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ORIGINAL ARTICLE

Genetic expression signatures of oral submucous fibrosis and oral cancer—A preliminary microarray report

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Abstract *Background/purpose:* Oral submucous fibrosis (OSF) is a potentially malignant disorder of oral squamous cell carcinoma (SCC). In this study, we obtained the genetic expression signatures of OSF and SCC by microarray analysis.
Materials and methods: Five patients with clinically evident OSF, five patients with SCC who also had existing OSF, and four normal volunteers who did not have a history of chewing betel quids were recruited. Biopsy specimens were obtained with an approved Institutional Review Board protocol. Total RNA from OSF or SCC was isolated and hybridized to a Human Oligo 1A (V2) Microarray (G4110B) chip against normal control RNA that was pooled from the four healthy volunteers.
Results: We found similar, but distinct genetic expression signatures for OSF and SCC. At the hierarchical clustering analysis, 24 known genes (23 upregulated and 1 downregulated) in OSF were differentially expressed consistently in all participants. Among the genes, *XRCC5* was cloned and transfected into oral cancer GNM cells. The results demonstrated that the overexpression of *XRCC5* increased the resistance of GNM cells to low-density X-ray irradiation and promoted the cell growth rate.
Conclusion: The distinct but similar genetic expression signatures seen in OSF and SCC suggested that this expression may be used as a supplemental diagnostic tool in pathology practice. This preliminary study showed that the *XRCC5* gene promoted GNM cell growth and conferred resistance to low-density X-ray irradiation. Further studies on the effect of *XRCC5* in oral cancer cells are in progress.

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Introduction

Oral submucous fibrosis (OSF) is a progressively scarring chronic disease of the oral mucosa. It is characterized by increasing mucosal rigidity that is caused by the proliferation of fibroelastic tissue and the deposition of dense fibrous connective tissue in the superficial submucosa resulting from increased collagen synthesis,[1,2] decreased collagenase activity,[3] or both. Oral submucous fibrosis is frequently associated with the habitual chewing of betel quid, which is a prevalent habit in Southeast Asia.[4] The disease affects 0.2—1.2% of the urban population that visits dental clinics in India.[5] A recent study on OSF showed that, of the 2 million people in Taiwan who habitually chew betel quid, 85.4% will develop OSF[6] and one-third of these patients may eventually develop squamous cell carcinoma (SCC). Therefore, OSF is a potentially malignant disorder of the oral mucosa that may develop into oral squamous cell carcinoma (SCC).[1]

Despite its potential for malignant transformation, it is difficult to predict on the basis clinical and histopathologic examinations alone whether OSF will develop into a malignancy. Previous studies of OSF on the molecular mechanism of premalignant transformation of the oral mucosa have identified some molecules such as COX-2,[7] type I plasminogen activator inhibitor,[8] p53,[9,10] keratinocyte growth factor-1,[11] interleukin-6,[12] tissue inhibitor of metalloprotease 1,[13] and adenomatous polyposis coli.[10] However, these studies were insufficient in discriminating OSF from SCC.

This study used a microarray analysis to identify potential biomarkers that can predict the malignant transformation of OSF. The results support the hypothesis that every cancerous or precancerous lesion may possess a specific genetic expression signature that can be distinguished from others after hierarchical clustering analysis.

Materials and methods

Case recruitment

Oral mucosal specimens were collected, as described by other researchers, from five patients with clinically evident OSF.[14,15] Clinical signs and symptoms include trismus, marble-like pallor on the buccal mucosa and a progressive stiffness of subepithelial tissue, and a grayish white oral mucosa.[1] All patients came to the Oral Surgery Clinic at the Chung Shan Medical University Hospital (Taichung City, Taiwan) for diagnosis and treatment. A review of their social habits showed all patients were smokers and areca nut chewers. Specimens of SCC were also collected from five patients who were undergoing surgical removal of the tumors at the same hospital. All five patients had SCC in the presence of clinically evident OSF and were all smokers and areca nut chewers. Normal tissues were obtained from the posterior mucobuccal fold of four healthy volunteers who did not have any history of chewing betel quid and were undergoing mandibular third molar extraction. All patients and volunteers were male and between the age of 30 and 55 years. The study protocol was approved by the Institutional Review Board of the Chung Shan Medical University Hospital. All patients with OSF or SCC and the healthy volunteers were provided with research information and informed consent was obtained from everyone before the study. All specimens were immediately frozen in −80°C until further use. The RNA extracted from the four healthy volunteers was pooled as one normal control for all microarray hybridization test, which is described later.

RNA extraction

The RNA extraction, labeling, and microarray analysis were performed in cooperation with DigiGenomics Co., Ltd. (Taipei county, Taiwan). In brief, the collected tissue samples were pulverized into fine powder in liquid nitrogen-filled mortar. They were then homogenized in TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA). The total RNA was extracted in accordance with the manufacturer's protocol. To remove residual genomic DNA, 50 μg or less of total RNA was treated with Ambion RNase-free DNase I (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. The enzyme was then removed by phenol/chloroform extraction. The RNA was recovered by ethanol precipitation. The integrity of RNA was checked in 0.9% agarose run in tris-acetate-EDTA (TAE) buffer to ensure that the RNA was of good quality for subsequent labeling steps.

Labeling and hybridization

The total RNA (1 μg) from each tissue sample was reverse transcribed into cDNA, further transcribed *in vitro* into cRNA, and labeled with CyDye by using the MessageAmp aRNA kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. The cRNA obtained from the RNA sample of the normal tissue was labeled with Cy3 and was the reference sample. The cRNA obtained from the RNA sample of the diseased tissue was labeled with Cy5 and was the experimental sample. The labeled cRNA of the reference and experimental sample was purified to remove uncoupled CyDyes, combined in equal amounts, and mixed with 2× hybridization buffer (based on the manufacturer's protocol) before hybridization onto Human Oligo 1A (V2) Microarray (G4110B) (Agilent Technologies, Inc., Santa Clara, CA, USA). The array contains approximately 22,000 oligonucleotides, representing more than 20,000 genes. Conditions and procedures for hybridization and washing followed the Agilent 60-mer oligo microarray processing protocol (Agilent Technologies, Inc., Santa Clara, CA, USA).

Microarray image analysis, data normalization, and data analysis

Microarray images were acquired by using the GenePix 4000B scanner (Axon Instruments, Union City, CA, USA). The image was analyzed by using GenePix Pro 5.1 software (Molecular Devices Corporation, Downingtown, PA, USA). Individual microarray data was normalized by using Expressionist Pro Refiner software (Genedata AG, Basel, Switzerland). A filter procedure was applied to eliminate nongene features such as positive controls, negative

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