Chapter 4: Results

4.1 GNA12

4.1.1 Validation of GNA12 expression in OSCC

The mRNA and protein expressions of GNA12 were studied in two separate sample sets. Majority of the cases in the two sample sets do not overlap, except for a few cases.

(A) Over-expression of GNA12 at the mRNA level

QPCR analysis on cDNA from 65 fresh frozen tissues indicated a significant elevation of GNA12 mRNA level in OSCC (Figure 4.1A). In comparison to the oral mucosa tissues that were used as controls, OSCC tissues demonstrated an over-expression of GNA12 with an averaged RQ value of 3.27 ± 0.55 (Range: -4.5 to 23). Using a cut-off point of RQ ≥ 2, over-expression of GNA12 was observed in 55% of OSCC (n=26) while there were 32% of OSCC showed unchanged GNA12 level and 13% showed down-regulation of this gene (Figure 4.1B).

(B) Over-expression of GNA12 at the protein level and the association with selected clinicopathological features

Protein expression of GNA12 was examined by IHC staining using GNA12 antibody that recognizes the N-terminus of the protein. IHC staining on TMaA tissues demonstrated high levels of GNA12 in 75% of OSCC tissues (n=33), whereby the tumour cells predominantly displayed intense expression of GNA12 in the cytoplasm (Figure 4.2). In contrast, GNA12 staining was low in 87% of the oral mucosa tissues used as control (n=20). The surrounding stromal tissues showed very weak staining of this protein. Statistical evaluation further indicated that high level of GNA12 protein is associated to oral cancer (p<0.001) (Table 4.1).

GNA12 expression in relation to the clinicopathological parameters was evaluated. The demographic features of the OSCC patient were summarized in Table 4.2. The average age of OSCC patient is 56 years, ranging from 28 to 80 years. Majority of the OSCC cases are presented at late stage. The Indians are more affected by the disease than other ethnic groups, as expected. GNA12 expression was high in OSCC tissues regardless of tumour site, involvement of LN metastasis, tumour size and staging. Pearson chi-square tests further revealed that GNA12 expression was not associated with these clinicopathological factors (Table 4.3). However, the high levels of GNA12 consistently detected in OSCC tissues prompted further examination of its role in oral cancer development in the following sections.

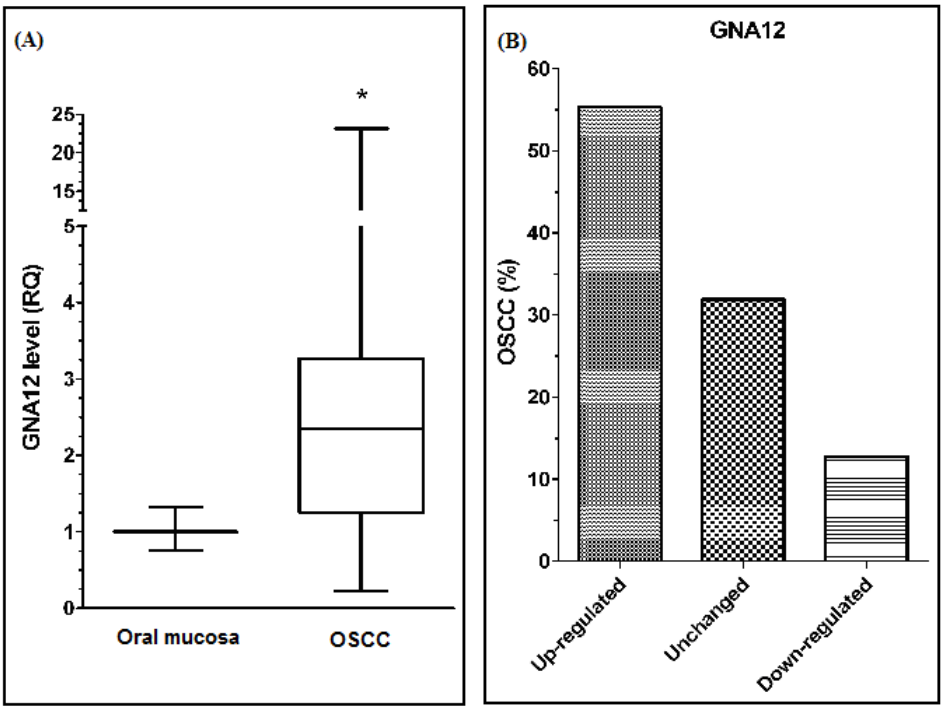

Figure 4.1: GNA12 mRNA levels in OSCC relative to oral mucosa tissues. (A) Box and whiskers plot showing comparative changes of GNA12 level in OSCC relative to the oral mucosa controls. OSCC tissues have high level of GNA12 (*$p < 0.001$). The band in the box is the median, and the ends of whiskers represent the minimum and maximum GNA12 levels. (B) Distribution of GNA12 mRNA expression in 47 OSCC patients. More than 50% OSCC cases exhibited over-expression of GNA12.

Table 4.1: Differential expression of GNA12 in oral tissues

Tissue type		GNA12 expression	
		Low (n, %)	High (n, %)
Oral mucosa*	**n = 23**	(20, 87.0)	(3, 13.0)
OSCC	**n = 44**	(11, 25.0)	(33, 75.0)

*FEP tissues and Tissues from the flap of gum excised during wisdom tooth extraction
Pearson chi-square test, p <0.001.

Table 4.2: Demographic data of 44 OSCC cases used for GNA12 IHC analysis

Variable		OSCC n	OSCC (%)	Mean	Standard deviation
Gender	Male	12	(27.3)		
	Female	30	(68.2)		
Age (year)				61	10
Ethnic	Malay	5	(11.4)		
	Chinese	4	(9.1)		
	Indian	34	(77.3)		
	Indigenous	1	(2.3)		
Risk habit	No habit	5	(11.4)		
	Betel quid	24	(54.5)		
	Smoking	4	(9.1)		
	More than 1 habit	11	(25.0)		

Table 4.3: Association of GNA12 expression to clinicopathological features of 44 patients with OSCC.

