เป็นหนังสือภาษาอังกฤษ

รายงานวิจัยฉบับสมบูรณ์

ความเกี่ยวพันกันของยีนหลากหลายทางพันธุกรรม โพรอินเฟลมมาทอรี่ไซโตคายยีนส์กับการเกิดมะเร็งช่องปาก ในประชากรภาคใต้

Involvement of polymorphisms in proinflammatory cytokine genes and development of oral cancer in Southern Thailand

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กิตติกรรมประกาศ

งานวิจัยนี้ ได้รับทุนอุดหนุนการวิจัยวิทยาเขตหาดใหญ่ ทุนอุดหนุนการวิจัยประเภทความร่วมมือกับต่างประเภทประจำปี 2550 (สัญญารับทุนเลขที่ DEN50200500350S)

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บทคัดย่อ

ปัจจุบันอุบัติการณ์มะเร็งช่องปาก ยังพบพบว่ามีสูงในประเทศแถบเอเชียตะวันออกเฉียงใต้ และก็ยังเป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทยโดยเฉพาะในภาคใต้ สาเหตุการเกิดมะเร็งชนิดนี้ เชื่อว่าเกี่ยวพันกับปัจจัยที่จำเพาะทางชีวภาพ สิ่งแวดล้อม และสัมพันธ์กับพฤติกรรมของประชากรในแถบภูมิภาคนี้ อาทิ การสูบบุหรี่ เคี้ยวหมาก เหน็บยาเส้น และการดื่มเหล้าเป็นต้น ปัจจุบันเชื่อว่าความหลากหลายทางพันธุกรรม (genetic polymorphisms) โดยเฉพาะของยีนส์ที่ควบคุมการสร้างโปรตีนในกระบวนการเมตาโบลิซึมของสารพิษ (xenobiotic metabolizing gene) ยีนส์ที่ควบคุมการซ่อมแซม DNA (DNA repair gene) รวมทั้งยีนส์ที่ควบคุมกระบวนการอักเสบและสร้างภูมิคุ้มกัน (inflammatory and immunomodulatory gene) มีความสำคัญในการทำนายความเสี่ยงของการเกิดมะเร็งหลายชนิดรวมทั้งมะเร็งช่องปากด้วย **วัตถุประสงค์** เพื่อหาความสัมพันธ์ของยีนส์ที่เกี่ยวข้องกับกระบวนการอักเสบและการควบคุมภูมิต้านทานของร่างกาย จำนวน 4 ยีนส์ คือ IL1α, IL1β, IL8, TNFα กับปัจจัยเสี่ยงสิ่งแวดล้อม คือ การสูบบุหรี่ ดื่มเหล้า และเคี้ยวหมาก กับโอกาสเสี่ยงต่อการเกิดมะเร็งช่องปากในประชากรไทยภาคใต้ **วิธีการวิจัยและประชากร** การวิจัยนี้เป็นการศึกษาแบบ case-control กลุ่มประชากรศึกษาเป็นผู้ป่วยมะเร็งช่องปากที่ได้รับการวินิจฉัยโรคทางจุลพยาธิวิทยาว่าเป็นมะเร็งช่องปากชนิด squamous cell carcinoma ผู้ป่วยมีประวัติการสูบบุหรี่ ดื่มเหล้า และหรือเคี้ยวหมาก เปรียบเทียบกับกลุ่มควบคุมที่เป็นอาสาสมัคร และเป็นประชากรในเขตพื้นที่ใกล้เคียงกันกับกลุ่มผู้ป่วย ประชากรกลุ่มควบคุมเป็นผู้ที่มีสุขภาพแข็งแรง การเปรียบเทียบกันโดยอาศัย เพศ อายุ (+/- 5 ปี) และพฤติกรรมการสูบบุหรี่ และดื่มเหล้า การวิเคราะห์ทางสถิติใช้ โปรแกรมสถิติสำเร็จรูป (R 2.8.1) **ผลการศึกษา** พบว่ากลุ่มประชากรผู้ป่วยมี 107 ราย กลุ่มประชากรควบคุมมี 157 ราย กลุ่มผู้ป่วยมีอายุเฉลี่ย 67.5 ปี กลุ่มควบคุมมีอายุเฉลี่ย 69.7 ปี การศึกษาของยีนส์ พบว่าผู้ป่วยมะเร็งช่องปาก มี "T" allele ของ $IL1\alpha^{+4845}$ ยีนส์หลากหลายพันธุกรรม (single nucleotide polymorphism: SNP) มากกว่ากลุ่มควบคุม (OR=2.0, 1.0-4.4) และในผู้ป่วยมะเร็งช่องปากที่มีพฤติกรรมเสี่ยงคือสูบบุหรี่ และดื่มเหล้าร่วมด้วย มี "T" allele ของ $IL1\beta^{+3953}$ SNP (OR=10.4, 1.1-93.2) หรือมี "C" allele ของ $TNF\alpha^{-1031}$ SNP (OR=3.4, 1.0-11.4) มากกว่ากลุ่มควบคุมอีกด้วย ซึ่งผลการศึกษานี้สนับสนุนสมมุติฐานที่ว่า ความแตกต่างกันของยีนส์หลากหลายพันธุกรรมของกลุ่มยีนส์สร้างโปรตีนควบคุมภูมิต้านทานในกระบวนการอักเสบ มีอิทธิพลต่อความไวการเกิดมะเร็งช่องปากในประชากรไทยภาคใต้ หว่าผลการศึกษานี้ยังเป็นการศึกษาเบื้องต้น ซึ่งมีประชากรตัวอย่างจำนานน้อย การยืนยันผลจำต้องมีการศึกษาในจำนวนประชากรตัวอย่างมากขึ้น อีกทั้งมีความน่าสนใจที่จะศึกษาเพิ่มเติมถึงความสัมพันธ์ ของปฏิกิริยาที่ซับซ้อนของภูมิต้านทานร่างกายในกระบวนการอักเสบ และการซ่อมแซมความเสียหายของเซลล์โดยยีนส์ซ่อมแซมดีเอ็นเอกับความไวต่อการเกิดมะเร็งช่องปาก รวมถึงจำเป็นที่ต้องทำการศึกษาวิจัยในห้องปฏิบัติการเพื่อให้มีความรู้คุณสมบัติและหน้าที่ของยีนส์หลากลายพันธุกรรมเหล่านี้ด้วย

Association of polymorphisms in proinflammatory cytokine genes with the development of oral cancer in Southern Thailand

Abstract

Oral squamous cell carcinoma (OSCC) is highly prevalent in southeastern Asia suggesting that region-specific environmental and biological factors contribute to the development of this cancer. Exposure to oral carcinogens (*i.e.* betel quid) and pathogenic agents (*i.e.* papilloma virus) is common among individuals that develop OSCC in countries such as Thailand, India etc. However, not all individuals with such exposures develop the disease suggesting that other factors further increase susceptibility to OSCC. It is therefore plausible that functional variants in DNA repair genes and/or genes controlling inflammation and immunological response play a role in determining susceptibility to OSCC. Previous studies (including ours) have found an association between variants in DNA repair genes and increased susceptibility to OSCC. By extension, the current study examined the association between SNPs in genes encoding proteins involved in inflammation and immunomodulation (IL1α, IL1β, IL8, TNFα) and OSCC. A total of 107 cases and 157 controls were analyzed. OSCC cases were more likely to carry the "T" allele at the $IL1\alpha^{+4845}$ SNP than controls (OR=2.0, 1.0-4.4). OSCC cases that smoke and drink were more likely to carry either the "T" allele at the $IL1\beta^{+3953}$ SNP (OR=10.4, 1.1-93.2) or the "C" allele at the $TNF\alpha^{-1031}$ SNP (OR=3.4, 1.0-11.4) than controls. These results support the hypothesis that variants in inflammatory or immunomodulatory genes influence susceptibility to OSCC in Thailand. Larger studies are needed to confirm these results and more importantly to properly investigate the complex interactions among genetic variants in DNA repair and inflammation and other non-genetic susceptibility factors. In addition, laboratory experiments designed to determine the functional properties of the genetic variants are needed.

Keywords: *gene polymorphism, genetic susceptibility, acute phase cytokines, inflammatory genes, oral cancer, molecular epidemiology, Thai population*

Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 275,000 new cases and 180,000 death around the world in 2002 (www.WHO.int). Among these cases, two-third occurred in males. In addition, the highest incidence is found in Southeast Asian countries, with closed to 200,000 new cases per year (Parkin et al., 2005). In Thailand, OSCC incidence is highest in the Southern region of the country where it is ranked the second in males among all cancers (Sriplung et al., 2005).

The high prevalence of OSCC in Asia may be due to the existence of certain life style factors such as the chewing of betel quids together with or without tobacco, alcohol consumption, cigarette smoking, infection with human papilloma virus and inadequate oral hygiene (Cancela et al., 2009; Chen et al., 2002; Gheit et al., 2009; Jayalekshmi et al., 2009; Kerdpon and Sriplung, 2001; Rahman et al., 2005; Syrjanen, 2005). In addition, genetic susceptibility may play an important role (Bau et al., 2007; Drummond et al., 2005; Hatagima et al., 2008; Kietthubthew et al., 2001; Kietthubthew et al., 2006; Marques et al., 2006). The information indicates that many risk factors contribute to the development of OSCC and additional factors remain to be determined.

Our current investigation is focused on the role of polymorphism of proinflammatory cytokine genes on OSCC in Thailand. The rationale is that an intimate relationship between chronic infection, inflammation and cancer development has been reported (Hussain and Harris, 2007). The prevalent assumption is that reactive oxygen and nitrogen species produced by inflammatory cells cause damage to important cellular components and

orchestrate a tumor-supporting microenvironment that support the initiation of cancer. Important components in this linkage are the cytokines produced by activated innate immune cells which stimulate tumor growth and progression. In addition, tobacco smoking creates oxidative stress environment which stimulates and amplifies the production of numerous proinflammatory cytokines such as interleukin–1β (IL1β), interferon-γ, tumor necrosis factor α (TNFα), etc. (Cooper and Magwere, 2008; Orosz et al., 2007). Later on, soluble mediators produced by cancer cells recruit and activate inflammatory cells and further stimulate tumor progression (Coussens and Werb, 2002; Hussain and Harris, 2007; Lin and Karin, 2007).

There are only few reports on the role of polymorphisms in proinflammatory cytokine genes and OSCC. These are reports among population in Taiwan (Liu et al., 2005), Greek and German (Vairaktaris et al., 2008; Vairaktaris et al., 2007; Vairaktaris et al., 2006) and Central and Eastern Europe (Campa et al., 2007). Along with other proinflammatory cytokines, TNFα induces nitric oxide synthtase in a cholangiocarcinoma cell line (Jaiswal et al., 2000). This enzyme produces nitric oxide, which can increase DNA damage by inhibiting sensitive DNA repair enzymes, and thereby contributes to an increase in genetic mutations (Jaiswal et al., 2000). In an oral epidermoid carcinoma KB CCL17 cell line, IL1 (α or β) and IL8 play important roles in the cascade of interacting cytokines (Cheng et al., 2000). Furthermore, an extract from the areca nut can directly alter inflammatory signaling (Chiang et al., 2008). Therefore, we have explored the effect of polymorphisms of several proinflammatory cytokine genes on the OSCC susceptibility in Thailand that has a very serious OSCC cancer burden. Polymorphic genes were selected based on their reported involvement in cancers risk (Campa et al., 2007; Lin and Karin, 2007; Liu et al., 2005; Patel et al., 2006; Vairaktaris et al., 2007; Wang et al., 2007). In our paper, we report an

association of SNPs: *IL1α*$^{+45845}$ G/T (rs17561), *IL1β*$^{+3953}$ C/T (rs1143634), *IL8*$^{-251}$ A/T (rs4073), *TNFα*$^{-1031}$ T/C (rs1799964), *TNFα*$^{-857}$ C/T (rs1799724), *TNFα*$^{-308}$ G/A (rs1800629) and *TNFα*$^{-238}$ G/A (rs361525) and the development of OSCC in a Southern Thai population. In addition, their interactions with the polymorphisms in xenobiotic metabolizing genes *GSTM1* +/-, *GSTT1* +/- (Kietthubthew et al., 2001), and DNA repair genes (*XRCC1*194 G/A and C/T; *XRCC3*241 C/T; *XPC* A/C and PAT; *XPD* 6 C/A, and A/C; and *MGMT* G/C and C/T (Kietthubthew et al., 2006) on OSCC susceptibility are also presented.

Materials and Methods

Subjects

The cases were patients with cancer in the oral cavity diagnosed as OSCC (ICD-10: C00-C06). The recruitment criteria and information collected were described previously (Kietthubthew et al., 2001); patients with histologically confirmed squamous cell carcinoma in the oral cavity were sequentially recruited from the Department of Radiology, Songklanagarind Hospital, Hatyai, Songkhla, Thailand, before they had chemo- and/or radio-therapy. The controls were recruited simultaneously from residents living in the similar geographic area (Songkhla Province and its vicinity). All individuals voluntarily participated in the study after they had provided informed consent. Each participant was personally interviewed with a questionnaire. Both the consent form and the questionnaire had been approved by the university ethics committee. The information collected and used in this study were related to past history of individual's life style habits (tobacco smoking, use of smokeless tobacco, betel chewing, alcohol drinking), other possible risk factors (occupational exposure to toxic substances, nutrition, oral infection), as well as personal and family history

of various cancers. A total of 114 cases and 192 controls were recruited. Since cases and controls were simultaneously and independently recruited, after matching on gender, 5-year age group, and behavior of cigarette smoking and alcohol drinking, only 107 cases and 157 controls were eligible for further analysis.

Genotyping

Leukocytes DNA from both the patients and the controls were used for genotyping. The TaqMan® method (Applied Biosystems Inc.; Foster City, CA) was used to determine genotypes for the selected SNPs in the candidate genes of interest. Genotypes for 7 SNPs in 4 candidate genes (*IL1β*, *IL1α*, *IL8* *TNFα*,) were determined using this approach. Approximately 10 ng of genomic DNA were used in each genotyping. PCR in a 10 ul total volume, and the manufacturer's suggested protocol was followed. The TaqMan Genotyping Buffer (ABI) was used in each reaction. We used a DNA Engine equipped with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories; Hercules, CA) for the PCR thermal cycling. All reactions were carried out in tube strips fitted with optical caps (Bio-Rad Laboratories). Negative (no template) controls were used in every run and when possible, individuals of known genotypes for the candidate SNPs were included. Reactions were performed in duplicate for each individual. Table1 provides information on the selected SNPs for the genes under study.

