Chapter 1: Introduction

1.1 Background

Oral cancer is a major health problem worldwide, accounting for 274 000 new cases and 145 000 death annually, of which two thirds occur in developing countries (Ferlay et al., 2001). Despite the advances in treatment modality, the prognosis for oral cancer remains unchanged. In particular, the 5-year survival rate is less than 50%, which is one of the lowest among many cancers (Hicks and Flaitz, 2000). The variability of clinical outcomes in patients and the heterogeneity of the disease, emphasize the importance to enhance our understanding on the molecular pathogenesis of oral cancer.

Lately, molecular profiling has proven to be valuable tools for the sub-classification of many human cancers, such as leukemia (Golub et al., 1999), breast cancer (Perou et al., 2000; van 't Veer et al., 2002) as well as head and neck squamous cell carcinoma (HNSCC) (Chung et al., 2004). These studies have contributed significantly to the development of clinical management and treatment decisions for cancer patients.

In order to understand the molecular mechanism underlying oral cancer, a microarray study was conducted on Malaysian oral cancer patients (Cheong et al., 2009). This study identified 281 genes that are differentially expressed in oral squamous cell carcinoma (OSCC) in comparison to proximal normal oral tissues. Notably, the Guanine nucleotide binding protein alpha 12 (GNA12) and Interferon-inducible transmembrane protein 3 (IFITM3) were among the genes that were found to be highly up-regulated in OSCC. This finding is in accordance to other molecular profiling studies on HNSCC (Arora et al., 2005; Braakhuis et al., 2006).

GNA12 and IFITM3 have been found to be implicated in many cancers including breast (Kelly et al., 2006a), prostate (Kelly et al., 2006b) and colorectal

malignancies (Hisamatsu et al., 1999; Andreu et al., 2006; Fan et al., 2008). To the best of our knowledge, their expressions and functions in oral cancer have not been reported. Preliminary bioinformatics analysis showed that these genes are not abundantly present in the five major organs: brain, lung, heart, kidney and liver (Nakamura, 2007); indicating that they could be potential therapeutic targets for oral cancer.

1.2 Aim

Given that GNA12 and IFITM3 were up-regulated in the previous microarray study (Cheong et al., 2009) and their implications in other cancers, we aim to examine the potential use of GNA12 and IFITM3 as molecular markers as well as therapeutic targets for oral cancer.

1.3 Specific objectives

This study is divided into two arms as the following:

(A) GNA12

1. To validate the mRNA expression of GNA12 in OSCC and non-malignant tissues.
2. To validate GNA12 protein expression in OSCC and non-malignant tissues, and to determine the association of its expression with selected clinicopathological features.
3. To exogenously express GNA12 in oral cancer cell line, and further determines its function in oral cancer.

(B) IFITM3

1. To validate the mRNA expression of IFITM3 in OSCC and non-malignant tissues.
2. To validate IFITM3 protein expression in OSCC and non-malignant tissues, and to determine the association of its expression with selected clinicopathological features.
3. To exogenously express IFITM3 in oral cancer cell line, and further determines its function in oral cancer.

Chapter 2: Literature Review

2.1. Oral cancer

2.1.1 Oral cancer incidence: global and Malaysia

Oral cancer ranks as the 9th most prevalent cancer worldwide in year 2000 (Stewart and Kleihues, 2003). In the same year, 266, 672 oral cancer cases have been reported (Barnes et al., 2005), and the World Health Organization (WHO) expects a worldwide rising oral cancer incidence in the next decades. The American Cancer Society estimated a total of 35, 720 new cases, and 7, 600 deaths from cancer of the oral cavity and pharynx are projected to occur in the United States of America in 2009 (Jemal et al., 2009). Although the incidence of oral cancer in Western countries is relatively low, it ranks amongst the three most common types of cancer in South-East Asia and Central Asia and the number of cases is expected to increase (Hirayama, 1966; Zienolddiny et al., 2004). Incidence of oral cancer showed extensive epidemiologic variations in geographical distribution. Differences across countries particularly relate to distinct risk profiles (Stewart and Kleihues, 2003). Notably, oral cancer is highly prevalent in India, where 90% of these patients are women who chew betel quid (Barnes et al., 2005)

The incidence of oral cancer differs between genders. Generally, the incidence is higher in men in the west. According to Stewart and Kleihues (2003), oral cancer ranks the eighth most common cancer worldwide in males. The incidence and mortality rates are higher in men than women in the West because men have heavier indulgence in tobacco and alcohol habits. However, in India, the incidence of oral cancer in women is greater than or equal to that of men because betel quid chewing and smoking are common in both gender.

The incidence of oral cancer increases with age (Levi et al., 1999). In most countries, oral cancer appears to be more confined to the elderly. In the West, 98% of cases were reported in patients over 40 years of age (Macpherson et al., 2003), whilst cases occur in young patients were also observed and these were linked to early exposure to tobacco and betel quid (Gupta and Ray, 2004).

Interestingly, there is also a difference in the site of the cancer between Western and Asian patients. The common sites of oral cancer found in the Western population are tongue and floor of the mouth, which is closely associated with the habit of smoking and alcohol drinking (Reibel, 2003). On the other hand, buccal mucosa represents the primary site for cancer development in India and South-East Asia where betel quid chewing is prevalent (Zain and Ghazali, 2001; Nagpal and Das, 2003).

According to the Ministry of Health in Malaysia, oral cancer is the second leading cause of death for cancers among males in public hospital (1998). The prevalence of oral cancer in the general population is at 0.04% and oral cancer lesions are found predominantly in Indians (Zain et al., 1997). Tongue and mouth cancers are common in the Indian population (Lim et al., 2008). The age standardized rate (ASR) of tongue and mouth cancers for Indian males and females in Malaysia were among the highest in global comparisons (Figure 2.1 and Figure 2.2) (Lim et al., 2008) and comparable to the Indian subcontinent such as Trivandrum and Madras where oral cancer is a known major problem (Lim et al., 2008). Meanwhile, the indigenous people of Sabah and Sarawak were also identified as high risk groups for oral cancer (Zain and Ghazali, 2001). The prevalence of oral cancer in these communities is evident and partly attributed to their cultural practice of betel quid chewing, as well as the use of tobacco and alcohol (Hirayama, 1966; Ramanathan and Lakshimi, 1976; Zain et al., 1997; Zain, 2001). In terms of site, the tongue and cheek are the most common sites affected in oral cancer (Norlida et al., 2008).

Although OSCC can appear de novo, a percentage of OSCC may begin as potentially malignant lesions that appear as white, red or ulcerated areas in the mouth. These lesions are usually not painful, but a small percentage may quietly and slowly become malignant (van der Waal, 2009). Hence, the disease is usually presented at a more advanced or late stage at the time of diagnosis. In Malaysia, 64% of oral cancer patients are presented at stages 3 and 4 (Norlida et al., 2008). Moreover, majority of these patients affected are of more than 60 years of age, hence contributing to more challenges in combating this disease. Thus, these patients not only suffer from poor quality of life, they also have relatively poor prognosis (Ministry of Health Malaysia, 2006).

