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Association between the polymorphisms in exon 12 of hypoxia-inducible factor-1α and the clinicopathological features of oral squamous cell carcinoma

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SUMMARY

Oral squamous cell carcinoma (OSCC) is a common malignancy. The incidence of OSCC is particularly high in some Asian countries because of the popularity of the habit of chewing areca. Hypoxia-inducible factor-1α (HIF-1α) is up-regulated in the hypoxic microenvironment to enhance tumor survival. Five polymorphisms have been identified in exon 12 of HIF-1α including the C1772T polymorphism causing P582S, and the G1790A polymorphism causing A588T of the HIF-1α protein. This study investigated the relationship between these functional polymorphisms and the risk of progression of OSCC. PCR and direct sequencing were utilized to compare the genotypic polymorphism and allelic frequency of HIF-1α in 96 normal controls and 305 OSCC patients. No statistically significant difference in C1772T and G1790A genotypes and allelic frequency between control and OSCC patients was found. However, multivariate analysis indicated that the A carrier of HIF-1α G1790A in OSCC patients was significantly higher in larger tumors than in the contrasting group with an adjusted odds ratio of 2.92. The T carrier of HIF-1α C1772T in buccal cancer patients was significantly higher in the non-areca-chewing group with an adjusted odds ratio of 0.111. The buccal cancer patients with C1772T or G1790A had lower recurrence frequency with an odds ratio of 0.266. These findings may suggest a correlation between the HIF-1α C1772T and G1790A polymorphisms and the growth of OSCC, and the decrease of OSCC recurrence frequency.

Introduction

Oral squamous cell carcinoma (OSCC) is a worldwide disease, especially in southern Asian countries. Its high incidence is closely linked to the popularity of the habit of areca (betel) nut chewing.[1] Areca nut chewing, alcohol drinking, and cigarette smoking are the greatest risk factors for OSCC in Taiwan.[2] OSCC is the fourth most frequent and has the fifth highest mortality of the male cancers in Taiwan. Recently, it has been shown that the age of OSCC patients is younger than before.[3] Carcinogenesis of OSCC involves alterations in cellular proliferation, anti-apoptosis, angiogenesis, migration, and invasion. These tumor progression phenotypes are intimately linked to the abnormalities in molecular regulation machinery.

Hypoxia triggers a cascade of molecular events including angiogenesis and cell-cycle control proteins during the development of human tumors. When tumors' diameters are longer than 2–3 mm, the centre of the tumor's microenvironment will become hypoxic.[4] Hypoxia-inducible factor-1 (HIF-1) is a key regulator of a broad range of cellular responses to hypoxia and acts in all mammalian cells. HIF-1 is associated with many physiological responses of cells, including proliferation, differentiation, and viability, and with the genesis and dynamic regulation of blood vessels, glucose, and energy metabolism.[5] HIF-1α is a member of the HIF family and its expression is regulated by the oxygen level.[6] HIF-1α is inhibited by Von-Hippel–Lindau (VHL) protein, which acts as an E3 ubiquitin

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ligase. VHL can target the N-terminal transactivation domain (N-TAD) within the oxygen-dependent degradation domain (ODDD) of HIF-1α. VHL and HIF-1α binding are dependent on hydroxylation of two conserved proline residues (Pro-402 and Pro-564), which are oxygen-sensitive.[7–10] Up-regulation of HIF-1α has been found in head and neck carcinoma (HNSCC) and carcinomas of the esophagus, lung, breast, stomach, pancreas, prostate, and kidney.[11–13] Immunohistochemistry studies demonstrated an association between HIF-1α expression and OSCC prognosis.[14] In addition, nuclear HIF-1α predicts the progression and survival of OSCC.[15]

