Chapter 5: Discussion

5.1 GNA12

5.1.1 Expression of GNA12 in oral cancer

This study confirmed that GNA12 is indeed up-regulated at the transcript and protein levels in OSCC tissues. We have shown that GNA12 was present at high levels in OSCC but not in the oral mucosa tissues, which thus validated the previous microarray findings reported from our laboratory (Cheong et al., 2009). Also, this finding is in line with other studies which showed that GNA12 was consistently highly expressed in various cancer types including NPC and adenocarcinomas of the breast and prostate (Kelly et al., 2006a; Kelly et al., 2006b; Liu et al., 2009). The over-expression of GNA12 may be required for the amplification of G12 signaling to promote the transforming effects of this oncogene.

Although our IHC analysis indicated that expression of GNA12 was not associated with LN involvement in OSCC, studies in breast and prostate cancers however demonstrated that GNA12 levels were significantly higher in *in situ* carcinoma tissues and invasive tumours, compared to benign tumours (Kelly et al., 2006a; Kelly et al., 2006b). In NPC, GNA12 transcript was also found to be elevated in the primary tumours of patients with LN metastasis, compared to those that do not (Liu et al., 2009). Furthermore, microarray profiling on HNSCC also identified GNA12 as one of the genes up-regulated in metastatic tumour (Braakhuis et al., 2006). There are a few possible explanations to this discrepancy observed. Firstly, tumours of the oral cavity have been suggested to be more heterogenous and distinct from other sub-sites of HNSCC (Chung et al., 2004), hence GNA12 alone may not be sufficient to distinguish between tissues with tumour spread to LN and to those that did not. Secondly, the aggressiveness of OSCC can differ depending on the occurrence site in the oral cavity (Woolgar, 2006), hence our observation that GNA12 was not associated to LN could be

attributed to the fact that the tumours of different sub-sites of the oral cavity were not adequately represented in the TMaA constructs, as more than 80% of the tumours examined here were from the cheek and gum which were suggested to be less aggressive than the tongue and FOM (Woolgar, 2006).

5.1.2 Effects of GNA12 in oral cancer

Having established that GNA12 is over-expressed in oral cancer, its role in oral carcinogenesis was further determined by *in-vitro* functional studies. As the activated GNA12 has been shown to be more potent in malignant transformation than the over-expression of the wild-type alone (Chan et al., 1993; Xu et al., 1993), the activated form of GNA12 which is a GTPase-deficient mutant (Gα12QL) was exogenously expressed in oral cancer cells. In this study, it was observed that the activated GNA12 could not be over-expressed at very high levels in the oral cells, as many cells did not survive after the virus transduction, suggesting that high levels of G-proteins activation can be cytotoxic (Xu et al., 1993).

However, it is evident that constitutive activation of GNA12 significantly induces oral cancer cell migration and invasion. This is consistent to that reported in NPC, breast and prostate cancers, where expression of the activated GNA12 constantly induced cancer cell migration and invasion (Kelly et al., 2006a; Kelly et al., 2006b; Liu et al., 2009). Moreover, the field of developmental studies also described that G12 family is important for cell-cell interaction, cell shape changes and movement during the gastrulation process in embryo development (Parks and Wieschaus, 1991; Lin et al., 2005). Although the mechanism underlying GNA12-mediated OSCC invasion have not been investigated due to time constraint in this project, other studies have suggested that the thrombin or LPA-induced G12/Rho/ROCK/JNK pathway is involved in cell-cell junction disruption to promote cellular invasion and migration (Marinissen et al., 2004;

Gavard and Gutkind, 2008). It has been discovered that GNA12 has the ability in modulating the reorganization of actin cytoskeleton (Buhl et al., 1995; Needham and Rozengurt, 1998; Gohla et al., 1999; Sah et al., 2000; Meyer et al., 2003; Xu et al., 2003) and epithelial-to-mesenchymal transition (EMT) for the conversion of cells from epithelial to fibroblastoid morphology (Liu et al., 2009). Although G12-mediated signaling often dependent on Rho activation, there are evidence showing that G12 family can directly interact and inactivate E-cadherin to promote cell migration in a Rho-independent manner (Meigs et al., 2001; Meigs et al., 2002).

In this study, GNA12 did not promote oral cancer cell growth and this is in concordance with that reported by Kelly and colleagues (2006a & 2006b) in breast and prostate cancers. However, GNA12 may be an important promoter for cell growth in certain cell types, as GNA12 in its wild-type and activated-form have been shown to be involved in fibroblast cells proliferation (Chan et al., 1993; Jiang et al., 1993; Xu et al., 1993; Voyno-Yasenetskaya et al., 1994). This may be due to the intrinsic properties of the tumour and also the differences in cellular origin such as the mesenchymal versus epithelial cells, since GNA12 may exert specific effects through the different down-stream signaling pathways (Fromm et al., 1997).