Figure 2.1: Mouth cancer international comparisons. Incidence of mouth cancer highlighted in red demonstrates that the incidence in Malaysian Indian females and males is among the highest in the world (Lim et al., 2008).

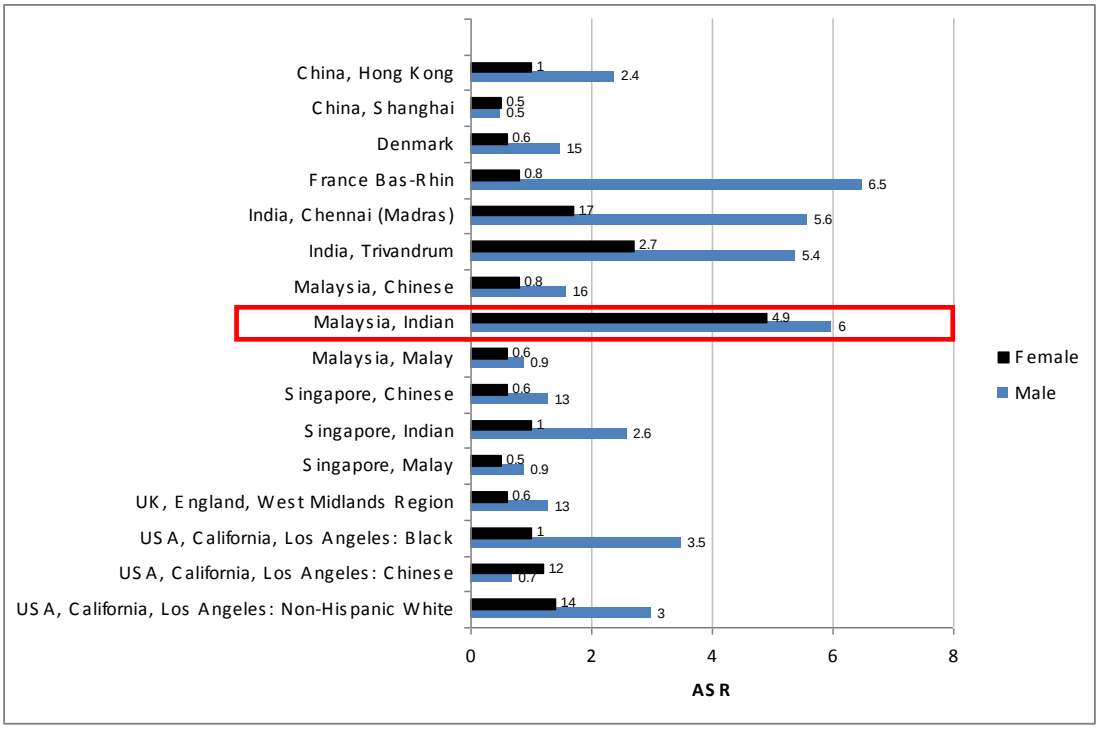


Figure 2.2: Tongue cancer international comparisons. Incidence of tongue cancer highlighted in red demonstrates that the incidence in Malaysian Indian females and males is among the highest in the world (Lim et al., 2008).

2.1.2 Classification of oral cancer

Oral cancer is a subgroup of head and neck malignancies that occur in the oral cavity which extends from the lips to the palatoglossal folds. As determined by the World Health Organization (WHO) International Classification of Disease version 10 (ICD-10), oral cancer is classified under C00-06, which comprise of tumours arising from the lips, anterior two-thirds of the tongue, salivary glands, gingiva, floor of the mouth, buccal surfaces and hard palate (Barnes et al., 2005). Tumours of the oral cavity can be epithelial, mesenchymal or haematolymphoid, depending on the type of tissues origin, however, more than 90% of malignant neoplasm of the oral cavity are squamous cell carcinomas (SCC) of the lining mucosae (Barnes et al., 2005). OSCC is an invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to spread to the lymph node (LN). Cancers of the salivary gland and sarcoma account

for less than 10% of all oral cancers and are aetiologically and biologically distinct from OSCC, so they will not be discussed in this study.

In clinical practice, treatment planning and prognosis for OSCC patients are mainly based on the tumour, node and metastasis (TNM) classification. Tumour staging has a crucial influence on the outcome of the patient, in particular the 5-year survival rate markedly decreases with the increase of disease stage (Lo et al., 2003).

2.1.3 Risk factors associated to oral cancer

In general, OSCC is considered as a disease which results from long term mucosal exposure to various carcinogens and co-factors (Zain and Ghazali, 2001). Risk behaviors, particularly the use of tobacco and areca nut have been recognized to be directly related to oral cancer incidence worldwide. Meanwhile, alcohol is considered as co-factor in the etiology of oral cancer (Barnes et al., 2005).

Tobacco use is estimated to account for about 41% of oral and/or pharyngeal cancer cases in men and 11% in women (Stewart and Kleihues, 2003), and its continuous usage is associated with tumour recurrence and aggravated periodontal breakdown (Nagpal and Das, 2003). More than 300 compounds present in tobacco have been identified to be carcinogenic (Schwartz, 2000; Das and Nagpal, 2002). The risk of oral cancer greatly increases when tobacco is used in combination with alcohol and/or betel quid chewing (Ko et al., 1995).

Betel quid chewing is common in the population of the Indian subcontinent and South-East Asia (Gupta and Ray, 2004). The areca nut and tobacco used in betel quid preparation have been identified as carcinogens (Zain et al., 1999). Exposure to alkaloids and polyphenols from areca nut during betel quid chewing contributes to genomic instability and increase risk to oral cancer (Zienolddiny et al., 2004). Hence,

oral cancer associated with betel quid chewing has been correlated with poorer prognosis (Massano et al., 2006).

Heavy intake of alcoholic beverages has been associated with nutrient deficiency, increased susceptibility to carcinogens and immune suppression. Moreover, acetaldehyde present in alcohol has been identified as tumour promoter (Petersen, 2009). Alcohol and tobacco showed a synergistic interaction in the etiology of oral cancer (Barnes et al., 2005), as alcohol can serve as solvent to enhance the penetration of carcinogens from tobacco into target tissues. Notably, alcohol and tobacco accounted for 75% of the disease burden of oral and oropharyngeal malignancies in Europe, America and Japan (Barnes et al., 2005).

Despite the large numbers of people using tobacco and alcohol, only a small percentage of them develop cancer. Many have attributed this to the differential ability of different individuals in breaking down and detoxifying carcinogens found in these substances. With this in mind, many groups have studied the role of genetic polymorphisms, particularly in xenobiotic enzymes in contributing to the risk of oral cancer development (Hung et al., 1997; Nair et al., 1999). Moreover, OSCC in individuals younger than 40 years of age has been reported to be on the increase. Although many of these were still claimed to be due to high exposure to risk factors including smoking and alcohol consumption (Llewellyn et al., 2003; Llewellyn et al., 2004), some of these individuals do not have any apparent risk habits (Sarkaria and Harari, 1994; Mork et al., 1999; Oliver et al., 2000), suggesting that there may be other genetic predisposition factors at play in these individuals. Other risk factors for oral cancer include infection of Human papilloma virus (HPV) genotype -16 and -18. Of interest, two papers have reported that the presence of HPV infection in Malaysia oral cancer patients is common (Hamid et al., 2007; Saini et al., 2010).

