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Expression of L-type Amino Acid Transporter 1 (LAT1) and 4F2 Heavy Chain (4F2hc) in Oral Squamous Cell Carcinoma and its Precusor Lesions

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Abstract. *Background: Amino acid transporters play an important role in supplying organic nutrients to cells. The expression of L-type amino acid transporter 1 (LAT1) and its subunit 4F2 heavy chain (4F2hc) was evaluated to determine the alterations of these transporters in oral normal mucosa (ONM), oral precancerous lesion (OPL) and oral squamous cell carcinoma (OSCC). Materials and Methods: Sections from formalin-fixed, paraffin-embedded samples of ONM, OPL or OSCC were examined using immunohistochemical staining to detect LAT1 and 4F2hc proteins. Results: The LAT1 and 4F2hc expression increased progressively from ONM to hyperplastic and to dysplastic lesions and OSCC. In particular, LAT1 may be a more specific indicator of tumor progression than 4F2hc. Conclusion: LAT1 and 4F2hc may have an important role in the early stages of multistep oral carcinogenesis. In addition, the specific inhibition of LAT1 and 4F2hc might be a new rationale to suppress oral cancer progression.*

It is well known that tumor cells use organic nutrients for their energy requirements. There are several reports suggesting that the requirement for amino acids increase in the proliferation process of tumor cells in order to support protein biosynthesis (1-3). Amino acid transport System L is a major nutrient transport system that is responsible for the Na^+-independent transport of large neutral amino acids including several essential amino acids (4,5). In malignant tumors, the first isoform of the system L, L-type amino acid transporter 1 (LAT1), which Kanai *et al.* (6) cloned, is up-regulated to support tumor cell growth. LAT1 is highly expressed in cultured cells as well as in malignant tumors, presumably to support their continuous growth (6-8). In addition, it was previously reported that the immunoreactivity of TA1/E16, a partial sequence corresponding to LAT1, was strong in human colon adenocarcinoma tissues but was barely detected in the surrounding normal colon tissues (9). Therefore, it has been proposed that LAT1 expression is related to the growth and proliferation of tumor cells (6, 8). LAT1 requires an additional protein, a heavy chain of the cell surface antigen 4F2 (4F2hc), for functional expression (6, 10, 11). The 4F2 antigen (CD98) was originally identified as a cell-surface antigen associated with lymphocyte activation (12, 13). It was later shown to be involved in various cellular activities such as cell proliferation, cell transformation and cell adhesion (14).

Therefore, the aim of this study was to examine the expression pattern of LAT1 and 4F2hc in the human oral normal mucosa (ONM), different degrees of oral precancerous lesion (OPL) and oral squamous cell carcinoma (OSCC) of different stages of differentiation, using paraffin-embedded tissue sections.

Materials and Methods

Specimens. The specimens were selected from archived paraffin blocks obtained from the Department of Oral Pathology, Chosun University School of Dentistry, Korea. The group of OPLs (21 males and 15 females, aged between 20 and 75 years) showing hyperplastic (n=11), mild (n=9), moderate (n=9) and severe (n=7) dysplastic changes was analyzed. The sites included buccal mucosa (n=14), mouth floor (n=9), tongue (n=8), palate (n=3) and retromolar pad (n=2). Thirty-seven cases of OSCC (20 males and 17 females, aged between 44 and 82 years), arising in the gingiva (n=15), mouth floor (n=7), tongue (n=6), palate (n=5) and retromolar pad (n=4), were examined. The OSCCs included were categorized histologically as follows: well- (n=14), moderately- (n=13) and poorly-differentiated (n=10). Nine normal subjects (6 males and 3 females, aged between 16 and 80 years) were included providing tissues from the gingiva and alveolar ridge (n=4), buccal mucosa (n=2), palate (n=2) and tongue (n=1).

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Key Words: LAT1, oral squamous cell carcinoma, 4F2hc.