Characteristics		Patients (n)	GNA12 expression Low (n, %)	GNA12 expression High (n, %)	*p*-value
Broder's Grading	Well	14	(2, 14.3)	(12, 85.7)	0.438[+]
	Moderate	24	(7, 29.2)	(17, 70.8)	
Stage	I and II	13	(2, 15.4)	(11, 84.6)	0.456[+]
	III and IV	30	(9, 30.0)	(21, 70.0)	
Lymph nodes	Negative	23	(4, 17.4)	(19, 82.6)	0.180[+]
	Positive	19	(7, 36.8)	(12, 63.2)	
Tumour size	T1 and T2	22	(5, 22.7)	(17, 77.3)	0.661[++]
	T3 and T4	21	(6, 28.6)	(15, 71.4)	
Site	Cheek and gum	38	(9, 23.7)	(29, 76.3)	0.630[+]
	Tongue and FOM	6	(2, 33.3)	(4, 66.7)	

[+] Fisher exact test

[++] Pearson Chi-square test

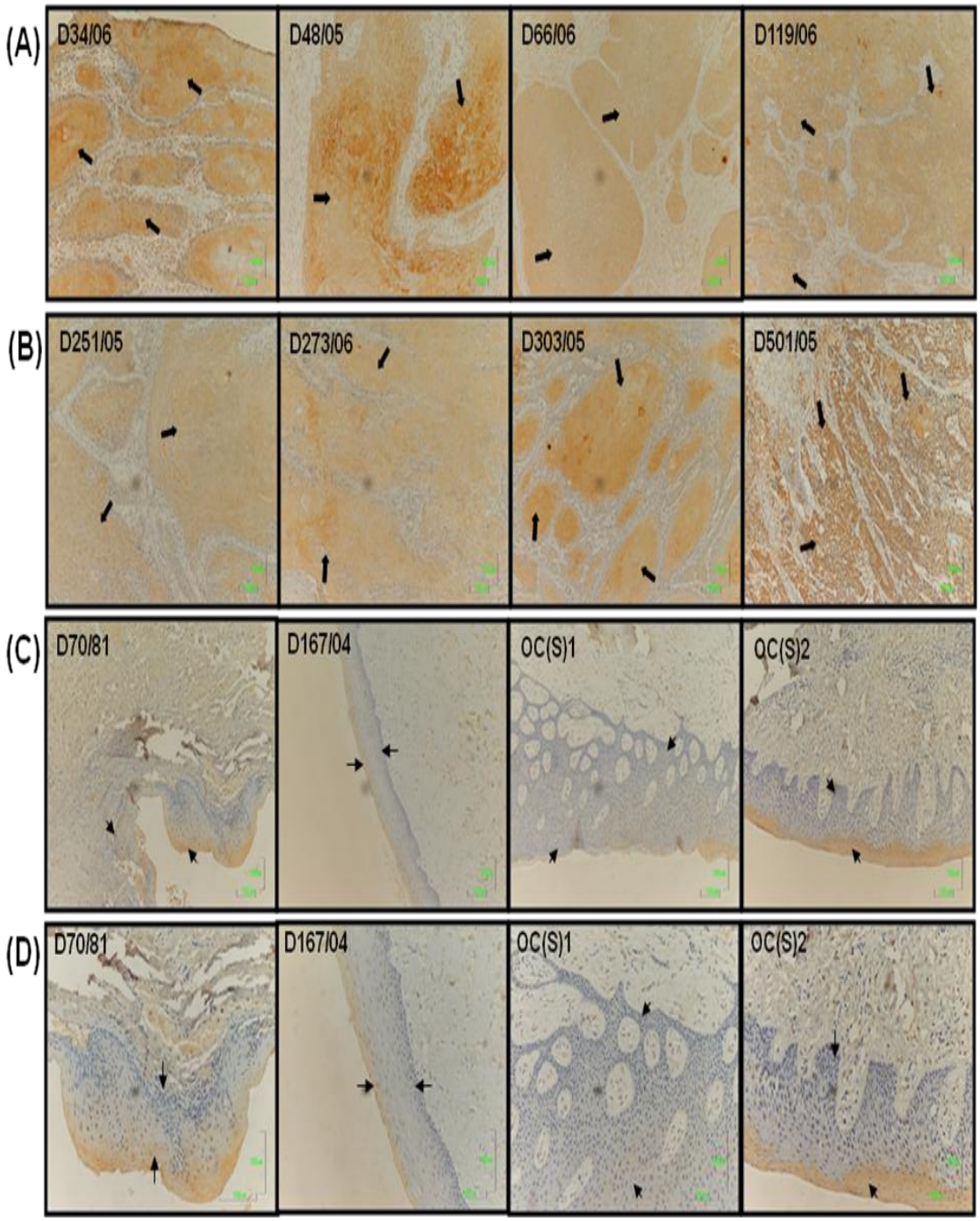

Figure 4.2: IHC staining of GNA12 in TMaA. High expression of GNA12 was detected in the tumour, as indicated by block arrows (Panel A & B, 100x magnification). Oral mucosa tissues showed negative staining, as indicated by arrow heads (Panel C, 100x magnification; Panel D, 200x magnification).

4.1.2 Modification of oral cancer cell line for GNA12 expression

(A) GNA12 plasmid propagation and extraction

Expression plasmids pLXRN and pLXRN/Gα12QL, as well as the packaging pVSV-G plasmid were propagated in XL1-Blue *E. coli* competent cells. The purified pLXRN, pLXRN/Gα12QL and pVSV-G plasmids showed the expected band size of 6.4kb, 7.6kb and 6.5kb respectively in agarose gel electrophoresis (Figure 4.3). The pLXRN/Gα12QL plasmid was used to express the 1.2kb activated GNA12 in oral cancer cell lines with low level of endogenous GNA12, whereas, the pLXRN plasmid was used as control.

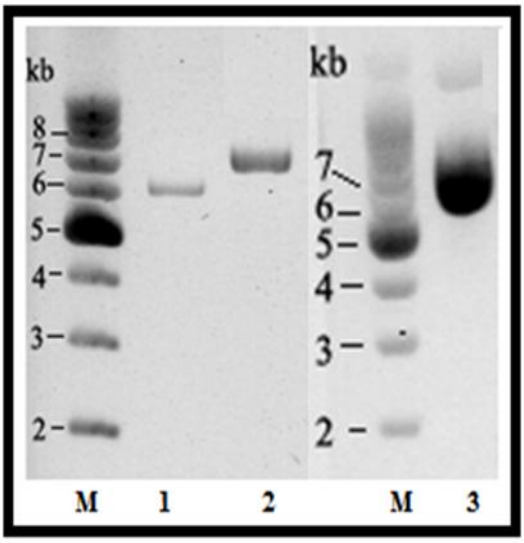

Figure 4.3: Agarose gel electrophoresis of purified plasmids. M: supercoiled DNA ladder, 1: pLXRN vector, 2: pLXRN/Gα12QL, 3: pVSVG vector.