Table1 Information regarding the selected SNPs under study in the candidate genes of interest

Gene	SNP	Location	Polymorphism	rs ID	ABI TaqMan Assay
IL1β	$IL1\beta^{+3953}$	Exon 5	C>T	rs1143634	C___9546517_10
IL1α	$IL1\alpha^{+4845}$	Exon 5	G>T	rs17561	C___9546471_10
IL8	$IL8^{-251}$	5′ UTR	T>A	rs4073	C__11748116_10
TNFα	$TNF\alpha^{-1031}$	5′ UTR	T>C	rs1799964	C___7514871_10
TNFα	$TNF\alpha^{-857}$	5′ UTR	C>T	rs1799724	C__11918223_10
TNFα	$TNF\alpha^{-308}$	5′ UTR	G>A	rs1800629	C___7514879_10
TNFα	$TNF\alpha^{-238}$	5′ UTR	G>A	rs361525	C___2215707_10

Statistical Analysis

The qualified 107 cases and 157 controls were stratified by gender, 5-year age stratum, cigarette smoking and alcohol drinking behaviors. Since the ratios between cases and controls varied among matching strata (Table2), conditional logistic regression was used to compromise the differences and to find odds ratios (ORs) of the association between polymorphisms and oral cancer. In multivariate analysis of such associations, adjusted ORs were calculated from the final minimum models which were different when the data were stratified by smoking and drinking behaviors. P-value at the level of 0.05 was considered significant and that greater than 0.05 but less than 0.10 was considered probably significant since our sample size might not be large enough to make the predictors statistically significant at the level of 0.05. We also use the term 'probably significant' when AIC and likelihood ratio tests confirm significant difference of the final minimal model and the larger one. Odds ratio (OR) for all three combinations of genotypes; wild-type, heterozygous and

homozygous mutations, was calculated separately. When one of the numbers of cases or controls in the homozygous mutation group was zero and another was less than five, both heterozygous and homozygous mutations were combined to get a more robust OR estimate. Referring to our previous studies on interactions of OSCC susceptibility and SNPs xenobiotic metabolizing genes (Kietthubthew et al., 2001), and DNA repair genes (Kietthubthew et al., 2006), all of them were analyzed in multivariate analysis. Trend test for each SNPs was calculated for trend in risk of having cancer in the 3 genotypes; wild-type, heterozygous and homozygous variants, respectively. All the analysis was done under R 2.8.1 environment (Team, 2008).

Results

Table2 demonstrates matching strata with different case-control ratios. Strata are arranged by cigarette smoking and alcohol drinking behaviors. The data indicate that smokers and/or drinkers were mostly males while non-smokers and non-drinkers were mostly females.

Table2 Matching strata and frequencies of cases and controls by exposure to cigarette and alcohol

Sex	Age group	Cases [107]	Controls [157]
Exposure to both cigarette and alcohol			
Male	45-49	3	1
Male	50-54	8	8
Male	55-59	9	9
Male	60-64	7	5
Male	65-69	9	8
Male	70-74	10	15
Male	75-79	13	6
Male	80-84	2	4
No exposure to cigarette and alcohol			
Male	55-59	1	1
Male	65-69	1	2
Male	70-74	3	1
Female	45-49	1	2
Female	55-59	2	1
Female	60-64	2	8
Female	65-69	5	11

Female	70-74	8	12
Female	75-79	3	21
Female	80-84	3	11
Female	85-89	3	3
Exposure to cigarette, not alcohol			
Male	55-59	2	2
Male	60-64	2	2
Male	65-69	1	8
Male	70-74	3	6
Male	75-79	1	4
Male	80-84	3	4
Female	70-74	1	1
Exposure to alcohol, not cigarette			
Male	70-74	1	1

A summary of our statistical analysis of the cases and controls is shown in Table3. Mean age of cases was 67.5 with standard deviation of 9.8 years and that of controls was 69.7 with standard deviation of 9.0 years. The percentage of males was higher among cases than controls. The majority (57.0%) of cases were smokers and drinkers while 46.5% of controls were non-smokers and non-drinkers.

Table3 Characteristics of cases and controls

	Matched subjects	
	Cases (107)	Controls (157)
	No. (%)	No. (%)
Sex		
Male	79 (73.8)	87 (55.4)
Female	28 (26.2)	70 (44.6)
Smoking		
Yes	74 (69.2)	83 (52.9)
No	33 (30.8)	74 (47.1)
Drinking		
Yes	62 (56.9)	57 (36.3)
No	45 (41.3)	100 (63.7)
Smoking/Drinking		
Both behaviors	61 (57.0)	56 (35.7)
One behavior	14 (13.1)	28 (17.8)
None	32 (29.9)	73 (46.5)
Betel chewing		
Yes	50 (46.7%)	50 (31.9%)
No	57 (53.3%)	107 (68.1%)

Genotype distribution and allele frequencies of each SNP in the controls were tested, and they were found to fit Hardy-Weinberg equilibrium expectations. Allele frequencies of the SNPs are shown in Table4

Table4 Allele frequencies of the pro-inflammatory cytokine SNPs

SNPs	Allele frequencies
$IL1\beta^{+3953}$ (C>T)	C = 0.9436 T = 0.0546
$IL1\alpha^{+4845}$ (G>T)	G = 0.8673 T = 0.1327
$IL8^{-251}$ (T>A)	T = 0.6349 A = 0.3651
$TNF\alpha^{-1031}$ (T>C)	T =0.7178 C = 0.2822
$TNF\alpha^{-857}$ (C>T)	C = 0.9371 T = 0.0629
$TNF\alpha^{-308}$ (G>A)	G = 0.9207 A = 0.0793
$TNF\alpha^{-238}$ (G>A)	G = 0.9708 A = 0.0292

In a crude analysis (Table5), the most significant observation is the association between oral cancer and the $IL1\alpha^{+4845}$ SNP where heterozygous and homozygous (GT/TT) genotypes increased the risk by 2.0 (95%CI: 1.1-3.9, $p < 0.03$) times for all cases and 2.7 (95%CI: 1.0-7.2, $p < 0.05$) times among those who smoke and drink. The heterozygous (TA) genotype of $IL8^{-251}$ gene is associated with a decreased risk of oral cancer, OR = 0.4 (95%CI: 0.2-1.0, $p < 0.04$), but not among the homozygotes, OR = 0.8 (95%CI: 0.3-2.2). The other genotypes in this study did not show an association with oral cancer and their ORs are either closed to 1 or have wide confidence intervals.

Table5 Crude analysis association of genotypes of candidate genes and risk of oral cancer

SNP		Matched-cases		Matched-controls		cOR	95% CI		p-value	p trend
		No.	%	No.	%		lower	upper		
Overall (no & ever smoke and drink)										
IL1β^{+3953}	CC	82	88.2	126	88.7	1.00				0.66#
	CT/TT	11	11.8	16	11.3	1.16	0.48	2.81	0.75	
IL1α^{+4845}	GG	59	66.3	107	79.3	1.00				0.08#
	GT/TT	30	33.7	28	20.7	2.02	1.06	3.87	0.03*	
IL8^{-251}	TT	32	50.8	34	34.3	1.00				0.04#*
	TA	21	33.3	49	49.5	0.44	0.20	0.98	0.04*	
	AA	10	15.9	16	16.2	0.80	0.29	2.18	0.66	
TNFα^{-1031}	TT	48	52.2	67	48.9	1.00				0.89#
	TC	36	39.1	60	43.8	0.95	0.53	1.72	0.87	
	CC	8	8.7	10	7.3	1.11	0.40	3.11	0.84	
TNFα^{-857}	CC	82	85.4	129	86.6	1.00				-
	CT/TT	14	14.6	20	13.4	0.84	0.37	1.92	0.68	
TNFα^{-308}	GG	83	85.6	133	87.5	1.00				0.50#
	GA/AA	14	14.4	19	12.5	0.55	0.14	2.10	0.38	
TNFα^{-238}	GG	92	94.8	141	92.8	1.00				-
	GA/AA	5	5.2	11	7.2	1.51	0.09	24.8	0.77	
Ever smoke and drink										
I IL1β^{+3953}	CC	46	86.8	48	92.3	1.00				-
	CT/TT	7	13.2	4	7.7	1.90	0.46	7.79	0.37	
IL1α^{+4845}	GG	34	65.4	42	84.0	1.00				0.16#
	GT/TT	18	34.6	8	16.0	2.69	1.01	7.19	0.05*	
IL8^{-251}	TT	17	47.2	11	26.8	1.00				0.13#
	TA	15	41.7	23	56.1	0.44	0.16	1.24	0.12	
	AA	4	11.1	7	17.1	0.38	0.08	1.70	0.20	
TNFα^{-1031}	TT	26	49.1	29	55.8	1.00				0.40#
	TC	22	41.5	20	38.4	1.43	0.61	3.35	0.41	
	CC	5	9.4	3	5.8	1.85	0.39	8.90	0.44	
TNFα^{-857}	CC	45	83.3	41	77.4	1.00				-
	CT /TT	9	16.7	12	22.6	0.59	0.21	1.71	0.33	
TNFα^{-308}	GG	49	90.7	48	88.9	1.00				-
	GA /AA	5	9.3	6	11.1	0.55	0.14	2.10	0.38	
TNFα^{-238}	GG	53	98.1	53	98.1	1.00				-
	GA /AA	1	1.9	1	1.9	1.51	0.09	24.8	0.77	
Neither smoke nor drink										
I IL1β^{+3953}	CC	27	90.0	58	87.9	1.00				0.82#
	CT/TT	3	10.0	8	12.1	1.01	0.23	4.45	0.99	
IL1α^{+4845}	GG	18	69.2	50	78.1	1.00				-
	GT/TT	8	30.8	14	21.9	1.77	0.59	5.29	0.31	
IL8^{-251}	TT	12	60.0	17	36.2	1.00				0.13#
	TA	4	20.0	21	44.7	0.35	0.09	1.36	0.13	
	AA	4	20.0	9	19.1	0.78	0.17	3.64	0.75	
TNFα^{-1031}	TT	15	51.7	33	51.6	1.00				0.96#
	TC	12	41.4	26	40.6	1.03	0.41	2.60	0.95	
	CC	2	6.9	5	7.8	0.80	0.13	4.82	0.81	
TNFα^{-857}	CC	27	84.4	64	90.1	1.00				-
	CT/TT	5	15.6	7	9.9	1.58	0.42	6.00	0.50	
TNFα^{-308}	GG	23	71.9	61	84.7	1.00				0.67#

	GA/AA	9	28.1	11	15.3	1.50	0.47	4.80	0.49	
$TNF\alpha^{-238}$	GG	32	100	65	90.3	1.00				-
	GA/AA	0	0.0	7	9.7	-	-	-	-	

OR = crude odds ratio without adjustment by the effect of other genes, p-value = p-value Wald test,
* = with statistical significance. # p trend calculated for three levels of genotypes

Testing for trends in associations between genotypes and OSCC susceptibility (i.e. wild-type, heterozygous, and homozygous variant genotypes), the trends were probable for $IL1\alpha^{+4845}$ ($p < 0.08$) but significant for $IL8^{-251}$ ($p < 0.04$). However, in subgroup analysis, the trends in the association with oral cancer disappeared.

Table6 Association of genotypes of candidate genes and risk of oral cancer adjusted for other genes

SNP		Matched-cases		Matched-controls		cOR	aOR	95% CI		p-value
		No.	%	No.	%			lower	upper	
Overall (no & ever smoke and drink)										
$IIL1\alpha^{+4845}$	GG	59	66.3	107	79.3	1.00	1.00			
	GT/TT	30	33.7	28	20.7	2.02	2.00	0.91	4.41	0.08
$IL8^{-251}$	TT	32	50.8	34	34.3	1.00	1.00			
	AT	21	33.3	49	49.5	0.44	0.92	0.33	2.60	0.88
	AA	10	15.9	16	16.2	0.80	0.49	0.22	1.10	0.08
Ever smoke and drink										
$IL1\beta^{+3953}$	CC	46	86.8	48	92.3	1.00	1.00			
	CT	7	13.2	4	7.7	1.90	10.4	1.16	93.2	0.04*
$IL8^{-251}$	TT	17	47.2	11	26.8	1.00	1.00			
	AT	15	41.7	23	56.1	0.44	0.28	0.08	0.91	0.03*
	AA	4	11.1	7	17.1	0.38	0.25	0.05	1.32	0.10
$TNF\alpha^{-1031}$	TT	26	49.1	29	55.8	1.00	1.00			
	CT	22	41.5	20	38.4	1.43	3.40	1.01	11.4	0.05*
	CC	5	9.4	3	5.8	1.85	2.03	0.26	15.9	0.50

cOR = crude odds ratio, aOR = adjusted odds ratio, p-value = Wald test

Multivariate analyses among the studied genes showed a probable association of genotypes in the SNPs $IL1\alpha^{+4845}$ and $IL8^{-251}$ all OSCC cases, p-value between 0.08 and 0.1, and neither of them could be omitted from the final minimal models (Table6). Among those who smoke and drink, the heterozygous (CT) genotype of $IL1\beta^{+3953}$ and $TNF\alpha^{-1031}$ significantly increased the risk of oral cancer (OR = 10.4 (95%CI: 1.16 – 93.2, $p < 0.04$) and

OR = 3.40 (95%CI: 1.01 – 11.4, p< 0.05). In contrast, the heterozygous genotype (AT) of the *IL8*$^{-251}$ decreased the oral cancer risk by 0.3 times (95%CI: 0.08 – 0.91, p< 0.03). However, the trend analysis did not confirm these statistical results. None of the variants in these genes were associated with oral cancer among non-smokers and non-drinkers.

In our series of studies, we have investigated the association between different groups of susceptibility genes and OSCC. These genes belong to those involved with xenobiotic metabolism and DNA repair, and the current study on inflammatory response. When all previously studied genes were included and adjusted in the statistical model (Table7), the involvement of specific genotypes was identified and for different conditions. For all OSCC cases, the association with the two DNA repair genes, *XRCC1*194 and *XPD6* was significant (p< 0.05, 95% CI:1.02 – 6.14 and p< 0.02, 95% CI: 1.21 – 6.60, respectively). In addition, genotypes in the IL1α and IL1β genes still had an effect on the model. Although the p values were not significant, the likelihood ratio of the statistical models in the three subgroups changed dramatically when *IL1*α^{+4845} was removed.