2.1.4 Prognosticator for oral cancer

Despite the advancement in technologies, the prognosis for late stage oral cancer is poor, with a 5-year survival rate of less than 50% (Hicks and Flaitz, 2000). Moreover, about 40-50% of patients with advance disease experience recurrence and approximately 80% of these recurrences occur within the first two years (Jones et al., 1992; Takes et al., 1997). To date, prognostication for oral cancer patients relies on the histopathological assessment of surgical resection specimen. Tumour features that can adversely affect the prognosis of oral cancer patient are tumour site, tumour size/thickness (depth), lymph nodes (LN) metastasis, histological grading, invasive front grading, lymphovascular and perineural invasion (Barnes et al., 2005; Woolgar, 2006).

Generally, LN metastasis is widely accepted as one of the major prognostic factors in oral cancer. Patients with LN metastases tend to have higher recurrence rates and significantly lower survival rates than those with disease-free nodes (Barnes et al., 2005; Massano et al., 2006). The association between tumour site and survival is explained by tumour site's influence on nodal metastasis and local spread. Some anatomic sites such as the superior gingivolabial sulcus, tongue and retromolar region, are linked with poorer outcome owing to the rich lymphatic drainage and the local extension being hard to evaluate and manage (Barnes et al., 2005; Woolgar, 2006). Tumour thickness was found to be a more accurate histological prognosticator than tumour size in predicting LN metastases (O-charoenrat et al., 2003; Massano et al., 2006). In Broders' classification, tumours are categorized into 3 groups based on the differentiation or maturation of the tumor cell population, namely well, moderate and poorly differentiated. However, a lack of correlation between Broders' grades and the prognosis of oral cancer have been reported (Bryne, 1998). This is most probably because cancer cells usually exhibit a heterogenous cell population with differences in

invasiveness and metastatic behavior. Hence, focusing on the pattern of invasion at the invasive front has been shown to be a more reliable prognosticator.

Although these parameters for prognostication have been useful in the clinical management of oral cancer patients, there are limitations in current diagnosis and prognosis methods. Clinicians have observed that not all tumours of the same stage behave similarly or respond to treatment equally. Therefore, identification of disease-related molecular markers may provide more accuracy in defining tumour behavior (Brinkman and Wong, 2006). Several *in-vitro* markers of malignancy such as loss of contact inhibition, tumour cell mobility and secretion of proteolytic enzymes were found to correlate with LN metastasis (Woolgar, 2006). Combination of these molecular markers with histological features is expected to enhance the sensitivity in predicting prognosis and treatment response.

2.1.5 Treatment

Current treatment regimes for oral cancer include surgery, radiotherapy, chemotherapy or a combination of the three modalities. Several factors can influence the choice of treatment including patients' age and health condition. In addition, the characteristics of the tumour are also taken into consideration before deciding on a treatment option and these are the site and size of the primary tumour, presence of nodal metastasis, spread of tumour outside the LN capsule, perineural and bone invasion by the primary tumour (Das and Nagpal, 2002).

The utilization of imaging technology such as computerized tomography and magnetic resonance imaging has increased visualization of the extent of the primary tumour and has improved assessment of the cervical LN to aid treatment decision (Ord and Blanchaert, 2001). Surgical resection is still the mainstay for oral cancers. Patients with advance stage cancer are often given radiotherapy and/or chemotherapy in

conjunction with surgery, either as adjuvant therapy to improve overall survival or as neoadjuvant therapy to shrink the size of tumour in order to facilitate surgery (Das and Nagpal, 2002). Currently used chemotherapy agents are cisplastin, carboplatin, 5-fluorouracil and the taxanes (Ord and Blanchaert, 2001).

There are many challenges in treating oral cancer. Local and regional tumour recurrence develops in approximately one-third of patients, despite definitive treatment. Successful therapy of oral cancer has been significantly hindered by the subsequent development of second primary tumors, the major determinant for treatment failure and death (Das and Nagpal, 2002). Oral cancer is one of the most severely debilitating forms of malignancy. The sequelae of oral cancer and its treatment include not only of facial mutilation, with its resultant social and psychological implications, but also impairment of salivary gland function, diminution of immune function and detrimental effects on speech, swallowing and breathing (Das and Nagpal, 2002). As elder patients are predominantly affected by oral cancer, there is a need for the development of less invasive treatment strategy such as molecular targeted therapy in the form of vaccine and antibody, in curative and palliative settings to improve the quality of life. Targeted therapy such as the anti-Her-2 monoclonal antibody (Herceptin) has showed increased in the survival of relapsed patients with Her-2-overexpressing breast tumors (Baselga, 2001). The success of Herceptin serves as a benchmark for cancer research where understanding the molecular mechanisms underlying the development of cancer can provide a rational foundation to explore new treatment modalities and to stratify patients that may benefit from these therapies.

2.1.6 Molecular alterations in oral cancer

Gene expression studies have been proven to be useful to improve characterization and/or sub-classification of human cancers such as breast cancer and leukemia (Golub et al., 1999; Perou et al., 2000). Such findings have important implications for in the prediction of clinical outcomes in cancer patients and tumour metastasis (Garber et al., 2001; Ramaswamy et al., 2003; Nguyen et al., 2009). For example, Mamaprint was developed based on molecular changes in breast cancer for the prediction of tumour metastasis and disease prognostication, to facilitate therapeutic strategies (van 't Veer et al., 2002).

Oral cancer is one of the few cancer types where it is possible to obtain biopsies at all stages of cancer progression, hence it is possible to define a genetic progression model of this disease by genetic profiling (Nagpal and Das, 2003). Several studies on head and neck squamous cell carcinoma (HNSCC) have demonstrated that a plethora of genes were differentially expressed in normal and malignant oral epithelium (Leethanakul et al., 2000a; Leethanakul et al., 2000b; Alevizos et al., 2001; Kuo et al., 2003; Leethanakul et al., 2003; Tsai et al., 2004; Warner et al., 2004; Miyazaki et al., 2006; Cheong et al., 2009). In particular, a study on HNSCC had successfully categorized these tumours into four distinct subtypes: possible epidermal growth factor receptor (EGFR)-pathway signature, a mesenchymal-enriched subtype, a normal epithelium-like subtype and a subtype with high levels of antioxidant enzymes (Chung et al., 2004). In addition, they also showed that the expression pattern can be used to predict LN metastasis at 80% accuracy. In another study, (Patel et al., 2008) showed that a subset of proteins are differentially abundant within normal oral squamous epithelia and tumours, and these subsets of protein correlated with differentiation grading of tumour, in that it is able to effectively distinguish among well, moderate and poorly differentiated tumours.