(B) Exogenous expression of GNA12 in oral cancer cell line ORL150

The mRNA of GNA12 was present at low levels in majority of the oral cancer cell lines tested, and similarly these cells showed basal expression of GNA12 protein (Appendix X). However, since GNA12 mRNA level was found to be consistently down-regulated in ORL150 oral cancer cell line and the respective tissue where it is derived, hence it was selected for the exogenous expression of activated form of GNA12

The pLXRN/Gα12QL plasmid was delivered into ORL150 by retrovirus transduction and stable clones were selected by G418 treatment. The expression of GNA12 after transduction was evaluated and ORL150/Gα12QL cells showed a slight increased in the mRNA (RQ = 1.55) and protein (1.22-fold) levels, relative to the pLXRN-control cells (Figure 4.4A). Since RhoA is a well-known down-stream target for GNA12 in G12/13 signaling, we also assessed if RhoA expression will be affected in the transformed-ORL150 cells. Similarly, Western blot for RhoA revealed a minute increased protein expression of 1.37-fold in ORL150/Gα12QL cell (Figure 4.4B). These results indicated that high level of activated GNA12 cannot be achieved and may be toxic to cells, as it was consistently observed that cell survival was very low after retrovirus transduction. These transformed cells were subsequently used to study the effect of GNA12 activation on cell growth, migration and invasion.

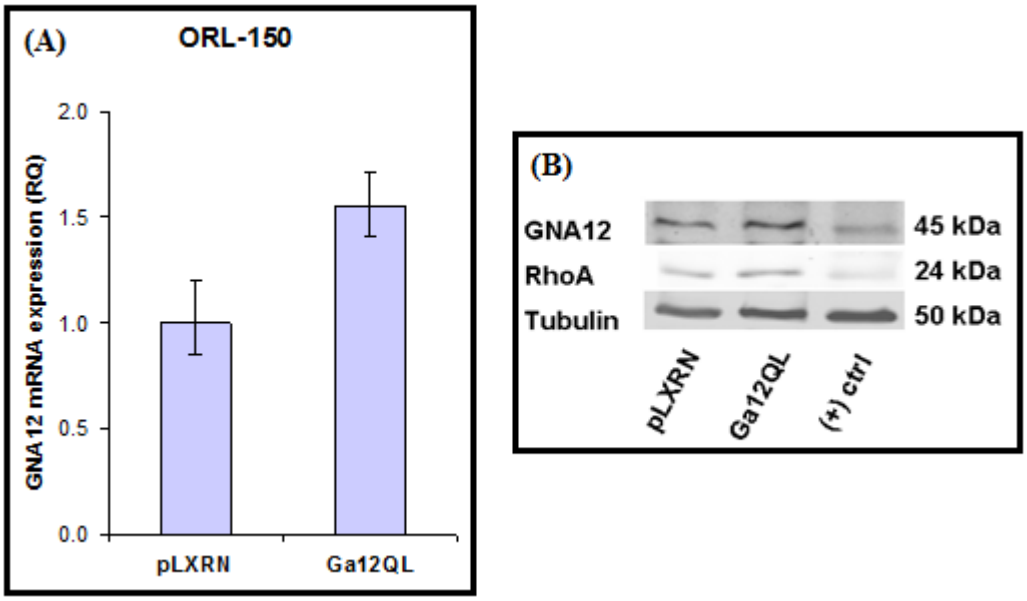

Figure 4.4: GNA12 expression in the transformed-ORL150 cells after 14 days of G418 selection. (A) mRNA level of GNA12 was elevated in ORL150/Gα12QL cells relative to ORL150/pLXRN control. (B) Western blot analysis showed slight increased of GNA12 and RhoA proteins expression in ORL150/Gα12QL cells. Tubulin was included as a control for protein loading.

4.1.3 *In-vitro* functional assays to determine the effects of GNA12 expression in oral cancer cells

(A) Effects of GNA12 expression on oral cancer cell proliferation

In this study, the growth of ORL150/Gα12QL cells and vector control cells were measured every 24 hours up to 9 days. The results showed that activated GNA12 has no measurable effect on oral cancer cell proliferation, as there was no significant difference in cell proliferation between the ORL150/ Gα12QL and ORL150/pLXRN control cells (Figure 4.5). We obtained consistent results in repeated experiments, hence confirmed that GNA12 does not play a role in oral cancer cell proliferation.

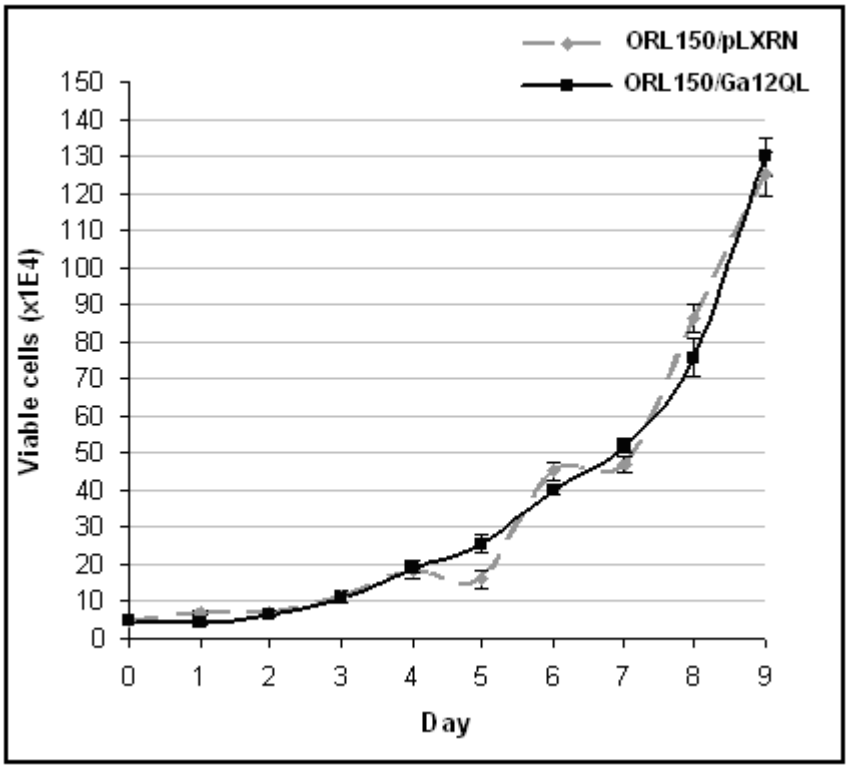

Figure 4.5: Growth curve for ORL150/Gα12QL and ORL150/pLXRN. The ORL150/Gα12QL cells grow at a similar rate to that of the vector control.

(B) Effects of GNA12 on cell migration and invasion

The migratory effect of ORL150/Gα12QL cells was first studied using the monolayer scratch assay. As wound closure can be attributed by both the migratory and proliferating effects of the cells; hence cells were pre-treated with Mitomycin C to negate effects contributed by cell proliferation. In comparison to the vector control cells, the ORL150/Gα12QL cells showed a faster wound closure rate, resulting in significant reduction of open wound area ($p<0.001$). At 18 hours post-scratching, the ORL150/Gα12QL cells had a wound gap that was 71% that of open gap measured at 0 hour, while the vector control cells had a bigger gap of 88% (Figure 4.6A). Consistently, it was observed that GNA12 activation indeed induces cell migration in the repeated experiments. Similarly, when these cells were tested on the Transwell migration assay, it was found that activated GNA12 also induces cell migration although this was not statistically significant (Figure 4.6B).