Several single nucleotide polymorphism (SNP) sites have been found in HIF-1α. However, the density of SNP appears particularly high in exon 12, which includes three synonymous SNP (C1720T, G1768A, and A1828T) and two non-synonymous SNP (C1772T and G1790A). These non-synonymous SNP result, respectively, in amino acid changes from proline 582 to serine (P582S) and those from alanine 588 to threonine (A588T). Exon 12 translates a portion of HIF-1α encompassing the N-TAD domain and the partial ODDD region, and has been reported to increase HIF-1α expression, even under normoxia.[16,17] The association between HIF-1α C1772T or G1790A and the risk or progression of carcinoma of the cervix, endometrium, and the colorectum, and of the esophagus, bladder, and kidney has been reported.[18–20] Both HIF-1α C1772T or G1790A have been demonstrated to increase HIF transcription activator function and the numbers of microvascular in HNSCC.[16,21] The frequency of HIF-1α C1772T or G1790A has been found to be higher in OSCC relative to controls.[22] However, only G1790A has been found to be associated with the risk and prognosis in a cohort of early-stage OSCC.[23] The present study further stratified that HIF-1α C1772T was associated with the size of the OSCC.

Materials and methods

Subjects

Nine OSCC cell lines were used in the study. SAS, OECM-1, OC3, and SCC25 were kind gifts from Dr. K.-W. Chang (National Yang-Ming University), and HSC3, TW206, SCC4, Cal27, and Ca922 were kindly gifted by Dr. M.-C. Kao (China Medical University). These OSCC cell lines were cultured using previously used protocols.[24–26] Three hundred and five OSCC cases without previous treatment and 96 healthy controls were randomly selected from patients who presented for physical examination in the Department of Oral and Maxillofacial Surgery at Mackay Memorial Hospital without prior neoplastic operation, immune disorder or oral lesion. The tumors were staged according to the TNM classification of malignant tumors defined by the AJCC (2002). People with or without oral cancer risk factors including areca chewing, cigarette smoking, and alcohol consumption were defined according Ko et al.[2] The study was approved by an institutional review board.

Genotyping

Peripheral blood cells or cell lines underwent DNA isolation using the Blood Mini Kit (Qiagen, Valencia, CA, USA). The amplicons containing both HIF-1α C1772T and G1790A were obtained by PCR reaction with the following primers: Forward: 5′-GACACAGATTTAGACTTGGAG-3′, Reverse: 5′-TGGGTAGGAGATGGAGATGC-3′. The PCR reaction condition was 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, with a final step at 72 °C for 10 min to allow complete extension of the PCR fragments. After confirming the integrity of the amplicons, genotyping was performed using direct sequencing. After purification of amplicons using a gel extraction kit (Qiaex II Gel Extraction Kit; Qiagen), sequencing was performed using a 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) as instructed by the manufacturer.[27] The sequencing primer is the same as the reverse primer used for the PCR reaction. ChromasPro software (Technelysium Pty Ltd, Tewantin, QLD, Australia) was used for the reading of sequences on chromatograms.

Statistical analysis

The Hardy–Weinberg equilibrium was assessed with a χ^2 test module. Unpaired *t*-test, Fisher's exact test, χ^2 analysis, binary logistic regression analysis, odds ratio and 95% confidence intervals

