Screen and analysis of key disease genes for precancerous lesions of oral buccal mucosa induced by DMBA in golden hamsters

DAN CHEN, KAI YANG, GUODONG ZHANG, JIE MEI and LI XIANG

Department of Oral and Maxillofacial Surgery, the First Affiliated Hospital, Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. 7,12-Dimethylbenz(a)-anthracene (DMBA)-induced oral buccal mucosa squamous cell carcinoma in Syrian golden hamsters was used to establish precancerous lesions. Agilent rat whole-genome microarray and biological information analysis were used to screen for genes related to key diseases during the transformation of normal buccal mucosa to precancerous lesions in golden hamsters. DMBA acetone solution (0.5%) was used to establish a model of precancerous lesions in oral buccal mucosa in golden hamsters. The results showed that a total of 1331 genes were differentially expressed, including 1278 known, 53 unknown, 747 up-regulated and 584 down-regulated genes. Analysis revealed a total of 14 gene interaction pathways that significantly associated with the 1278 known differentially expressed genes ($P<0.05$). In conclusion, the occurrence of precancerous lesions in the oral buccal mucosa of golden hamsters was caused by a number of genetic changes that resulted in changes to their respective pathways. Key candidate genes for the formation of precancerous lesions in oral buccal mucosa included *Cyp2b13*, *Orc1L*, *casp8*, *CCL5*, *CXCL12*, *CCL20*, *Serping1*, P518/*Qrfp*, *F5*, *TFPI*, *Vcam1*, *Fn1*, *Angpt2*, *Lcp2*, *Cxadr*, *Lyn*, *Hck*, *Btk*, RGD1564385/*fes*, *Vav1* and *IL5ra*.

Introduction

Oral cancer accounts for approximately 2% of systemic malignant tumors, of which 90% are squamous cell carcinomas (1). Buccal cancer is one of the most common oral cancers. Although great progress has been made in a variety of oral cancer treatment methods, the 5-year survival rate for buccal and oral cancer following treatment is only 55-60% (1,2). Previous studies confirmed that oral cancer is a complex, multiphase, multi-step pathological process involving multiple genetic changes (3,4), where a number of genes play various roles at different stages of cancer development. The majority (80%) of oral cancers originate from precancerous lesions (5). At present, no effective treatment method for the prevention of the occurrence of precancerous lesions exists. If effective intervention to prevent the occurrence of precancerous lesions at the molecular level is achieved, the early and effective prevention of oral cancer may also be achieved.

The development of microarray technology allows for whole-genome microarray technology to provide a comprehensive understanding of genetic changes during carcinogenesis and screen cancer-related genes to determine cellular gene expression profiles. Microarray technology is currently used in studying oral tumor markers (6,7), tumor molecular typing (8,9), drug screening (10) and the comparative analysis of discrepancies in gene expression patterns between cancer tissues and adjacent or normal tissue (11,12). However, no reports are currently available regarding the analysis of disease-related genes during the transformation of normal buccal mucosa to precancerous lesions on a whole-genome level.

An experimental animal model of 7,12-dimethylbenz(a)-anthracene (DMBA)-induced oral buccal mucosa squamous cell carcinoma in Syrian golden hamsters was first reported in 1954 by Salley (13). The animal model was later shown to exhibit carcinogenesis, growth characteristics and biological behavior similar to that of human oral mucosal epithelial cells (14,15). Thus, it is considered to be an ideal animal model for studying oral mucosal carcinogenesis.

In this study, the DMBA-induced oral buccal mucosa squamous cell carcinoma in Syrian golden hamsters was used to establish precancerous lesions. Agilent whole-genome microarray containing 41,000 genes/EST sequences and bioinformatics analysis were used to screen and analyze precancerous disease-related genes during the transformation of normal buccal mucosa to precancerous lesions in order to identify cellular gene expression profiles. This procedure may provide data and methodology for the exploration of the molecular mechanisms underlying precancerous lesions and their prevention.

