Increased expression of galectin-1 is associated with human oral squamous cell carcinoma development

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Abstract. Galectin-1 (Gal-1) is a newly found immuno-regulatory carbohydrate-binding protein in cancer biology. The purpose of this study was to evaluate the effects of Gal-1 on oral squamous cell carcinoma (OSCC) development. Immuno-histochemistry, Western blotting and RT-PCR were carried out in 62 primary OSCC, 38 oral leukoplakia (OPL) tissues to detect the Gal-1 expression in both protein and mRNA levels. Ten normal oral mucosa (NOM) tissues were used as control. Gal-1 protein was significantly overexpressed in OSCC cancer cells and OPL prickle cells compared to NOM ($P<0.05$). In accordance with Gal-1 protein, Gal-1 mRNA was also up-regulated in OSCC tissues and OPL tissues. Furthermore, both the Gal-1 protein and mRNA in OSCC tissues were higher than in OPL tissues ($P<0.05$). Our data supports the important roles of Gal-1 in OSCC development and suggests that Gal-1 upregulation in the OSCC and OPL tissues might be a predictor of early oral carcinogenesis.

Introduction

OSCC is one of the most common head and neck cancers, its development is a multistep process characterized by accumulation of genetic, epigenetic, and metabolic changes due to exposure to carcinogens (1,2). Many of these changes have been identified in the last decade, and led to great improvement in the understanding of the potential biology of this disease. But compared with other cancers, OSCC still has one of the poorest 5-year survival rates: only 52% of people diagnosed with OSCC survive 5 years (3). The poor prognosis of OSCC is usually due to its late detection and diagnosis, the 5-year survival rate is 81% for persons diagnosed with early-stage OSCC but only 22% for those diagnosed with advanced stage cancer (3). However, little improvement has been made during the past two decades in improving early detection of OSCC, only 35% of OSCC is detected at the earliest stage (4). So, there is a strong need to evaluate OSCC development and determine early carcinogenesis biomarkers that can help clinicians formulate proper treatment plans.

OPL is the most common oral premalignancy in human. It was reported that the malignant transformation rates of OPL range from 17% to 24% of the patients with median follow-up more than 7 years (5). So far there are no clinical parameters that can predict the potential of malignant transformation in patients with OPL. One purpose of this study was to determine a role of Gal-1 in predicting OSCC development in patients with OPL.

Galectins are a family of carbohydrate-binding proteins with an affinity for ß-galactoside. Gal-1, a prototype member of this family, exists as a homodimer composed of one carbohydrate-recognition domain of approximately 130 amino acids. Gal-1 is widely distributed in many normal and pathological tissues and appears to be functionally polyvalent, such as regulating cell proliferation, differentiation and apoptosis, mediating tumor transformation, growth and so on. There are several Gal-1 ligands, including laminin, lysosome-associated membrane proteins, and fibronectin, the specific functions of Gal-1 depend on what ligands it binds to (6).

It is widely reported that Gal-1 is overexpressed in many different types of tumors, including melanoma, astrocytoma, prostate cancer, ovarian cancer, hypopharyngeal and laryngeal squamous cell carcinomas and so on (7-9). However, there are relatively few reports associated with this theme in OSCC, especially with oral carcinogenesis. The aim of this study was to detect the Gal-1 mRNA and protein expression in OSCC and OPL tissues, and then deducing the underlying mechanism of OSCC development. As a result, we provide evidence that Gal-1 is significantly upregulated in OSCC and OPL tissues compared to NOM. Gal-1 mRNA and protein level becomes higher gradually from NOM, OPL to OSCC tissues, cancer-associated stroma cells also had positive staining. We suggest that Gal-1 may serve as a marker for OSCC development.

Materials and methods

Sixty-two primary OSCC, 38 OPL and 10 NOM tissues were included in this study. Immediately after specimens were

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taken from the operation rooms, three sets of corresponding tissues (weight, 100 mg) were stored using the following methods: i) two pairs of samples were frozen in liquid nitrogen for RNA and protein extraction; ii) the third pair was fixed in 10% buffered formaldehyde solution for 12-24 h and embedded in paraffin wax until the immunohistochemical (IHC) study. A total of 110 patients seen in the year 2007 at the Department of Stomatology, Union hospital and Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology were enrolled. Written informed consent was obtained from all participating patients. No patients had received chemotherapy or radiotherapy before operations. All cancers were histologically graded as well-differentiated, moderately-differentiated or poorly-differentiated, according to the World Health Organization (WHO) classification. Tumor pathological stage was classified according to the AJCC system.