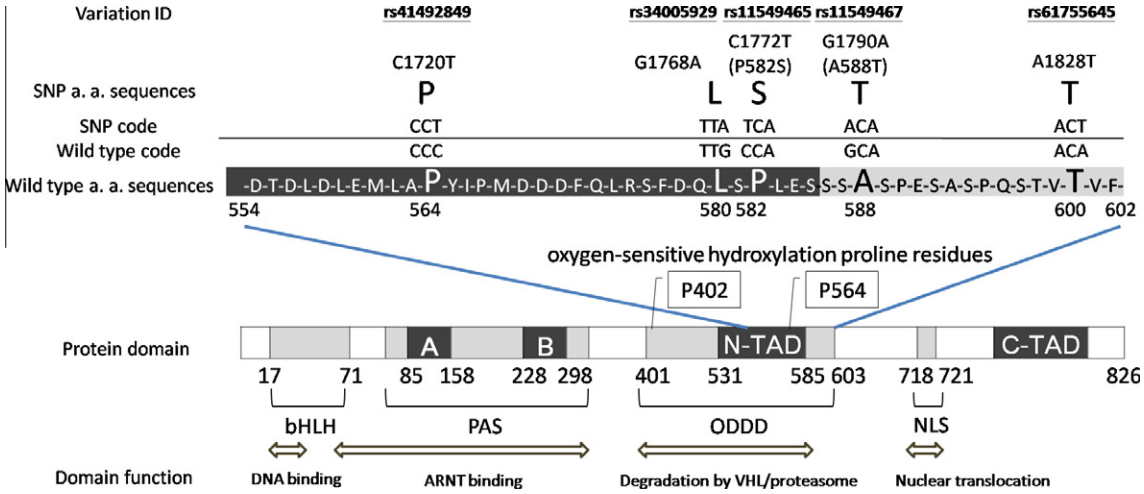

Figure 1 Protein domains and five SNP in exon 12 of HIF-1α. The domains of the HIF-1α polypeptide from the N to the C terminal: basic helix–loop–helix (bHLH), PER-ARNT-SIM (PAS) domains, which are involved in HRE binding and dimerization with ARNT (aryl hydrocarbon nuclear receptor translocator); oxygen-dependent degradation domain (ODDD) contains two proline residues (P402 and P564), two transacting domains (TAD), and a nuclear localization signal (NLS). Five SNP in the 12th exon of HIF-1α: C1720T, G1768A, C1772T (P582S), G1790A (A588T), and A1828T and each variation ID, amino acid sequences, and translation codes. This figure was modified according to Tanimoto et al.[16] and Park (2004).