Materials and methods

Main reagents and instruments. The following reagents and instruments were utilized: DMBA (Sigma Corporation, USA),

Correspondence to: Dr Kai Yang, Department of Oral and Maxillofacial Surgery, the First Affiliated Hospital, Chongqing Medical University, Chongqing 400016, P.R. China
E-mail: cqfyyk@hotmail.com

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20 Syrian golden hamsters (Chengdu Institute of Biological Products, China), PCR instrument (PTC-100, MJ Company), hybrid furnace (G-2545A, Agilent Technologies, Palo Alto, CA, USA), agilent scanner (G2565AA, Agilent Technologies), single-labeled agilent rat cDNA microarray (Agilent Technologies), Cy3 NHS ester (GE Healthcare, PA13105), aaUTP (Ambion, AM8436), low RNA input linear amplication kit (Agilent, 5184-3523), gene expression hybridization kit (Agilent, 5188-5242), gene expression wash buffer kit (Agilent, 5188-5327), stabilization and drying solution (Agilent, 5185-5979), gasket slide (Agilent, G2534-60003), hybridization chamber (Agilent, G2534A), RNeasy mini kit (Qiagen, 74106) and RT-PCR kit (Bioflux). The animals and experimental procedures were approved by the Management Committee of Laboratory Animals Use, Institute of Laboratory Animal, Chongqing Medical University, China.

Establishment of hamster model with cheek pouch precancerous lesions. A total of 20 Syrian golden hamsters (10 males and 10 females; weight, 90-120 g; age, 6-8 weeks) were randomly divided into 2 groups (10 per group). Group I was considered the normal control group and group II the experimental group. The hamsters in group II were fixed on a self-made mouse shelf and the cheek pouches were opened. The bilateral hamster cheek pouches were painted with 0.5% DMBA acetone solution using a No. 1 oil pen every Monday, Wednesday and Friday afternoon for 6 weeks. The animals were then starved for 2 h. Group I was not treated. The animals were sacrificed in the 6th week and cheek pouch tissue samples were removed. Half of the tissue was immediately placed in liquid nitrogen for preservation and the remaining half was fixed using a 10% formalin solution. The fixed tissue samples were then embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and mounted. The pathological examination was performed via observation using light microscopy.

Diagnostic criteria for precancerous lesions. The precancerous lesions were diagnosed according to the 12 criteria set by the World Health Organization (WHO) (16). These criteria included: i) the disappearance of basal cell polarity, ii) stratified-like changes in basal cells, iii) increased ratio of nucleus to cytoplasm, iv) drop-shaped epithelial nail protrusion, v) disorder of the epithelial layer, vi) increased and abnormal mitosis, vii) increased mitosis (1/2 epithelium undergoing mitosis), viii) pleomorphic cells, ix) hyperchromatic nuclei, x) enlarged nucleolus, xi) diminished or lack of intercellular adhesion and xii) dyskeratosis. The diagnostic classification was based on the observation of the aforementioned criteria: 1-2 criteria, mild dysplasia; 3-4, moderate epithelial dysplasia; and ≥5, severe dysplasia or carcinoma *in situ*. In this study, moderate dysplasial tissues were used for the experiments.

Extraction and purification of RNA from normal hamster cheek pouch mucosa and precancerous lesion samples. Total RNA from samples from groups I and II was extracted using the TRIzol extraction kit (Invitrogen, Carlsbad, CA, USA). The Qiagen RNeasy kit was used to purify total RNA, according to the manufacturer's instructions. Equivalent amounts of total RNA from groups I and II were dissolved in Milli-Q water and preserved at -80˚C.

cRNA labeling and synthesis. Extracted RNA samples were single-labeled with Cy3 using reverse transcription. The concrete steps were: 2 μg of RNA from the samples obtained from the 2 groups was added to two test tubes (1.5 ml) and 5 μl of primer was added. RNase-free water was added for a total volume of 11.5 μl and the contents were combined. The solution was incubated in a 60˚C water bath for 10 min and cooled in ice water for 5 min. Then, 2 μl DDT (100 mmol/l), 1 μl dNTP, 4 μl 5 first strand buffer, 0.5 μl RNaseOUT and 1 μl MMLV RT were added to the RNA samples and combined. cRNA was synthesized in a 2-h reaction performed at 40˚C, with a thermal cover heated to 65˚C. The synthetic cRNA was labeled using aaUTP and purified using the Qiagen RNeasy mini kit. The cRNA concentration was measured using a spectrophotometer. The cRNA was then labeled and repurified.

cRNA fragmentation and microarray hybridization. Approximately 875 ng of Cy3-labeled cRNA extracted from the normal mucosa and precancerous lesions was added to 11 μl blocking agent and 2.2 μl fragmentation buffer. Nuclease-free water was added to make the total volume 55 μl. The mixture was incubated at 60˚C for 30 min for fragmentation, following which 55 μl GEX hybridization buffer was added. Then, 100 μl of the above solution was added to the microarray for rolling hybridization at 65˚C for 17 h at a speed of 10 rpm. The microarray was removed and washed in lotion 1 for 1 min and then washed with lotion 2 for 1 min at 37˚C.