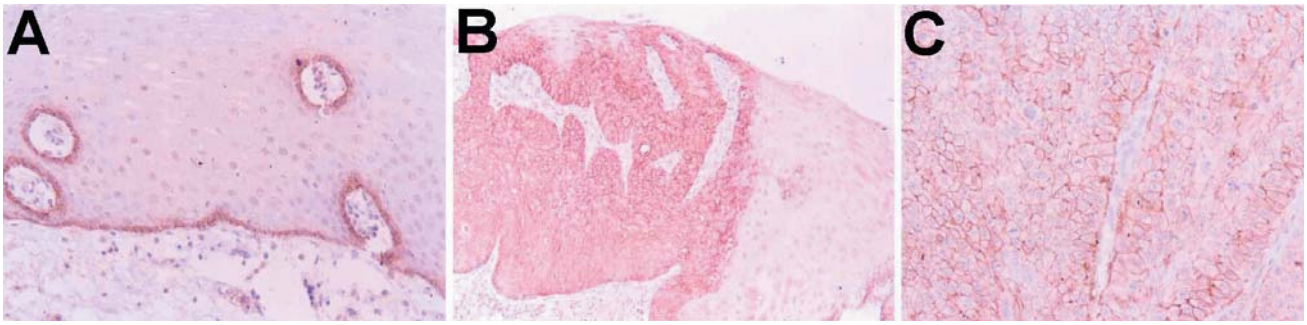

Figure 1. *LAT1 expression. (A) Positive staining of the basal keratinocytes alone in the ONM. (B) LAT1 expressed in the cytoplasm as well as plasma membrane of the severe dysplasia of OPLs. (C) OSCC exhibiting strong membranous expression.*
ONM: Oral normal mucosa, OPL: Oral precancerous lesion, OSCC: Oral squamous cell carcinoma.

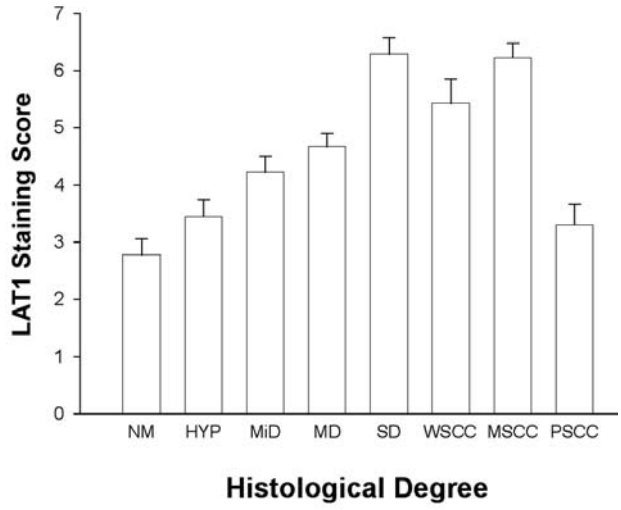

Figure 2. *The mean values of the immunohistochemical staining score of LAT1 expression in the different stages of oral carcinogenesis.*
N: Normal oral mucosa, H: Hyperplastic epithelium, MiD: Mild dysplasia, MD: Moderate dysplasia, SD: Severe dysplasia, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately-differentiated squamous cell carcinoma, PSCC: Poorly-differentiated squamous cell carcinoma. Bars *mean±SD.*

Immunohistochemistry. All the tissue had previously been fixed in 10% neutral buffered formalin and embedded in paraffin. The 5 μM sections were cut and mounted on poly-lysine-coated glass slides and air-dried overnight at room temperature. After the sections had been deparaffinized in xylene and rehydrated through a graded ethanol series, they were immersed in 3% hydrogen peroxide in methanol (V/V) for 15 min in order to quench their endogenous peroxidase activity. Antigen retrieval was performed using an autoclave. The sections were then washed in Tris-HCl and incubated with normal 1% BSA (bovine serum albumin in Tris-HCl) for 1 h, in order to reduce the non-specific antibody binding. After washing in Tris-HCl, the sections were incubated overnight with LAT1 (1:100, Kumamoto Immunochemical Laboratory, Transgenic, Kumamoto, Japan) and 4F2hc (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies at 4˚C.

After incubation with the primary antibodies, the sections were washed thoroughly in Tris-HCl and exposed to a streptavidin-biotin complex (Dako Corp., Carpinteria, CA, USA) according to the manufacturer's instructions. Diaminobenzidine (Dako Corp.) was used as the chromogen and counterstaining with Mayer's hematoxylin was performed.

