CHAPTER 5

Discussion

Nowadays, there have been many attempts to develop biomarkers of OSCC as diagnostic, prognostic, or therapeutic molecules. ADAM9 has been previously reported to be overexpressed as well as suggested to be useful as a targeted therapeutic molecule for some other cancers.[61-63] However, there have so far been a few studies about ADAM9 and OSCC, and these studies only emphasize on the chromosomal alterations and mRNA levels of ADAM9 expression. Therefore, to extend the findings from these studies, several *in vivo* and *in vitro* experiments have been performed in this study to determine expression of ADAM9 protein in OSCC, which is considered the final and functional product of *ADAM9* gene.

By immunohistochemistry, a significant increase in ADAM9 protein expression in OSCC tissues compared with that in normal oral tissues was shown. This is in agreement with the findings from other previous studies that demonstrated ADAM9 protein overexpression in several different types of cancer from various organs.[5,45,47-50,57,64-66] Particularly for squamous cell carcinoma, ADAM9 protein overexpression is shown in cancers of the cervix, the esophagus, the pharynx, the larynx, and the skin around the head and neck region.[5,57,65,66] Furthermore, a few previous studies have demonstrated increased expression of ADAM9 mRNA in OSCC tissues,[5,66] which corresponds with our immunohistochemical finding. However, none of these two studies has demonstrated overexpression of ADAM9 protein in oral cancer tissues.

A significant and positive correlation was demonstrated between ADAM9 expression and an increasing degree of oral cancer cell differentiation. This finding is similar to the results shown in mouse prostate and breast cancers that demonstrate higher ADAM9 mRNA expression in well-differentiated than in poorly-differentiated lesions.[54] Moreover, well-, moderately- and poorly-differentiated prostate cancers were

observed in *ADAM9*-transgenic mice with the highest ADAM9 expression demonstrated in the well-differentiated prostate cancer.[54,67] Therefore, our finding of significantly greater ADAM9 protein expression in the well- and the moderately-differentiated OSCC than in the poorly-differentiated OSCC may suggest a possible role for ADAM9 in carcinogenesis and in the transition from well-differentiated to poorly-differentiated OSCC. Poorly-differentiated OSCC is generally related to cancer aggressiveness due to its highest recurrence rate and worst disease-free survival rate, compared with well- and moderately-differentiated OSCC.[68] As a result, it is speculative that decreased ADAM9 expression in OSCC may regulate a change from low-grade to high-grade cancer that results in increased cancer aggressiveness and severity. However, this study was based on only the histopathological reports, so the lack of clinical information, including disease severity, treatment outcome, and survival data, is a limitation of this study. Therefore, it is interesting to further determine whether or not distinct degrees of ADAM9 expression are associated with tumor aggressiveness in OSCC.

The significantly increased ADAM9 protein expression in well-and in moderately-differentiated OSCC also corresponds with ADAM9 expression in the suprabasal layers of normal oral epithelium and epidermis[31] (Fig. 5.1), indicating an association between ADAM9 expression and epithelial cell differentiation. Calcium is a major regulator of keratinocyte differentiation both *in vivo* and *in vitro*.[69] Normally, keratinocytes fail to differentiate under low calcium (0.03 mM) condition, and their differentiation process is initiated when the calcium concentration is above 0.1 mM,[69] whereas the physiologic calcium concentration can be up to 1.2 mM.[70] To confirm the association between ADAM9 and epithelial differentiation in this study, serum-free keratinocyte growth medium with three different doses of calcium, 0.03, 0.15 or 1.2 mM, was used to treat HOKs for 48 hours, and expression of filaggrin was used to indicate the terminal differentiation of HOKs. As anticipated, undifferentiated HOKs expressed a low level of ADAM9 protein, whereas ADAM9 protein expression was considerably greater in differentiated HOKs than in undifferentiated HOKs. This result is consistent with the finding from a previous study in immortalized keratinocytes, HaCaT cells, by Zigrino and co-workers (Fig. 5.2).[31] Therefore, ADAM9 expression is related to epithelial differentiation in both oral carcinoma and normal epithelial cells.