From clinical perspectives, this study suggests that GNA12 may be important for tumour dissemination and the inhibition of GNA12 in reducing local invasion and metastasis warrants further investigation. GPCRs that are up-stream of the G12 family have also been found to be over-expressed in many cancers and they currently represent the premier drug targets of 50-60% of all marketed therapeutic agents (Dorsam and Gutkind, 2007). Since many of the different GPCRs require G12 protein signaling to mediate cell invasion, it may not be feasible to block each individual receptor with different antagonist. Hence, GNA12 could be an alternative target for therapy and specific approaches to achieve this have been described by Holler (1999).

5.2 IFITM3

5.2.1 IFITM3 expression in oral cancer

This study demonstrated that IFITM3 was consistently over-expressed in OSCC at the mRNA and protein levels, thus validated the microarray findings reported on OSCC and HNSCC (Arora et al., 2005; Cheong et al., 2009). The up-regulation of IFITM3 in OSCC is in agreement with that observed in various other cancers (Hisamatsu et al., 1999; Andreu et al., 2006; Seyfried et al., 2008). In particular, elevation of IFITM3 levels is consistently reported in colorectal cancer and this has been proposed to be a potential biomarker for the early detection and/or disease progression for colorectal cancer (Hisamatsu et al., 1999; Andreu et al., 2006; Fan et al., 2008). While the mechanism of IFITM3 over-expression in OSCC is currently unknown, study in the mouse model for colorectal cancer has demonstrated that IFITM3 induction in was associated to the dysregulation of the Wnt/β-catenin signaling (Andreu et al., 2006). It is also possible that genetic and epigenetic changes at chromosome 11p15.5, where IFITM3 is located, can lead to abnormal expression of genes in HNSCC (Rainho et al., 2001).

Despite that OSCC significantly showed high levels of IFITM3, this had no significant association to the selected clinicopathological parameters. This is consistent with findings in colorectal cancer whereby IFITM3 mRNA levels were high regardless of tumour stage (Andreu et al., 2006). Moreover, elevation of IFITM3 levels in OSCC was also not associated with LN metastasis. As IHC evaluation on IFITM3 expression in HNSCC have not been reported in the literature, it is intriguing to note that IFITM1 which is a close family member of IFITM3, was detected to be highly expressed in the invasive front of HNSCC tumour (Hatano et al., 2008). Since the invasive front of the OSCC tumours was not specifically incorporated in the TMaA constructs, hence the expression of IFITM3 at the tumour invasive front was not determined in this study.

Interestingly, this study also demonstrated that the expression of IFITM3 in OSCC tissues was mainly detected in the cytoplasm even though plasma membrane staining was seen in very few cases. In contrast, IFITM3 staining on colorectal cancer was more confined to the plasma membrane (Appendix V). Since IFITM3 being a transmembrane protein, its presence in the cytoplasm is still not fully understood.

5.2.2 Effects of IFITM3 on oral cancer

Currently, studies on the role of IFITM3 in cancer are limited. This study examined if IFITM3 expression could contribute to any neoplastic phenotypes in oral cancer. It is intriguing to observe that IFITM3 negatively regulated oral cell growth. Consistently, up-regulation of IFITM3 was also found to be responsible for the growth inhibition of androgen-independent prostate cancer by estradiol (Coleman et al., 2006), and IFN-α-treated melanoma cells (Brem et al., 2003). Furthermore, Hisamatsu and colleagues (1999) suggested that the up-regulation of IFITM3 may play a role in the protection against the proliferation of inflammation-mediated cells or tumour cells. While this study have not identify the mechanism through which IFITM3 over-expression results in the suppression of cell growth, studies in melanoma cells in fact demonstrated that expression of IFITM3 protein alone is sufficient to inhibit cell growth (Brem et al., 2003). In addition, the anti-proliferative effects by IFITM3 can be possibly mediated through cell differentiation, and growth arrest (El-Tanani et al., 2009).

We further determined if the IFITM3-induced reduction in cell proliferation was caused by apoptosis. The small increased of apoptotic cell population detected in IFITM3 over-expressing cell may be due to a decreased proliferation rather than increased apoptosis, as being suggested by Ropolo et al. (2004). Interestingly, IFITM2 which is another close family member of IFITM3 has been shown to be a pro-apoptotic gene and that the induction of apoptosis is dependent on caspase activities and not on

p53 expression (Daniel-Carmi et al., 2009). In order to confirm if IFITM3 indeed induces apoptosis, the expressions of apoptosis-related proteins such as caspases need to be further examined.