It is evident that the understanding and identification of differentially expressed genes can help us tailor better management strategies. Recently, the metastatic signatures for primary oral and oropharynx SCC can identify tumours that have metastasized to the LN (Chung et al., 2004; Schmalbach et al., 2004; O'Donnell et al., 2005; Roepman et al., 2005). Strikingly, a study in HNSCC had revealed that gene expression patterns in tumour cells metastasized to the LN are most similar to the corresponding primary tumors from which they arose (Roepman et al., 2006). This indicated that metastatic properties are acquired early during tumorigenesis and sustained through cancer progression and therefore, the identification of these changes in the primary tumour can predict the LN status in these patients.

The molecular profile of oral cancer patient in Malaysia was reported in a previous study (Cheong et al., 2009). This study demonstrated that although there were common pathways identified in oral cancers, some of the gene expression changes observed can be attributed to the aetiological factors associated with the initiation and development of these cancers. Notably, Cheong et al. (2009) identified 281 genes to be differentially expressed between tumour and normal oral mucosa tissues, and amongst these genes, GNA12 and IFITM3 were identified to be up-regulated in OSCC.

2.2 Guanine nucleotide binding protein alpha-12 (GNA12)

2.2.1 Heterotrimeric guanine nucleotide binding proteins (G proteins)

Heterotrimeric G proteins are found in all classes of eukaryotes and have essential roles in mediating cell signal transduction. They are distinct from the monomeric G proteins commonly known as the Ras superfamily GTPases, because heterotrimeric G proteins comprised of two functional signaling units, a guanine nucleotide binding α-subunit which contains the intrinsic GTPase activity; and a high affinity βγ-dimer (Rens-Domiano and Hamm, 1995; Wennerberg et al., 2005).

Heterotrimeric G proteins are important secondary messenger for G protein-coupled receptors (GPCRs) in channeling external stimuli such as growth factors, hormones, neurotransmitters, chemokines, photons, local mediators, and sensory stimuli (Figure 2.3), to elicit a wide variety of physiological responses in the cell through the activation of various effectors (Strathmann and Simon, 1991; Hamm, 1998; Offermanns, 2001; Wettschureck and Offermanns, 2005). Upon ligand binding to the GPCR, the conformation changes in the receptor catalyses the activation of heterotrimeric G-proteins whereby the α-subunit releases its bound guanosine diphosphate (GDP) and bind to guanosine triphosphate (GTP). This event leads to the dissociation of the heterotrimeric G protein into a free GTP-bound α-subunit and a free βγ-dimer. Both GTP-bound α subunit and the βγ-dimer then initiate cellular responses by stimulating the activity of various downstream effectors such as adenylyl cyclases, phopholipases and ion channels (Hepler and Gilman, 1992; Hamm, 1998). The duration of G protein signaling is controlled by the lifetime of Gα-subunit in the GTP-bound form. The intrinsic GTPase activity of the Gα-subunit hydrolyzes the bound-GTP to GDP thus deactivating itself (Arshavsky and Pugh, 1998). This deactivation event can be enhanced by Phospholipase C (PLC) (McCudden et al., 2005) and the regulators of G protein signaling (Stadel et al.) (Siderovski et al., 1996; Wettschureck and Offermanns, 2005) for rapid shut-down of signaling. Then, the GDP-bound Gα-subunit re-associates with βγ-dimer and resets itself to interact with another molecule of GPCR (Figure 2.4), and the cycle starts again to amplify the signal in a short period of time (Hepler and Gilman, 1992; Hamm, 1998).

In the mammalian system, 23 Gα-subunits, 5 β-subunits and 12 γ-subunits have been identified (Simon et al., 1991; Dhanasekaran et al., 1998; McCudden et al., 2005). However, heterotrimeric G proteins are classified based on the amino acid sequences and functions of the α-subunit, and thereby is categorized into four subfamilies: Gs, Gi,

Gq, G12 (Table 2.1) (Rens-Domiano and Hamm, 1995; Kozasa et al., 1998). In particular, the G12 family will be further discussed in the next section because it is the only heterotrimeric Gα-subunits that have potent transforming capabilities when over-expressed (Chan et al., 1993).

Table 2.1: G-proteins: their receptors and effectors (Dhanasekaran et al., 1998).

Class/sub-family	Gα-subunit	Receptors	Effectors
Gs	$G\alpha_s$	β1,2-Adrenergic, Glucagon, Dopamine, Seratonin, α2-Adenosine, ACTH, LH, FSH, MSH, GNRH, and TSH	Adenylyl Cyclase Ca^{++} Channel Na^{+} Channel
	$G\alpha_{olf}$	Odorant receptors	Adenylyl Cyclase
Gi	$G\alpha_{i1}$ $G\alpha_{i2}$ $G\alpha_{i3}$	α2-Adrenergic, thrombin, m2-, 4-Muscarinic acetylcholine, α1-Adenosine, Vasopressin, Somatostatin	Adenylyl Cyclase K^{+} Channel Ca^{++} Channel Phospholipase C
	$G\alpha_{oA,B}$	Muscarinic	Phospholipase A2 K^{+} Channel Ca^{++} Channel Phospholipase C
	$G\alpha_{t1,2}$	Opsins	cGMP-Phosphodiesterase
	$G\alpha_g$	Taste receptors	cGMP-Phosphodiesterase Phospholipase C-β
	$G\alpha_z$	Dopaminergic, 5-HT1A, Seratonin	Adenylyl cyclase
Gq	Gαq $G\alpha_{11}$ $G\alpha_{16}$ $G\alpha_{14}$ $G\alpha_{15}$	α1-Adrenergic, LPA, Chemokine, Bradykinin, m1-, m-3, m-5-Muscarinic acetylcholine, Thrombin, LPA, Vasopressin, Thromboxane, Chemokine Histaminergic	Phospholipase C-β
G12	$G\alpha_{12}$	Thrombin, Thromboxane, LPA, Neurokinin-1	GEFs, GDIs, GAPs
	$G\alpha_{13}$	Thrombin, Thromboxane, Bradykinin, LPA, Neurokinin-1, Angiotensin II-AT1	GEFs, GDIs, GAPs