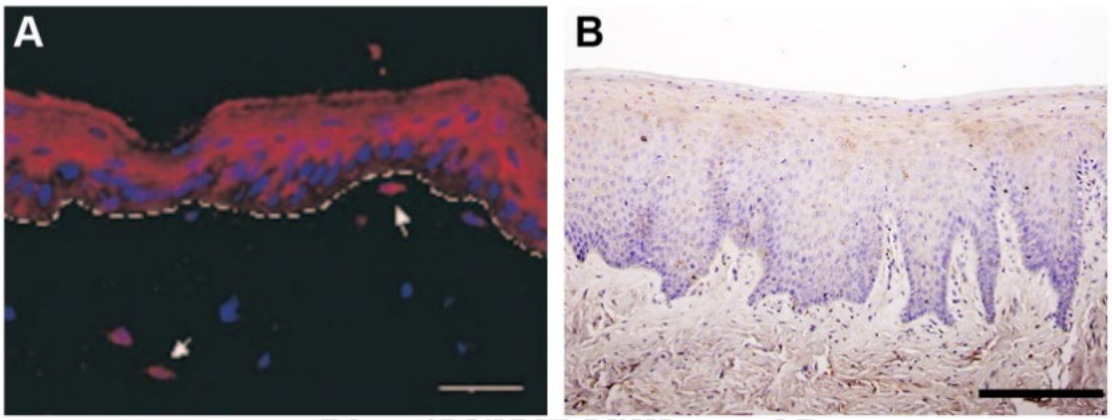

Figure 5.1 ADAM9 expression pattern in normal skin and oral epithelium. (A) Immunofluorescence of ADAM9 staining (red) in human skin reveals ADAM9 protein expression predominantly found in the suprabasal layers.[31] (B) Immunohistochemistry of ADAM9 staining in normal oral mucosa shows a similar pattern to that in human skin. Bars = 200 micron.

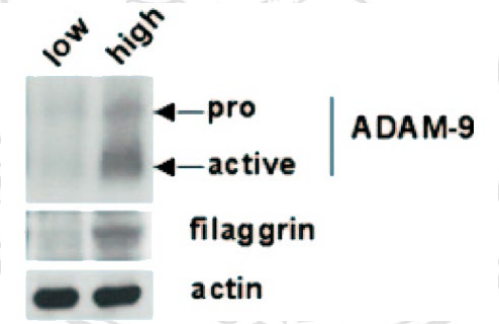

Figure 5.2 Enhanced ADAM9 protein expression in a HaCaT cell line under a high calcium concentration. A representative blot of HaCaT cells shows increased ADAM9 and filaggrin expression under a high calcium condition.[31]

Consistent with ADAM9 protein expression on the surface of cancer cells in OSCC tissues, expression of membrane ADAM9 was detected in three of four tested oral cancer cell lines by flow cytometry (Fig. 4.7). Furthermore, by Western blot hybridization, expression of membrane ADAM9 (an active form) at 84 kDa was significantly enhanced in two of the three oral cancer cell lines (Fig. 4.9), which were positive for membrane ADAM9 expression by flow cytometry. These results are not only similar to those of Uehara and co-workers[6] that demonstrated increased ADAM9

mRNA expression in some oral cancer cell lines but also extend their results by demonstrating the presence of membrane ADAM9 in oral cancer cell lines and a significant increase in membrane ADAM9 expression that can possibly function as a shedding enzyme for HB-EGF.[36,57] In addition, varying degrees of active ADAM9 expression among different oral cancer and HOK cell lines are similar to different degrees of ADAM9 mRNA expression in other oral cancer and normal epithelial cell lines (Fig. 5.3).[6]

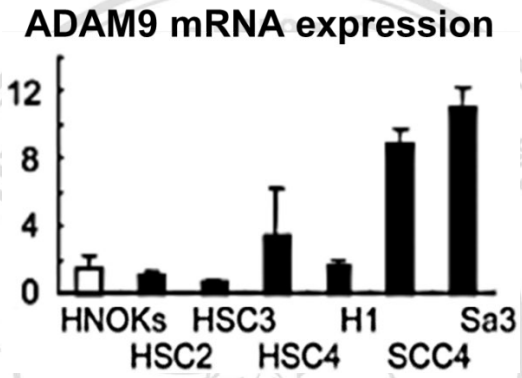

Figure 5.3 Varying ADAM9 mRNA expression in OSCC cell lines from a previous study.[6]

At present, the best-defined function of ADAMs is proteolytic activity with most of the putative substrates identified as transmembrane proteins.[4] In squamous cell carcinoma of the skin and the esophagus, ADAM9 has been reported to cleave the pro-HB-EGF precursor that leads to anti-apoptotic activity and cancer invasion through the EGFR/Akt pathway (Fig. 5.4).[57,71] In this light, overexpression of active ADAM9 found in oral cancer cell lines is in line with the result from a previous study that has demonstrated overexpression and activation of Akt by phosphorylation in the same oral cancer cell lines (Fig. 5.5).[59,72] Hence, the possible function of active ADAM9 as a sheddase to activate EGFR and Akt by phosphorylation in these oral cancer cell lines warrants further investigations. It is also necessary to examine the behavior or aggressiveness of the two oral cancer cell lines, HN6 and HN008, whose ADAM9 expression was significantly enhanced, compared with that of the other cell line, *i.e.*, HN5, which minimally expressed ADAM9.