In Transwell invasion assays, the number of cells that invaded through the matrigel barrier was consistently higher in ORL150/Gα12QL than the vector control cells. Notably, ORL150/Gα12QL cells showed 4-fold increased in invasion in respond to 3T3-conditioned medium after 24 hours (Figure 4.6C). Taken together, these results strongly suggested that GNA12 may function to regulate the migration and invasive ability of OSCC cells.

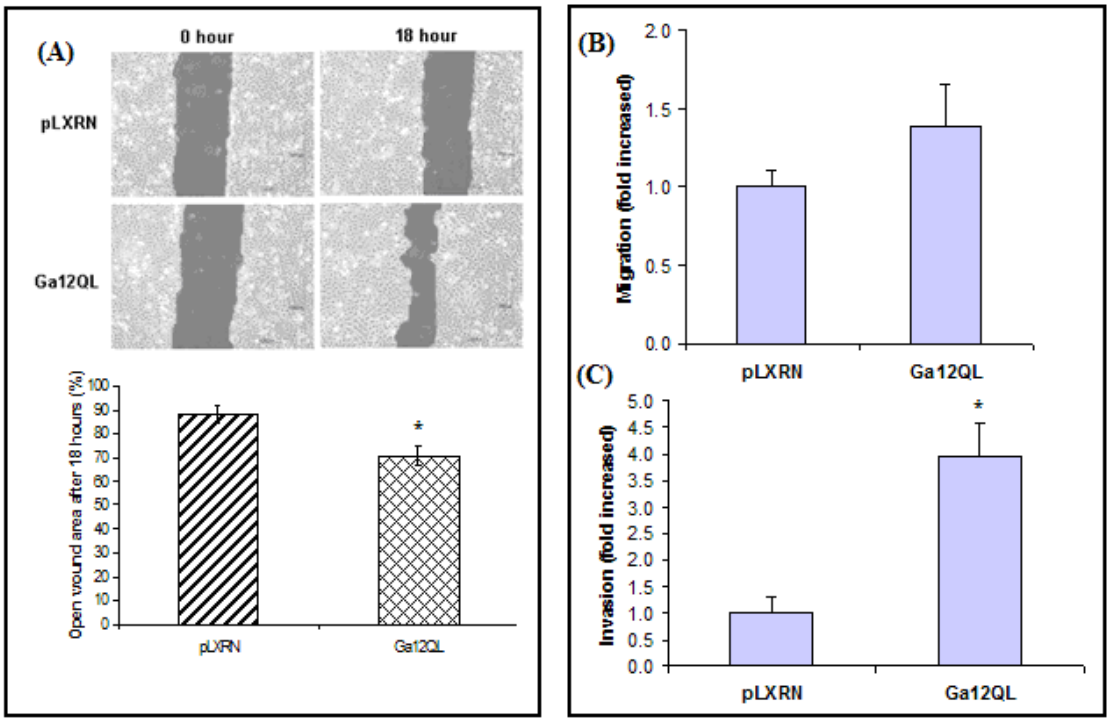

Figure 4.6: GNA12 induces cancer cell migration and invasion. (A) Monolayer wound healing assay showed expression of Gα12QL in ORL150 induces migration as indicated by reduced open wound area ($p < 0.001$). (B) Transwell migration assay showed increased cell migration by Gα12QL ($p = 0.262$). (C) Transwell matrigel invasion showed Gα12QL induces cell invasion ($p = 0.015$).

4.2 IFITM3

4.2.1 Validation of IFITM3 expression in OSCC

The expression of IFITM3 was validated in the same set of OSCC specimens used in the study for GNA12. However, due to the availability of the specimens, only 46 OSCC cases were studied using QPCR and 43 OSCC cases studied using IHC, respectively.

(A) Over-expression of IFITM3 at the mRNA level

The transcript level of IFITM3 in 46 OSCC and 18 oral mucosa tissues were compared by QPCR. OSCC tissues exhibited different levels of IFITM3 in the range of RQ = 0.46 to 18. However, IFITM3 expression was significantly greater in OSCC tissues (mean RQ: 2.9 ± 0.46) in relative to the oral mucosa tissues used as controls (mean RQ: 1.0 ± 0.02) (Figure 4.7A). Using the defined cut-off value of RQ ≥ 2 for IFITM3 over-expression, 46% of OSCC (n=21) showed up-regulation of IFITM3. While 28% of OSCC (n=13) showed unchanged levels and another 26% (n=12) showed down-regulation (Figure 4.7B).

(B) Over-expression of IFITM3 at the protein level

To validate the protein expression of IFITM3 in the pathogenesis of OSCC, IHC analysis was conducted using primary antibody that recognized the full length IFITM3 protein. Positive IFITM3 immunoreactivity was observed predominantly in the cytoplasm of tumour cells, although plasma membrane staining was also detected in some cases. In general, tumour cells exhibited specific and intense staining than the oral mucosa tissues controls (Figure 4.8). Consistent to the up-regulation of IFITM3 mRNA in OSCC reported in section 4.2.1(A), our IHC finding also demonstrated that IFITM3 protein expression was significantly higher in OSCC compared to the oral mucosa

tissues ($p < 0.001$). IFITM3 was highly expressed in 79% OSCC (n=34), while 83% of oral mucosa tissues (n=18) showed low levels of IFITM3 expression (Table 4.4).

Following these observations, statistical method was used to determine if the expression of IFITM3 is associated to the clinicopathological factors of the OSCC cases examined. The demographic features of OSCC patients used for IFITM3 study (Table 4.5) are similar to that reported in section 4.1.1(B). The expression of IFITM3 showed no association to any of the clinicopathological characteristics (Table 4.6). Nevertheless, increased IFITM3 levels in OSCC tissues indicated that it may be involved in tumorigenesis. Thus, the role of IFITM3 in oral cancer was further investigated.