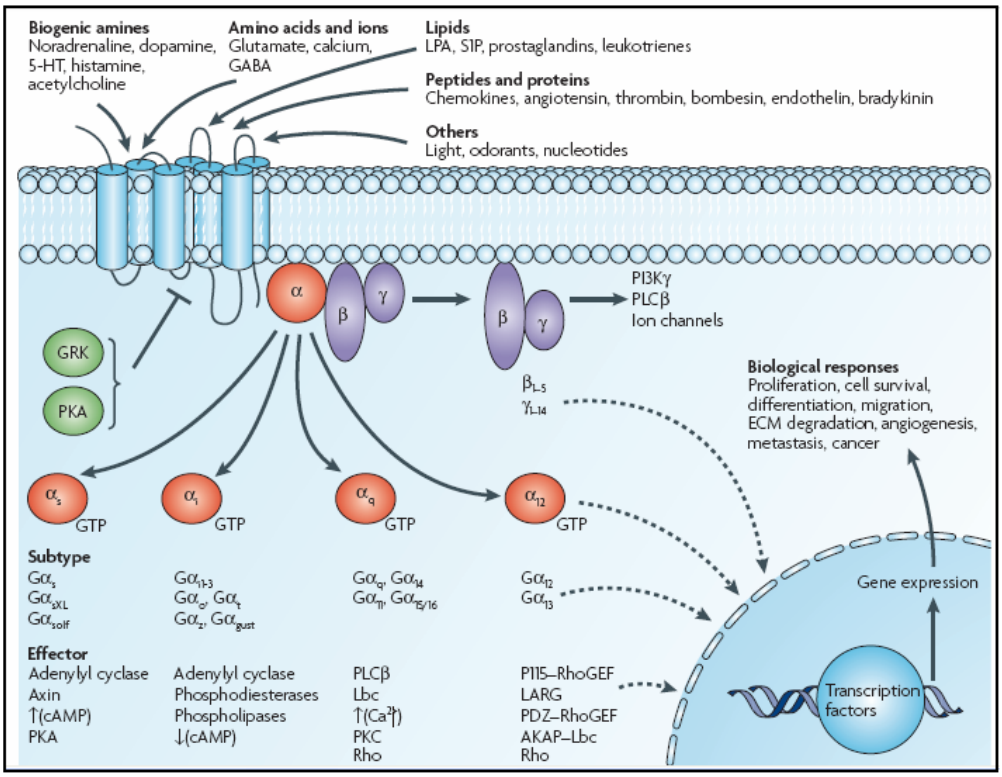


Figure 2.3: Diversity of GPCR and G-proteins signaling. G-proteins are classified into 4 sub-families, namely: Gs, Gi, Gq, Gα12. These G-proteins interacts with different effectors upon receiving extracellular signal through various GPCRs for the regulation of many cellular functions that are involved in cancer progression (Dorsam and Gutkind, 2007).

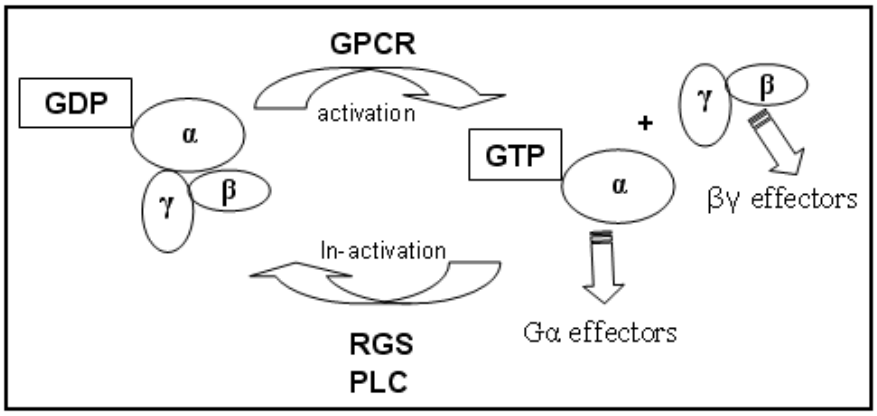


Figure 2.4: Activation and inactivation of G-protein signaling. GPCRs convert the heterotrimeric G-protein from the inactive GDP-bound, to active GTP-bound state. Activated G-protein further stimulates downstream effectors for signaling. RGS hydrolyzes the GTP-bound G-protein to terminate the signaling (Adapted from Milligan and Kostenis, 2006).

2.2.2 Heterotrimeric G proteins of the G12 family

G12 proteins were first isolated from a soft tissue sarcoma cDNA library by Chan et al. in 1993. It was classified as an oncogene due to its ability to induce transformation in rodent fibroblasts and tumour formation in animals (Chan et al., 1993; Xu et al., 1993). There are 2 members in the G12 family: GNA12 and GNA13, in which both members share 67% amino acid identity (Strathmann and Simon, 1991). The C-terminal of G12 proteins are involved in the interaction with GPCR, while their N-terminal short sequences which have low amino acid sequence homology, are reported to determine the selectivity of coupling to receptors as well as interacting with the βγ-dimer (Yamaguchi et al., 2002).

The G12-mediated signaling pathways are involved in a variety of physiological responses, including embryonic development (Ruppel et al., 2005), cell growth (Parks and Wieschaus, 1991), cell polarity and migration (Xu et al., 2003; Lin et al., 2005), angiogenesis (Offermanns et al., 1997), platelet activation (Moers et al., 2003), immune response (Girkontaite et al., 2001), apoptosis (Berestetskaya et al., 1998) and neuronal responses (Katoh et al., 1998). Notably, mouse embryos lacking G12 are embryonic lethal, indicating the functions of G12 members are equally critical for the regulation of many biological responses (Offermanns et al., 1997).

G12 family signal thorough GPCRs such as receptors for thrombin, lysophosphatidic acid (LPA) and thromboxane A2 (Riobo and Manning, 2005). Activation of G12 then leads to the activation of the small GTPases Ras, Rac, CDC42 and in particular RhoA which are the direct down-stream effectors, for a variety of intracellular processes (Hall, 1998). G12 proteins regulate RhoA via the family of guanine nucleotide exchange factors (GEF) for RhoA, including p115RhoGEF (Kozasa et al., 1998), PDZ-RhoGEF (Fukuhara et al., 1999) and LARG (Booden et al., 2002). These effectors posses the Dbl-homology (DH), pleckstrin-homology (PH) and the

regulators of G-protein signaling domains for the nucleotide exchange activity of Rho and for stimulation of the intrinsic GTPase accelerating protein (GAP) activity of G12. Abberation of G12-signaling was found in diseases such as leukemia, hypertension and ataxia (Suzuki et al., 2009).

The earliest identified role of G12 family in carcinogenesis was the ability to promote cell growth and induce neoplastic transformation (Chan et al., 1993; Jiang et al., 1993; Xu et al., 1993; Voyno-Yasenetskaya et al., 1994). Both the wild type and/or oncogenically activated mutant form of G12 caused malignant transformation in fibroblast cells where these cells demonstrated increased cell proliferation, anchorage-independent growth, attenuation of apoptotic signals, and neoplastic cytoskeletal changes. Many of the growth promoting and transforming effects of G12 are mediated by RhoA (Buhl et al., 1995). Stimulation of RhoA promotes the activation of JNK (c-Jun N-terminal kinases) (Marinissen et al., 2004), p38 MAPK (Marinissen and Gutkind, 2001), STAT3 (Kumar et al., 2006b) and PDGFα receptor (Kumar et al., 2006a) as well as serum response element-regulated transcription (Hill et al., 1995), NFκB-regulated transcription (Perona et al., 1997) and expression of COX-2 (Dermott et al., 1999; Slice et al., 1999). However, the G12 family is much more potent stimulators of fibroblast transformation than the RhoA (Fromm et al., 1997). In addition, G12 family can regulate transformation in a RhoA-independent manner, such as through Rac (Vaiskunaite et al., 2000), ERK5 (Fukuhara et al., 2000), and the cadherin-β-catenin complex (Meigs et al., 2001; Meigs et al., 2002).