Table7 Association of genotypes of candidate genes and risk of oral cancer adjusted for other genes

Genes		Matched-cases		Matched-controls		aOR	95% CI		p-value
		No.	%	No.	%		lower	upper	
Overall (no & ever smoke and drink)									
*IL1*α^{+4845}	GG	59	66.3	107	79.3	1.00			
	TT/GT	30	33.7	28	20.7	1.80	0.75	4.30	0.18
IL8^{-251}	TT	32	50.8	34	34.3	1.00			
	AT	21	33.3	49	49.5	0.49	0.21	1.18	0.11
	AA	10	15.9	16	16.2	0.98	0.33	2.91	0.98
GST T1	wild	39	37.1	85	54.8	1.00			
	null	66	62.9	70	45.2	2.07	0.94	4.55	0.07*
*XRCC1*194	CC	40	38.1	72	46.7	1.00			
	CT	50	47.6	63	40.9	2.50	1.02	6.14	0.05**
	TT	15	14.3	19	12.3	3.51	0.84	14.7	0.08*
XPD6	CC	45	43.3	77	50.3	1.00			
	CA/AA	59	56.7	76	49.7	2.76	1.21	6.60	0.02**
Ever smoke and drink									
*IL1*β^{+3953}	CC	46	86.8	48	92.3	1.00			
	CT/TT	7	13.2	4	7.7	93.4	3.28	2658	0.01**

IL1α^{+4845}	GG	34	65.4	42	84.0	1.00			
	GT/TT	18	34.6	8	16.0	6.10	0.66	56.6	0.11
IL8^{-251}	TT	17	47.2	11	26.8	1.00			
	AT	15	41.7	23	56.1	0.05	0.00	0.46	0.01**
	AA	4	11.1	7	17.1	0.01	0.00	0.33	0.01**
TNFα^{-857}	CC	45	83.3	41	77.4	1.00			
	CT/TT	9	16.7	12	22.6	0.003	0.00	1.16	<0.01**
TNFα^{-308}	GG	49	90.7	48	88.9	1.00			
	GA/AA	5	9.3	6	11.1	0.04	0.00	1.38	0.07*
*XRCC1*194	CC	22	36.7	33	60.0	1.00			
	CT/TT	38	63.3	22	30.0	8.15	1.91	34.7	<0.01**
XRCC3241	CC	49	81.7	49	87.5	1.00			
	CT/TT	11	18.3	7	12.5	93.4	3.28	2658	0.01**
Neither smoke nor drink									
IL1α^{+4845}	GG	18	69.2	50	78.1	1.00			
	GT/TT	8	30.8	14	21.9	2.43	0.68	8.60	0.17
GST T1	wild	10	33.3	38	53.5	1.00			
	null	20	66.7	33	46.5	2.89	0.93	8.97	0.06*
XPD6	CC	10	31.2	35	49.3	1.00			
	CA/AA	22	68.8	36	50.7	5.59	1.58	19.7	0.01**

aOR = adjusted odds ratio, * significant at $p < 0.10$, ** significant at $p < 0.05$

For OSCC cases who ever smoked or drank, *IL1β*$^{+3953}$, *XRCC1*194 and *XRCC3*241 were significantly associated with OSCC, p< 0.01, 95%CI: 3.28 – 2658; p< 0.01, 95%CI: 1.91 – 34.7; p< 0.01, 95%CI: 3.28 – 2658, respectively. The variants at SNPs *TNFα*$^{-857}$ and *TNFα*$^{-308}$ appear to be significant in the subgroup of smokers and drinkers though the odds ratios are very low and confidence intervals are very wide.

Among the OSCC cases who had never smoked nor drank, only the *XPD6* susceptibility gene showed a significant association (p< 0.01, 95%CI: 1.58 – 19.7).

Discussion

Our data support previous studies demonstrating the involvement of pro-inflammatory cytokine genes in the development of OSCC although there remain inconsistencies among these studies. For example, our observed, significant association between *IL1β*$^{+3953}$ (Table6) and OSCC contrasts with the study of Vairaktaris et al (2008). The reverse holds for the

TNFα^{-308} SNP (Yapijakis et al., 2009). We observed that the *IL8*$^{-251}$ polymorphism decreased the risk for OSCC. This finding disagrees with the previous report in the German and Greek population (Vairaktaris et al., 2007). The different results can be caused by the studies using different ethnic groups with different environmental and genetic backgrounds. The association of the variants in the SNPs, *IL1α*$^{+4845}$ and *IL8*$^{-251}$, with oral cancer is worth noting especially among those who smoke and drink even if the association is not strong. The lack of trend in association with the genotypes in *IL1α*$^{+4845}$ and *IL8*$^{-251}$ in the subgroup analysis even when the genes seem to be significant in the overall analysis can best be explained by the reduction in sample size when the subgroups defined by smoking and drinking behavior are considered.

From our multi-genotype association analyses (Table7), some meaningful results can be derived from the study. For all OSCC cases, the association was strongest with the apparent susceptibility genotypes in the DNA repair SNPs, *XRCC1*194 and *XPD*6. For cases who had ever smoked or drank, the strongest association was with *IL1β*$^{+3953}$, *XRCC1*194 and *XRCC3*241. For those who had never smoked nor drank, the association of *XPD*6 was the strongest.

The involvement of different susceptibility DNA repair genes in OSCC illustrates the critical role of DNA repair in the cancer process. In addition, the data indicate that different repair genes may play a more important role under different environmental conditions, e.g. in smokers and non-smokers for the repair of different types of DNA damage.

The observed interactions between the smoking habits and susceptibility pro-inflammatory genes for OSCC are supportive of other observations. For example, chemicals

in cigarette smoke have been reported to affect immune function and affect the response to different types of infections (Gualano et al., 2008; Harel-Meir et al., 2007; Herr et al., 2009). More specifically, smoking can affect the expression of the genes that we have investigated, e.g. *TNFα, IL1β* and *IL8* (Chen et al., 2007; Mian et al., 2009).

In conclusion, certain SNPs in genes that are involved in inflammation and immune response, and that were investigated in this study appear to be associated with the development of OSCC. Subtle differences among individuals in their ability to metabolize xenobiotics, repair damaged DNA, and properly manage inflammatory responses due to common genetic differences all appear to play a role in the development of OSCC. In conjunction with previous studies, these latest findings illustrate the critical interactions between genes and the environment that lead to the development of cancer, such as OSCC. In addition, our findings should lead to more focused investigations into the different mechanisms influencing susceptibility to OSCC under different environmental conditions.

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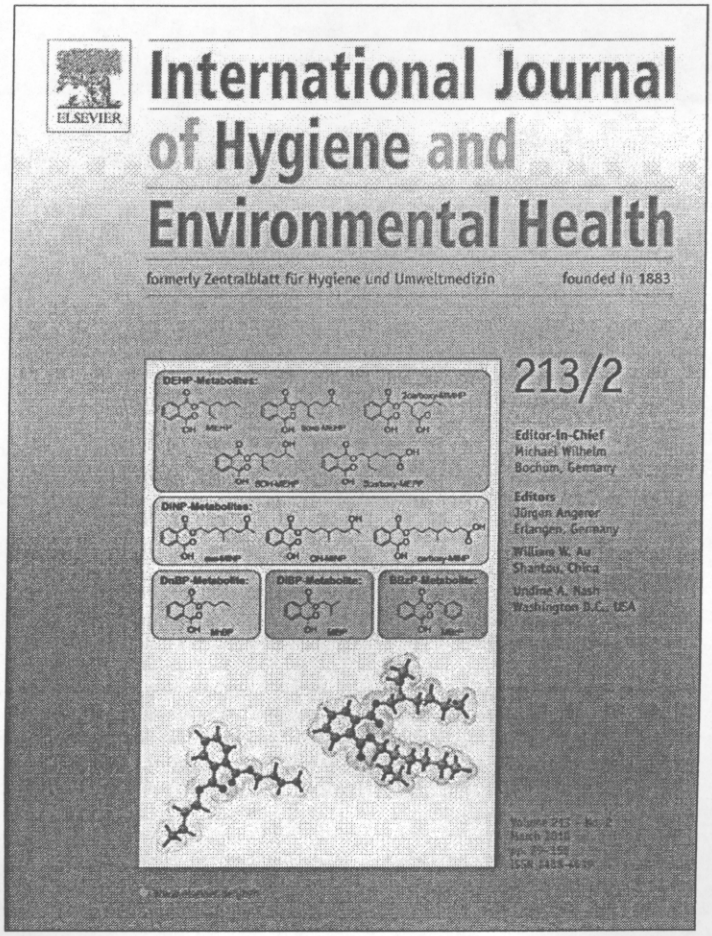
Appendix

(published papers related to the current report)



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Association of polymorphisms in proinflammatory cytokine genes with the development of oral cancer in Southern Thailand

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is highly prevalent in southeastern Asia suggesting that region-specific environmental and biological factors contribute to the development of this cancer. Exposure to oral carcinogens (*i.e.* betel quid) and pathogenic agents (*i.e.* papilloma virus) is common among individuals that develop OSCC in countries such as Thailand, India etc. However, not all individuals with such exposures develop the disease suggesting that other factors further increase susceptibility to OSCC. It is therefore plausible that functional variants in DNA repair genes and/or genes controlling inflammation and immunological response play a role in determining susceptibility to OSCC. Previous studies (including ours) have found an association between variants in DNA repair genes and increased susceptibility to OSCC. By extension, the current study examined the association between SNPs in genes encoding proteins involved in inflammation and immunomodulation (IL1α, IL1β, IL8, TNFα) and OSCC. A total of 107 cases and 157 controls were analyzed. OSCC cases were more likely to carry the "T" allele at the $IL1\alpha^{+4845}$ SNP than controls (OR=2.0, 1.0–4.4). OSCC cases that smoke and drink were more likely to carry either the "T" allele at the $IL1\beta^{+3953}$ SNP (OR=10.4, 1.1–93.2) or the "C" allele at the $TNF\alpha^{-1031}$ SNP (OR=3.4, 1.0–11.4) than controls. These results support the hypothesis that variants in inflammatory or immunomodulatory genes influence susceptibility to OSCC in Thailand. Larger studies are needed to confirm these results and more importantly to properly investigate the complex interactions among genetic variants in DNA repair and inflammation and other non-genetic susceptibility factors. In addition, laboratory experiments designed to determine the functional properties of the genetic variants are needed.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 275,000 new cases and 180,000 death around the world in 2002 (www.WHO.int). Among these cases, two-third occurred in males. In addition, the highest incidence is found in Southeast Asian countries, with closed to 200,000 new cases per year (Parkin et al., 2005). In Thailand, OSCC incidence is highest in the Southern region of the country where it is ranked the second in males among all cancers (Sriplung et al., 2005).

The high prevalence of OSCC in Asia may be due to the existence of certain life style factors such as the chewing of betel quids together with or without tobacco, alcohol consumption, cigarette smoking, infection with human papilloma virus and inadequate oral hygiene (Cancela et al., 2009; Chen et al., 2002; Gheit et al., 2009; Jayalekshmi et al., 2009; Kerdpon and Sriplung, 2001; Rahman et al., 2005; Syrjanen, 2005). In addition, genetic susceptibility may play an important role (Bau et al., 2007; Drummond et al., 2005; Hatagima et al., 2008; Kietthubthew et al., 2001, 2006; Marques et al., 2006). The information indicates that many risk factors contribute to the development of OSCC and additional factors remain to be determined.

Our current investigation is focused on the role of polymorphism of proinflammatory cytokine genes on OSCC in Thailand. The rationale is that an intimate relationship between chronic infection, inflammation and cancer development has been reported (Hussain and Harris, 2007). The prevalent assumption is that reactive oxygen

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and nitrogen species produced by inflammatory cells cause damage to important cellular components and orchestrate a tumor-supporting microenvironment that support the initiation of cancer. Important components in this linkage are the cytokines produced by activated innate immune cells which stimulate tumor growth and progression. In addition, tobacco smoking creates oxidative stress environment which stimulates and amplifies the production of numerous proinflammatory cytokines such as interleukin−1β (IL1β), interferon-γ, tumor necrosis factor α (TNFα), etc. (Cooper and Magwere, 2008; Orosz et al., 2007). Later on, soluble mediators produced by cancer cells recruit and activate inflammatory cells and further stimulate tumor progression (Coussens and Werb, 2002; Hussain and Harris, 2007; Lin and Karin, 2007).

There are only few reports on the role of polymorphisms in proinflammatory cytokine genes and OSCC. These are reports among population in Taiwan (Liu et al., 2005), Greek and German (Vairaktaris et al., 2008, 2007, 2006) and Central and Eastern Europe (Campa et al., 2007). Along with other proinflammatory cytokines, TNFα induces nitric oxide synthtase in a cholangiocarcinoma cell line (Jaiswal et al., 2000). This enzyme produces nitric oxide, which can increase DNA damage by inhibiting sensitive DNA repair enzymes, and thereby contributes to an increase in genetic mutations (Jaiswal et al., 2000). In an oral epidermoid carcinoma KB CCL17 cell line, IL1 (α or β) and IL8 play important roles in the cascade of interacting cytokines (Cheng et al., 2000). Furthermore, an extract from the areca nut can directly alter inflammatory signaling (Chiang et al., 2008). Therefore, we have explored the effect of polymorphisms of several proinflammatory cytokine genes on the OSCC susceptibility in Thailand that has a very serious OSCC cancer burden. Polymorphic genes were selected based on their reported involvement in cancers risk (Campa et al., 2007; Lin and Karin, 2007; Liu et al., 2005; Patel et al., 2006; Vairaktaris et al., 2007; Wang et al., 2007). In our paper, we report an association of SNPs: $IL1\alpha^{+45845}$ G/T (rs17561), $IL1\beta^{+3953}$ C/T (rs1143634), $IL8^{-251}$ A/T (rs4073), $TNF\alpha^{-1031}$ T/C (rs1799964), $TNF\alpha^{-857}$ C/T (rs1799724), $TNF\alpha^{-308}$ G/A (rs1800629) and $TNF\alpha^{-238}$ G/A (rs361525) and the development of OSCC in a Southern Thai population. In addition, their interactions with the polymorphisms in xenobiotic metabolizing genes *GSTM1* +/−, *GSTT1* +/− (Kietthubthew et al., 2001), and DNA repair genes (*XRCC1*194 G/A and C/T; *XRCC3*241 C/T; *XPC* A/C and PAT; *XPD* 6 C/A, and A/C; and *MGMT* G/C and C/T (Kietthubthew et al., 2006) on OSCC susceptibility are also presented.

Materials and methods

Subjects

The cases were patients with cancer in the oral cavity diagnosed as OSCC (ICD-10: C00-C06). The recruitment criteria and information collected were described previously (Kietthubthew et al., 2001); patients with histologically confirmed squamous cell carcinoma in the oral cavity were sequentially recruited from the Department of Radiology, Songklanagarind Hospital, Hatyai, Songkhla, Thailand, before they had chemo- and/or radio-therapy. The controls were recruited simultaneously from residents living in the similar geographic area (Songkhla Province and its vicinity). All individuals voluntarily participated in the study after they had provided informed consent. Each participant was personally interviewed with a questionnaire. Both the consent form and the questionnaire had been approved by the university ethics committee. The information collected and used in this study were related to past history of individual's life style habits (tobacco smoking, use of smokeless tobacco, betel chewing, alcohol drinking), other possible risk factors (occupational exposure to toxic substances, nutrition, oral infection), as well as personal and family history of various cancers. A total of 114 cases and 192 controls were recruited. Since cases and controls were simultaneously and independently recruited, after matching on gender, 5-year age group, and behavior of cigarette smoking and alcohol drinking, only 107 cases and 157 controls were eligible for further analysis.