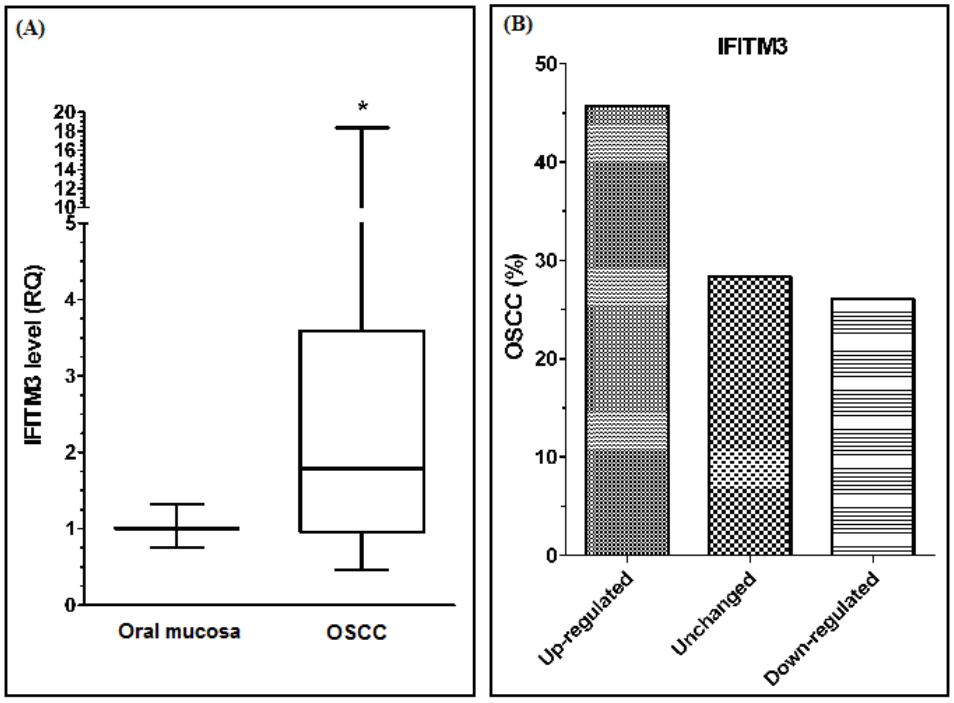

Figure 4.7: IFITM3 mRNA levels in OSCC relative to oral mucosa tissues. (A) Box and whiskers plot showed comparative changes in IFITM3 expression in OSCC in comparison to oral mucosa controls. OSCC tissues have high level of IFITM3 (*p = 0.003). The band in the box is the median, and the ends of whiskers represent the minimum and maximum IFITM3 levels. (B) Distribution of IFITM3 mRNA expression in 46 OSCC patients. More than 40% OSCC showed over-expression of IFITM3.

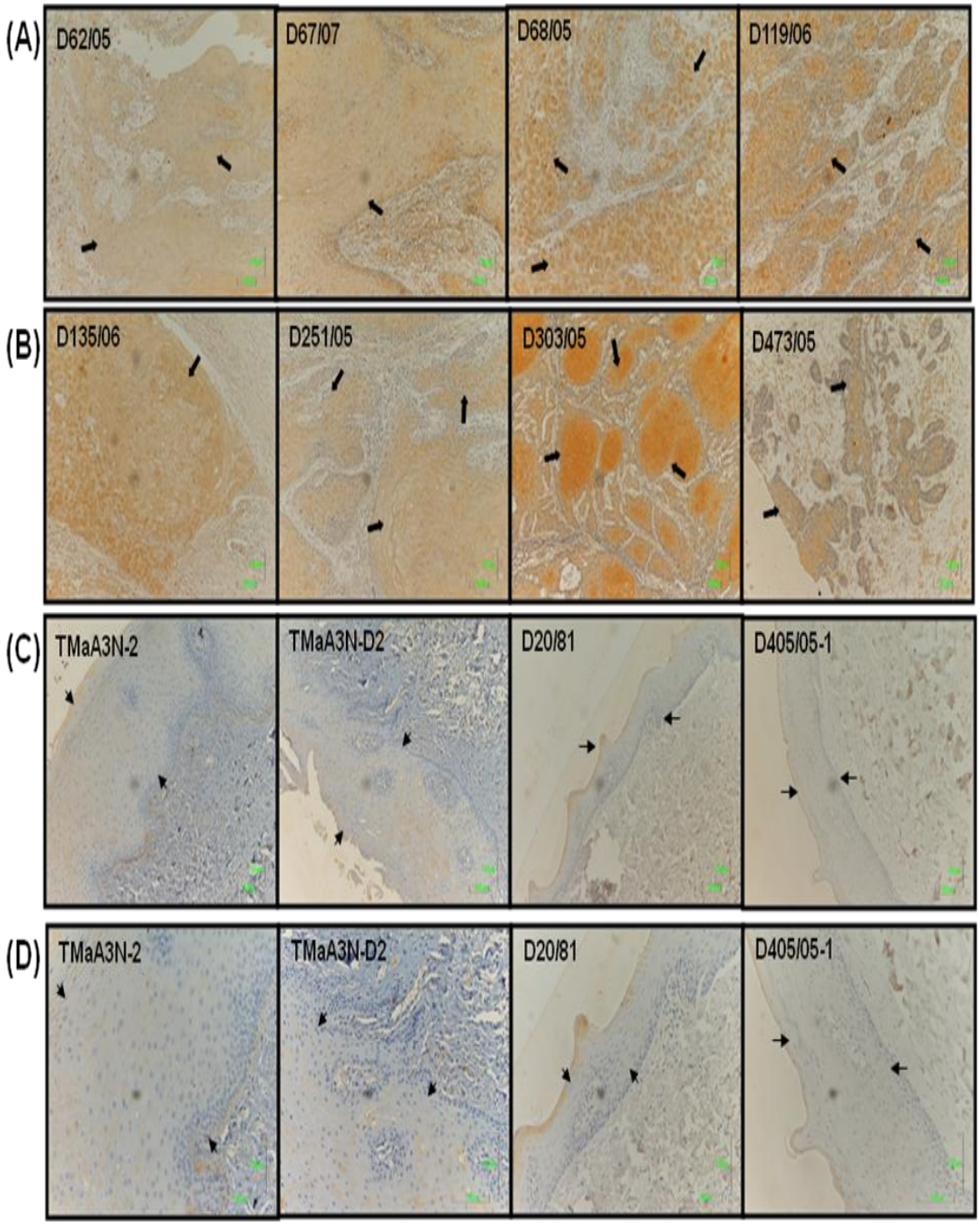

Figure 4.8: IHC staining of IFITM3 in TMaA. High expression of IFITM3 was detected in the tumour, as indicated by block arrows (Panel A & B, 100x magnification). Oral mucosa tissues showed negative staining, as indicated by arrow heads (Panel C, 100x magnification; Panel D, 200x magnification).