Immunohistochemistry. Tissues samples were fixed in formaldehyde solution and embedded in paraffin. The sections were dewaxed twice in xylene for a total of 10 min, and rinsed in alcohol and gradient alcohol/water mixtures for 5 min in each solution. After rinsing in distilled water, antigen retrieval was done by boiling in 10 mM citrate buffer (pH 6.0) twice for 3 min in a microwave oven at high power. Endogenous peroxidase was blocked in hydrogen peroxide for 5 min and washed with PBS twice for 5 min. Immunohistochemistry (IHC) analysis was performed using a streptavidin-biotin complex system (Dako Corporation, Carpinteria, CA). Slides were then incubated with anti Gal-1 monoclonal antibody (Novocastra laboratories Ltd., CL-GAL1) with 1:80 to 1:200 dilution overnight at 4˚C. Sections were washed and incubated with biotinylated secondary antibody for 1 h at room temperature and the color was developed with AEC substrate chromogen for 10 min at room temperature. Negative control slides were performed for each separate specimen group using normal goat IgG, or second antibody alone. The staining reactions were determined by microscopic examination.

Statistical analysis. The results of immunohistochemical staining were interpreted by two experienced pathologists and categorized with a semi-quantitative scale as 0 (negative staining); 1+, (0~30% positive); 2+, (30~60% positive); or 3+ (>60% positive). The staining results were further categorized into 'weak' and 'strong' groups, which represents the group of 0 and 1+, and the group of 2+ and 3+, respectively. One way ANOVA and t test were used to look for differences in protein expression between different groups. All P-values were 2-sided; $P \leq 0.05$ was considered statistically significant.

mRNA extraction and RT-PCR. Briefly, RNA was isolated from each sample using the 'RNeasy Protect Mini Kit' (Qiagen, Cat.No.74124). First-strand cDNA synthesis was performed using 'SuperScript® First-Strand Synthesis System' with oligo (dT)-primers (Invitrogen, Cat.No.11904-018) according to the manufacturer's protocols. The following PCR amplifications were done in a 20 μl reaction mix containing 1 μl 10 pmol of sense and antisense primers respectively, 1 μl 0.6 μg/μl template DNA and 17 μl PCR supermix (Invitrogen Cat.No.10572-014). The antisense sequence primer is 5'-CAGGCTGGAAGGGAAAGA-3', the sense sequence is 5'-GGTGGCTCCTGACGCTAA-3', the size of PCR product is 179 bp. The PCR conditions included initial denaturation at 94˚C for 5 min, and 35 cycles of denaturation for 30 sec and annealing at 55˚C for 30 sec, followed by extension at 72˚C for 30 sec. Final extension of PCR products was carried out at 72˚C for 7 min. The products were resolved by electrophoresis using a 2.0% agarose gel buffered with 1X TAE. Bands were visualized by ethidium bromide (0.5 mg/ml) and products of expected size were confirmed with a 100 bp ladder (Sigma).

Protein extraction and Western blot analysis. Tissue samples (100 mg) were homogenized in 500 μl of a lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride). After 30 min at 4˚C, the lysate was centrifuged at 10,000 rpm for 30 min at 4˚C. For Western blot analysis, 4 μg protein was added to a loading buffer and boiled for 5 min. Subsequently, the protein sample was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a polyvinylidene difluoride membrane. The membrane was then blocked with 5% non-fat dry milk in TPBS (0.1% Tween PBS) buffer at 4˚C overnight. After decanting the blocking buffer, the membrane was incubated with anti Gal-1 monoclonal antibody (Novocastra laboratories Ltd., CL-GAL1) in PBS with 5% BSA for 1 h at 37˚C with agitation. After washing 4 times with TPBS, the membrane was incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at 37˚C. After washing, the membrane was submerged in ECL developing solution (Amersham) followed by autoradiography.

Results

Clinicopathological characteristics. The mean age of the enrolled OSCC and OPL patients was 56.1 and 46.2 years, respectively. The other related information can be obtained from Table I.