Genotyping

Leukocytes DNA from both the patients and the controls were used for genotyping. The TaqMan® method (Applied Biosystems Inc.; Foster City, CA) was used to determine genotypes for the selected SNPs in the candidate genes of interest. Genotypes for 7 SNPs in 4 candidate genes (*IL1β*, *IL1α*, *IL8 TNFα*,) were determined using this approach. Approximately 10 ng of genomic DNA were used in each genotyping. PCR in a 10 ul total volume, and the manufacturer's suggested protocol was followed. The TaqMan Genotyping Buffer (ABI) was used in each reaction. We used a DNA Engine equipped with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories; Hercules, CA) for the PCR thermal cycling. All reactions were carried out in tube strips fitted with optical caps (Bio-Rad Laboratories). Negative (no template) controls were used in every run and when possible, individuals of known genotypes for the candidate SNPs were included. Reactions were performed in duplicate for each individual. Table 1 provides information on the selected SNPs for the genes under study.

Statistical analysis

The qualified 107 cases and 157 controls were stratified by gender, 5-year age stratum, cigarette smoking and alcohol drinking behaviors. Since the ratios between cases and controls varied among matching strata (Table 2), conditional logistic regression was used to compromise the differences and to find odds ratios (ORs) of the association between polymorphisms and oral cancer. In multivariate analysis of such associations, adjusted ORs were calculated from the final minimum models which were different when the data were stratified by smoking and drinking behaviors. P-value at the level of 0.05 was considered significant and that greater than 0.05 but less than 0.10 was considered probably significant since our sample size might not be large enough to make the predictors statistically significant at the level of 0.05. We also use the term 'probably significant' when AIC and likelihood ratio tests confirm significant difference of the final minimal model and the larger one. Odds ratio (OR) for all three combinations of genotypes; wild-type, heterozygous and homozygous mutations, was calculated separately. When one of the numbers of cases or controls in the homozygous mutation group was zero and another was less than five, both heterozygous and homozygous mutations were combined to get a more robust

Table 1
Information regarding the selected SNPs under study in the candidate genes of interest.

Gene	SNP	Location	Polymorphism	rs ID	ABI TaqMan Assay
IL1β	$IL1\beta^{+3953}$	Exon 5	C>T	rs1143634	C___9546517_10
IL1α	$IL1\alpha^{+4845}$	Exon 5	G>T	rs17561	C___9546471_10
IL8	$IL8^{-251}$	5′ UTR	T>A	rs4073	C__11748116_10
TNFα	$TNF\alpha^{-1031}$	5′ UTR	T>C	rs1799964	C___7514871_10
TNFα	$TNF\alpha^{-857}$	5′ UTR	C>T	rs1799724	C__11918223_10
TNFα	$TNF\alpha^{-308}$	5′ UTR	G>A	rs1800629	C___7514879_10
TNFα	$TNF\alpha^{-238}$	5′ UTR	G>A	rs361525	C___2215707_10

Table 2
Matching strata and frequencies of cases and controls by exposure to cigarette and alcohol.

Sex	Age group	Cases	Controls
		[107]	[157]
Exposure to both cigarette and alcohol			
Male	45-49	3	1
Male	50-54	8	8
Male	55-59	9	9
Male	60-64	7	5
Male	65-69	9	8
Male	70-74	10	15
Male	75-79	13	6
Male	80-84	2	4
No exposure to cigarette and alcohol			
Male	55-59	1	1
Male	65-69	1	2
Male	70-74	3	1
Female	45-49	1	2
Female	55-59	2	1
Female	60-64	2	8
Female	65-69	5	11
Female	70-74	8	12
Female	75-79	3	21
Female	80-84	3	11
Female	85-89	3	3
Exposure to cigarette, not alcohol			
Male	55-59	2	2
Male	60-64	2	2
Male	65-69	1	8
Male	70-74	3	6
Male	75-79	1	4
Male	80-84	3	4
Female	70-74	1	1
Exposure to alcohol, not cigarette			
Male	70-74	1	1

Table 3
Characteristics of cases and controls.

	Matched subjects	
	Cases (107)	Controls (157)
	No. (%)	No. (%)
Sex		
Male	79 (73.8)	87 (55.4)
Female	28 (26.2)	70 (44.6)
Smoking		
Yes	74 (69.2)	83 (52.9)
No	33 (30.8)	74 (47.1)
Drinking		
Yes	62 (56.9)	57 (36.3)
No	45 (41.3)	100 (63.7)
Smoking/Drinking		
Both behaviors	61 (57.0)	56 (35.7)
One behavior	14 (13.1)	28 (17.8)
None	32 (29.9)	73 (46.5)
Betel chewing		
Yes	50 (46.7%)	50 (31.9%)
No	57 (53.3%)	107 (68.1%)

Table 4
Allele frequencies of the pro-inflammatory cytokine SNPs.

SNPs	Allele frequencies
$IL1\beta^{+3953}$ (C > T)	C=0.9436
	T=0.0546
$IL1\alpha^{+4845}$ (G > T)	G=0.8673
	T=0.1327
$IL8^{-251}$ (T > A)	T=0.6349
	A=0.3651
$TNF\alpha^{-1031}$ (T > C)	T=0.7178
	C=0.2822
$TNF\alpha^{-857}$ (C > T)	C=0.9371
	T=0.0629
$TNF\alpha^{-308}$ (G > A)	G=0.9207
	A=0.0793
$TNF\alpha^{-238}$ (G > A)	G=0.9708
	A=0.0292

OR estimate. Referring to our previous studies on interactions of OSCC susceptibility and SNPs xenobiotic metabolizing genes (Kietthubthew et al., 2001), and DNA repair genes (Kietthubthew et al., 2006), all of them were analyzed in multivariate analysis. Trend test for each SNPs was calculated for trend in risk of having cancer in the 3 genotypes; wild-type, heterozygous and homozygous variants, respectively. All the analysis was done under R 2.8.1 environment (Team RDC, 2008).

Results

Table 2 demonstrates matching strata with different case-control ratios. Strata are arranged by cigarette smoking and alcohol drinking behaviors. The data indicate that smokers and/or drinkers were mostly males while non-smokers and non-drinkers were mostly females.

A summary of our statistical analysis of the cases and controls is shown in Table 3. Mean age of cases was 67.5 with standard deviation of 9.8 years and that of controls was 69.7 with standard deviation of 9.0 years. The percentage of males was higher among cases than controls. The majority (57.0%) of cases were smokers and drinkers while 46.5% of controls were non-smokers and non-drinkers.

Genotype distribution and allele frequencies of each SNP in the controls were tested, and they were found to fit Hardy-Weinberg equilibrium expectations. Allele frequencies of the SNPs are shown in Table 4

In a crude analysis (Table 5), the most significant observation is the association between oral cancer and the $IL1\alpha^{+4845}$ SNP where heterozygous and homozygous (GT/TT) genotypes increased the risk by 2.0 (95%CI: 1.1–3.9, $p < 0.03$) times for all cases and 2.7 (95%CI: 1.0–7.2, $p < 0.05$) times among those who smoke and drink. The heterozygous (TA) genotype of $IL8^{-251}$ gene is associated with a decreased risk of oral cancer, OR=0.4 (95%CI: 0.2–1.0, $p < 0.04$), but not among the homozygotes, OR=0.8 (95%CI: 0.3–2.2). The other genotypes in this study did not show an association with oral cancer and their ORs are either closed to 1 or have wide confidence intervals.

Testing for trends in associations between genotypes and OSCC susceptibility (i.e. wild-type, heterozygous, and homozygous variant genotypes), the trends were probable for $IL1\alpha^{+4845}$ ($p < 0.08$) but significant for $IL8^{-251}$ ($p < 0.04$). However, in subgroup analysis, the trends in the association with oral cancer disappeared.

Multivariate analyses among the studied genes showed a probable association of genotypes in the SNPs $IL1\alpha^{+4845}$ and $IL8^{-251}$ all OSCC cases, p-value between 0.08 and 0.1, and neither of them could be omitted from the final minimal models (Table 6). Among those who smoke and drink, the heterozygous (CT) genotype of $IL1\beta^{+3953}$ and $TNF\alpha^{-1031}$ significantly increased the risk of oral cancer (OR=10.4 (95%CI: 1.16–93.2, $p < 0.04$) and OR=3.40 (95%CI: 1.01–11.4, $p < 0.05$). In contrast, the heterozygous genotype (AT) of the $IL8^{-251}$ decreased the oral

Table 5
Crude analysis association of genotypes of candidate genes and risk of oral cancer.

SNP		Matched-cases		Matched-controls		cOR	95% CI		p value	p trend
		No.	%	No.	%		lower	upper		
Overall (no & ever smoke and drink)										
IL1β^{+3953}	CC	82	88.2	126	88.7	1.00				0.66#
	CT/TT	11	11.8	16	11.3	1.16	0.48	2.81	0.75	
IL1α^{+4845}	GG	59	66.3	107	79.3	1.00				0.08#
	GT/TT	30	33.7	28	20.7	2.02	1.06	3.87	0.03*	
IL8^{-251}	TT	32	50.8	34	34.3	1.00				0.04#*
	TA	21	33.3	49	49.5	0.44	0.20	0.98	0.04*	
	AA	10	15.9	16	16.2	0.80	0.29	2.18	0.66	
TNFα^{-1031}	TT	48	52.2	67	48.9	1.00				0.89#
	TC	36	39.1	60	43.8	0.95	0.53	1.72	0.87	
	CC	8	8.7	10	7.3	1.11	0.40	3.11	0.84	
TNFα^{-857}	CC	82	85.4	129	86.6	1.00				–
	CT/TT	14	14.6	20	13.4	0.84	0.37	1.92	0.68	
TNFα^{-308}	GG	83	85.6	133	87.5	1.00				0.50#
	GA/AA	14	14.4	19	12.5	0.55	0.14	2.10	0.38	
TNFα^{-238}	GG	92	94.8	141	92.8	1.00				–
	GA/AA	5	5.2	11	7.2	1.51	0.09	24.8	0.77	
Ever smoke and drink										
IL1β^{+3953}	CC	46	86.8	48	92.3	1.00				–
	CT/TT	7	13.2	4	7.7	1.90	0.46	7.79	0.37	
IL1α^{+4845}	GG	34	65.4	42	84.0	1.00				0.16#
	GT/TT	18	34.6	8	16.0	2.69	1.01	7.19	0.05*	
IL8^{-251}	TT	17	47.2	11	26.8	1.00				0.13#
	TA	15	41.7	23	56.1	0.44	0.16	1.24	0.12	
	AA	4	11.1	7	17.1	0.38	0.08	1.70	0.20	
TNFα^{-1031}	TT	26	49.1	29	55.8	1.00				0.40#
	TC	22	41.5	2.	38.4	1.43	0.61	3.35	0.41	
	CC	5	9.4	3	5.8	1.85	0.39	8.90	0.44	
TNFα^{-857}	CC	45	83.3	41	77.4	1.00				–
	CT/TT	9	16.7	12	22.6	0.59	0.21	1.71	0.33	
TNFα^{-308}	GG	49	90.7	48	88.9	1.00				–
	GA/AA	5	9.3	6	11.1	0.55	0.14	2.10	0.38	
TNFα^{-238}	GG	53	98.1	53	98.1	1.00				–
	GA/AA	1	1.9	1	1.9	1.51	0.09	24.8	0.77	
Neither smoke nor drink										
IL1β^{+3953}	CC	27	90.0	58	87.9	1.00				0.82#
	CT/TT	3	10.0	8	12.1	1.01	0.23	4.45	0.99	
IL1α^{+4845}	GG	18	69.2	50	78.1	1.00				–
	GT/TT	8	30.8	14	21.9	1.77	0.59	5.29	0.31	
IL8^{-251}	TT	12	60.0	17	36.2	1.00				0.13#
	TA	4	20.0	21	44.7	0.35	0.09	0.36	0.13	
	AA	4	20.0	9	19.1	0.78	0.17	3.64	0.75	
TNFα^{-1031}	TT	15	51.7	33	51.6	1.00				0.96#
	TC	12	41.4	26	40.6	1.03	0.41	2.60	0.95	
	CC	2	6.9	5	7.8	0.80	0.13	4.82	0.81	
TNFα^{-857}	CC	27	84.4	64	90.1	1.00				–
	CT/TT	5	15.6	7	9.9	1.58	0.42	6.00	0.50	
TNFα^{-308}	GG	23	71.9	61	84.7	1.00				0.67#
	GA/AA	9	28.1	11	15.3	1.50	0.47	4.80	0.49	
TNFα^{-238}	GG	32	100	65	90.3	1.00				–
	GA/AA	0	0.0	7	9.7	–	–	–	–	

OR=crude odds ratio without adjustment by the effect of other genes, p-value=p-value Wald test, *= with statistical significance. # p trend calculated for three levels of genotypes.

cancer risk by 0.3 times (95%CI: 0.08–0.91, $p < 0.03$). However, the trend analysis did not confirm these statistical results. None of the variants in these genes were associated with oral cancer among non-smokers and non-drinkers.

In our series of studies, we have investigated the association between different groups of susceptibility genes and OSCC. These genes belong to those involved with xenobiotic metabolism and DNA repair, and the current study on inflammatory response. When all previously studied genes were included and adjusted in the statistical model (Table 7), the involvement of specific genotypes was identified and for different conditions. For all OSCC cases, the association with the two DNA repair genes, *XRCC1*194 and *XPD*6 was significant ($p < 0.05$, 95% CI:1.02–6.14 and $p < 0.02$, 95% CI: 1.21–6.60, respectively). In addition, genotypes in the IL1α and IL1β genes still had an effect on the model. Although the p values were not significant, the likelihood ratio of the statistical models in the three subgroups changed dramatically when *IL1α*$^{+4845}$ was removed.

For OSCC cases who ever smoked or drank, *IL1β*$^{+3953}$, *XRCC1*194 and *XRCC3*241 were significantly associated with OSCC, $p < 0.01$, 95%CI: 3.28–2658; $p < 0.01$, 95%CI: 1.91–34.7; $p < 0.01$, 95%CI: 3.28–2658, respectively. The variants at SNPs *TNFα*$^{-857}$ and *TNFα*$^{-308}$ appear to be significant in the subgroup of smokers and drinkers though the odds ratios are very low and confidence intervals are very wide.

Among the OSCC cases who had never smoked nor drank, only the *XPD*6 susceptibility gene showed a significant association ($p < 0.01$, 95%CI: 1.58–19.7).