Table 4.4: Differential expression of IFITM3 in oral tissues			
Tissue type		**IFITM3 expression**	
		Low (n, %)	**High (n, %)**
Oral mucosa*	**n = 23**	(19, 82.6)	(4, 17.4)
OSCC	**n = 43**	(9, 20.9)	(34, 79.1)

*FEP tissues and Tissues from the flap of gum excised during wisdom tooth extraction
Pearson chi-square test, $p < 0.001$

Table 4.5 : Demographic data of 43 OSCC cases used for IFITM3 IHC analysis					
Variable		**OSCC**		**Mean**	**Standard deviation**
		n	**(%)**		
Gender	Male	12	(27.9)		
	Female	31	(72.1)		
Age (year)				61	10
Ethnicity	Malay	5	(11.6)		
	Chinese	4	(9.3)		
	Indian	33	(76.7)		
	Indigenous people	1	(2.3)		
Risk habit	No habit	5	(11.6)		
	Betel quid	23	(53.5)		
	Smoking	4	(9.3)		
	More than 1 habit	11	(25.6)		

Table 4.6: Association of IFITM3 expression to clinicopathological features of 43 patients with OSCC.					
Characteristics		**Patients (n)**	**IFITM3 expression**		***p*-value**
			Low (n, %))	**High (n, %))**	
Broder's Grading	Well	14	(2, 14.3)	(12, 85.7)	0.687[+]
	Moderate	23	(5, 21.7)	(18, 78.3)	
Stage	I and II	13	(2, 15.4)	(11, 84.6)	0.695[+]
	III and IV	29	(7, 24.1)	(22, 75.9)	
Lymph nodes	Negative	23	(3, 13.0)	(20, 87.0)	0.147[+]
	Positive	18	(6, 33.3)	(12, 66.7)	
Tumour size	T1 and T2	22	(5, 22.7)	(17, 77.3)	1.000[+]
	T3 and T4	20	(4, 20.0)	(16, 80.0)	
Site	Cheek and gum	37	(6, 16.2)	(31, 83.8)	0.095[+]
	Tongue and FOM	6	(3, 50.0)	(3, 50.0)	

[+] Fisher exact test

4.2.2 Modification of oral cancer cell line for IFITM3 expression

(A) Cloning IFITM3 into lentiviral expression vector

The IFITM3 coding region was successfully amplified by PCR using the cDNA of ORL207 which has high mRNA level of IFITM3 (Appendix X). The PCR product was purified by gel extraction method (Figure 4.9) and subsequently cloned into pLenti6.3 vector. Both pLenti6.3/IFITM3 and pLenti6.3/lacZ control vectors were transformed into Stbl3 *E. coli* competent cells and positive clones that were resistant to carbenicillin were selected, as they carried the ampicillin resistance marker.

A total of 33 colonies were screened by culture PCR using vector primers, and 13 colonies have IFITM3 insert cloned in the correct orientation and in frame with the C-terminal tag (Figure 4.10). A clone was selected for sequencing to ensure that there is no mutation in the IFITM3 insert. Then, both pLenti6.3/IFITM3 and pLenti6.3/lacZ plasmids were propagated in Stbl3 and the harvested plasmids were of the correct size as determined by agarose gel electrophoresis (Figure 4.11).

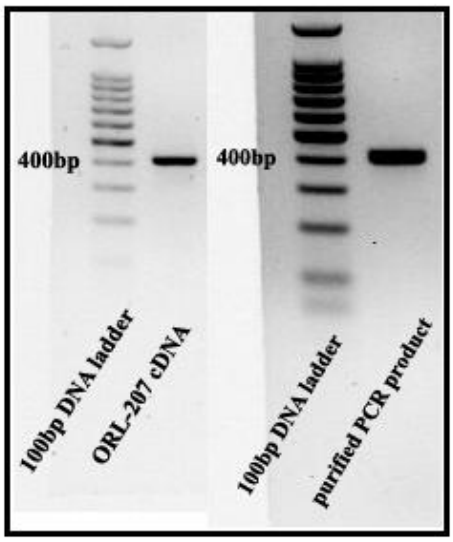

Figure 4.9: Agarose gel electrophoresis of IFITM3 purified PCR product. The expected band size for IFITM3 PCR product is 405bp.

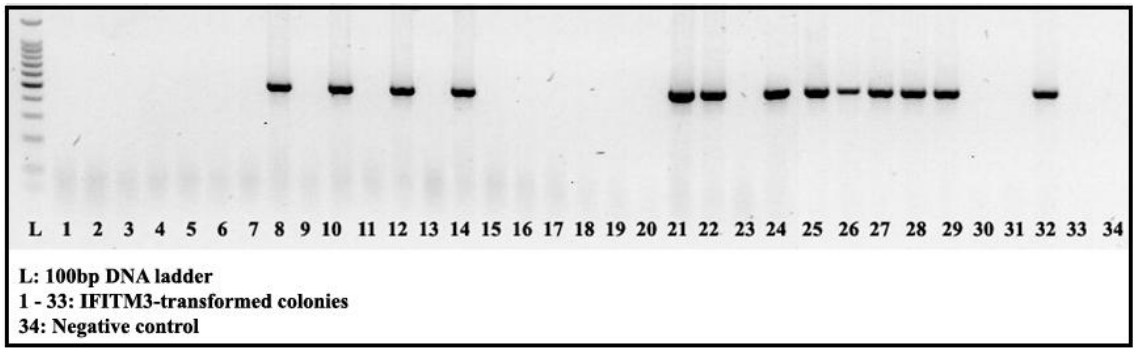

Figure 4.10: Analyzing IFITM3-transformed *E. coli* on agarose gel electrophoresis. Positive transformants are indicated by the presence of the IFITM3 PCR product band at 501bp.

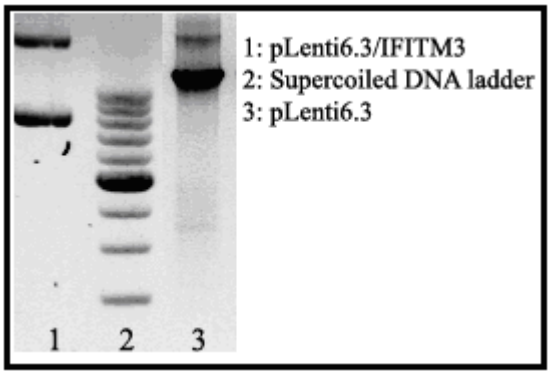

Figure 4.11: Agarose gel electrophoresis for IFITM3 expression plasmid. The pLenti6.3/IFITM3 and pLenti6.3 control plasmids have the band size of 8.1 kb, and 10.8 kb, respectively.

(B) Exogenous expression of IFITM3 in oral cancer cell line ORL188

IFITM3 was exogenously expressed in ORL188 which has been previously determined to have low level of IFITM3 (Appendix X). ORL188 was transduced with lentiviruses carrying IFITM3, and then stable clones were selected by blasticidin for 14 days. In comparison to the vector control, IFITM3 mRNA and protein levels increased to RQ 10.26 and 12.66, respectively (Figure 4.12A & B). Taken together these results showed successful over-expression of IFITM3 in ORL188.