Immunohistochemistry. We found the protein expression of Gal-1 increased gradually from NOM (Fig. 1), OPL to OSCC tissues. In the OPL tissues, positive staining was mainly found in prickle cell layer (Fig. 2). The OSCC cancer cells showed very strong staining (Fig. 3). The positive staining of Gal-1 was also found in cancer-associated stromal cells. Gal-1 was expressed mainly in the cytoplasm, but was also found in the nucleus (Fig. 3). There were significant differences in Gal-1 staining between NOM and OSCC cancer cells ($P<0.05$), as well as OPL prickle cells ($P<0.05$). Gal-1 expression in OSCC was also higher than in OPL tissues ($P<0.05$) (Figs. 4 and 5). The positive staining was significantly stronger in the poorly-differentiated tissues than in well-differentiated tissues ($P<0.05$). The correlation between Gal-1 expression and the clinicopathological variables in detected cases is summarized in Table I, the Gal-1 expression did not show any statistically significant changes according to gender, age, sites of tumor, or AJCC stage. This

Table I. The correlation between Gal-1 expression and the clinicopathological variables in 62 OSCC patients.

Variable	Case	-	+	Positive rate %	χ^2	P-value
Gender						
Male	38	7	31	81.58	0.384	>0.05
Female	24	6	18	75.00	0.384	>0.05
Age (years)						
<55	29	6	23	79.31	0.068	>0.05
≥55	33	7	26	78.79	0.068	>0.05
Sites of tumor						
Tongue	43	10	33	76.74	0.443	>0.05
Others	19	3	16	84.21	0.443	>0.05
Differentiation						
Well	36	11	24	69.44	4.762	<0.05
Moderately + Poorly	17+9	2+0	15+9	92.31	4.762	<0.05
AJCC stage						
I + II	22	4	18	81.82	0.160	>0.05
III + IV	40	9	31	77.5	0.160	>0.05

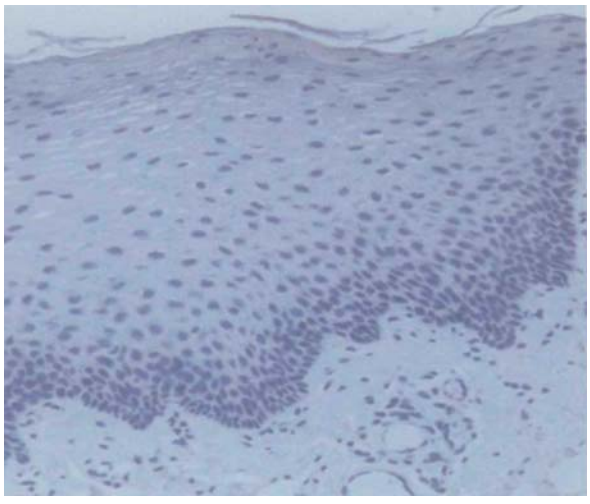

Figure 1. Gal-1 expression was negative in NOM (SPX200).

result implied that Gal-1 may overexpress in cancer cells and cancer-associated stromal cells during early oral carcinogenesis.

RT-PCR. From left to right, the Gal-1 RT-PCR bands are from OSCC, OPL and NOM respectively. GAPDH was used as internal control. The PCR band is 179 bp. The results demonstrated that the mRNA increased gradually from NOM, OPL to OSCC, which were coincident with the results of immunohistochemistry (Fig. 6).

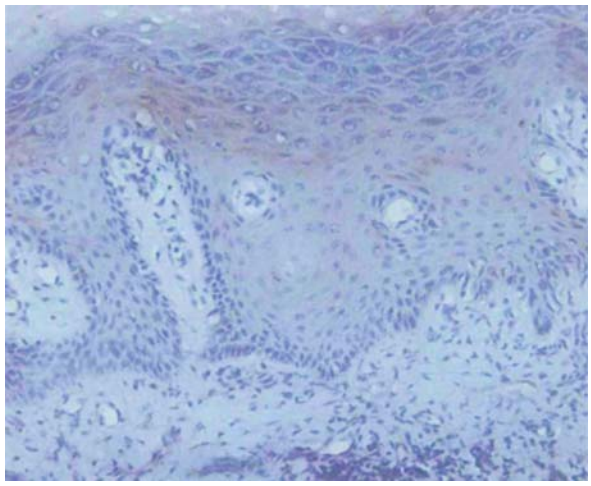

Figure 2. Gal-1 positive staining was mainly found in prickle cell layer in OPL tissues (SPX200).