Microarray scanning and data processing. Microarray hybridization results were scanned using an Agilent G2565AA fluorescence scanner and read by feature extraction software. The scanner resolution was 5 μm, and the photomultiplier tubes were set to 100 and 10% for 2 scans. The scan results were automatically merged by Agilent software and feature extraction was used for uniform treatment. The homogenization coefficient in this experiment was 0.853. The ratio of average signal intensity and average background of the microarray was >3, which was consistent with the manufacturer's standards.

Bioinformatics analysis. Ratios of ≥2 and ≤0.5 were the threshold values for screening differentially expressed genes. Differentially expressed genes underwent functional classification according to the Gene Ontology (GO) classification standard. The databases KECG, GENMAPP and BIOCARTA (www.biorag.com) were used for signal pathway analysis and identification of the signal pathways for abnormally expressed genes. $P<0.05$ was used both to determine the significance of pathways and screen for target pathways.

Verification of microarray results by RT-PCR. From the microarray results, the Atp6v0d2 up-regulation and Sfrp2 down-regulation were randomly selected for verification by RT-PCR. In accordance with the instructions, the TRIzol kit (Invitrogen) was used for total RNA extraction from samples from groups I and II. RNA (1 μg) was reverse transcribed to cDNA in a 20 μl reaction using a thermocycler. The process involved 3 min pre-denaturation at 94˚C; 30 cycles of 1 min denaturation at 94˚C, 30 sec annealing at 52˚C and 1 min extension at 72˚C; and after 10 min at 72˚C it was terminated. The PCR primers were designed by Primer 3.0 (Table I).

Table I. Primers for RT-PCR used to verify the microarray results.

Gene name	Gene library number	Primer sequences	Length of product (bp)
Atp6v0d2	NM_001011972	F: 5'-CGAGGGTGCAAAGCCAGCCT-3' R: 5'-AGCCGCAGTCCCTCCGGATA-3'	210
Sfrp2	NM_001100700.1	F: 5'-CTCCTGCCGCCCACAGAGGA-3' R: 5'-GATGCTGCGGGAGATGCGCT-3'	180
β-actin	NM_001101.2	F: 5'-CCCGCCACCAGTTCGCCAT-3' R: 5'-TGTGGGTGACCCCGTCTCCG-3'	240

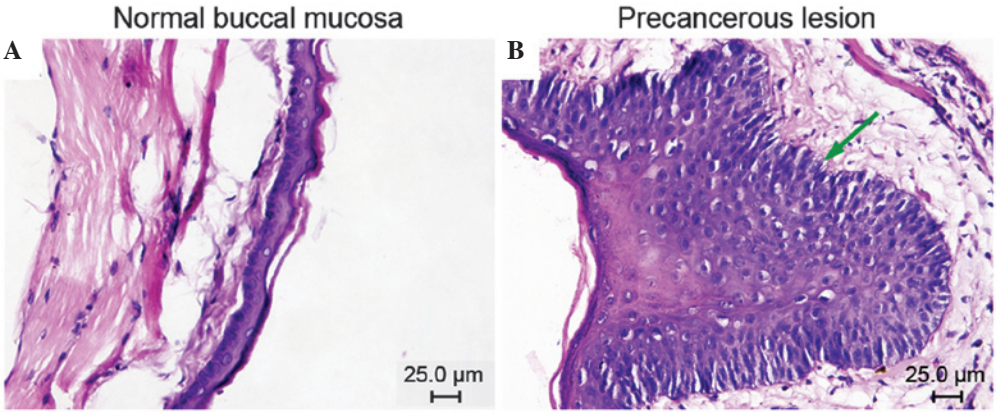

Figure 1 Pathological examination under light microscopy (H&E; magnification, x 400). (A) Normal buccal mucosa and (B) a precancerous lesion (moderate dysplasia). Light microscopy shows drop-shaped epithelial nail protrusion, clearly stratified-like changes in basal cells, the basement membrane is clearly visible, pleomorphic changes in cells and that a section of the nucleus to cytoplasm ratio is increased (arrow).