The absorption experiments were performed in order to verify the specificity of the immunoreactions. The blocking peptide for LAT1 was kindly provided by Kumamoto Immunochemical Laboratory and the blocking peptide for 4F2hc was obtained from

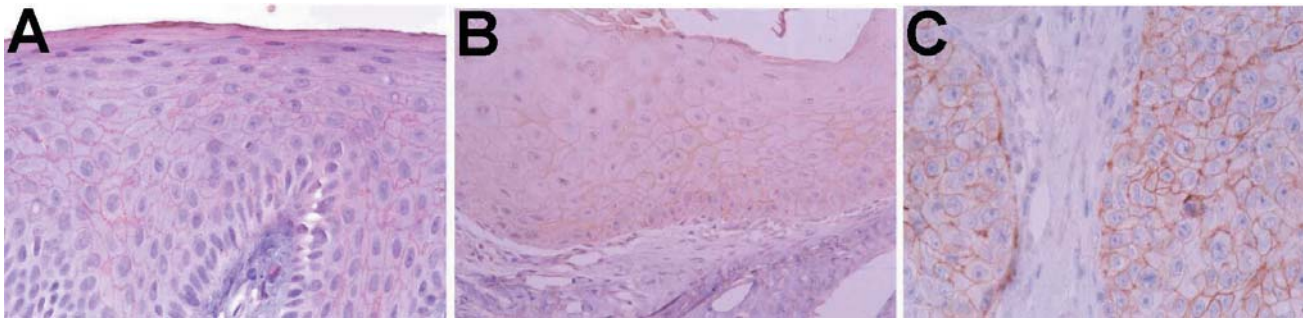

Figure 3. *4F2hc expression. (A) The ONM showing membranous expression in almost all the epithelial layers. (B) The dysplastic mucosa showing both cytoplasmic and membranous expression. (C) Strong 4F2hc expression in the tumor cells of the OSCC.*
ONM: Oral normal mucosa, OSCC: Oral squamous cell carcinoma.

Santa Cruz Biotechnology. They were added at a 10-fold mass excess to the antibodies and incubated for 2h at room temperature, and then incubated on the slides. The antibodies treated with the specific peptide were incubated with the sections.

Evaluation. The intensity of both LAT1 and 4F2hc in the epithelium and tumor cells was evaluated by dividing into four groups: 0 = no; 1 = mild; 2 = moderate; and 3 = strong staining intensity. The quantity of immunostainings were evaluated as follows: 0 = no positive immunostaining; 1 = <10%; 2 = 10-25; 3 = 25-50; and 4 = >50% of epithelium and tumor cells showing positive immunoreactivity. A combined score for both LAT1 and 4F2hc immunostainings, based on both qualitative and quantitative immunostaining, were composed by adding the qualitative to the quantitative score. The maximum score after adding was 7 and the minimum, 0.

The results are presented as mean±S.E.M. Statistical significance was analyzed by using the Student's *t*-test for two groups and one way analysis of variance for multi-group comparison. $P<0.05$ is considered statistically significant.

Results

The expression of both LAT1 and 4F2hc varied according to the cell layer and tumor differentiation, in the ONM, OPL and in OSCC, respectively. However, this was not related to the location of the tissue.

LAT1. In the ONM, LAT1 was expressed only in the basal cell layers, predominantly membranous (Figure 1A). Moreover, their LAT1 expression increased as the tissues progressed from being normal to hyperplasia, dysplasia and SCC. The LAT1-positive cells of the basal and parabasal layers increased as the tissue passed from being normal epithelium to hyperplastic and dysplastic stages. In particular, areas of severe epithelial dysplasia exhibited strong expression throughout the full thickness (Figure 1B). In the OSCCs, immunoreactivity was observed within most of the tumor cells, except for the extremely well-differentiated squamous epithelium and the keratinized area. However, the distribution of positively-stained cells was heterogeneous. Immunoreactive cells in the well- or moderately-differentiated SCC were predominantly observed in the front of the invading tumor islands (Figure 1C). In the poorly-differentiated SCC, however, only a few cells in the bulk of the tumors were positive for LAT1.