Also, studies have indicated the participation of G12 and Rho in cell junctions signaling and stress fiber formation assembly of contractile actin and myosin filaments that can lead to cytoskeletal reassembly, shape change and loss of cell adhesion, commonly observed in cancer cells that results in cell migration (Buhl et al., 1995;

Needham and Rozengurt, 1998; Gohla et al., 1999; Sah et al., 2000; Meyer et al., 2003; Xu et al., 2003).

Although the G12 family members have high sequence homology and share some common binding partners, their functions do not completely overlap. GNA12 is more potent in inducing oncogenic transformation, cell shape changes, migration and gastrulation processes (Jiang et al., 1993; Xu et al., 1993; Vara Prasad et al., 1994), while GNA13 is more potent in inducing apoptosis and angiogenesis (Althoefer et al., 1997; Berestetskaya et al., 1998). Therefore, remains a challenge to identify how the signaling specificity is controlled in this family of proteins.

2.2.3 GNA12 and cancer

GNA12 or sometimes referred as Gα12 or *gep* oncogene is located at chromosome 7p22.2. The expression of wild-type or an activated mutant of GNA12 (Gln229-Leu; Gα12QL) induces neoplastic transformation of fibroblast cell lines (Jiang et al., 1993; Xu et al., 1993), and stimulates anchorage-independent growth and tumorigenesis in animal models (Chan et al., 1993; Jiang et al., 1993; Xu et al., 1993; Voyno-Yasenetskaya et al., 1994). Recent studies have shown that GNA12 level was up-regulated in certain cancer types, such as breast cancer (Kelly et al., 2006a), prostate cancer (Kelly et al., 2006a; Kelly et al., 2006b), head and neck cancer (Braakhuis et al., 2006) and nasopharyngeal cancer (Liu et al., 2009), supporting the role of GNA12 in cancer development. Although mutation that inhibit the intrinsic GTPase activity of the Gα-subunits resulting in the constitutive signaling pathways have been reported in Gαs and Gαi in different forms of tumours (Dhanasekaran et al., 1998), however activated mutational changes in GNA12 have not been found in human tumours.

Kelly et al. (2006a, 2006b) clearly demonstrated that expression of the activated forms of GNA12 [Gα12(Q231L)] promoted cancer cell invasion and migration.

Inhibition of G12 signaling by stable expression of the RGS domain of p115RhoGEF (a guanine nucleotide exchange factor) in metastatic carcinoma cells reduced the fate of metastatic dissemination of breast cancer cells in mouse model. Studies by (Meigs et al., 2002) provided more evidence that activated GNA12 can block cadherin-mediated cell adhesion in breast cancer cells, to promote invasion. In addition, GNA12 also mediated ovarian cancer cell migration, which depends on Rho signaling and its down-stream effector Rho kinase (ROCK) (Bian et al., 2006).

Interestingly, the growth promoting activity of GNA12 was not consistently observed in all cancer types. Enforced expression of the activated form of GNA12, or thrombin induced activation of GNA12 have been demonstrated to induce apoptosis via MAPK kinase kinase 1 (MEKK1)-mediated JNK activation and apoptosis-signal regulating kinase 1 (ASK1) stimulation in COS-7 cells (Berestetskaya et al., 1998), indicating that high levels of activated GNA12 is toxic in certain cell types.

GNA12 signaling consists of three main key players: GPCR, GNA12, and RhoA. Notably, in addition to GNA12, GPCRs and RhoA have been shown to be activated and over-expressed in cancer resulting in cancer cell proliferation, invasion and migration (Figure 2.5) (Dhanasekaran et al., 1998; Sahai and Marshall, 2002; Dorsam and Gutkind, 2007; Gavard and Gutkind, 2008), which supports the fact that G-proteins signaling pathways have oncogenic potential and can induce tumour formation.

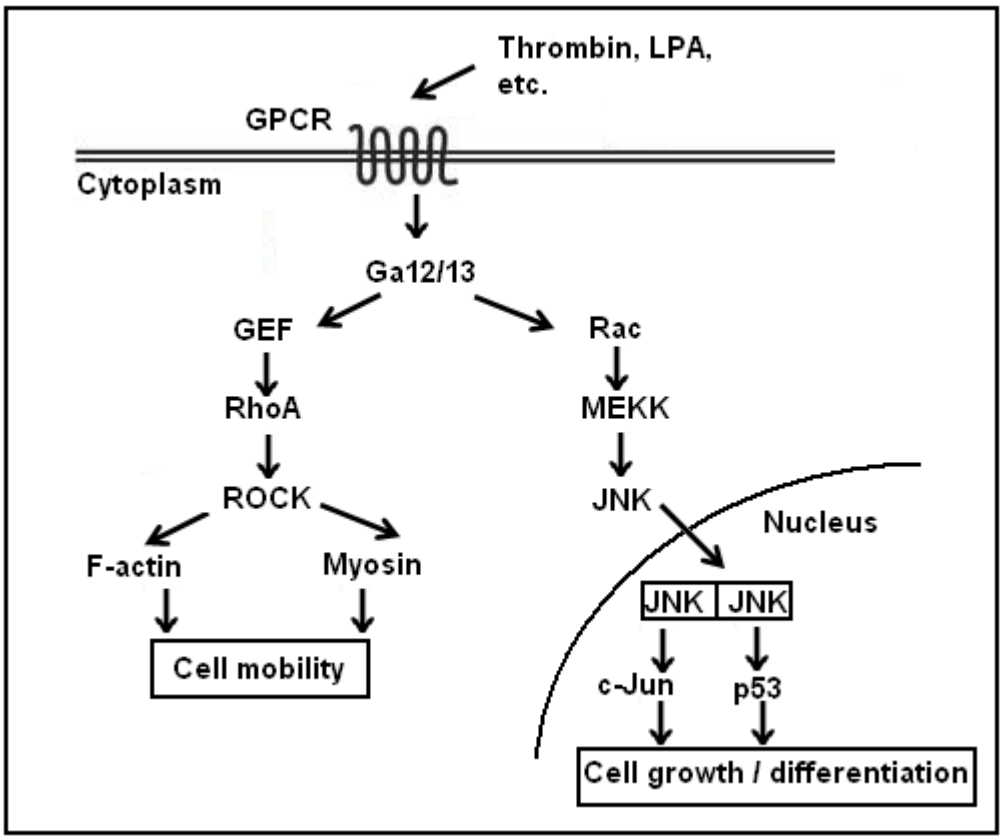


Figure 2.5: G12 signaling model. Ligands binding to GPCR triggers activation of Gα12/13 signaling for RhoA-mediated cell migration and/or Rac/JNK-induced cell growth, differentiation and transcription (Adapted from Gavard et al., 2008 and Liu et al., 2009).