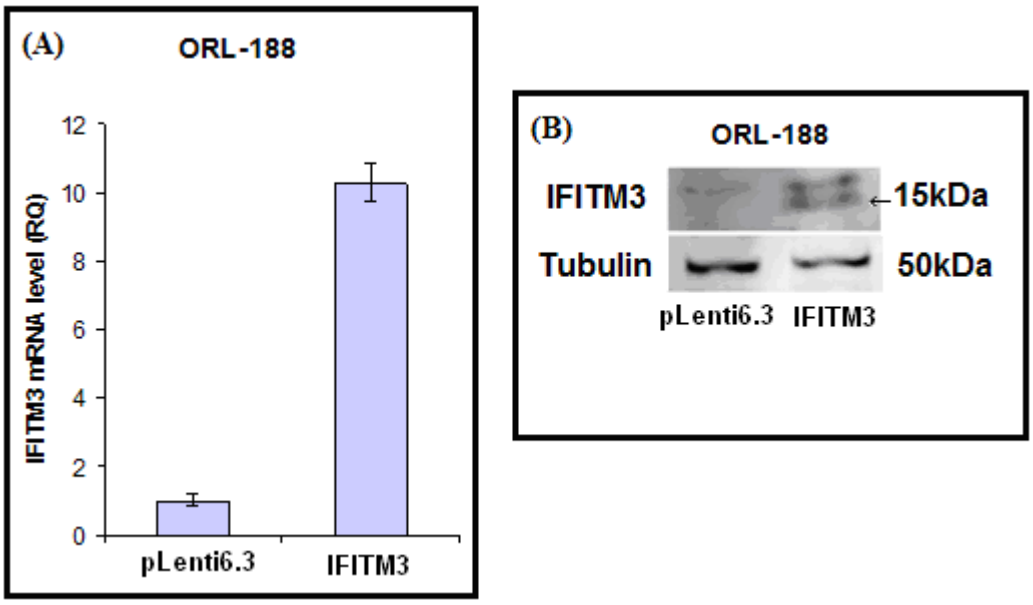

Figure 4.12: IFITM3 expression in ORL188 cells after blasticidin selection. (A) IFITM3 mRNA level was elevated in IFITM3-transformed ORL188 cells. (B) Western blot indicated over-expression of IFITM3 in ORL188/IFITM3 cells compared to ORL188/pLenti6.3.

4.2.3 *In-vitro* functional assays to determine the effects of IFITM3 expression in oral cancer cells

(A) Effects of IFITM3 expression on oral cancer cell proliferation

The effect of IFITM3 on oral cancer cell growth was monitored over 9 days and measurement was taken every 24 hours. Interestingly, the proliferation of IFITM3-transformed ORL188 cells was markedly slower than vector control cells. The population doubling time for ORL188/IFITM3 cells and the vector control cells were 35 hours and 32 hours respectively. Prolonged observations from day-6 onwards discovered that the viable cell number for ORL188/IFITM3 was markedly lower than vector control cells. These findings thus indicated that IFITM3 over-expression suppressed the growth of oral cancer cells (Figure 4.13).

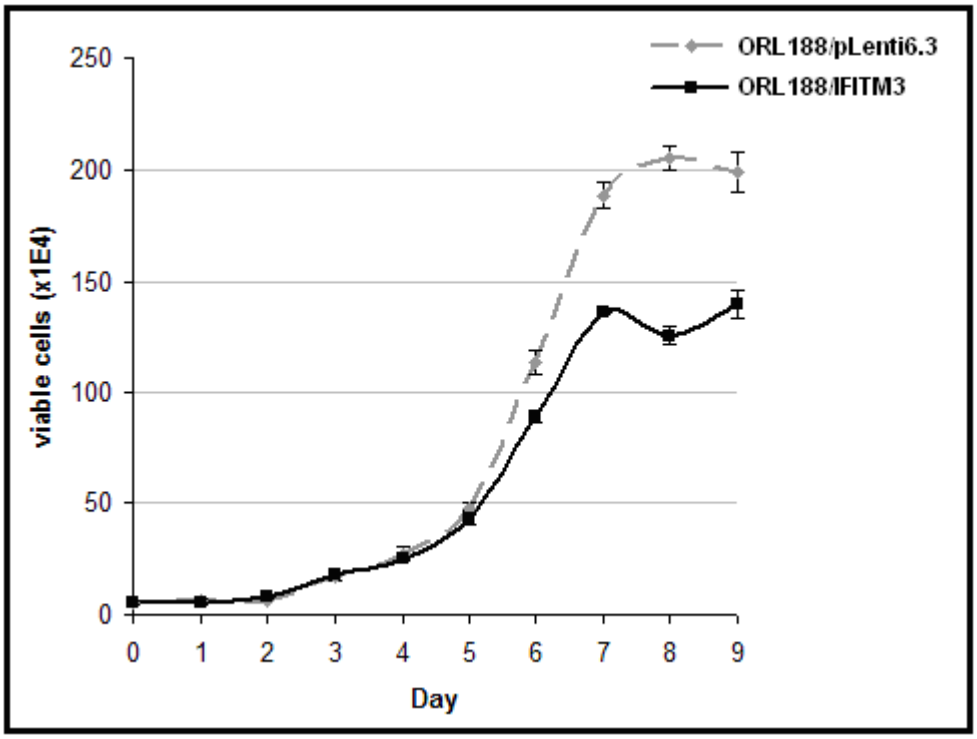

Figure 4.13: Effects of IFITM3 expression on oral cancer cell proliferation. ORL188/IFITM3 cells grew slower than the ORL188/pLenti6.3 vector control cells and had a longer population doubling time.

(B) Effects of IFITM3 over-expression on cell apoptosis

Since expression of IFITM3 resulted in suppression of cell proliferation, we next investigated if the growth inhibitory effect is due to apoptosis. ORL188/IFITM3 and ORL188/pLenti6.3 vector control cells were cultured for 48 hours until 70% confluent, and analyzed by flow cytometry to determine the population of apoptotic cells. We consistently found slight increased of apoptotic cell population in ORL188/IFITM3 cells compared to ORL188/pLenti6.3 vector control cells (Figure 4.14). Quantitative analysis of the data however showed that the difference in the percentage of apoptotic cells in the vector control and ORL188/IFITM3 cells was less than 10%.