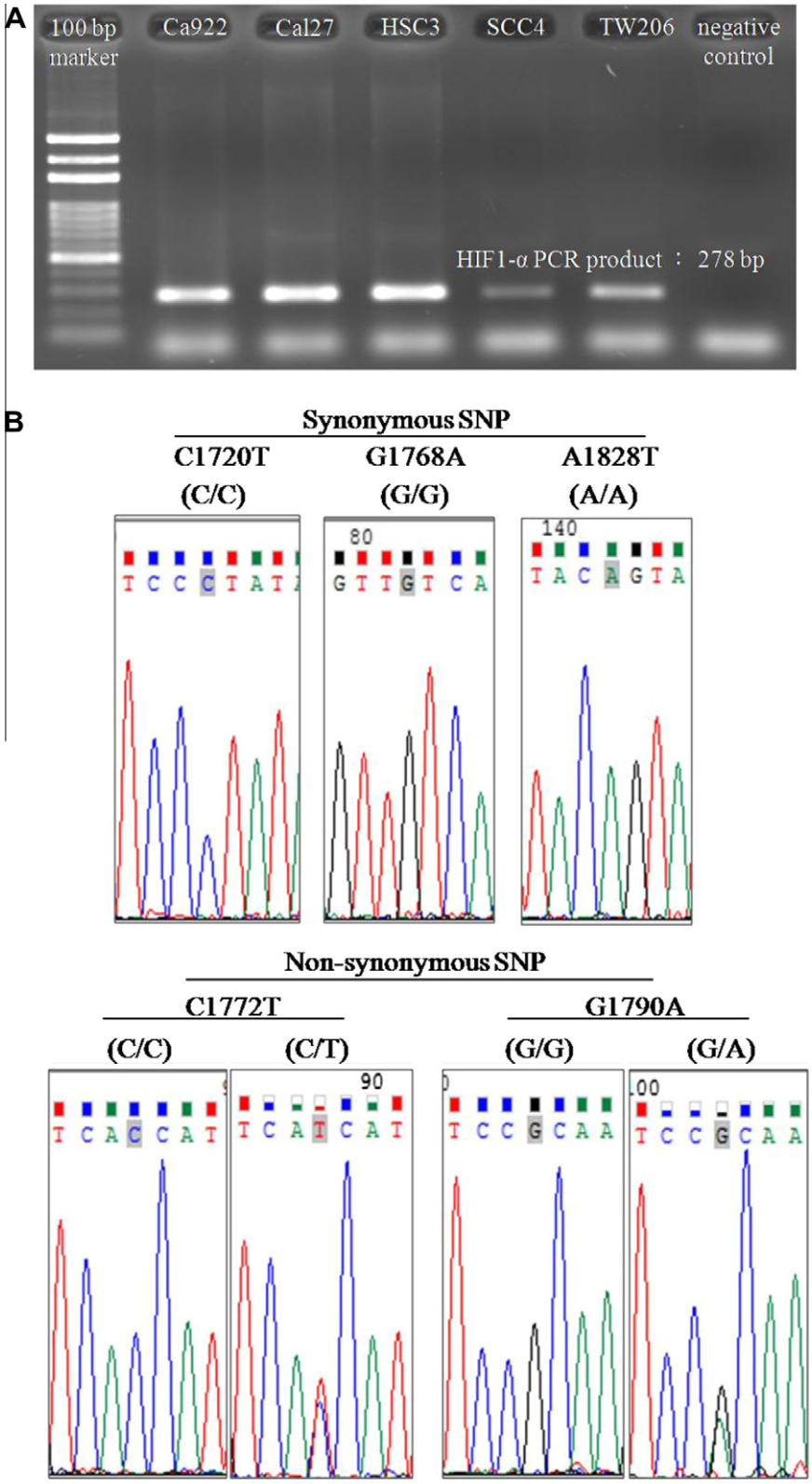

Figure 2 Amplification and genotyping of HIF-1α in OSCC cells. (A) PCR reaction. The HIF-1α amplicons were from five OSCC cell lines underwent electrophoresis on 2% agarose gel to evaluate the specificity and integrity. It showed a clear band with motility of ~278-bp. (B) Direct sequencing of amplicons. The letters marked with gray boxes were SNP sites. Upper panels, three homozygous wild-type synonymous SNP: C1720T (C/C), G1768A (G/G), and A1828T (A/A). Lower panels, non-synonymous homozygous wild-type SNP C1772T (C/C) and G1790A (G/G) as well as heterozygous SNP C1772T (C/T) and G1790A (G/A).

were performed using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA) or Prism 5.0 (GraphPad software, San Diego, CA, USA). Differences between the variants were considered significant when $p < 0.05$.

Table 1
Subjects and clinical characteristics.

Number of subjects	OSCC (n = 305)	Normal (n = 96)	
Age (years)			
Mean ± SD	52.8 ± 11.1	47.2 ± 9.4	$p < 0.0001$
Sex			
Male	287	81	$p = 0.003$
Female	18	15	
Areca chewing			
No	23	7	$p = 0.936$
Yes	282	89	
Cigarette smoking			
No	19	7	$p = 0.712$
Yes	286	89	
Alcohol consumption			
No	28	9	$p = 0.954$
Yes	277	87	
Site			
Buccal cancer	117		
Tongue	62		
Gingiva cancer	57		
Others	51		
Tumor size			
Small (T1, T2)	126		
Large (T3, T4)	179		
N stage			
$N = 0$	199		
$N \geqq 1$	106		
Stage			
Early (Stages I and II)	92		
Late (Stages III and IV)	213		
Differentiation			
Poor and moderate	161		
Well	144		
Perineural invasion			
No	273		
Yes	32		
Perivascular invasion			
No	274		
Yes	31		
Survival			
Live	227		
Dead	78		
Recurrence			
No	227		
Yes	78		

Unpaired t-test or Fisher's exact test.

Results

The HIF-1α amplicons were designed containing these five SNP sites in the study. The variation of each HIF-1α SNP is shown in Fig. 1. C1720T, G1768A, and C1772T were in N-TAD, while only C1772T and G1790A made translation codes change from CCA to TCA, and from GCA to ACA respectively. These two codes caused the amino acid number 582th proline (P) and 588th alanine (A) to change to serine (S) and threonine (T) respectively. The amplicons were first found by gel electrophoresis to be 278-bp in size (Fig. 2A), and were subjected to direct sequencing to reveal the status of all five SNP sites at the same time (Fig. 2B). Although the sequencing revealed clear distinctions among various genotypic patterns in screened OSCC cell lines, only HSC3 and TW206 cells were found to have heterozygous G/A genotype in nucleotide 1790. The other SNP alleles were all of homozygous wild-type. The genotypic analysis was limited to C1772T and G1790A in subjects.

Four hundred and one subjects were included in the study for HIF-1α genotyping. The demographic data of 305 OSCC patients and 96 controls are shown in Table 1. The ages (mean ± SD) of patients and controls were 52.8 ± 11.1 and 47.2 ± 9.4 years, respectively. There was a statistically significant difference between patients and controls. Nearly all patients were male (94.09%), which was significantly different from the control group (84.38%). No difference in oral habits including areca chewing, alcohol consumption and tobacco smoking was found between patients and controls. The most common site of OSCC was on the buccal mucosa, followed by the tongue and the gingiva. The clinicopathological parameters were carefully recorded and are shown in Table 1.

