. Fluoride 2000;33:66-73.
3 Xiang Q, Liang Y, Chen L, Wang C, Chen B, Chen X, et al. Effect of fluoride in drinking water on children's intelligence. Fluoride 2003;36:84-94.
4 Zhang WL, Cui YN, Gao S, Zhang XY, Li GS. Expression of proto- oncogenes c-fos and c-jun in osteoblasts activated by excessive fluoride. Chin J Prev Med 2003;37:246-50 [in Chinese].
5 Chen L, Tong A, Yu D, Chen R. Effects of fluoride on the expression of c-fos and c-jun genes and cell proliferation of rat osteoblasts. Chin J Prev Med 2000;34:327-9 [in Chinese].
6 Machalinski B, Baskiewicz-Masiuk M, Sadowska B, Machalinska A, Marchlewicz M, Wiszniewska B, et al. The influence of sodium fluoride and sodium hexafluorosilicate on human leukemic cell lines. Fluoride 2003;36:231-40.
7 Wang AG, Chu Q, He WH, Xia T, Liu JL, Zhang M, et al. Effects on protein and mRNA expression levels of p53 induced by fluoride in human embryonic hepatocytes. Toxic Lett 2005;158:158-63.
8 Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nature Genetics 2002; Suppl 32:490-5.
9 Hattori Y, Matsuda N, Sato A, Watanuki S, Tomioka H, Kawasaki H, et al. Predominant contribution of the G protein-mediated mechanism to NaF-induced vascular contractions in diabetic rats: association with an increased level of G(qalpha) expression. Pharmacol Exp Ther 2000;292:761-8.
10 Misra UK, Gawdi G, Pizzo SV. Beryllium fluoride-induced cell proliferation: a process requiring P21(ras)-dependent activated signal transduction and NF-kappaB-dependent gene regulation. J Leukoc Biol 2002;71:487-94.
11 Xu S, Khoo S, Dang A, Witt S, Do V, Zhen E, et al. Differential regulation of Mitogen-activated protein/ERK kinase (MEK)1 and MEK2 and activation by a Ras-independent mechanism. Mol Endocrinol 1997; 11:1618-25.
12 Refsnes M, Schwarze PE, Holme JA, Lag M. Fluoride-induced apoptosis in human epithelial lung cells (A549 cells): role of different G protein-linked signal systems. Hum Exp Toxicol 2003;22:111-23.
13 Thrane EV, Refsnes M, Thoresen GH, Lag M, Schwarze PE. Fluoride-induced apoptosis in epithelial lung cells involves activation of MAP kinases p38 and possibly JNK. Toxicol Sci 2001;61:83-91.
14 Xu H, Zhang JM, Zhang XY, Li GS. Differential expression of osteopontin and bax, bcl-2 by fluoride. Chin J Prev Med 2005;39:107-10 [in Chinese].
15 Hilger RA, Koller M, Konig W. Inhibition of leukotriene formation and IL-8 release by the paf-receptor antagonist SM-12502. Inflammation 1996;20:57-70.
16 Akashi M, Loussararian AH, Adelman DC, Saito M, Koeffler HP. Role of lymphotoxin in expression of interleukin 6 in human fibroblasts. Stimulation and regulation. J Clin Invest 1990;85:121-9.
17 Lau KH, Goodwin C, Arias M, Mohan S, Baylink DJ. Bone cell mitogenic action of fluoroaluminate and aluminum fluoride but not that of sodium fluoride involves upregulation of the insulin-like growth factor system. Bone 2000;30:705-11.
18 Susa M. Heterotrimeric G proteins as fluoride targets in bone [review]. Int J Mol Med 1999;3:115-26.
19 Refsnes M, Becher R, Lag M, Skuland T, Schwarze PE. Fluoride-induced interleukin-6 and interleukin-8 synthesis in human epithelial lung cells. Human Exp Toxicol 1999;18:645-52.
20 Refsnes M, Thrane EV, Lag M, Hege Thoresen G, Schwarze PE. Mechanisms in fluoride-induced interleukin-8 synthesis in human lung epithelial cells. Toxicology 2001;167:145-58.

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