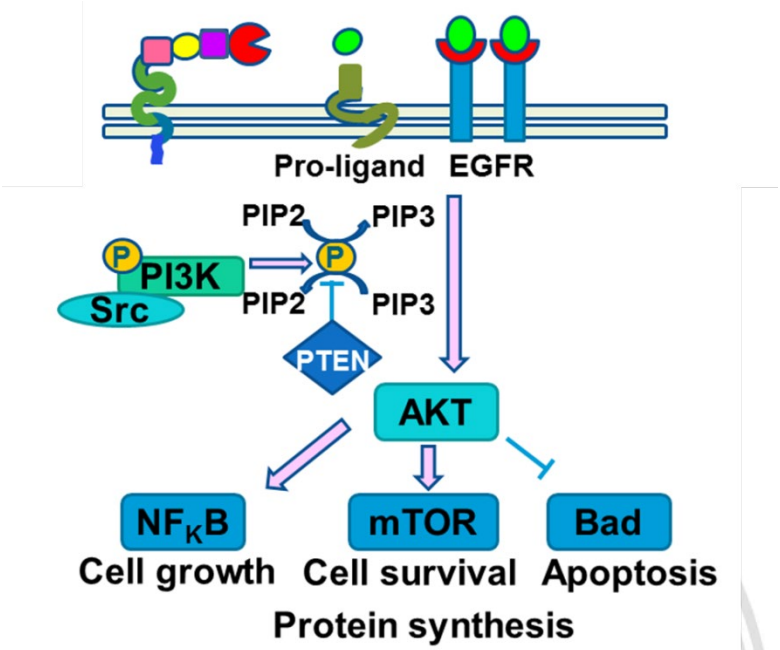

Figure 5.4 The ADAM9/EGFR/Akt pathway adapted from Liu *et al*., 2015.[57]

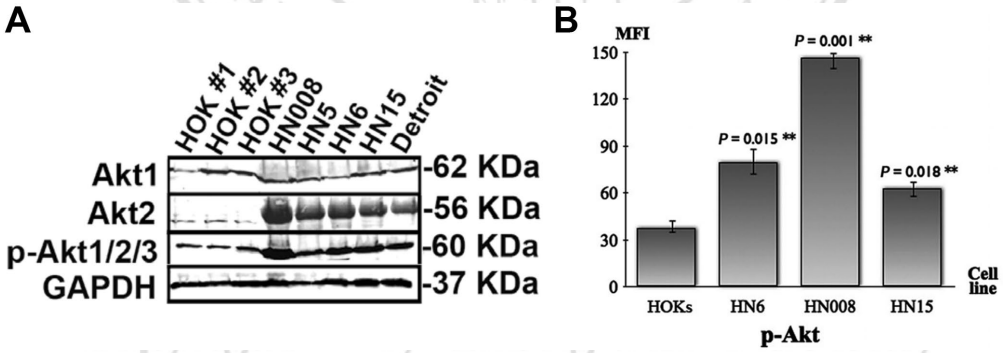

Figure 5.5 Increased expression of phosphorylated Akt (p-Akt) expression in the same OSCC cell lines. (A) A representative blot demonstrated overexpression and phosphorylation (p-Akt1/2/3) of Akt2 in five OSCC cell lines, including HN008, HN5, HN6, HN15, and Detroit.[59] (B) By flow cytometry, a bar graph demonstrated a significant increase in mean fluorescent index (MFI), reflecting increased expression of p-Akt in HN008, HN6 and HN15 compared with that in HOKs.[72]

Since ADAM9 does not appear to be essential in development, fertility or adult homeostasis, inhibition of ADAM9 in tumors may, therefore, cause little complication, which can be advantageous over current surgical, chemical and radiation therapies.[67] In recent decades, there have been many studies proposing the usage of ADAM9 as a prognostic or a therapeutic marker in various types of cancer. An example is a study conducted in high ADAM9- and low ADAM9-expressing gastric cancer cell lines. It has been found that monoclonal antibody specifically targeted against ADAM9 or small interfering RNA against ADAM9 can reduce cell proliferation and invasion in the high ADAM9-expressing cell line and in the hypoxia-induced ADAM9-expressing cell line, but not in the low ADAM9-expressing cell lines.[45] Consequently, different levels of ADAM9 expression in each cancer cell line may dictate the efficacy of monoclonal antibody or small interfering RNA targeted against ADAM9. Similarly, in the four oral cancer cell lines tested in this study, there were three high ADAM9-expressing cell lines (HN008, HN6 and HN15) and a low ADAM9-expressing cell line (HN5). Therefore, it is reasonable to assume that using monoclonal antibody or small interfering RNA to silence ADAM9 expression could prove useful to decrease aggressive behaviors in the high ADAM9-expressing oral cancer cell lines rather than in the low ADAM9-expressing oral cancer cell line. This warrants further investigations. Furthermore, it is also interesting to assess ADAM9 expression in OSCC tissues in order to develop ADAM9 as an OSCC biomarker for targeted therapy and personalized medicine.

In summary, ADAM9 is overexpressed in OSCC tissues and in some oral cancer cell lines and its expression is correlated with cancer cell differentiation, consistent with other cancer types and with increased ADAM9 expression in the suprabasal layers of the skin. Additional studies are still needed to explore the functional role of ADAM9 in the pathogenesis and aggressiveness of OSCC.