Analysis of the C1772T polymorphism indicated that in the patients 282 cases were type C/C (92.46%), and 23 cases were type C/T (7.54%); in the controls 89 cases were type C/C (92.71%), and 7 cases were type C/T (7.29%). No significant differences in genotypic and allelotypic frequencies for the C1772T polymorphism were observed between patients and controls. As for the G1790A polymorphism, in the patients 281 cases were type C/C (92.13%), and 24 cases were type C/T (7.87%); in the controls 7 cases were type G/G (92.71%) and 89 cases were type G/A (7.29%). No significant differences in genotypic and allelotypic frequencies for the G1790A polymorphism were observed between patients and controls. Compared with individuals with at least one of the two polymor-

Table 2
HIF-1α polymorphism in OSCC patients and controls.

Nucleotide	Amino acid	Genotype or allelotype	OSCC cell lines	Patients (%)	Controls (%)
C1772T	P582S	C/C	100.00% (9/9)	92.46% (282/305)	92.71% (89/96)
		C/T	0.00% (0/9)	7.54% (23/305)	7.29% (7/96)
		T/T	0.00% (0/9)	0.00% (0/305)	0.00% (0/96)
Genotype (C/C): (C/T+T/T)					
$P = 1$ OR = 0.9643 95% CI = 0.4003–2.323					
		C	100.00% (18/18)	96.23% (587/610)	96.35% (185/192)
		T	0.00% (0/18)	3.77% (23/610)	3.65% (7/192)
Allelotype C:T					
$P = 1$ OR = 0.9657 95% CI = 0.4077–2.287					
G1790A	A588T	G/G	77.78% (7/9)	92.13% (281/305)	92.71% (89/96)
		G/A	22.22% (2/9)	7.87% (24/305)	7.29% (7/96)
		A/A	0.00% (0/9)	0.00% (0/305)	0.00% (0/96)
Genotype (G/G): (G/A+A/A)					
$P = 1$ OR = 0.9209 95% CI = 0.3838–2.210					
		G	88.89% (16/18)	96.07% (586/610)	96.35% (185/192)
		A	11.11% (2/18)	3.93% (24/610)	3.65% (7/192)
Allelotype G: A					
$P = 1$ OR = 0.9239 95% CI = 0.3916–2.179					

Fisher's exact test.

phisms, C1772T or G1790A, of the HIF-1α gene, there were no significant differences between patients and controls (data not shown). The genotypic distribution of C1772T and G1790A in both patients and controls exhibited no deviations from the Hardy–Weinberg equilibrium (Table 2).

The association between HIF-1α polymorphisms and the clinicopathological features was analyzed using binary logistic regression analysis adjusting for age. Genotypic distribution of C1772T was not associated with any clinicopathological parameters (Table 3). The frequency of the heterozygous G1790A (G/A) genotype was significantly higher in larger tumors (T3 and T4 tumors; adjusted OR = 2.920, 95% CI = 1.044–8.173; Table 3). The cross analysis of C1772T and G1790A polymorphisms showed a significant increase in double heterozygous genotypes (C/T or G/A) in larger tumors (adjusted OR = 2.161, 95% CI = 1.071–4.361). The late-stage tumors had a greater increase in heterozygous G/A genotypes than early-stage tumors. However, the difference was not statistically significant (Table 3).

Areca chewing is a major risk factor for OSCC and is the most frequent cause of buccal cancer in Taiwan. We identified 117 buccal cancer patients in this study and analyzed using binary logistic regression analysis by adjusting age and sex. No significant differences in genotypic and allelotypic frequency for the G1790A polymorphism were observed between patients and controls (data not shown). However, OSCC patients carrying C1772T polymorphisms showed a significant increase in heterozygous genotypes (C/T) in non-areca-chewing patients (adjusted OR = 0.111, 95% CI = 0.016–0.789). The frequency of double heterozygous genotypes (C/T or G/A) in buccal cancer is lower recurrence (adjusted OR = 0.266, 95% CI = 0.071–1.002; Table 4).

Discussion

The stability of HIF-1α is dependent on proline residues in ODDD. The hydroxylation of HIF-1α on proline residues in normoxia are liable to be recognized by VHL for degradation.[7–10] Five

Table 3
HIF-1α genotype compared with clinical parameters.