Results

Conventional hematoxylin and eosin staining of normal buccal mucosa and precancerous lesions. Group I comprised normal buccal mucosa. Cases in group II were classified according to the WHO criteria for precancerous lesions: 3 showed mild dysplasia and 7 moderate dysplasia (Fig. 1).

Microarray hybridization results and analysis of biological data

Screen of differentially expressed genes in normal buccal mucosa and precancerous lesions. A total of 1331 genes, including 1278 known, 53 unknown, 747 up-regulated and 584 down-regulated genes, were found to be differentially expressed during the transformation of normal buccal mucosa to precancerous lesions in golden hamsters.

Gene Ontology functional classification. A total of 1331 differentially expressed genes were divided into 8 major functional groups according to the GO classification criteria (Table II).

Pathway analysis. Pathway analysis revealed a total of 14 gene interaction pathways significantly associated with the 1278 known differentially expressed genes ($P<0.05$). Only 21 genes in the 1278 differentially expressed genes enhanced the 14 pathways (Table III).

RT-PCR verification results. Atp6v0d2 up-regulation and Sfrp2 down-regulation were verified using RT-PCR (Fig. 2). The optical density values of the PCR bands were analyzed and compared with β-actin. Expression of Atp6v0d2 and Sfrp2 in cheek pouch mucosa precancerous lesions was 6.21 and 0.231 times, respectively, that of the normal cheek pouch mucosa, which was consistent with the microarray results (Table IV).

Discussion

This study identified 1331 genes that were differentially expressed during the transformation of normal buccal mucosa to precancerous lesions in golden hamsters. Additionally, the genes were found to act in 14 different pathways, suggesting that the occurrence of precancerous oral buccal mucosa lesions in golden hamsters is a complicated pathological process that involves a number of genetic changes that affect numerous pathways. GO analysis revealed that the differentially expressed genes can be divided into 8 functional groups, in which 30.78% of the genes identified are involved in the regulation

Table II. Gene Ontology classification for differentially expressed genes.

Gene Ontology functional classification	No. of up-regulated genes	No. of down-regulated genes	Total no.	Percentage
Genes related to regulation of cell physiological processes	239	187	426	30.78
Genes related to cell structure	232	181	413	29.84
Genes related to molecular location	87	68	155	11.20
Genes related to macromolecule metabolism	61	48	109	7.88
Genes related to immune regulation	58	46	104	7.51
Genes related to signal transduction	38	30	68	4.91
Genes related to material transfer	31	25	56	4.04
Unkown genes	36	17	53	3.83

Each gene may play a variety of roles.

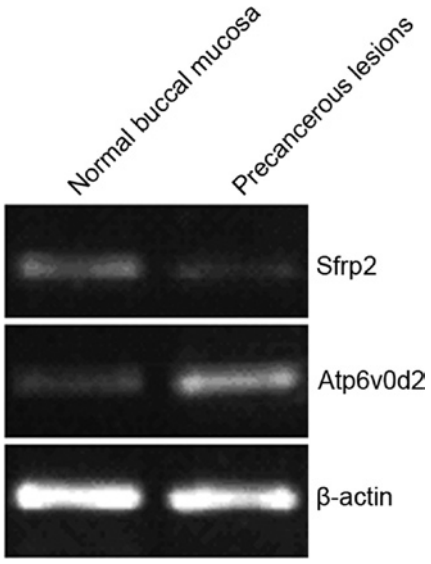

Figure 2. Verification of RT-PCR results.

of cell physiological processes and 29.84% are related to cell structure. Together, these two functional groups account for 60.62% of the 1331 genes identified, indicating that these genes are crucial in the formation of oral precancerous lesions.

Pathway analysis identified 14 gene interaction pathways that were significantly correlated to the 1278 known differentially expressed genes. Only 21 of the 1278 differentially expressed genes were enhanced in the 14 pathways. Since pathways express interactions between proteins, we suggest that the following candidate genes are key in the development of precancerous oral buccal mucosal lesions: *Cyp2b13*, *Orc1L*, *casp8*, *CCL5*, *CXCL12*, *CCL20*, *Serping1*, P518/*Qrfp*, *F5*, *TFPI*, *Vcam1*, *Fn1*, *Angpt2*, *Lcp2*, *Cxadr*, *Lyn*, *Hck*, *Btk*, RGD1564385/*fes*, *Vav1* and *IL5ra*.