Table 6
Association of genotypes of candidate genes and risk of oral cancer adjusted for other genes.

SNP		Matched-cases		Matched-controls		cOR	aOR	95% CI		p-value
		No.	%	No.	%			lower	upper	
Overall (no & ever smoke and drink)										
IL1α^{+4845}	GG	59	66.3	107	79.3	1.00	1.00			
	GT/TT	30	33.7	28	20.7	2.02	2.00	0.91	4.41	0.08
IL8^{-251}	TT	32	50.8	34	34.3	1.00	1.00			
	AT	21	33.3	49	49.5	0.44	0.92	0.33	2.60	0.88
	AA	10	15.9	16	16.2	0.80	0.49	0.22	1.10	0.08
Ever smoke and drink										
IL1β^{+3953}	CC	46	86.8	48	92.3	1.00	1.00			
	CT	7	13.2	4	7.7	1.90	10.4	1.16	93.2	0.04*
IL8^{-251}	TT	17	47.2	11	26.8	1.00	1.00			
	AT	15	41.7	23	56.1	0.44	0.28	0.08	0.91	0.03*
	AA	4	11.1	7	17.1	0.38	0.25	0.05	1.32	0.10
TNFα^{-1031}	TT	26	49.1	29	55.8	1.00	1.00			
	CT	22	41.5	20	38.4	1.43	3.40	1.01	11.4	0.05*
	CC	5	9.4	3	5.8	1.85	2.03	0.26	15.9	0.50

cOR=crude odds ratio, aOR=adjusted odds ratio, p-value=Wald test

Table 7
Association of genotypes of candidate genes and risk of oral cancer adjusted for other genes.

Genes		Matched-cases		Matched-controls		aOR	95% CI		p-value
		No.	%	No.	%		lower	upper	
Overall (no & ever smoke and drink)									
IL1α^{+4845}	GG	59	66.3	107	79.3	1.00			
	TT/GT	30	33.7	28	20.7	1.80	0.75	4.30	0.18
IL8^{-251}	TT	32	50.8	34	34.3	1.00			
	AT	21	33.3	49	49.5	0.49	0.21	1.18	0.11
	AA	10	15.9	16	16.2	0.98	0.33	2.91	0.98
GST T1	wild	39	37.1	85	54.8	1.00			
	null	66	62.9	70	45.2	2.07	0.94	4.55	0.07*
*XRCC1*194	CC	40	38.1	72	46.7	1.00			
	CT	50	47.6	63	40.9	2.50	1.02	6.14	0.05**
	TT	15	14.3	19	12.3	3.51	0.84	14.7	0.08*
XPD6	CC	45	43.3	77	50.3	1.00			
	CA/AA	59	56.7	76	49.7	2.76	1.21	6.60	0.02**
Ever smoke and drink									
IL1β^{+3953}	CC	46	86.8	48	92.3	1.00			
	CT/TT	7	13.2	4	7.7	93.4	3.28	2658	0.01**
IL1α^{+4845}	GG	34	65.4	42	84.0	1.00			
	GT/TT	18	34.6	8	16.0	6.10	0.66	56.6	0.11
IL8^{-251}	TT	17	47.2	11	26.8	1.00			
	AT	15	41.7	23	56.1	0.05	0.00	0.46	0.01**
	AA	4	11.1	7	17.1	0.01	0.00	0.33	0.01**
TNFα^{-857}	CC	45	83.3	41	77.4	1.00			
	CT/TT	9	16.7	12	22.6	0.003	0.00	1.16	<0.01**
TNFα^{-308}	GG	49	90.7	48	88.9	1.00			
	GA/AA	5	9.3	6	11.1	0.04	0.00	1.38	0.07*
*XRCC1*194	CC	22	36.7	33	60.0	1.00			
	CT/TT	38	63.3	22	30.0	8.15	1.91	34.7	<0.01**
XRCC3241	CC	49	81.7	49	87.5	1.00			
	CT/TT	11	18.3	7	12.5	93.4	3.28	2658	0.01**
Neither smoke nor drink									
IL1α^{+4845}	GG	18	69.2	50	78.1	1.00			
	GT/TT	8	30.8	14	21.9	2.43	0.68	8.60	0.17
GST T1	wild	10	33.3	38	53.5	1.00			
	null	20	66.7	33	46.5	2.89	0.93	8.97	0.06*
XPD6	CC	10	31.2	35	49.3	1.00			
	CA/AA	22	68.8	36	50.7	5.59	1.58	19.7	0.01**

aOR=adjusted odds ratio, *significant at $p < 0.10$, **significant at $p < 0.05$.

Discussion

Our data support previous studies demonstrating the involvement of pro-inflammatory cytokine genes in the development of OSCC although there remain inconsistencies among these studies. For example, our observed, significant association between *IL1β*$^{+3953}$ (Table 6) and OSCC contrasts with the study of Vairaktaris et al (2008). The reverse holds for the *TNFα*$^{-308}$ SNP

(Yapijakis et al., 2009). We observed that the $IL8^{-251}$ polymorphism decreased the risk for OSCC. This finding disagrees with the previous report in the German and Greek population (Vairaktaris et al., 2007). The different results can be caused by the studies using different ethnic groups with different environmental and genetic backgrounds. The association of the variants in the SNPs, $IL1\alpha^{+4845}$ and $IL8^{-251}$, with oral cancer is worth noting especially among those who smoke and drink even if the association is not strong. The lack of trend in association with the genotypes in $IL1\alpha^{+4845}$ and $IL8^{-251}$ in the subgroup analysis even when the genes seem to be significant in the overall analysis can best be explained by the reduction in sample size when the subgroups defined by smoking and drinking behavior are considered.

From our multi-genotype association analyses (Table 7), some meaningful results can be derived from the study. For all OSCC cases, the association was strongest with the apparent susceptibility genotypes in the DNA repair SNPs, *XRCC1*194 and *XPD6*. For cases who had ever smoked or drank, the strongest association was with $IL1\beta^{+3953}$, *XRCC1*194 and *XRCC3*241. For those who had never smoked nor drank, the association of *XPD6* was the strongest.

The involvement of different susceptibility DNA repair genes in OSCC illustrates the critical role of DNA repair in the cancer process. In addition, the data indicate that different repair genes may play a more important role under different environmental conditions, e.g. in smokers and non-smokers for the repair of different types of DNA damage.

The observed interactions between the smoking habits and susceptibility pro-inflammatory genes for OSCC are supportive of other observations. For example, chemicals in cigarette smoke have been reported to affect immune function and affect the response to different types of infections (Gualano et al., 2008; Harel-Meir et al., 2007; Herr et al., 2009). More specifically, smoking can affect the expression of the genes that we have investigated, e.g. *TNFα*, *IL1β* and *IL8* (Chen et al., 2007; Mian et al., 2009).

In conclusion, certain SNPs in genes that are involved in inflammation and immune response, and that were investigated in this study appear to be associated with the development of OSCC. Subtle differences among individuals in their ability to metabolize xenobiotics, repair damaged DNA, and properly manage inflammatory responses due to common genetic differences all appear to play a role in the development of OSCC. In conjunction with previous studies, these latest findings illustrate the critical interactions between genes and the environment that lead to the development of cancer, such as OSCC. In addition, our findings should lead to more focused investigations into the different mechanisms influencing susceptibility to OSCC under different environmental conditions.

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Polymorphism in DNA repair genes and oral squamous cell carcinoma in Thailand

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Abstract

DNA repair capacity is essential in maintaining cellular functions and homeostasis. However, the repair capacity can be altered based on DNA sequence variations in DNA repair genes and thus may cause cancer susceptibility. We investigated associations between polymorphisms in DNA repair genes and oral squamous cell carcinoma (OSCC) in a Thai population. Nine known single nucleotide polymorphisms (SNPs) in five common DNA repair genes were investigated: *XRCC1* (Arg194Trp and Arg399Gln); *XRCC3* (Thr241Met); *XPC* (PAT and Lys939Gln); *XPD* (exon 6, and Lys751Gln); and *MGMT* (Trp65Cys and Leu84Phe). We studied 106 cases and 164 healthy controls that were frequency-matched by age (±5 years), gender, and cigarette smoking and alcohol drinking habits. The genotype assays were performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The R version 2.0.1 statistical software was applied for statistical analysis of association. Based on multivariate analyses, we found that the variant genotypes with *XRCC3* 241Met exhibited a >3-fold elevated risk (OR = 3.3, 95% CI = 1.31–8.36, $p = 0.01$) for OSCC. There was a marginally significant risk observed in variants with *XRCC1* 194Trp (OR = 1.81, 95% CI = 0.91–3.63, $p = 0.09$) and *XPD* exon 6 (OR = 1.71, 95% CI = 0.93–3.16, $p = 0.09$). Combination of the variant genotypes of these three susceptibility genes was associated with a highly significant risk for OSCC (OR = 9.43, 95% CI = 1.98–44.9, $p < 0.01$). From further multivariate analyses, the variants with *XRCC1* 194Trp and possibly *XRCC3* 241Met interacted with tobacco and alcohol to further increase the risk (OR = 3.37 95% CI = 1.41–8.02, $p < 0.01$; OR = 2.92, 95% CI = 0.94–9.04, $p = 0.06$). On the other hand, increased risk was detected in non-betel chewers (OR = 2.88, 95% CI = 1.31–6.31, $p < 0.01$; OR = 2.61, 95% CI = 0.97–7.11, $p = 0.06$) who carry the two variant genotypes, respectively. Males with the variants *XRCC1* 194Trp or *XRCC3* 241Met had a higher risk of developing OSCC than males with the corresponding wild-type genotypes (OR = 2.72, 95% CI = 1.34–5.52, $p < 0.01$; OR = 2.95, 95% CI = 1.12–7.75, $p < 0.05$). Such association was not detected in females. Interestingly, the risk increased in female carriers of *XPD* exon 6 (OR = 3.93, 95% CI = 1.14 – 13.6, $p < 0.05$). We could not demonstrate a significant interaction of these SNPs with age in this study. Our data indicate that the variant genotypes with *XRCC3* 241Met and possibly *XRCC1* 194Trp and *XPD* exon 6 contribute to OSCC

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development in a Thai population. In addition, these SNPs influence the repair of DNA damage that is caused by environmental risk factors for oral cancer.

Keywords: DNA repair; Gene polymorphisms; Oral squamous cell carcinoma; Oral cancer; Head and neck cancer; Genetic susceptibility; Smoking

Introduction

On a daily basis, various DNA repair mechanisms function continuously to correct damaged DNA that is caused by exposure to either endogenous or exogenous toxic substances. Recent reviews indicate that there are at least 130 functional DNA repair genes in humans, which are grouped into five major DNA repair pathways (Wood et al., 2001; Yu et al., 1999). (1) Direct repair pathway such as O^6-methylguanine DNA methyltransferase (*MGMT*). (2) Base-excision repair (BER) pathway including X-ray repair cross-complementing group 1 (*XRCC1*), apurinic/apyrimidinic endonucleases (*APE*), DNA glycosylases, DNA polymerase-β and DNA ligases (I-IV). (3) Nucleotide-excision repair (NER) pathway including many genes, such as xeroderma pigmentosum complementing groups: *XPA–XPG*. (4) Double-strand break (DSB) repair pathway such as *XRCC3*, *BRCA1*, *BRCA2* and *LIG4*. (5) Mismatched repair (MMR) pathway including 6 genes in human: h*MSH2*, h*MSH3*, h*MSH6*, h*MLH1*, h*PMS1*, and h*PMS2*. Recently, there has been considerable interest in understanding genetic variability in DNA repair genes and their influence on modifying an individual's susceptibility to cancer. This topic has been reviewed (e.g. Spitz et al., 2003). Polymorphisms in *XRCC1*, *XRCC3*, *XPC*, *XPD*, and *MGMT* have been identified and reported to be associated with cancers of lung, head and neck, upper aerodigestive tract, urinary bladder, nasopharynx, etc. (Benhamou et al., 2004; Cho et al., 2003; Olshan et al., 2002; Shen et al., 2001, 2002, 2003; Spitz et al., 2003; Sturgis et al., 1999, 2000, 2002; Tae et al., 2004). Unfortunately, many of these observations have not been consistent (Goode et al., 2002; Au et al., 2004). The discrepancy can be due to the small sample size and the difference in ethnicity of populations investigated. More molecular epidemiological studies need to be conducted, using more vigorous study protocols, to provide a better understanding of genetic susceptibility to environmental related cancers, such as oral cancer. A topic that has received insufficient attention is genetic susceptibility to oral cancer.

Genetic susceptibility publications on oral squamous cell carcinoma (OSCC) have frequently been included in either that of head and neck cancer or cancer of the upper aerodigestive tract (including oropharynx and larynx) (Benhamou et al., 2004; Olshan et al., 2002; Shen et al., 2001, 2002; Spitz et al., 2003; Sturgis et al., 1999, 2000, 2002; Tae et al., 2004). Although most of these investigations have studied Caucasian populations, two were for Asian populations (Hsieh et al., 2003; Tae et al., 2004). It is possible that such investigations using broadly defined cancer sites may contribute to the discrepant observations because of the potential differences in etiology. Therefore, focusing studies on more homogeneous cancer sites may reduce variations.

In Thailand, the estimated incidence rate of cancer of the oral cavity (ICD-10 codes; C00-C06), including lip, buccal mucosa, gum, tongue, floor of mouth, and palate is 6.8 and 4.8 per 100,000 population in males and in females, respectively, becoming the 4th and 7th leading cancers for the two genders (Fritz et al., 2000; Sriplung, 2003). The major risk factors are tobacco, alcohol and betel consumption habits (Kerdpon et al., 2001). Information on genetic susceptibility to OSCC in Thailand is limited although we have reported previously that the *GSTM1* null genotype increased OSCC risk in this ethnic group (Kietthubthew et al., 2001). Here, we report our investigation on associations of nine SNPs in five DNA repair genes and OSCC risk in a Thai population.

Materials and methods

Study population

The population included in this case-control study was ethnically Thai. A total of 112 cases and 192 controls were recruited. The recruitment criteria were described previously (Kietthubthew et al., 2001). They were matched as described below (see Statistical analysis). In brief, patients with histologically confirmed squamous cell carcinoma in the oral cavity (tongue, buccal, palate, floor of mouth, and lip) were sequentially recruited from the Department of Radiology, Songkhlanagarind Hospital, Hatyai, Songkhla, Thailand, before they had chemo- and/or radiotherapy. The controls were recruited simultaneously from residents living in the similar geographic area (Songkhla province and its vicinity). All individuals voluntarily participated in the study after they had provided informed consent. Each participant was personally interviewed with a questionnaire that had

been approved by the university ethics committee. The information collected and used in this study was related to past history of individual's life style habits (tobacco smoking, use of smokeless tobacco, betel chewing, alcohol drinking), other possible risk factors (occupational exposure to toxic substances, nutrition, oral infection), as well as personal and family history of various cancers.