	C1772T Genotype		G1790A Genotype		C1772T and G1790A Genotype	
	C/C	C/T	G/G	G/A	C/C or G/G	C/T or G/A
Site						
Buccal cancer	107	9	105	11	97	19
Non-buccal cancer	175	14	176	13	162	27
P	0.943		0.41		0.73	
OR (95% CI)	0.969	0.403–2.330	1.42	0.612–3.310	1.12	0.589–2.129
Tumor size						
Small (T1, T2)	116	8	121	5	113	13
Large (T3, T4)	164	15	160	19	146	33
P	0.838		0.04		0.031	
OR (95% CI)	1.1	0.433–2.806	2.92	1.044–8.173	2.161	1.071–4.361
N stage						
N = 0	183	16	184	15	168	30
N = 1	99	7	97	9	91	15
P	0.660		0.77		0.989	
OR (95% CI)	0.812	0.322–2.050	1.14	0.480–2.692	1.005	0.519–1.943
Stage						
Early (Stages I and II)	85	7	89	3	82	10
Late (Stages III and IV)	197	16	192	21	177	63
P	0.838		0.062		0.129	
OR (95% CI)	1.1	0.433–2.807	3.283	0.994–11.415	1.8	0.843–3.845
Differentiation						
Poor and moderate	150	11	147	14	136	25
Well	132	12	134	10	123	21
P	0.569		0.56		0.791	
OR (95% CI)	1.283	0.545–3.018	0.78	0.333–1.811	0.903	0.426–1.918
Perineural invasion						
No	252	21	253	20	233	40
Yes	30	2	28	4	26	6
P	0.715		0.3		0.581	
OR (95% CI)	0.755	0.168–3.403	1.83	0.581–5.743	1.308	0.504–3.391
Perivascular invasion						
No	255	19	252	22	234	40
Yes	27	4	29	2	25	6
P	0.214		0.75		0.463	
OR (95% CI)	2.087	0.654–6.658	0.78	0.174–3.497	1.43	0.551–3.716
Survival						
Alive	208	19	210	17	192	35
Dead	74	4	71	7	67	11
P	0.388		0.69		0.815	
OR (95% CI)	0.612	0.201–1.867	1.21	0.479–3.033	0.916	0.440–1.910
Recurrence						
No	209	18	209	18	192	35
Yes	73	5	72	6	67	11
P	0.558		0.97		0.695	
OR (95% CI)	0.734	0.261–2.063	0.98	0.372–2.579	0.863	0.413–1.805

Binary logistic regression analysis (adjusted for age).

Table 4
HIF-1α genotype compared with risk factors and recurrence in buccal cancer.

	C1772T Genotype		G1790A Genotype		C1772T and G1790A Genotype	
	C/C	C/T	G/G	G/A	C/C or G/G	C/T or G/A
Areca chewing						
No	7	2	18	0	24	2
Yes	110	7	99	11	93	17
P	0.028		0.999		0.234	
OR (95% CI)	0.111	0.016–0.789	94,321,330	0–0	0.318	0.048–2.099
Cigarette smoking						
No	11	1	14	0	21	1
Yes	106	8	103	11	96	18
P	0.996		0.999		0.996	
OR (95% CI)	0.000	0–0	18,559,156	0–0	0.000	0–0
Alcohol consumption						
No	16	1	8	0	26	1
Yes	101	8	109	11	91	18
P	0.566		0.999		0.786	
OR (95% CI)	0.519	0.055–4.892	128,982,514	0–0	1.357	0.151–12.196
Recurrence						
No	79	9	82	8	82	16
Yes	38	0	35	3	35	3
P	0.999		0.544		0.050	
OR (95% CI)	0.000	0–0	0.647	0.159–2.639	0.266	0.071–1.002

Binary logistic regression analysis (adjusted for age and sex).