In the 14 pathways, caspase-8 (casp8) is formed from pro-casp8 following pro-casp8 activation in the apoptosis pathway. The caspase family of proteases is a group of aspartate-specific hydrolases containing cysteine. Following cascade activation of the caspases, the Fas receptor is activated and induces apoptosis, for which casp8 activation is the first step in cascade activation (17,18). In this study, casp8 was down-regulated, resulting in cell apoptosis.

The association of licensing factors with the pre-replicative complex pathway was primarily correlated to the regulation of DNA replication. DNA synthesis is required to induce a post-replicative state in the cell. Origin recognition complex (Orc) is a protein complex involved in the replication of eukaryotic DNA that regulates cell proliferation and the cell cycle (19). Orc1 is a key member of this protein complex (20) and is a crucial factor in the cell cycle progression from the G0 to G1/S phase. In this study, Orc1L was up-regulated in two pathways and promoted cell proliferation.

Chemokine receptors bind chemokines, and rhodopsin-like receptor pathways are subordinate branches of the G protein-coupled receptor (GPCR) signaling pathway. GPCRs are members of the seven-pass transmembrane domain receptor superfamily. GPCRs, combined with chemokines, activate PKC kinases and Ras and Rho family members. Moreover, these receptors play a key role in cell growth, adhesion and directional migration. Previous studies showed that a high expression of chemokines CCL5 and CXCL12 promotes cell proliferation and cancer cell metastasis (21,22). CCL5 and CXCL12 were up-regulated in the three preceding pathways (signaling by GPCR, chemokine receptors bind chemokines and rhodopsin-like receptor pathways) in this study. CCL20 is known to be involved in the directional migration of dendritic and T cells. CCL20 promotes the chemotaxis of immature dendritic cells to tumor areas, activates T cells and triggers an immune response, thus inhibiting tumor growth (23). Previous studies showed that CCL20 is highly expressed in tumors (24). However, in this study, CCL20 in precancerous lesions was expressed at low levels in the three preceding pathways. This low expression may have led to a decreased local immunity, thereby promoting lesion development. P518/*Qrfp* is an endogenous GPCR ligand and its involvement in tumor development has not previously been reported.

Formation of the fibrin clot is a branch of the hemostasis pathway (25). *F5* encodes coagulation factor V and the *Serping1* gene encodes the C1 inhibitor. *TPFI* inhibits the extrinsic coagulation process and activation of FIX by the FVIIa tissue factor complex. In this pathway, F5 and Serping1 expression were up-regulated and TFPI expression was down-regulated,

Table III. Pathway analysis.

Pathway	P-value	Gene symbol
Caspase-8 is formed from pro-caspase-8	0.0366	Up: 0 Down: Casp8
Activation of pro-caspase-8	0.0366	Up: 0 Down: Casp8
Switching of origins to a post-replicative state	0.0364	Up: *Orc1L* Down: 0
Association of licensing factors with the pre-replicative complex	0.0366	Up: *Orc1L* Down: 0
Signaling by G protein-coupled receptor	0.0224	Up: *CCL5*; *CXCL12* Down: *CCL20*; P518/*Qrfp*
Class A/1(rhodopsin-like receptors)	0.0224	Up: *CCL5*; *CXCL12* Down: *CCL20*; P518/*Qrfp*
Chemokine receptors bind chemokines	0.0106	Up: *CCL5*; *CXCL12* Down: *CCL20*
Formation of fibrin clot	0.0158	Up: *F5*; *Serping1* Down: *TFPI*
Integrin cell surface interactions	0.0321	Up: *Vcam1*; *Fn1* Down: 0
Signaling in immune system	0.0447	Up: *Angpt2*; *Lcp2*; *Vcam1* Down: *Cxadr*
Arachidonic acid metabolism	0.0120	Up: 0 Down: *Cyp2b13*
Metabolism of xenobiotics by cytochrome P450	0.0400	Up:0 Down: *Cyp2b13*
IL5	0.0313	Up: *Lyn*; *IL5ra*; *Hck*; *Btk* Down: 0
IL6	0.0302	Up: RGD1564385 (fes); *Btk*; *Vav1*; *Lyn*; *Hck* Down: 0

Up, up-regulated; Down, down-regulated.

Table IV. Comparison of RT-PCR results with microarray results.