2.3 Interferon inducible transmembrane protein 3 (IFITM3)

2.3.1 Interferon and interferon inducible genes

Interferons (IFNs) are multifunctional cytokines produced by a wide variety of cell types including leukocytes, fibroblast, natural killer and activated T-cells. They play a critical role in innate and adaptive immunity for the defense against viral and parasite infection as well as immune surveillance by inducing anti-proliferative and differentiating activities in cells (Isaacs and Lindenmann, 1957; Borden et al., 2007). IFNs are classified into two groups based on their binding receptors. The type I IFNs includes IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω; while type II IFN

consist only IFN-γ. Today, IFN-α, IFN-β and IFN-γ are used in various clinical settings in human (Pestka et al., 2004). Their antitumour effects were later discovered and demonstrated in certain cancer types including chronic leukemia, lymphoma, bladder carcinoma, melanoma, and renal carcinoma (Isaacs and Lindenmann, 1957; Gresser et al., 1969; Borden et al., 2000; de Veer et al., 2001). In the event of IFNs signaling to mediate the host defense mechanism, cascades of intracellular signaling pathways are activated, leading to up-regulation of many interferon-stimulated genes (ISGs) within the cell (de Veer et al., 2001; Martensen and Justesen, 2004).

ISGs are a diverse group of more than 300 genes that mediate the biological effects of IFN-stimulation (de Veer et al., 2001). Most of the ISGs have conserved interferon stimulable response element (ISRE) in their gene sequence that confer the responsiveness to interferon (Martensen and Justesen, 2004), and play important roles in host defense (Borden et al., 2007). Small ISGs have molecular mass between 10-20 kDa. These are further categorized into three families based on their amino acid sequence similarity: the ISG12 (6-16, ISG12, and ISG12-S), 1-8 (9-27/Leu, 1-8U and 1-8D) and ISG15 (ISG15/UCRP). These ISGs are widely expressed predominantly upon type I IFN induction (Martensen and Justesen, 2004).

2.3.2 The 1-8 ISG family

The human 1-8 ISG family includes IFITM1 (9-27/Leu), IFITM2 (1-8D) and IFITM3 (1-8U). They are positioned on chromosome 11p15.5, in the order of IFITM3, followed by IFITM1 and IFITM2 in reverse orientations. Members of the 1-8 ISG family have more than 90% sequence homology in over 70% of the coding sequence (Lewin et al., 1991). IFITM3 showed very high similarity to IFITM2, except that the latter contains additional 68 bp tandem duplication at the 3' non-coding region. The 1-8 ISG family carries the conserved ISRE with the sequence of GGAAAN(N)GAAAC at

the promoter/enhancer region that confer the responsiveness to IFNs (Figure 2.6). In particular, IFITM1 and IFITM3 mRNA are highly inducible by type I and II IFN, however IFITM2 may not response to IFNs as there is a nucleotide transition in the ISRE (Lewin et al., 1991).

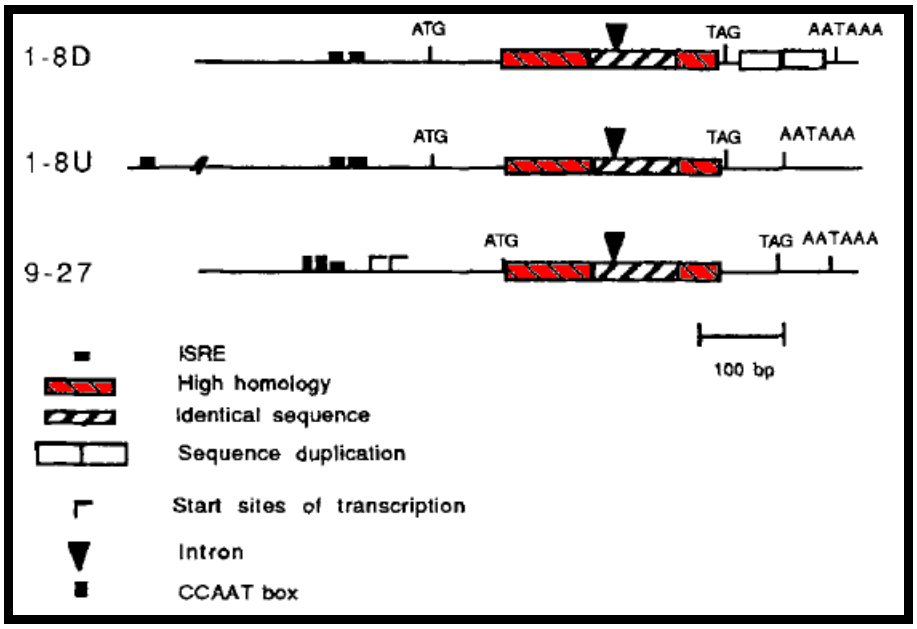


Figure 2.6: Sequence and main features of the 1-8 groups of genes. The 3 members of 1-8 ISG family have a very similar structure spanning 1.5kb with one intron. Each contain the ISREs in the immediately 5' flanking sequence (Lewin et al., 1991).

The 1-8 ISG family members are short surface proteins with two transmembrane helixes of high core sequence similarity but with more divergent amino (N) and carboxy (C) termini ends exposed to the extracellular environment (Figure 2.7B) (Brem et al., 2003; Ropolo et al., 2004). Interestingly, these proteins have a unique region in 25 amino acid intracellular domain at amino acid position 80 to 108, that is highly conserved across different species (Lange et al., 2003; Ropolo et al., 2004). The protein features of the 1-8 ISG family are as described in Table 2.2.

The 1-8 ISG family has been suggested to function in a variety of contexts, including cell growth (Deblandre et al., 1995; Brem et al., 2003), immune cell

regulation (Evans et al., 1993; Smith et al., 2006), carcinogenesis (Andreu et al., 2006; Tirosh et al., 2007; Hatano et al., 2008), somitogenesis and germ cell development (Lange et al., 2003; Saitou et al., 2003; Tanaka et al., 2004; Tanaka et al., 2005). During embryo development, the mouse *fragilis* family of ISGs that are homologous to the human 1-8 ISG family, are expressed in pluripotent embryonic stem cells (Lickert et al., 2005) and germ cell precursor (Saitou et al., 2003; Tanaka et al., 2005). They are responsible for the specification of germ cell fate in mice. Interestingly, *Fragilis* can repress the homeobox genes and ultimately program the cells to become totipotent germ cells (Saitou et al., 2003). In contrary, recent study on mice carrying a deletion of the *fragilis* gene family cluster showed no detectable effects on development of the germ line, viability or fertility (Lange et al., 2008). Although the up-regulation of the *fragilis* genes may be a standard response of tissues during systemic or local immune system stimulation, however the lack of these genes may affect the ability of these mutant mice to cope with different pathogenic challenges.

