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Original Research

Identification of TPD54 as a candidate marker of oral epithelial carcinogenesis

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ABSTRACT

The purpose of this study was to identify a novel marker of oral carcinogenesis in order to make the early diagnosis of malignant potential of oral lesion. In this study, we analyzed using tissue proteomics that relies on a functional relationship between laser microdissection (LMD) and nano-flow liquid chromatography and mass spectrometry and protein identification by tandem mass spectrometry (LC/MS/MS). A microdissected formalin-fixed paraffin-embedded tissue block of a single case of oral squamous cell carcinoma (OSCC) in the lower gingiva was analyzed by LC/MS/MS resulting in more than 400 unique protein identifications. In comparison with each protein profiling list from normal epithelium, dysplastic epithelium and OSCC, a new candidate marker of oral carcinogenesis, tumor protein D54 (TPD54), was identified. We performed real-time PCR, the Western blotting and immunohistochemical analysis for validation about TPD54 in various tissues including normal epithelium, hyperplastic, dysplastic epithelium and OSCC. As the result of real-time PCR analysis, the level of TPD54 expression in cancerous region was statistically higher than that in normal epithelia. In the Western blotting analysis, expression of TPD54 was high in OSCC cell, but was little seen in keratinocyte and fibroblast cells. And in the immunohistochemical analysis, the ratio of TPD54 expression increased according to the degree of carcinogenesis in epithelial lesions. From these results, we concluded that TPD54 was a candidate marker of oral epithelial carcinogenesis.

1. Introduction

Oral cancer is a significant public health threat accounting for 270,000 new cases annually worldwide [1]. Early diagnosis and treatment of OSCC may have a major impact on survival and quality of life. Clinically, oral potentially malignant disorders may appear as a white or red lesion, designated as leukoplakia or erythroplakia, respectively. It has been estimated that approximately 1–2% of all leukoplakia progress into cancer per year [2]. There is an urgent need to identify a novel marker underlying the malignant transformation from precancerous to cancerous tissue, and thus to identify novel early diagnostic targets.

In the present study, we identified TPD54, which is a member of the TPD52-like family [3,4], as a novel candidate marker of oral epithelial carcinogenesis in order to make the early diagnosis of malignant potential of oral lesion using tissue proteomics that relies on a functional relationship between laser microdissection (LMD) and nano-flow liquid chromatography and mass spectrometry and protein identification by tandem mass spectrometry (LC/MS/MS). In order to evaluate whether TPD54 was the effective marker for diagnosis, we further investigated the expression of TPD54 in oral normal epithelium, hyperplasia, dysplasia and OSCC.

2. Materials and methods

2.1. LMD and LC/MS/MS

We analyzed the case of an 84-year-old male's lower gingival squamous cell carcinoma, which underwent surgery for OSCC as a primary treatment without previous radiation therapy or

☆ Asian AOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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chemotherapy, with LMD and LC/MS/MS. The patient provided informed consent before enrollment in the study, in accordance with the protocol approved by the Institutional Review Board at Showa University Hospital. For LMD, 3 regions (the cancerous region, the adjacent dysplastic region and the histologically normal epithelium) were determined as the target regions from formalin-fixed paraffin-embedded tissues section of HE staining. The target regions in the sections were microdissected with a Laser Microdissection System (P.A.L.M., Munich, Germany). The specimens were extracted peptides by Liquid Tissue Protein MS Prep Kit according to manufacturer's protocol. LC/MS/MS analysis were performed by nano-flow reverse phase liquid chromatography followed by tandem MS, using an LTQ linear ion-trap mass spectrometer (Thermo Fischer, San Jose, CA, USA). Protein identification was also based on the assignment of at least two peptides. False positive rates for the data sets generated in this study were evaluated using Mascot and a randomized database based on Swiss-Prot (decoy function).