Genotyping assay

We assayed nine polymorphisms in five DNA repair genes: *XRCC1* (Arg194Trp and Arg399Gln); *XRCC3* (Thr241Met); *XPC* (PAT and Lys939Gln); *XPD* (exon 6, and Lys751Gln); and *MGMT* (Trp65Cys and Leu84Phe). These genes are involved in four of the five major DNA repair pathways as described in the Introduction. In addition, some of the variant genotypes were selected because they have a functional repair deficiency (Au et al., 2004): *XRCC1* 399Gln and *XRCC3* 241Met are defective in BER whereas *XRCC1* 194Trp is proficient in BER and *XPD* 751Gln is deficient in NER. *XPD* exon 6 was selected because the variant genotypes (heterozygous and homozygous) had lower DNA repair capacity than the wild type although the polymorphism does not result in an amino acid change at codon 156 (Wei et al., 1993). The *XPC* PAT polymorphism has been reported to increase risk for head and neck squamous cell carcinoma (HNSCC) although the exact biological effect of the SNPs is still unclear (Shen et al., 2001). *XPC* exon 15 was selected because it is in linkage

Table 1. Summary on PCR-RFLP assay of polymorphisms in DNA repair genes

Genes	Primer sequence (5′–3′)	Annealing PCR		RE, condition	Fragments size (bp)	References
		Temp (°C)	Product (bp)			
XRCC1						
Arg194Trp	Upper: gccagggccccctcctccaa Lower: taccctcagacccacgagt	63	485	*Pvu* II, 1 unit, 37 °C, 3 h	CC: 485 CT: 485, 396, 89 TT: 396, 89	Sturgis et al. (1999)
Arg399Gln	Upper: caagtacagccaggtcctag Lower: ccttcctcatctggagtac	55	248	*Nci* I, 10 unit, 37 °C, 3 h	GG: 159, 89 GA: 248, 159, 89 AA: 248	Au et al. (2003)
XRCC3						
Thr241Met	Upper: ggtcgagtgacagtccaaac Lower: tgcaacggctgagggtctt	58	455	*Nla* III, 1 unit, 37 °C, 3 h	CC: 315, 140 CT: 315, 210, 140, 105 TT: 210, 140, 105	Smith et al. (2003)
XPC						
PAT, intron9	Upper: tagcacccagcagtcaaag Lower: tgtgaatgtgcttaatgctg	66	266, 344	ND	−/−: 266 −/+: 344, 266 +/+: 344	Shen et al. (2001)
exon 15	Upper: ggaggtggactctcttctgatg Lower: tagatcccagcagatgacc	55	765	*Pvu*II, 5 units, 37 °C, 3 h	AA: 765 AC: 765, 585, 180 CC: 585, 180	
XPD (ERCC2)						
exon 6	Upper: tggagtgctatggcacgatctct Lower: ccatgggcatcaaattcctggga	63	644	*Tfi* I, 0.5 unit, 65°C, 1 h	CC: 587, 57 CA: 587, 474, 113, 57 AA: 474, 113, 57	Sturgis et al. (2000)
Lys751Gln	Upper: tcaaacatcctgtccctact Lower: ctgcgattaaaggctgtgga	54	344	*Pst* I, 10 unit, 37 °C, 3 h	AA: 234, 110 AC: 234, 171, 110, 63 CC: 171, 110, 63	
MGMT						
Trp65Cys	Upper: ctaagccccctgttctcactttt Lower: acatccgcagatggcttagttac	56	201	*Mva* I, 1 unit, 37 °C, 3 h	GG: 110, 91 GC: 201, 110, 91 CC: 201	Abe et al. (1997)
Leu84Phe	Upper: ctaagccccctgttctcactttt Lower: acatccgcagatggcttagttac	56	201	*Ear* I, 1 unit, 37 °C, 3 h	CC: 128, 53, 20 CT: 128, 73, 53, 20 TT: 128, 73	

PCR – polymerase chain reaction; RE – restriction enzymes; bp – base pair; ND – not done; *XRCC1* – X-ray complementing group 1; *XRCC3* – X-ray complementing group 3; *XPD* – Xeroderma pigmentosum complementing group D; MGMT – O^6–methylguanine-DNA methyl transferase.

disequilibrium with *XPC* PAT (Khan et al., 2000). There is little information on the association of *MGMT* polymorphisms and cancer risk. However, there is a report indicating that *MGMT* protein expression was lost in HNSCC (Zuo et al., 2004).

Blood collection and DNA sample preparation procedures were described in our previous report (Kietthubthew et al., 2001). Briefly, archived DNA from selected subjects was genotyped using the PCR-RFLP method. The PCR primers used in our assays were adopted from published reports (Table 1). The primers were designed to amplify the regions of DNA that contain the polymorphic sites of interest for *XRCC1* Arg399Gln in exon 10 (G→A), and Arg194Trp in exon 6 (C→T); *XRCC3* Thr241Met in exon 7 (C→T); *XPC* in exon 15 (A→C) and PAT, a poly [AT] insertion /deletion in intron 9; *XPD* in exon 6 (C→A), and Lys751Gln in exon 23 (A→C); and *MGMT* Trp65Cys (G→C) and Leu84Phe (C→T). The PCR conditions consisted basically of the following: (i) activation of Taq polymerase at 95 °C for 9 min, (ii) 35 cycles of denaturation at 95 °C for 20 s; annealing for 20 s at appropriate temperatures (Table 1); elongation at 72 °C for 20 s, (iii) extension at 72 °C for 5 min. The amplified products were examined on 2% agarose gels. The PCR products were subsequently digested by appropriate restriction enzymes (New England BioLabs, USA). The restriction enzymes used and the conditions for digestion are listed in Table 1. All the RFLP fragments, except for the *MGMT* Leu84Phe, were determined on 2.5–3% agarose gels. The *MGMT* Leu84Phe was examined using 10% polyacrylamide gel electrophoresis (PAGE). Fragment sizes of the polymorphisms are also shown in Table 1.

Statistical analysis

Statistical analyses were performed using the R version 2.0.1 (http://cran.r-project.org/), an open-source statistical software. Since controls were loosely selected from the general population based on the frequency of sex, age and smoking and drinking habits of cases, the following procedure was used for matching. In the analysis phase, all subjects were pooled and then stratified into small groups by the combination of the following four factors: sex, 5-year age groups, smoking and drinking habits. Strata containing only cases or controls were dropped. Thus, cases and controls were matched within the same subgroups. By this method, 106 from a total of 112 cases and 164 from a total of 192 controls were frequency matched. As the study was a matched design, conditional logistic regression was used to find association between genetic factors and oral cancer. Since betel chewing behavior was not matched at the designed phase, this factor was treated as a confounding factor in all analyses. In the first stage, all selected cases were used and only one gene polymorphism was analyzed to find an association at the level of random error of 10%. Genes that showed an association to oral cancer were put into a multivariate model and the p-value was then set at 0.05. However, Akaike information criterion (AIC) was also used to determine whether a factor should be included in or excluded from the final model. Then subjects were subdivided into subgroups based on smoking and drinking habit, betel chewing habit, sex, and age groups. The analysis was done again for these subgroups to identify genes playing roles in different subgroups of the population.

Tests for Hardy–Weinberg equilibrium (HWE) among cases and controls were calculated on observed and expected genotype frequencies using Pearson's χ^2-square test with one degree of freedom.

Results

The characteristics of the patients and controls are shown in Table 2. There were 106 cases and 164 frequency-matched healthy controls. The mean ages of the patients and the controls were 67.1 (range 35–87 years) and 68.4 (range 35–88 years) years, respectively. The distribution of the genotypes of DNA repair genes and their risk for OSCC are shown in Table 3. The risk was calculated based on crude OR analyses that were adjusted for betel quid chewing. The homozygous variant genotype of *XRCC1* 399Gln reduced the OSCC risk (OR = 0.30, 95%

Table 2. Characteristics of cases and controls

		Matched subjects*	
		Cases 106 (%)	Control 164 (%)
Sex	Male	77 (72.6)	91 (55.5)
	Female	29 (27.4)	73 (44.5)
Age	<66	42 (39.6)	52 (31.7)
	66–75	41 (38.7)	67 (40.9)
	>75	23 (21.7)	45 (27.4)
Smoking	No	34 (32.1)	79 (48.2)
	Yes	72 (69.7)	85 (51.8)
Drinking	No	45 (42.5)	105 (64.0)
	Yes	61 (57.5)	59 (36.0)
Betel chewing	No	56 (52.8)	114 (69.5)
	Yes	50 (57.2)	50 (30.5)
Religion	Buddhism	96 (90.6)	159 (97.0)
	Islam & Christian	10 (9.4)	5 (3.0)

% – percentages of the study population.
*p-value > 0.05.

Table 3. Distribution of DNA repair gene polymorphisms and OSCC risk

	Matched-cases		Matched-controls		Crude OR[a] (95% CI)	*p*-value
	No.	%	No.	%		
XRCC1 Arg194Trp						
CC	40	37.7	77	47.0	1.00	
CT	50	47.2	67	40.9	2.26 (1.20–4.28)	0.01
TT	16	15.1	20	12.2	1.97 (0.86–4.51)	0.11
XRCC1 Arg399Gln						
GG	55	51.9	67	40.9	1.00	
GA	45	42.5	74	45.1	0.64 (0.35–1.16)	0.14
AA	6	5.7	23	14.0	0.30 (0.10–0.88)	0.03
XRCC3 Thr241Met						
CC	83	78.3	140	85.4	1.00	
CT	22	20.8	23	14.0	2.31 (1.09–4.91)	0.03
TT	1	0.9	1	0.6	0.66 (0.04–10.92)	0.77
XPC-PAT						
−/−	60	56.6	89	54.3	1.00	
+/−	36	34.0	66	40.2	0.83 (0.46–1.48)	0.52
+/+	10	9.4	9	5.5	1.60 (0.55–4.66)	0.39
XPC exon 15						
AA	59	55.7	87	53.0	1.00	
AC	37	34.9	67	40.9	0.87 (0.48–1.55)	0.63
CC	10	9.4	10	6.1	1.35 (0.50–3.92)	0.53
XPD exon 6						
CC	45	42.5	82	50.0	1.00	
CA	52	49.1	62	37.8	1.74 (0.94–3.22)	0.08
AA	9	8.5	20	12.2	0.85 (0.30–2.37)	0.75
XPD Lys751Gln						
AA	83	79.0	126	76.8	1.00	
AC	21	20.0	36	22.0	0.69 (0.35–1.39)	0.31
CC	1	1.0	2		2.04 (0.19–21.66)	0.55
MGMT Trp65Cys						
GG	106	100.0	164	100.0	—	—
GC	0	0.0	0	0.0	—	—
CC	0	0.0	0	0.0	—	—
MGMT Leu84Phe						
CC	84	79.2	130	79.3	1.00	
CT	21	19.8	33	20.1	1.11 (0.54–2.26)	0.78
TT	1	0.9	1	0.6	0.37 (0.01–15.73)	0.60

[a]Crude OR = crude odds ratio conditional on matched set adjusted for betel quid chewing.

CI = 0.10−0.88, $p = 0.03$). The heterozygous genotypes for *XRCC1* 194Trp and *XRCC3* 241Met significantly increased the risk (OR = 2.26, 95% CI = 1.20−4.28, $p = 0.01$; OR = 2.31, 95% CI = 1.09−4.91, $p = 0.03$, respectively). There was a marginally higher risk for the heterozygous *XPD* exon 6 (OR = 1.74, 95% CI = 0.94−3.22, $p = 0.08$). However, there was no apparent association for the other genes investigated. In our population, the *MGMT* did not exhibit polymorphisms at the codon 65 (Trp/Cys). The three at risk genotypes as detected in the univariate analysis were further explored by using multivariate analysis and adjusted for an effect of other genes studied. The data are shown in Table 4. Only the *XRCC3* 241Met exhibited a significantly higher risk (OR = 3.3, 95% CI = 1.31−8.36, $p = 0.01$). The *XRCC1* 194Trp and the *XPD* exon 6 caused a marginally significant increase in risk (OR = 1.81, 95% CI = 0.91−3.63, $p = 0.09$; OR = 1.71, 95% CI = 0.93−3.16, $p = 0.09$, respectively). When variant susceptibility genotypes were

Table 4. Association of *XRCC1*, *XRCC3*, *XPD* and OSCC risk

	Matched-cases		Matched-controls		Adjusted OR[a] (95% CI)	*p*-value
	No. (106)	%	No. (164)	%		
XRCC1 Arg194Trp						
CC	40	37.7	77	47.0	1.00	
CT/TT	66	62.3	87	53.0	1.81 (0.91–3.63)	0.09
XRCC3 Thr241Met						
CC	83	78.3	140	85.4	1.00	
CT/TT	23	21.7	24	14.6	3.3 (1.31–8.36)	0.01
XPD exon 6						
CC	45	42.5	82	50.0	1.00	
CA/AA	61	57.5	82	50.0	1.71 (0.93–3.16)	0.09

[a]Adjusted OR – odds ratio conditional on matched set and adjusted for other genes and betel quid chewing.

Table 5. Association of *XRCC1* 194Trp, *XRCC3* 241Met, *XPD* exon 6 polymorphisms and OSCC risk, interactions of three genes

Genes			Adjusted OR[a] (95% CI)	*p*-value
Genotypes				
XRCC1 194Trp	*XRCC3* 241Met	*XPD* exon 6		
CC	CC	CC	1.00	
CC	CC	CA/AA	1.24 (0.47–3.31)	0.67
CC	CT/TT	CC	3.33 (0.64–17.2)	0.15
CC	CT/TT	CA/AA	1.23 (0.22–6.87)	0.82
CT/TT	CC	CC	1.42 (0.55–3.69)	0.47
CT/TT	CC	CA/AA	3.83 (1.42–10.3)	<0.01
CT/TT	CT/TT	CC	3.68 (0.88–15.5)	0.08
CT/TT	CT/TT	CA/AA	9.43 (1.98–44.9)	<0.01

[a]Adjusted OR – odds ratio conditional on matched set and adjusted for other genes and betel quid chewing.

combined in pairs or in triplets, they increased the risk for OSCC further compared with the effect of each single gene (Table 5). The triplet was associated with the maximum and significant increase in risk (OR = 9.43, 95% CI = 1.98−44.9, $p<0.01$).