The mean values of LAT1 expression increased gradually from ONM (2.78±0.28) to hyperplastic (3.45±0.28) and more to mild (4.67±0.28), moderate (5.43±0.24) and severe (6.29±0.29) dysplastic lesions. The difference between LAT1 expression in the ONM and that in the mild dysplasia is statistically significant ($p<0.05$), while the difference between normal and moderate dysplasia is highly significant ($p<0.01$), as is that between normal and severe dysplastic lesions ($p<0.001$) (Figure 2).

The mean±S.E.M.s of LAT1 staining score in the different grades (well, moderate, poorly) of OSCC were 5.43±0.42, 6.23±0.26 and 3.30±0.37, respectively. In the poorly-differentiated OSCC, the LAT1 staining score decreased compared to that of well- and moderately-differentiated OSCC (Figure 2).

4F2hc. In the ONM, 4F2hc was present in cells of almost all layers of the epithelium including keratin, but the basal cell was weakly stained. A positive reaction was found in both the cell membrane and cytoplasm (Figure 3A). Similar to LAT1 expression, there was evidence of increased expression during the progress to moderate and severe dysplasia in the dysplastic lesion (Figure 3B). In the area of well- or moderately- differentiated OSCCs, 4F2hc showed significantly increased expression in the cytoplasm and membrane as well as the keratinized area of the tumor cells (Figure 3C). In the poorly- differentiated OSCCs, the immunoreaction was focally positive.

The mean values of 4F2hc expression increased gradually from ONM (3.78±0.32) to hyperplastic (4.90±0.49), mild (4.90±0.35) and more to moderate (5.33±0.35), and severe (6.14±0.26) dysplastic lesions. The difference between 4F2hc expression in the ONM and that in the moderate dysplasia is statistically significant ($p<0.05$), while the difference between normal and severe dysplasia is highly significant ($p<0.01$). However, there is no association between normal and hyperplastic or mild dysplastic lesions (Figure 4).

The mean±S.E.M.s of 4F2hc staining score in the different grades (well, moderate, poorly) of OSCC were 5.36 + 0.30, 5.00 + 0.25 and 3.80 + 0.40, respectively. The mean values of 4F2hc expression decreased gradually from well- to moderate- and poorly- differentiated OSCC (Figure 4).

Discussion

This study investigated the expression of both LAT1 and 4F2hc, the L-type amino acid transporters, in ONM, OPL and OSCC. To the best of the authors' knowledge, this is the first demonstration of the expression pattern of these L-type amino acid transporters in ONM, OPL and OSCC using human paraffin- embedded material.

The results showed that LAT1 was expressed only in the basal cell layer of the ONM, and its expression pattern was predominantly membranous. However, LAT1 immunoreactivity was found to be strong in human OPLs and OSCCs. In addition, LAT1 expression gradually increased in the cytoplasm and membrane during the progression from hyperplastic to more dysplastic lesions. This suggests that more advanced precancerous lesions exhibit higher LAT1 expression levels in addition to a dysregulated control of cell proliferation. The gradually increasing LAT1 expression detected during the multistep progressive change indicates that the LAT1 protein can play an important role in the early stages of oral squamous epithelial carcinogenesis. It is in agreement with the observation

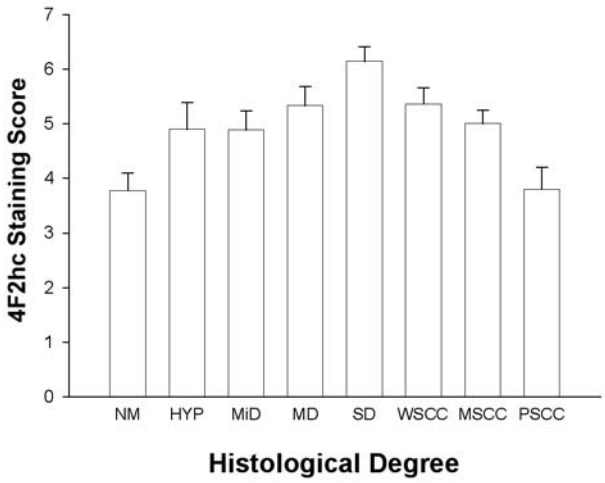

Figure 4. *The mean values of the immunohistochemical staining score of 4F2hc expression in the different stages of oral carcinogenesis.*
N: Normal oral mucosa, H: Hyperplastic epithelium, MiD: Mild dysplasia, MD: Moderate dysplasia, SD: Severe dysplasia, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately-differentiated squamous cell carcinoma, PSCC: Poorly-differentiated squamous cell carcinoma. Bars *mean±SD.*