2.2. Real-time semi-quantitative PCR

To confirm the difference of TPD54 mRNA expression between normal epithelia and cancerous lesion, we used 4 cases of OSCC specimens for real-time PCR analysis. For real-time RT-PCR analysis, total RNA was extracted from laser-microdissected cells using the RNeasy Micro Kit (QIAGEN, Germany). cDNA from total RNA were synthesized using the High Capacity cDNA Archive according to the manufacture's instruction. The relative quantification (RQ) of mRNA was performed with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The High Capacity RNA-to-cDNA Master Mix reagents (Applied Biosystems) were used for PCR amplification. Reverse transcription was done using 16 μl of total RNA from patient samples. The resulting cDNA was stored at −20 °C until further use. From this cDNA solution, 2 μl was removed to be subsequently used for the real-time RT-PCR reaction, 10 μl the TaqMan® Gene Expression Master Mix reagents (Applied Biosystems) containing 1 μl TaqMan® gene expression assay (Applied Biosystems) was used at 20 μl/tube. The RT-PCR assay was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) with the following profile; 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 50 cycles each at 95 °C for 15 s and 50 °C for 1 min. With the threshold cycle (Ct) values detected by this system, relative quantification of TPD54 (Assay ID: Hs00162876) mRNA level compared with internal control gene GAPDH (Assay ID: HS99999905) was calculated according to the ΔCt method.

2.3. Western blot

To confirm the difference of TPD54 protein expression, human OSCC (HSC-2, HSC-3, HSC-4, SAS), keratinocyte (KC; Male, DS PHAMA BIOMEDICAL, Osaka, Japan), and fibroblast cell (Male, DS PHAMA BIOMEDICAL, Osaka, Japan) were evaluated. Each cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2. Rabbit polyclonal anti-human TPD54 (Protein Tech Group, Inc, Chicago) was used in the experiments. Collected cells were lysed with TritonX-100 lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.5% TritonX-100, 5 mM EDTA, 1 mM sodium *o*-vanadate) supplemented with Complete Mini™ protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured using the Bio-Rad quick start protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Whole cell lysates (25 μg protein) were separated with a 10–20% Ready Gel J (Bio-Rad Laboratories) and transferred to a Hybond PVDF membrane (Amersham, Arlington Heights, IL, USA). The membrane was blocked with bovine serum albumin/PBS and reacted with the primary antibody for 90 min at room temperature. The blots were visualized using a Western Lightning Plus (PerkinElmer, Waltham, MA, USA).

2.4. Immunohistochemistry

Twenty-nine OSCC (male: 17, female: 12, tongue: 27, lower gingival: 2), 32 epithelial hyperplasia, 27 epithelial dysplasia (mild dysplasia: 5, moderate dysplasia: 7, severe dysplasia: 5) and 5 normal epithelia were evaluated. Epithelial dysplasia was graded according to the WHO classification (2005) [5]. All the patients provided informed consent before enrollment in the study, in accordance with the protocol approved by the Institutional Review Board at Showa University Hospital. All the specimens were fixed in 10% neutral-buffered formalin. After embedding in paraffin, 5-μm sections were cut and mounted. Sections were then dried, de-paraffinized and rehydrated. After quenching endogenous peroxidase activity and blocking non-specific binding sites, slides of specimens were incubated at 4 °C overnight with 1:200 dilution of primary antibody directed against TPD54 (Protein Tech Group, Inc, Chicago). Immunostaining was performed using the Envision system (Dako, Japan) according to the manufacturer's instructions. Peroxidase activity was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxidase. The sections were then counterstained with hematoxylin, dehydrated, cleared and finally mounted. The control oral mucosa consisted of 5 normal epithelia obtained during third molar removal. Immunostaining was evaluated in a coded manner without knowledge of the clinical and pathological parameters by two independent observers (A.F. and G.Y.). For each section, 10 high-power fields were chosen, and a total of at least 1000 cells were evaluated. The results were expressed as the percentage of positive cells counted. To confirm the reproducibility, 25% of the slides were chosen randomly and scored twice. All duplicates were similarly evaluated. The percentage of positive cells in each case was semiquantitatively evaluated between "positive (50% < positive cells)" and "negative (50% > positive cells)".

2.5. Statistical analysis

All values are expressed as means and ±standard deviation. The statistical significance of differences between groups was analyzed using the unpaired Student's *t*-test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Protein identification by LC/MS/MS

Fig. 1 indicates the numbers of proteins identified by LC/MS/MS analysis. 22 kinds of proteins, some of which have been known as oral tumor marker, were common in the cancerous region and the adjacent dysplastic region, not in the normal epithelia. In the 22 kinds of proteins, we identified TPD54, which was quantified as much as the proteins of oral tumor markers (date not shown), as a novel candidate marker of oral epithelial carcinogenesis.

3.2. Gene expression of TPD54

To confirm whether the expression was a transcriptional level or not, real-time semiquantitative reverse transcription PCR was performed with a set of human-specific primers and template cDNA generated by reverse-transcription. Similar to the LC/MS/MS results, the level of TPD54 expression in cancerous region was significantly higher than that in normal epithelia (Fig. 2).