Gene name	Gene mRNA/β-actin mRNA (mean ± SD)		B/A ratio	Microarray ratio
	A	B		
Atp6v0d2	0.1292±0.0023	0.8023±0.0143	6.21	6.70
Sfrp2	0.4201±0.0034	0.09702±0.0007	0.231	-4.65

A, normal buccal mucosa; B, mucosa with precancerous lesions; SD, standard deviation.

suggesting that the main characteristics of the vascular changes in precancerous lesions enhanced coagulation and inhibited anticoagulation.

The integrin cell surface pathway primarily mediates adhesion between cells and the extracellular matrix (ECM). Vcaml is a member of the immunoglobulin superfamily and high expression levels of Vcam1 enhance endothelial cell migration and promote angiogenesis (26). High expression levels of Fn, a macromolecular glycoprotein with a double-stranded structure (27), increase cell-cell and cell-ECM adhesion. In

the integrin pathway, Vcam1 and Fn1 are highly expressed, enhancing the adhesion between cells and the ECM, thereby promoting angiogenesis.

We identified four abnormally expressed genes (*Angpt2*, *Lcp2*, *Vcam1* and *Cxadr*) that were enhanced in signaling pathways involved in the immune system. A high expression of Angpt2 and Vcam1 promotes angiogenesis (26,28). Lcp2 plays a key role in T cell activation; however, its role in tumor formation has not previously been reported. Cxadr is a cell adhesion molecule and its decreased expression weakens intercellular adhesion (29). During precancerous lesion formation, we observed that *Angpt2*, *Lcp2* and *Vcam1* were up-regulated, whereas *Cxadr* was down-regulated. The combined effect of the three genes may promote angiogenesis.

The arachidonic acid (AA) and cytochrome P450 (CYP450)-dependent xenobiotic metabolic pathways are subordinate branches of biological oxidation pathways. AA is an essential fatty acid for the human body and previous studies have shown that abnormal AA in the cyclooxygenase and lipoxygenase metabolic pathways is associated with tumor occurrence and development (30,31). CYP450 is a group of isoenzymes with a similar structure and function encoded by a superfamily of genes. CYP450 plays a key role in the metabolism of endogenous and external carcinogens, chemical poisons and toxins. CYP450 induces genetic mutations or inhibits gene expression (32). Cyp2b13 is a member of a second CYP450 family and its expression in the two pathways was down-regulated. This down-regulation may lead to the decreased metabolism of exogenous toxins such as DMBA, and promote the formation of precancerous lesions.

The IL5 and IL6 pathways are correlated to the regulation of intracellular protein tyrosine kinase (PTK) activity. PTKs are crucial for cell signal transduction and the regulation of cell growth, proliferation and differentiation (33). Lyn, Btk, Hck and Fes are members of the non-receptor-type PTK family. A high expression of Lyn, Btk, Hck and Fes results in cell proliferation, anti-apoptosis and the promotion of angiogenesis (34-36). Up-regulation of Vav1 and IL5ra is correlated to tyrosine phosphorylation and the activation of signal transduction. Up-regulation of Lyn, IL5ra, Hck, Btk, RGD1564385 (fes) and Vav1 in the pathway enhances tyrosine kinase activity, thereby promoting cell proliferation and angiogenesis, and inhibiting apoptosis.

This study showed that precancerous buccal mucosal lesions in hamsters not only involved the abnormal expression of a number of genes, but that the identified differentially expressed genes induce the occurrence of precancerous lesions through various pathways. The overall mechanism involved in the formation of hamster buccal mucosal precancerous lesions requires that the chemical carcinogen DMBA act on the oral buccal mucosa. This activity results in changes in gene expression including that of Cyp2b13. Subsequently, DMBA metabolism (with cell and genetic toxicity) is reduced through the metabolism of xenobiotics by the CYP450 and AA metabolic pathways. The dual role of cell metabolism disorders and DMBA toxicity likely results in changes in the aforementioned genes and pathways, including activation of DNA replication (Orc1L up-regulation) and inhibition of the cell apoptosis pathway (casp8 down-regulation). The gene expression changes induced cell proliferation, angiogenesis, apoptosis inhibition, cell cycle regulation disorders and decreased local immunity, resulting in the occurrence and development of precancerous lesions. Therefore, we hypothesize that *Cyp2b13*, *Orc1L*, casp8 and the remaining 21 genes are candidates in the development of oral precancerous lesions. Inhibitors that regulate these genes or pathways effectively may improve treatment methods and chemoprophylaxis of precancerous lesions.

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