of colon cancer tissue using TA1/E16, a partial sequence corresponding to LAT1 (8). On the other hand, 4F2hc expression was present in the cells of almost all layers of ONM. Its expression was gradually increased in the cytoplasm and membrane in OPLs as well as OSCCs, which suggests that 4F2hc is also related to tumor growth. However, since LAT1 expression was limited to the basal cell layer of the ONM, and its expression was higher in human OPLs and SCCs, it is believed that LAT1 is a more specific indicator of tumor growth than 4F2hc.

This report demonstrated a marked correlation between LAT1 expression and the transformed phenotype in OPLs and OSCCs, which is in contrast to ONM. This might make LAT1 useful as a proliferation marker. Singh and Siegal (15) suggested that the amino acid transporters activated by hormonal signals, such as estrogen and epidermal growth factor (EGF), took in some amino acids in order to up-regulate ornithine decarboxylase (ODC) and matrix metalloprotease enzymes, which are closely related to the proliferation and invasion of tumor cells. Since these pathways have also been described in OSCC (16,17), the relationship between LAT1 and these pathways to the transformation process in general remains to be investigated. Further study is also needed to correlate the presence of LAT1 with the other biomarkers in OSCC. On the basis of the small number of dysplastic lesions in this study, the results are promising, but preliminary. Therefore, a larger study of OPLs is needed to determine the extent to which LAT1 correlates with the malignant change.

Because LAT1 together with 4F2hc were up-regulated in the dysplastic lesions and OSCCs compared to ONM, and both LAT1 and 4F2hc showed simultaneously reduced expression in the poorly-differentiated OSCC, there is thus the possibility that, in cooperation with 4F2hc the ability of LAT1 to promote tumor growth is enhanced (1). Recently, Fenczik *et al.* (18) reported that 4F2hc, is an important regulator of integrin activation, which is involved in cell growth. In addition, 4F2hc expression was predominantly observed in the keratin layer of ONM as well as in the keratinized area of OSCC. This suggests that 4F2hc expression recapitulated the pattern seen in the ONM, indicating that 4F2hc protein may be modulated primarily during the terminal differentiation pathway of normal and neoplastic keratinocytes. Alternatively, 4F2hc protein expressed in the keratin might be a cross interaction between intricate intermediate filament molecules in the keratinocytes. However, in the poorly- differentiated OSCC, both LAT1 and 4F2hc expressions were rather lower than in a dysplastic lesion and a well- to moderately-differentiated OSCC. It was thought that oral carcinogenesis may involve an early expression and a late suppression of both LAT1 and 4F2hc protein, when cells are stimulated to enter the cell cycle. Although the involvement of LAT1 and 4F2hc in early stages of oral carcinogenesis has been suggested, it is not known whether or not overexpression in some other malignant cell lineages, such as the bladder and colon cancer cell lines (8, 19), results in a reduced tumorigenicity and metastatic potential of these cells. Alternatively, it is possible that there are different mechanisms for regulating LAT1 and 4F2hc gene expression or other amino acid transporters in the poorly-differentiated OSCC. The exact mechanisms underlying these events should be delineated further.

In summary, both LAT1 and 4F2hc are up-regulated in dysplastic lesions and OSCC as compared to ONM. Moreover, it is possible that the ability of LAT1 to promote tumor growth is enhanced in cooperation with 4F2hc. It is believed that LAT1 may be a useful biomarker for a better understanding of multistep oral carcinogenesis. In the future, the specific inhibition of LAT1 might be a new rationale to suppress tumor cell growth in oral cancer.

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