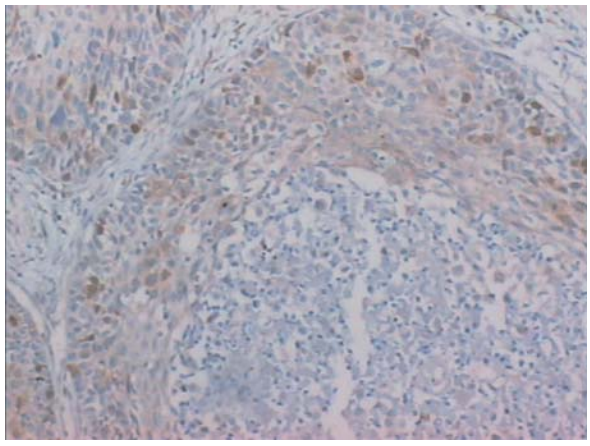

Figure 3. Strong positive of Gal-1 in OSCC cancer cells locates mainly in cytoplasm (SPX200).

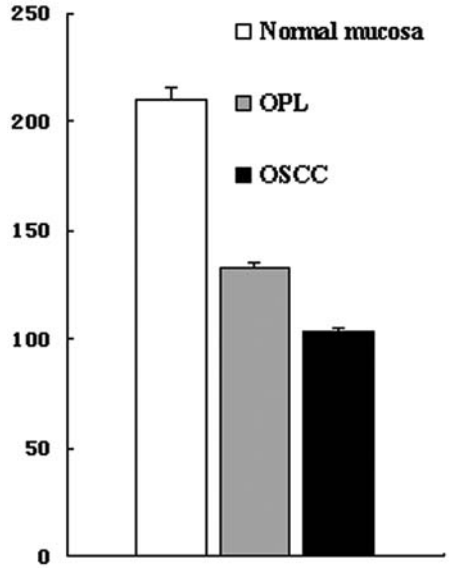

Figure 4. Gray scale of Gal-1 positive staining in NOM, OPL, OSCC has significant difference.

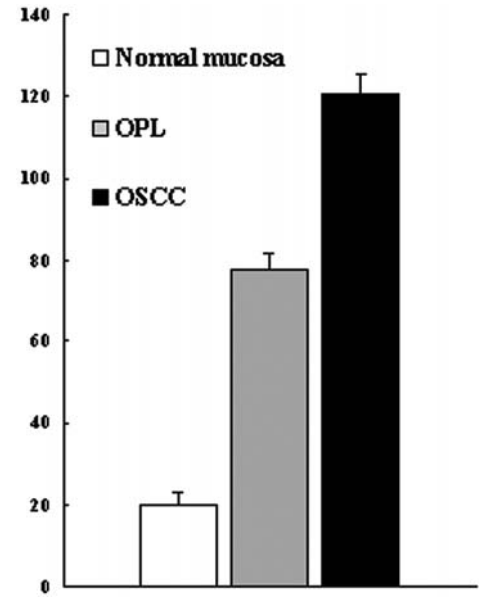

Figure 5. Positive area of Gal-1 in NOM is much smaller than that in OPL and OSCC.

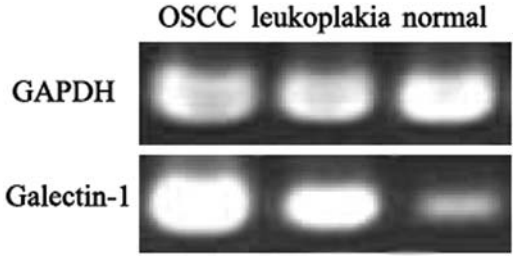

Figure 6. Gal-1 RT-PCR results of OSCC, OPL, NOM tissues.

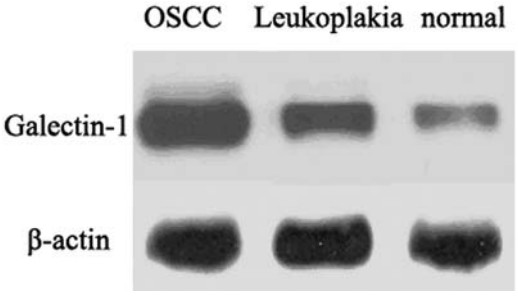

Figure 7. Gal-1 Western blot results of OSCC, OPL, NOM tissues.