This study also revealed that over-expression of IFITM3 resulted in the suppression of oral cancer cell invasion and migration, which is in agreement to the many findings that have reported the down-regulation of this protein in metastatic and invasive cancer cell lines (Huang et al., 2000; Brem et al., 2001; Varambally et al., 2005; Kang et al., 2009). Consistently, high levels of IFITM3 detected in urinary bladder cancer cells also suggested that it is involved in the suppression of the metastatic phonotype (Jung et al., 2002). In breast cancer, IFITM3 directly inhibited osteopontin, which eventually inhibited osteopontin-mediated cell adhesion, cell invasion and colony formation (El-Tanani et al., 2009). These also suggested that, IFITM3 seems to behave in opposing effect to IFITM1 which promoted HNSCC and gastric cancer invasion (Yang et al., 2005; Hatano et al., 2008).

Expression of IFITM3 seems to have inhibitory response on cell growth, invasion and migration. Despite the fact that IFITM3 is over-expressed in OSCC, this study was unable to address what causes IFITM3 to have functions which closely resembled to that of anti-tumour gene. Since IFITMs are immune-related genes, their expression could be affected by IFNs and cytokines (Lewin et al., 1991; Saetre et al., 2007). As IFN therapy is not currently used to treat oral cancers in Malaysia, it would not be possible that our patients are exposed to IFNs. However the 1-8 ISGs can be induced through IFN-independent manner, including ionizing radiation (Clave et al., 1997). Currently, this study have not examined if the up-regulation of IFITM3 in our OSCC patients is an effect after being exposed to radiotherapy. Furthermore, it is also unclear if the presence of IFITM3 could be due to an immune response towards inflammation at the site of cancer. To date, no mutation was detected in IFITM3 and its

ISRE near the promoter. It was proposed that the dysregulation of IFITM3 may be due to unknown post-translational modification such as phosphorylation, and also epigenetic changes (Ropolo et al., 2004).

It appears that over-expression of IFITM3 in OSCC does not support its role as shown in the *in-vitro* functional studies. This is perhaps not surprising as other members of the 1-8 ISG family have also been reported to have tumour suppressor properties despite their over-expression in several different cancer types. Notably, despite the fact that IFITM1 was found to be over-expressed in many cancers (Yang et al., 2005; Rogers et al., 2006; Hatano et al., 2008; Seyfried et al., 2008), other studies reported that IFITM1 exerted anti-proliferative effects in p53-dependent manner and suppression of this gene conferred tumorigenicity to a non-malignant hepatocyte in nude mice (Yang et al., 2007).

Therefore, further studies are required to further confirm the functional role of IFITM3 in OSCC, and further determine if it could be a useful therapeutic target.

Chapter 6: Conclusion

6.1 Summary

This study determined the expression of GNA12 and IFITM3 in oral cancer as well as their roles in oral carcinogenesis.

In this investigation, the expressions of GNA12 and IFITM3 were validated in Malaysia oral cancer patients. To the best of our knowledge, this is probably the largest study to describe and validate the expression of GNA12 and IFITM3 at the mRNA and protein levels. GNA12 and IFITM3 mRNA levels are confirmed to be significantly over-expressed in OSCC in comparison to oral mucosa control tissues. High levels of GNA12 and IFITM3 protein expressions were detected in OSCC by IHC staining on TMaA tissues. Statistical analyses revealed that high levels of GNA12 and IFITM3 are associated with OSCC, suggesting that they could be potential molecular markers for oral cancer. However, expression of GNA12 and IFITM3 showed no significant association to selected clinicopathological parameters.

GNA12 and IFITM3 were exogenously expressed in oral cancer cell lines. *In-vitro* studies indicated that GNA12 is involved in oral cancer cell invasion and migration, but has no effect on cell proliferation. Over-expression of IFITM3 in oral cancer cell line has inhibitory effects on cell growth, invasion and migration. Taken together, these evidence add substantially to our understanding of the roles and expressions of GNA12 and IFITM3 in oral cancer.

In conclusion, GNA12 may act as an oncogene in driving oral cancer invasion and its potential as therapeutic target needs to be further explored. Meanwhile, the role of IFITM3 remains to be further determined as high levels of this protein demonstrated anti-tumour effects.