SNP sites have been found in exon 12 within ODDD of the HIF-1α protein. We found that two out of nine OSCC had the G1790A G/A genotype of HIF-1α. Since no polymorphism was found in the synonymous SNP in OSCC cells, the importance of these synonymous SNP was excluded from the risk assessment of OSCC. Further dissection in OSCC patients and controls also excluded the association between G1790A and the risk of OSCC. One study reported a significant association between G1790A and OSCC susceptibility.[22] Munoz-Guerra et al.[23] found an association between G1790A and the risk of T1/T2 OSCC. Although the discrepancy between our studies and previous studies may represent a difference in race or study cohort, it is also likely that our control cohort is too small to signify a statistical difference for risk assessment.[22,23] The lack of correlation identified between the HIF-1α polymorphism and OSCC risk could be supported by the fact that HIF-1α expressed in the upper layers of normal squamous epithelium could be associated with squamous differentiation rather than oncogenesis.[28]

Studies have demonstrated that C1772T or G1790A can affect the normal physiological function of HIF-1α. Both C1772T or G1790A increase HIF-1α stability and HIF transcription activities[16,17,21] and this might underlie the increased microvascularity in HNSCC and OSCC.[16,21] Our results implicated G1790A polymorphism in the growth of OSCC as reflected by the presence of a higher frequency of the G/A genotype and the A allelotype in T3/T4 tumors in relation to contrasting groups. There was also a borderline increase in the A allelotype in T4 tumors relative to the remaining tumors ($p = 0.0551$; OR = 2.407, 95% CI = 0.9962–5.813; detailed analysis not shown). Munoz-Guerra et al.[23] addressed a correlation between the A allelotype and relapse or the short survival of T1/T2 patients. This study further specified the marked association between the G/A genotype and the A allelotype and the tumor size. Since HIF-1α is up-regulated during hypoxia, which benefits tumor progression in at the late stage, it is postulated that tumors carrying the G1790A polymorphism might exhibit HIF-1α activity for growth even when the oxygen level is sufficient when the tumor size is small.[10] This postulation needs to be clarified by a study of expression. Our analysis indicated no increase in the G/A genotype and the A allelotype in N+ subjects relative to N0 subjects; therefore, the increase in the G/A genotype in late-stage subjects could be due mainly to the impact of tumor size.

The higher nuclear HIF-1α labeling indices in tissues significantly correlate with OSCCs of larger size, lymph node metastasis, and more advanced clinical stage[15]; and C1772T could provide HIF-1α with higher stability.[17] Our findings were in agreement with previous studies in OSCC denoting no association between C1772T and the risk or progression of such tumors.[22,23] However, these findings conflict with those in HNSCC and carcinomas in the endometrium, esophagus, colorectum, and prostate.[16–20] Although HIF-1α expression modulates tumor progression and survival in hypoxic microenvironments, there are additional crucial factors, whose regulation is independent from hypoxia, being involved in the OSCC progression.[24,29] The interaction between these factors and HIF-1α for OSCC progression requires clarification.

Most of the OSCC patients have buccal cancer, which is caused by betel nut chewing in Taiwan. The recurrence rate of buccal cancer patients with C/T or G/A heterozygous HIF-1α genotypes is low. The result was different to HIF-1α expression in breast cancer, which was significantly predictive of metastasis risk and of relapse.[30] Both C1772T and G1790A HIF-1α make HIF-1α protein more stable to wild-type (C/C and G/G) to increase the HIF transcription activator function.[16,17,21] Factors inhibiting the HIF-1 (FIH-1) protein that interact with amino acid 531–826 of HIF-1α include the C1772T and G1790A polymorphism sites.[31] Both polymorphism sites might decrease the binding affinity between FIH-1 and HIF-1α and increase the transcription activator function of HIF-1 protein. The expression of HIF-1α affects many physiological functions that have complicated regulation mechanisms.[32] The mechanism of endometrial carcinoma recurrence with higher HIF-1α expression but lower microvessel density is still unclear.[32,33] The induction of HIF-1 will increase mRNA expression of the N-Myc downstream regulated gene-1 (NDRG1, tumor suppressor gene).[34] Whether NDRG1 was specifically induced by HIF-1 in buccal cancer is still unknown .These suppositions need more evidence and information before they are proven.

In conclusion, C1772T and G1790A of the HIF-1α gene were important factors for enhancing the betel nut effect of the buccal site and increasing the tumor size in oral cancer respectively. Carry

anyone of these polymorphism sites of buccal cancer patients were low recurrence.

Conflicts of interest statement

None declared.

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