Western blot analysis. To further confirm the protein level of Gal-1 in the three groups described above, we also analyzed its expression by Western blotting. As shown in Fig. 7, OSCC, OPL, and NOM tissues express high, intermediate, and low amounts of Gal-1 respectively. The band corresponding to OSCC was markedly more prominent in NOM. On the other hand, OPL tissues showed intermediate expression level compared with the other two groups (Fig. 7).

Discussion

This study focused on evaluating the Gal-1 function in OSCC development. We showed that Gal-1 protein was significantly upregulated in OPL prickle cell layer compared to NOM, the highest level of Gal-1 was found in cancer cells of OSCC, which corresponded with Gal-1 mRNA. Together with all the results, we guess Gal-1 may serve as a potential biomarker to predict the risk for OSCC development in patients with OPL.

As described above, functional Gal-1 is a homodimer and performs a variety of functions (10,11). Gal-1 is known to be an important contributor to T cell homeostasis (12-16). It modulates immune cell functions by controlling the proliferation and apoptosis of effector T cells, as well as blocking T cell activation (12-17). Stillman *et al* (15) proved that Gal-1 bound cell surface glycoprotein receptors to induce T cell death. In an experimental study of chronic inflammatory diseases, recombinant Gal-1 has been shown to suppress Th1-dependent responses and to enhance T cell susceptibility to apoptosis (16). Gal-1 also greatly increases the secretion of Th2 cytokines, including interleukin (IL)-4, IL-5, IL-10, and IL-13 (18,19). At present Gal-1 is considered as a novel regulator of immune cell homeostasis.

Recent studies demonstrated that Gal-1 plays important roles in carcinogenesis and cancer progression in many types of tumors (7,20). Its overexpression has been correlated with several aspects of cancer biology, including modulation of cancer cells apoptosis, migration and adhesion (21). Gal-1 was found overexpressed in hypopharyngeal and laryngeal squamous cell carcinomas (9). It also increased the adhesion of prostate cancer cells and ovarian cancer cells to extracellular matrix (22). Exogenous Gal-1 was proved to enhance the locomotivity of malignant glioma cells *in vitro* (23).

Regarding OSCC, Gal-1 has been proved to be an OSCC-related protein and gene (24). Chiang *et al* (25) showed that the overexpression of Gal-1 at OSCC invasive front was associated with poor prognosis. But there has been no report of Gal-1 effect on OSCC development. Our study demonstrated that the Gal-1 mRNA and protein levels were significantly higher in OSCC cancer cells compared to NOM epithelium. Cancer-associated stromal cells also had strong positive staining, Gal-1 was significantly upregulated in poorly differentiated and moderately differentiated tissues than in well-differentiated tissues. We did not find any obvious correlation between Gal-1 expression in cancer cells and clinicopathologic parameters such as gender, age, sites of tumor or AJCC stage. We found Gal-1 mRNA and protein were also frequently higher in OPL than NOM, but lower than in OSCC. Associating our results with previous findings, we consider that Gal-1 promotes OSCC development. The mechanism is still unknown, but there is a good prospect that it is associated with T cells apoptosis induced by Gal-1. Tumor development involves many complicated factors, the most important of which is the ability of cancer cells to escape detection and elimination by the host immune system. T cells play a pivotal role in host immune response against cancer cells, but Gal-1 secreted by cancer cells can induce T cells to apoptosis, so it is believed that Gal-1 promotes

tumor immune privilege (11). Argentinian scientists reported that the higher the Gal-1 was in mouse melanoma cells, the faster the tumors grew. Blocking Gal-1 expression in tumor tissues would result in decreased tumor volume, and stimulation of the formation of tumor-specific T cytological *in vivo*. So, Gal-1 might help the tumor to obtain immune privilege by regulating the survival or polarization of effector T cells (11). Blois *et al* proved the pivotal role for Gal-1 in fetomaternal tolerance (26), suggesting that Gal-1 inducing T lymphocytes to apoptosis was closely-related with immune suppression.

Although it is not yet fully elucidated, the role of Gal-1 in OSCC development is certainly an interesting issue due to the highlighted significance of Gal-1 in squamous cell carcinoma (24,25). Collectively, we consider Gal-1 a potential biomarker to predict the risk for OSCC development in patients with OPL.

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