6.2 Study Limitations

The findings in this study are subject to a few limitations. Firstly, the current project only investigated a relatively small number of specimens that are available, hence may have limited the statistical power to detect significant differences of GNA12 and IFITM3 expressions in association to the selected clinicopathological parameters. Secondly, in the *in-vitro* functional studies, the roles of GNA12 and IFITM3 were only demonstrated in one representative oral cancer cell line respectively, and require further confirmation in at least another oral cancer cell line. An issue that was not addressed in this study was whether the inhibition of target gene by siRNA techniques would complement to the results as observed in the over-expression studies. Finally, the interacting pathways of GNA12 and IFITM3 responsible for oral cancer have not been elucidated in this study.

6.3 Recommendations for future research

To address these study limitations, future studies should therefore concentrate on several of the recommendations stated below:

6.3.1 GNA12

Although functional studies supported that GNA12 is involved in cell invasion and migration, however this was not reflected in the IHC analysis as no association was found between GNA12 expression and LN involvement. It has been observed that OSCC on the tongue appeared to be more aggressive and often caused tumour spread to LN, in comparison to OSCC occurring at other sites of the oral cavity (Woolgar, 2006). Therefore, it would be interesting to assess the clinical relevance of GNA12 expression in tongue cancer by increasing the number of tissues in a more comprehensive study, and to further determine if there is an association to nodes invasion. In view that only

primary tumour was selected for IHC analysis in the present study, another approach is to examine the OSCC cells that are present in the LN to determine if these cells would also have high expression of GNA12.

The functional implication of GNA12 in cancer invasion and metastasis need to be further confirmed in another oral cancer cell line that has low level of this protein in order to determine if the observed phenotypes can be reproduce. Knock-down of GNA12 by short hair-pin RNA (shRNA) would be a good way to find out if this can revert oral cancer cells to non-malignant phenotype, and if they have "oncogene addiction" to GNA12 for the maintenance of the malignant phenotype.

Members of G12 family are highly homologous and that they share the common signaling mechanism with Gq, hence it remains a challenge to determine if they are equally important for the specific disease. The use of specific shRNA to GNA12 will enable us to differentiate the effects mediated by GNA12 from the other G-proteins, to determine the specific effects of this gene.

Inhibition of GNA12-mediated signaling could be capitalized for the treatment of invasive oral cancer. Notably, the G12 signaling can be blocked to inactivate down-stream signaling to the effectors by the expression of RGS protein, which in turn inhibited cancer cell invasion (Kozasa and Ye, 2004). Thus, further investigations using the RGS domain should be evaluated in the *in-vivo* system to determine if blocking GNA12 signaling is sufficient to prevent oral cancer cell invasion and/or migration.

GNA12-mediated signalings have oncogenic potential. Many of its interacting partners such as the GPCRs, RhoA, ROCK, JNK and MAPK have been reported to be dysregulated in cancers (Gavard and Gutkind, 2008; Liu et al., 2009). Hence, combination therapy can be designed to target multiple molecules of that pathway from a given state of "oncogene addiction". Given that GNA12 is involved in many pathways for regulating essential biological responses, careful dissection of its signaling

mechanism is crucial to define the aberrated pathway that is responsible for the invasive phenotype in cancer to minimize toxicity.

6.3.2 IFITM3

Despite the fact that IFITM3 was found to be over-expressed in OSCC, *in-vitro* experiments demonstrated that expression of IFITM3 has functions that closely resemble that of an anti-tumour gene. IHC analysis on IFITM3 should be examined in more detail to find out if the high level of IFITM3 present in OSCC could be due to the radiotherapy received by patients. In addition, post-translational modification and epigenetic changes in IFITM3 in OSCC patients remain to be investigated.

The functional roles of IFITM3 in oral cancer also requires confirmation in another transformed cell line to determine its tumour suppressive functions as well as to investigate the mechanisms that is also involved in IFITM3 anti-tumour effects. Studies have hinted the need to investigate the function of IFITM3 as a tumour antigen. This is because peptides derived from IFITMs were found to be antigenic and immunogenic, and can potential targets for a cytotoxic T-cells mediated immunotherapy in colon carcinoma (Tirosh et al., 2007). In addition, the effects of IFITM3 knock-down should be evaluated by si-RNA to confirm its anti-tumour effects in oral cancer.

IFITM proteins are well known to be present at low levels in most cells, and at higher level in cells exposed to IFNs (Friedman et al., 1984). Although studies on melanoma have demonstrated that IFITM3 level can be induced by IFN for anti-proliferative effects, it was thought that IFITMs have more specific effects than IFNs, as they could respond independently of IFNs. Therefore, factors affecting IFITM3 expression need to be examined before we can further determine its presence.