Molecular profiling on many cancer studies have demonstrated that the 1-8 ISG family was dysregulated (Wu et al., 2005; Yang et al., 2005; Fernandez-Cobo et al., 2006; Rogers et al., 2006; Fumoto et al., 2008; Hatano et al., 2008; Seyfried et al., 2008). However, the signaling mechanism involving 1-8 ISG family in tumourigenesis is yet to be elucidated. In this review, only IFITM3 will be discussed, in view that it is over-expressed in oral cancer.

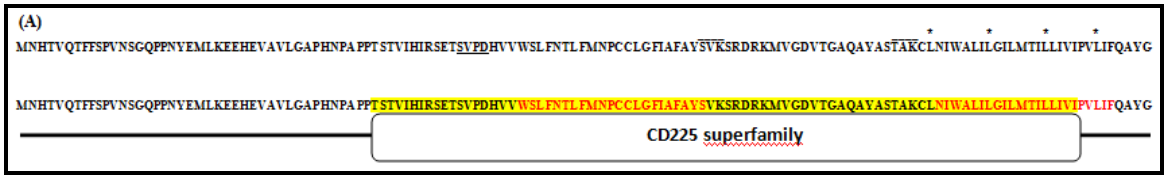
Table 2.2: Protein features of 1-8 ISG group (Martensen and Justesen, 2004)

1-8 family	Amino acid size	Transmembrane domain	Induction by IFN			Virus	Others
			-α	-β	-γ		
IFITM1	125	37-59 85-107	++	++	++	+VSV, +EBV, +HSV1, +CMV	IRF-1, BRG1, 5-aza-CdR, x-ray
IFITM2	132	57-79 105-127	-	-	ND	+EBV	LPS, BRG1, x-ray
IFITM3	133	58-80 106-128	++	ND	ND	+EBV	IRF-1, LPS
++: highly up-regulated; +: up-regulated; ND: not detected; -:not up-regulated							

2.3.3 Features of IFITM3 protein sequence and interacting partners

IFITM3 is also known as 1-8U, contains a conserved domain that belongs to the CD225 superfamily that suppresses cell growth in respond to IFNs (Friedman et al., 1984; Lewin et al., 1991). Functional motifs sequence analysis identified the presence of a leucine zipper, two potential protein kinase C (PKC) and one potential casein kinase II phosphorylation sites in the highly conserved region of all three molecules, but no signal peptides that are important for protein export are reported in these sequences (Figure 2.7A) (Lewin et al., 1991; Martensen and Justesen, 2004).

Although the signaling pathway for IFITM3 is not fully understood, some of its interacting partners have been described. For example, the SP1 transcription factor recruites BAF complex, to alter the chromatin structure of the IFITM3 promoter for IFITM3 activation (Liu et al., 2002). In addition, IFITM3 also responds to interleukin-6 (IL-6) signaling through activation of signal transducer and activator of transcription (STAT3) and Brahma-related gene 1 (BRG1) resulting in acetylation at the promoter of IFITM3 to facilitate transcription (Ni and Bremner, 2007).



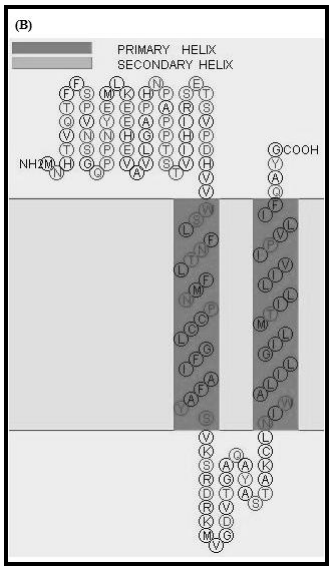


Figure 2.7 (A): Amino acid sequence of IFITM3. Possible casein kinase II (SVPD) phosphorylation site is underlined; protein kinase C (SVK, TAK) phosphorylation sites are overlined; (*) leucine zipper motif; red text indicate transmembrane region; conserved domain is highlighted in yellow. (Adapted from Lewin et al., 1991).

Figure 2.7 (B): IFITM3 secondary structure. Both the N- and C-termini are exposed to the extracellular environment. Predicted by classification and secondary structure prediction of membrane protein (http://bp.nuap.nagoya-u.ac.jp/sosui/)

2.3.4 IFITM3 and cancer

Members of the 1-8 ISG family including IFITM1 and IFITM2 have been found to be over-expressed in cancer (Tirosh et al., 2007; Hatano et al., 2008). Similarly, over-expression of IFITM3 has been shown to be associated with colorectal cancer (Hisamatsu et al., 1999; Andreu et al., 2006; Fan et al., 2008), urinary bladder cell carcinoma (Jung et al., 2002), breast cancer (Abba et al., 2004; Fernandez-Cobo et al., 2006) and oral cancer (Arora et al., 2005). The first reported incidence of IFITM3 over-expression was observed in colorectal cancer (Zhang et al., 1997; Hisamatsu et al., 1999), whereby IFITM3 was strongly expressed in sporadic and ulcerative colitis associated colon cancer and may be related to the activation of Wnt/β-catenin signaling (Andreu et al., 2006). Expression of this protein in severely inflamed colonic mucosa suggested the possibility of these tissues progressing into a tumour behavior. Hence, the potential use of IFITM3 as marker for early detection of colon cancer has also been strongly suggested (Fan et al., 2008).

IFITM3 was reported to be markedly over-expressed in androgen-independent prostate cancer cell lines, benign prostate hyperplasia tissues (Vaarala et al., 2000) and drug resistant melanoma cells (Wittig et al., 2002; Brem et al., 2003). In addition, it was suggested that over-expression of IFITM3 confers insensitivity to growth inhibitory effects of IFN, when the tetraspanin receptors formed by IFITM3, CD81, CD82 and major histocompatibility complex (MHC) proteins are saturated and hindered the transmission of anti-proliferative signals to the nucleus (Brem et al., 2003).

Down-regulation of IFITM3 has also been detected in cancer. Notably, IFITM3 was down-regulated in invasive and metastatic oral cancer cells (Kang et al., 2009). Moreover, the mouse and rat homologues of IFITM3 are consistently down- regulated in tumour compared to untransformed precursor cell line (Zuber et al., 2000; Brem et al., 2001a; Brem et al., 2001b). IFITM3 expression can be restored upon treatment with IFN

and anti-inflammatory drugs and this suppresses the tumour growth such as myeloma (Cheriyath et al., 2007) and gastric carcinoma (Mima et al., 2005). There are accumulating evidence that increase expression of IFITM3 has a negative effect on cell growth, such as in epithelial cells of prostate gland undergoing senescence (Untergasser et al., 2002), in mature human dendritic cells (Martensen and Justesen, 2004) and in polyploidization of megakaryocytes (Raslova et al., 2007).

From current evidences, it appears that IFITM3 could have both tumour promoting and suppressive properties, and this may be influenced by the genetic background of the tumour and the tumour microenvironment, therefore this warrants further investigation.