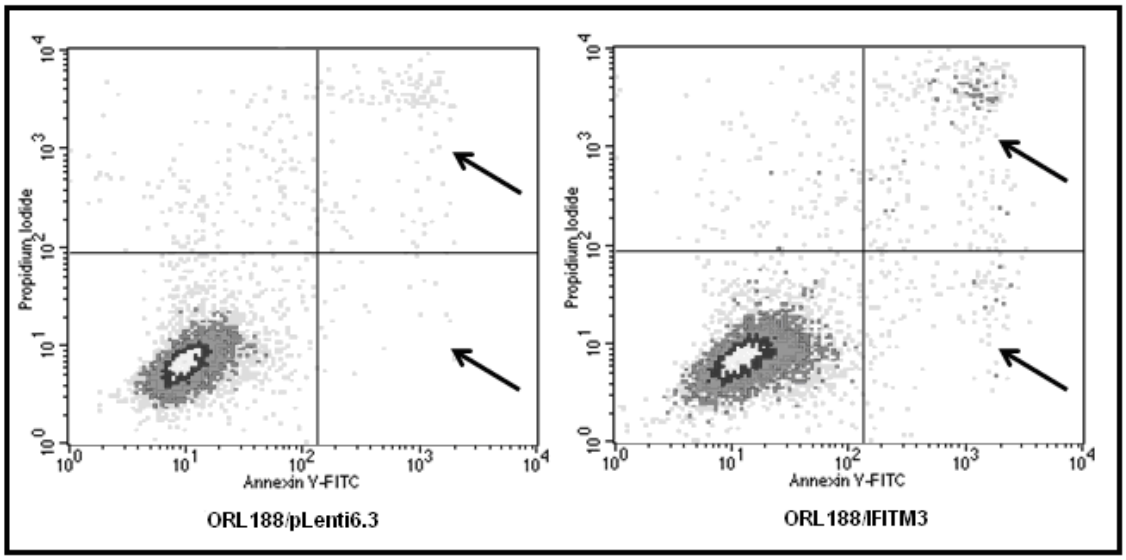

Figure 4.14: Cell apoptosis analyzed by flow cytometry. The population apoptotic cell was indicated by arrows. ORL188/IFITM3-transformed cells have slight increased in apoptotic cells than the vector control cells.

(C) Effects of IFITM3 on cell migration and invasion

The migratory effect of IFITM3 on oral cancer cell was only evaluated using Transwell migration method. The monolayer wound healing assay was not applicable to the transformed-cells as ORL188/IFITM3 cells were unable to form a confluent monolayer in the culture dish (Figure 4.15). These cells gradually detached from the dish when the cell confluency reaches 70%. In Transwell migration assays, ORL188/IFITM3 cells exhibited significant reduction in cell migration by 80% compared to the vector control cells. Similarly, ORL188/IFITM3 cells also showed a decrease in cell invasion by 93% compared to vector control cells (Figure 4.16). Taken together, these results suggested that the expression of IFITM3 inhibited the migration and invasion in oral cancer cells.

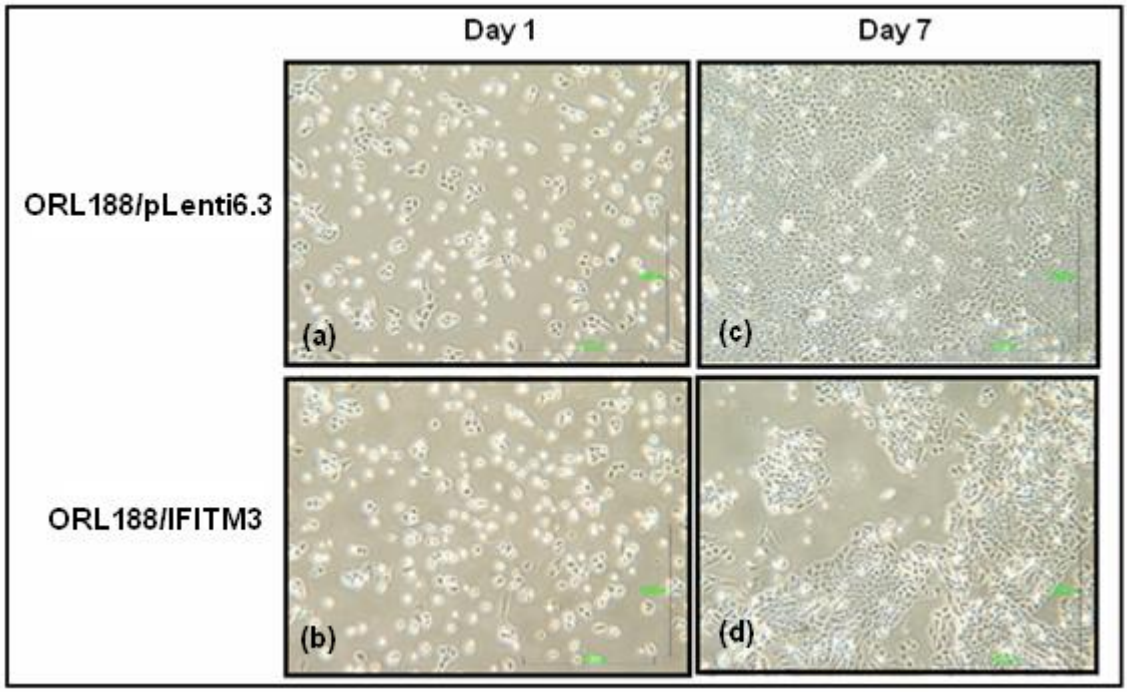

Figure 4.15: ORL188/IFITM3 and ORL188/pLenti6.3 in cell culture. ORL188/IFITM3-transformed cells were unable to form confluent monolayer in comparison to ORL188/pLenti6.3 (d *vs.* c).

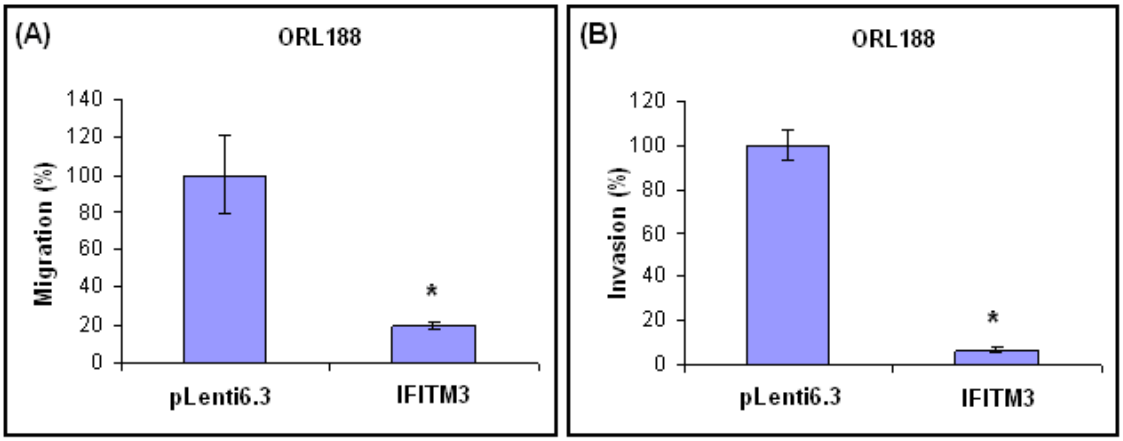

Figure 4.16: Effect of IFITM3 on cell migration and invasion. (A) IFITM3 expression inhibited cell migration (*p = 0.019). (B) Expression of IFITM3 reduced cell invasion (*p = 0.004).