Analyses were conducted to investigate the interactions between genotypes and life-style factors. As shown in Table 6, only the variants *XRCC1* 194Trp interacted with the smoking and drinking habits to significantly elevate the risk (OR = 3.37, 95% CI = 1.41−8.02, $p<0.01$), and positive interactions were detected in individuals with the non-betel chewing habit (OR = 2.88, 95% CI = 1.31−6.31, $p<0.01$). A similar but marginally significant effect was found with *XRCC3* 241Met (OR = 2.92, 95% CI = 0.94−9.04, $p = 0.06$ for smoking and drinking; OR = 2.61, 95% CI = 0.97−7.11, $p = 0.06$ for non-betel chewing). The *XPD* exon 6 variants exhibited a significantly elevated risk in non-smokers and non-drinkers (OR = 4.10, 95% CI = 1.20−14.0, $p = 0.03$). As shown in Table 7, males with variants *XRCC1* 194Trp and *XRCC3* 241Met had significantly higher risk (OR = 2.72, 95% CI = 1.34−5.52, $p<0.01$; OR = 2.95, 95% CI = 1.12−7.75, $p<0.05$, respectively). The effect was, however, not detected in females. On the other hand, the variant *XPD* exon 6 exerted a higher risk in females (OR = 3.93, 95% CI = 1.14−13.6, $p<0.05$). The distributions of the variant genotypes were in the HWE for both the patients and the controls (data not shown).

Discussion

From our investigation in a Thai population, we found that OSCC risk was increased with inheritance of three of the nine studied variant genotypes: *XRCC1* 194Trp, *XRCC3* 241Met and *XPD* exon 6. The risk increased further in individuals with *XRCC1* 194Trp and *XRCC3* 241Met who had the smoking and drinking but not the betel chewing habits. In carriers with *XPD* exon 6 variants the increased risk was prominent among

Table 6. Association of life style habits, *XRCC1* 194Trp, *XRCC3* 241Met, *XPD* exon 6 polymorphisms and OSCC risk

Life style habits / Genes	Genotypes	Adjusted OR[a] (95% CI)	*p*-value
No smoking & drinking			
XRCC1 194Trp	CT/TT	1.30 (0.46–3.70)	0.62
XRCC3 241Met	CT/TT	1.47 (0.43–5.00)	0.54
XPD exon 6	CA/AA	4.10 (1.20–14.0)	0.03
Smoking & drinking			
XRCC1 194Trp	CT/TT	3.37 (1.41–8.02)	<0.01
XRCC3 241Met	CT/TT	2.92 (0.94–9.04)	0.06
XPD exon 6	CA/AA	1.48 (0.64–3.44)	0.36
No betel chewing			
XRCC1 194Trp	CT/TT	2.88 (1.31–6.31)	<0.01
XRCC3 241Met	CT/TT	2.61 (0.97–7.11)	0.06
XPD exon 6	CA/AA	1.50 (0.68–3.30)	0.31
Betel chewing			
XRCC1 194Trp	CT/TT	1.20 (0.42–3.44)	0.74
XRCC3 241Met	CT/TT	2.85 (0.72–11.3)	0.14
XPD exon 6	CA/AA	2.20 (0.81–5.99)	0.12

[a]Adjusted OR – OR adjusted for other genes.

Table 7. DNA repair gene polymorphisms and OSCC, stratified by sex

Genes	Adjusted OR[a] (95% CI)	
	Sex	
Genotypes	Male	Female
XRCC1 Arg194Trp CT/TT	2.72 (1.34–5.52)[a]	1.10 (0.37–3.28)
XRCC1 Arg399Gln GA/AA	0.59 (0.30–1.16)	0.49 (0.17–1.42)
XRCC3 Thr241Met CT/TT	2.95 (1.12–7.75)[b]	1.37 (0.41–4.61)
XPC-PAT +/–/+/+	0.81 (0.41–1.58)	1.14 (0.42–3.10)
XPC exon 15 AC/CC	0.88 (0.45–1.71)	1.05 (0.38–2.88)
XPD exon 6 CA/AA	1.12 (0.56–2.22)	3.93 (1.14–13.6)[b]
XPD Lys751Gln AC/CC	0.82 (0.36–1.85)	0.58 (0.17–2.05)
MGMT Leu84Phe CT/TT	1.22 (0.55–2.74)	0.78 (0.19–3.25)

[a]Adjusted OR- OR adjusted for other genes.
[a]*p*-value <0.01.
[b]*p*-value <0.05.

non-smokers. This study provides support of the concept that DNA repair gene polymorphisms play important roles in modifying individual susceptibility to environmental cancers (Benhamou et al., 2004; Cho et al., 2003; Goode et al., 2002; Olshan et al., 2002; Shen et al., 2001, 2002, 2003; Spitz et al., 2003; Sturgis et al., 1999, 2000, 2002; Tae et al., 2004; Au and Salama, 2005). To our knowledge, there was only one report of a molecular epidemiological-based study in an Asian population on association between DNA repair gene polymorphisms and HNSCC (Tae et al., 2004). They reported that *XRCC1* 194Trp but not *XRCC1* 399Gln affected the risk for HNSCC in a Korean population (OR = 2.6, 95% CI = 1.53–4.46).

Our observation of genetic susceptibility to OSCC has good support from other studies. For example, *XRCC3* 241Met has been reported to be functionally deficient in the repair of X-ray but not UV-light induced chromosome aberrations, indicating that the variant genotype is defective in BER, but not NER (Au et al., 2003). Among the nine polymorphisms investigated, this variant genotype is associated with the most significant risk for OSCC (Table 4). In our study, the risk was mainly found in males (Table 7) and among those with the smoking and drinking habits (Table 6) although Shen et al. (2002) reported significant risk among females in a broader group of cancer patients (HNSCC). In another broad category of cancers, upper aerodigestive tract cancer, Benhamou et al. (2004) did not observe any significant risk with the *XRCC3* 241Met variant.

We found that the OSCC risk from the homozygous and heterozygous *XRCC1* 194Trp variant genotypes was marginally significant ($p = 0.09$). However, the risk was significantly increased in males and among smokers and drinkers (Tables 6 and 7). In addition, the risk with *XRCC1* 194Trp was enhanced in combination with the other two susceptibility genotypes, *XPD* exon 6 or *XRCC3* 241Met compared with the effect of single genes. Combination of the three susceptibility genotypes elevated the risk significantly (Table 5). The data indicate that there were synergistic interactions among the susceptibility genotypes. Our findings have support from a functional study (Au et al., 2003), in which *XRCC1* 194Trp was demonstrated to have a reduced repair capacity for X-ray- or UV light-induced chromosome aberrations. In HNSCC patients, the variant genotype was associated with reduced risk among Caucasians (Sturgis et al., 1999) but increased risk for Koreans (Tae et al., 2004).

With respect to the *XPD* exon 6 polymorphism, we observed that the variant genotype was associated with borderline significant increase in risk (Table 4), especially among the non-smokers and females (Tables 6 and 7). From previous reports, no associated risk with the variant genotype was found for HNSCC (Sturgis et al., 2000) and a borderline risk for basal cell carcinoma (Vogel et al., 2001). The variant genotype was reported to have lower DNA repair capacity than the wild type

(Wei et al., 1993) although the SNP does not result in an amino acid change at the codon 156.

In our study, the variant *XRCC1* 399Gln was associated with reduced risk for OSCC although the genotype has been reported to be associated with reduced BER but proficient NER activities (Au et al., 2003). Similarly, a marked reduction in risk was reported for HNSCC among a US population (OR = 0.01, 95% CI = 0.0004–0.3, Olshan et al., 2002) and among Koreans (Tae et al., 2004).

Although genetic susceptibility analyses are expected to provide novel and valuable information regarding the induction and development of cancer, many of these studies have not produced reproducible results (Au et al., 2004; Au and Salama, 2005). The major reason is that many parameters affect the outcome of such investigations, e.g. specificity of the tumor sites, sample size, ethnic differences in genotype distribution and life style habits, and interactions with other susceptibility genes. The distributions of DNA repair genes are remarkably different in different ethnic populations for some SNPs. For example, the distribution of the *XRCC1* 194Trp variant genotypes (Arg/Trp and Trp/Trp) were 40.9% and 12.2%, respectively, in our Thai population (Table 3); 26.9% and 3.5%, respectively in a Korean population (Tae et al., 2004); 6.2% and 0% (Olshan et al., 2002) and 14.4% and 0% (Sturgis et al., 1999) in US non-Hispanic white populations. For *XRCC3* Thr241Met, the distribution for the variant Thr/Met and Met/Met genotypes were 14.0% and 0.6%, respectively, in our population (Table 3), 53.6% and 18.1% (Benhamou et al., 2004); 48.0% and 12.2% (Shen et al., 2002); and 54% and 13% (Shen et al., 2003) in US Caucasian populations.

Besides DNA repair genes, a variety of other polymorphic genes may contribute to susceptibility to OSCC (Au and Salama, 2005). We have previously reported that the *GSTM1* null metabolizing gene is associated with risk for OSCC (Kietthubthew et al., 2001). The thymidylate synthase 5′- and 3′-untranslated region polymorphism which is involved in folate metabolism is associated with risk for HNSCC (Zhang et al., 2004). The heme oxygenase-1 promoter polymorphism is related to risk for OSCC among males who chewed areca (Chang et al., 2004). Consequently, unexpected results can be generated and these may not be indicative of wrong observations. In our study, we observed gender effects in susceptibility. In addition, the variant *XRCC1* 399Gln, which was associated with defective BER, was associated with reduced risk for OSCC and for HNSCC in other studies. A possibility is that the susceptibility genotype is in linkage disequilibrium with other genotypes that compensate the repair deficiency. Therefore, comprehensive investigations involving major contributing factors are needed to be conducted to have a better understanding of the susceptibility phenomenon to OSCC.

The present study shows that three polymorphisms in DNA repair genes are involved in OSCC susceptibility in a Thai population: *XRCC1* 194Trp, *XRCC3* 241Met and *XPD* exon 6. The interesting point is that enzymes from each of the three genes are known to participate in different DNA repair pathways. This observation is consistent with the induction of multiple DNA lesions by cigarette smoke and by other environmental agents that require different pathways for repair. Therefore, our study represents an important addition to previously published work on polymorphism of DNA repair genes and susceptibility of cancer. Further studies with a larger number of subjects and simultaneous measurement of different polymorphic genes are needed to provide a better understanding of the susceptibility phenomenon (Au and Salama, 2005). In the meantime, we are evaluating the interactions between susceptibility chemical metabolizing and DNA repair genes for risk in OSCC.

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RESEARCH COMMUNICATION

The *p53* Codon 72 Polymorphism and Risk of Oral Cancer in Southern Thailand

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Abstract

The codon 72 polymorphism of the *p53* tumor suppressor gene has been investigated extensively for its association with various cancers around the world. However, its influence has not been elucidated in the Thai population. Therefore, a case-control study with 97 patients and 97 matched controls was conducted to elucidate the association between the polymorphic p53 and oral cancer risk in a Southern Thai population. The frequencies of the Arg/Arg, Arg/Pro, and Pro/Pro genotypes were 36%, 35%, and 29%, respectively in the controls and 33%, 45% and 22%, respectively in the patients. This study shows that there was no significant association between the p53 codon 72 polymorphism and oral cancer risk. There was also no link with respect to smoking or drinking habits. However, our data suggest that for individuals who were younger than 65 years old, the Pro/Pro genotype may offer some protection against oral cancer (OR = 0.13, 95%CI 0.04-1.10). This is the first report on p53 polymorphism and oral cancer in Thailand.

Key Words: p53 codon 72 polymorphism - oral cancer - tobacco smoking - alcohol consumption - betel chewing - genetic susceptibility

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Introduction

Oral cancer, mainly oral squamous cell carcinoma (OSCC), is one of the ten most common cancers in the world (Scully and Bedi, 2000) and the disease is predicted to increase in prevalence during the next several decades (Sciubba, 2001). In Thailand, the incidence for the disease is the highest in the southern part of the country where it is ranked the second in males and the sixth in females among all cancers (Srivatanakul et al., 1999). While the major risk factors for oral cancer are cigarette smoking and alcohol consumption in Western countries, the risk factors in Southern Thailand are alcohol drinking, tobacco smoking, betel quid chewing and the use of smokeless tobacco (Kerdpon et al., 2001). The latter practices are similar to those reported in other Asian countries (Ko et al., 1995; Balaram et al., 2002). Similar to many other malignancies, oral cancer is caused by the interactions between genetic and certain epigenic or environmental factors. The genetic component may influence an individual's susceptibility to cancer. This component includes polymorphic genes that modulate chemical metabolism, DNA repair, and cell cycle control (Sreelekha et al., 2001; Topcu et al., 2002). In our investigation of genetic susceptibility to oral cancer in Southern Thailand, we have reported that inheritance of the *GSTM1* null allele conferred an increased risk for oral cancer. The increased risk is particularly obvious among those who have had life-style risk habits such as alcohol drinking, tobacco smoking and betel chewing (Kietthubthew et al., 2001).

The *p53* tumor suppressor gene contributes to the maintenance of genomic stability by controlling cell cycle and facilitating DNA repair in response to DNA damage (Hollstein et al., 1991). It is a gatekeeper or guardian gene

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of cell division (Levine, 1997). Therefore, alterations in p53 function are critical events in carcinogenesis. In fact, mutations of p53 are frequently found in a variety of human cancers including oral cancer (Partridge et al., 2000; Oswald et al., 2000; Hsieh et al., 2001).

The *p53* gene was found to be polymorphic with a single nucleotide polymorphism in the exon 4 in codon 72 (Matlashewski et al., 1987). The substitution of G to C changes the amino acid from arginine to proline. It gives rise to three genotypes, Arg/Arg, Arg/Pro and Pro/Pro. More recently, the p53Arg and p53Pro proteins are reported to be biologically and biochemically different from each other (Thomas et al., 1999; Martin et al., 2000). For example, Storey et al demonstrated that p53Arg was more readily degraded by the E6 oncoprotein of the high-risk human papilloma virus strains (HPV16 and 18) than the p53Pro and the Arg/Arg genotype increased the risk of HPV-related cervical cancer (Storey et al., 1998). Several other studies in the association of the *p53* polymorphism on cancer susceptibility were reported recently, however, the results have not been consistent (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). So far, there were only few reports on the association of the *p53* codon 72 polymorphism and oral cancer (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Nagpal et al., 2002; Shen et al., 2002). Summersgill et al (2000) reported that there was no association between the *p53* codon 72 polymorphism and the risk for oral cancer among Caucasians. Nagpal et al (2002) observed that the Arg/Arg genotype conferred susceptibility to HPV infection and oral carcinogenesis among an Eastern Indian population. Shen et al (2002) reported that although the *p53* polymorphism was not associated with head and neck cancer among a group of non-Hispanic white population; the Pro allele was associated with an early age of onset of the cancers, particularly oral cancer. The controversy could be due to differences in ethnic composition of the studied populations and to their associated risk factors as mentioned earlier. Since such an investigation has not been reported in Thailand, we have studied the *p53* codon 72 polymorphism and oral cancer with adjustment based on the presence of other significant risk factors.

Materials and Methods

Recruitment of study participants

In this case - control study, the criteria for recruitment of patients and controls were the same as those used in our previous study (Kietthubthew et al., 2001). The two groups were one-to-one matched by sex and by age ($\pm$ 5 years), smoking status and drinking status. The study subjects included 97cases and 97 controls. Briefly, patients with cancer in the oral cavity, histologically squamous cell carcinoma, were sequentially recruited from August 1998 to May 2001 from the Department of Radiology, Songkhlanagarind Hospital, before they had chemo- and/or radio-therapy. The controls were recruited from residents living in the similar geographic area (Songkhla Province and its vicinity). All individuals voluntarily participated in the study after they had provided informed consent. Each participant was personally interviewed with a questionnaire that had been approved by the university ethic committee. The information collected and used in this study were related to past history of individual's demographic background, tobacco smoking, use of smokeless tobacco, betel chewing, alcohol drinking, and other possible risk factors as occupational exposure, nutrition, oral hygiene as well as personal and family history of various cancers.

Blood collection and DNA extraction

Peripheral blood samples from the qualified participants were collected. Each blood sample was centrifuged at 2,000 rpm, blood cells were kept frozen (-20°C) until whole blood DNA extraction was performed. A non-organic DNA extraction procedure was used to isolate DNA specimens (Sambrook et al., 1989).

Analysis of the p53 codon 72 polymorphism

The exon 4 of the *p53* gene was amplified by the PCR procedure. The upper and the lower primer was 5' - CCCGGACGATATTGAACA- 3' and 5'- AGAAGCCCAGACGGAAAC- 3', respectively. The reaction conditions were: activation of polymerase at 95°C for 9 min, then followed by 40 cycles of 94°C for 1 min; 61°C for 1 min; 72°C for 1 min. The PCR product was a 203 base pair DNA fragment. The fragment was then digested for two hours by a restriction enzyme, using either of these enzymes: *ACCII* (Takara, Japan), at 37°C or *BstUI* (New England BioLabs, USA) at 60°C. The genotypes were determined by electrophoresis on 3% agarose gel and visualized with ethidium bromide under UV light. The enzymes cut the PCR product of the Arg allele into two fragments, 125 bp and 78 bp, while the PCR product of the Pro allele remained uncut (Fig 1) .

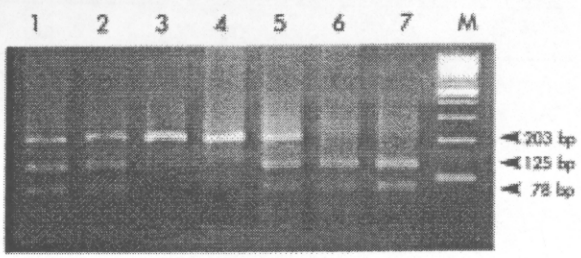

Figure 1. *p53* Polymorphism on 3% Agarose Gel Evaluated by the Restriction Enzyme Analysis. The Arg allele showing two small bands at 125 bp and 78 bp (lanes 6,7). The Pro allele showing a single band of 203 bp (lane 3,4). The heterozygous form has three bands at 203, 125 and 78 bp (lanes 1,2,5). M is the DNA 100 bp marker.

Statistical Analysis

The data were analysed by using Stata statistical software (Stata version 7.0). Chi-squared test was used for the comparison of proportion of categorical variables and t-test was used to compare the age. Due to the skewness of the data, the difference in alcohol consumption (gm-ethanol), tobacco smoking (pack-yr), and betel quid chewing (quid-yr) between cases and controls were tested by rank-sum (Mann-Whitney) test. This study was a matched design case-control, therefore, conditional logistic regression was applied appropriately to obtain odd ratios (ORs) of the association between oral cancer and the *p53* polymorphism, crude and adjusted for demographic and risk behavior variables and their 95% confidence intervals. Stratified analysis by smoking and drinking status with adjustment for levels of exposure cigarette, alcohol and betel chewing gave the risk of oral cancer for Arg/Pro and Pro/Pro genotypes of the *p53*, using the Arg/Arg genotype as a reference.

Results

As shown in Table 1, the study subjects including 97cases and 97 controls. They were well matched based on the selection criteria (age and gender) and the following results were obtained.

Table 2 shows the distribution of genotypes for the *p53* codon 72 polymorphism. The genotype frequency for Arg/Arg, Arg/Pro, and Pro/Pro were 33%, 45%, 22%, respectively in the patients, and were 36%, 35% and 29% in the controls, respectively. The distributions of genotype in both groups were in Hardy-Weinberg equilibrium. Furthermore, there was no significant difference ($p = 0.07$) in the allele frequencies between the control and case groups. Smoking and alcohol drinking behavior did not alter the risk for oral cancer based on the *p53* polymorphism. In subjects who consumed both tobacco and alcohol, the risk of oral cancer for Arg/Pro and Pro/Pro genotypes of *p53* was not significant (OR = 1.69, 95%CI = 0.61-4.71 and OR = 1.40, 95%CI = 0.41-4.73, respectively) using the Arg/Arg genotype as the reference. In subjects who neither smoked nor drank alcohol, the stratified analysis also demonstrated no association of oral cancer and the Arg/Pro or Pro/Pro genotypes (OR = 0.58, 95%CI = 0.09-3.81 and OR = 0.14, 95%CI = 0.01-2.17, respectively). Since there were few individuals who had a smoking or drinking habit alone, these two strata were excluded.

Table 1. Characteristics of the Subjects

		Case (97)	Control (97)	p-value
Sex	male	67	67	1.000[1]
	female	30	30	
Age	mean	67.4	67.8	0.812[2]
	SD	9.8	9.5	
Alcohol (gm-ethanol)	Median	9.8	10.45	0.674[3]
	Q1, Q3	0, 46.58	0, 29.00	
Smoking (pack-yr)	Median	200	164	0.622[3]
	Q1, Q3	0, 440	0, 410	
Betel Quid (quid-yr)	Median	0	0	< 0.001[3]
	Q1, Q3	0, 2880	0, 2640	

Q1 = first quatile , Q3 = third quatile, 1 = Chi-squared, 2 = t-test, 3 = rank-sum test

To assess the age-dependent development of oral cancer and the influence of the *p53* gene, we have subdivided the patient population into three age categories, irrespective of smoking and drinking habits: < 65, 66-75 and >75 years old, respectively. The data, as shown in Table 3, confirms that the *p53* variant genotype does not increase the risk for oral cancer. The Pro/Pro genotype seems to protect against oral cancer (using Arg/Arg as reference) in individuals younger than 65 years but statistical significance is not achieved (OR = 0.13, 95%CI = 0.02–1.10).

Discussion

The *p53* codon 72 polymorphism has been extensively investigated for its association in many diseases, including

Table 2. Association of *p53* Codon 72 Polymorphism and Risk Behavior and Oral Cancer

p53	Case	Control	OR	95%CI	Adj.OR*	95% CI
Arg/Arg(%)	32(33)	35(36)	ref.		ref.	
Arg/Pro(%)	44(45)	34(35)	1.37	0.74-2.53	1.29	0.58-2.86
Pro/Pro(%)	21(22)	28(29)	0.81	0.40-1.66	0.93	0.37-2.36
both smoke and drink						
Arg/Arg (%)	16(31)	20(39)	ref.		ref.	
Arg/Pro (%)	25(48)	19(37)	1.48	0.65-3.37	1.69	0.61-4.71
Pro/Pro (%)	11(21)	12(24)	1.11	0.43-2.91	1.40	0.41-4.73
never smoke never drink						
Arg/Arg (%)	11(37)	9(30)	ref.		ref.	
Arg/Pro (%)	13(43)	11(37)	1.00	0.31-3.24	0.58	0.09-3.81
Pro/Pro (%)	6(20)	10 (33)	0.43	0.10-1.87	0.14	0.01-2.17

* adjusted for past exposure (10 years before) to amount of cigarette smoking, ethanol consumption, and betel chewing.

Table 3. Association of *p53* and Age of Onset of Oral Cancer

p53 polymorphism	Age group		
	<= 65	66 - 75	> 75
Arg/Arg	ref	ref	ref
Arg/Pro	0.66 (0.17-2.58)	1.86 (0.48-7.33)	1.29 (0.23-7.33)
Pro/Pro	0.13 (0.02-1.10)	3.59 (0.67-19.17)	1.23 (0.26-5.92)

oral cancer (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002), however, the results are inconsistent. The discrepancy could be caused by the different ethnic composition of the studied populations (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002) and by the different risk habits in various regions (Summersgill et al., 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). The present study on oral cancer of the Southern Thai population indicates that there is no significant association between the *p53* codon 72 polymorphism and oral cancer. The *p53* polymorphism does not show evidence of interaction with any frequently practiced risk habits on oral cancer risk. This finding is concordant with some previous reports (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Shen et al., 2002). Although this is a negative study of *p53* polymorphism and oral cancer, this is the first report in Southeast Asian region, where the risks exposure and modifying factors for oral carcinogenesis may be different from the previous studies.

The information on the interaction of the *p53* codon 72 polymorphism and oral cancer remains obscure. There were six publications in the literature on the relationship between the *p53* polymorphism and oral cancer (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Nagpal et al., 2002; Shen et al., 2002). McWilliam et al (2000), Hamel et al (2000) and more recently Shen et al (2002) did not find that the p53 codon 72 polymorphism plays a role in the risk for squamous cell carcinoma of the head and neck (SCCHN) among Caucasians. Although, Shen et al (2002) suggested that the Pro/Pro genotype was associated with an early onset in oral cancer (p=0.046), they commented that the observation needed to be confirmed by a larger sample size. In another study in a Caucasian population, Summersgill et al (2000) also failed to demonstrate the relationship between the *p53* polymorphism and oral cancer either with or without HPV infection. There were two reports concerning interaction of the *p53* polymorphism and oral cancer from Indian population. Tandle et al (2001) did not observe an association of the *p53* genotype and oral cancer susceptibility whereas Nagpal et al (2002) indicated that the Arg/Arg genotype increased susceptibility to HPV infection and associated with oral carcinogenesis. The latter study was investigated in 110 patients who were highly addicted to tobacco and betel quid chewing. As mentioned earlier, the major differences in the prevalence of the variant alleles and the associated risk factors may affect the risk association. The genotypes frequency of the normal control in many studies showed that there were significant differences in the prevalence of the *p53* codon 72 polymorphism among different ethnic populations (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). Our current analysis reveals that in the Southern Thai population, the frequencies of the *p53* codon 72 polymorphism (Arg/Arg, Arg/Pro, and Pro/Pro) are 36%, 35%, and 29%, respectively. The distribution pattern is similar to those reported in most Asian populations [Japanese 36%, 44% and 20 % (Kuroda et al., 2003); Indian 14%, 65% and 20% (Tandle et al., 2001); Taiwanese 37.0%, 45.7% and 17.3% (Lee et al, 2000)]. In one Chinese study (Guimaraes et al.,2001), the frequency of the p53 Pro/Pro genotypes was extremely high (42.1%) while a report of the Eastern Indian (Nagpal et al., 2002), very low Pro/Pro genotype was observed However, the sample sizes of these two studies were very small, i.e. 57 and 26, respectively. In Asian populations, the distribution of the heterozygous form (Arg/Pro) was more common than the homozygous genotypes. This distribution pattern was different from the Caucasian populations which showed that the Arg/Arg was the most common genotype, with frequencies ranging from 53% (Shen et al., 2002) to 79% (Connor et al., 2001) whereas the frequencies of the homozygous Pro/Pro genotype were very low 3.5% (Connor et al., 2001), 7.2% (Shen et al., 2002). Such major differences in the distribution of the variant alleles in different ethnic populations may impact their contribution to the susceptibility to environmental related cancers, like oral cancer.

Infection with human papilloma virus (HPV), especially the high risk types (HPV16/18), is involved in oral carcinogenesis (Nagpal et al., 2002; Miller et al., 1989; Schwart et al., 1998). This relationship has support from mechanistic observation which indicated that the E6 protein of the HPV16/18 binds to *p53*, thus facilitating the development of cancer (Storey et al., 1998). Recently, Nagpal et al (2002) revealed that the p53 polymorphism increased the susceptibility of the HPV-related oral carcinogenesis of Eastern Indians who were highly addicted to chewing tobacco and betel quid. They indicated that Arg/

Arg genotype caused more susceptible to oral cancer than Pro/Pro genotype. This data was contradictory to an earlier published data from a Caucasian study (Summersgill et al., 2000). In our study, we did not investigate the infection with HPV in our patients and controls. However, the interaction between the E6 and the p53 proteins may contribute to a significant influence on the risk for oral cancer. Of particular relevance is the observation that the p53Arg protein is more readily degraded by E6 than the p53Pro. Under this scenario, inactivation of the Arg/Arg genotype by E6 becomes the major risk factor, leaving the Arg/Pro and the Pro/Pro genotypes to interact with other risk factors for their contribution to oral cancer. With this consideration, our observed interactions between smokers/drinkers and the Arg/Pro and Pro/Pro genotypes for risk for oral cancer are possible. Furthermore, the protective effect of the Pro/Pro genotype is also possible.

In conclusion, the *p53* codon 72 polymorphism is not associated with oral cancer in Southern Thailand. The Pro/Pro genotype may offer some protective effect on oral cancer. The interactions between the HPV E6 protein and the p53 proteins from the different alleles need to be considered to understand more precisely the risk factors for oral cancer. Furthermore, the relationship between the *p53* polymorphism and polymorphisms in xenobiotics metabolizing genes will need to be considered when the sample size is large enough for such interactive analysis.

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Genetic and Environmental Interactions on Oral Cancer in Southern Thailand

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Many countries are interested in understanding the relationship between genetic susceptibility and their prevalent environmental cancers for disease prevention. In Thailand we conducted a population-based case-control study of 53 matched pairs to assess the risk of oral cancer in relation to genetic polymorphism of the glutathione-S-transferase genes (GSTM1 and GSTT1) in cigarette smokers, alcohol drinkers, and betel quid chewers. Interaction of the genes with other potential risk factors such as local bean consumption were also elucidated. Homozygous deletion of GSTM1 has a frequency of 56.6% (n = 30 over 53) among the patients and 30.2% (16/53) among the controls. This gene is associated with a 2.6-fold higher risk for development of oral cancer (95% CI 1.04–6.5). Among the null GSTM1 individuals, those who smoke, consume alcohol, and/or chew betel quid have a significantly increased risk for oral cancer with an odd ratio (OR) = 4.0 (95% CI = 1.2–13.7), OR = 7.2 (95% CI = 1.5–33.8), and OR = 4.4 (95% CI = 1.1–17.8), respectively. Interactions between any two of the lifestyle habits for oral cancer risk, however, are not found. The frequency of the GSTT1 null genotype is 34.0% (18/53) among the patients and 47.2% (25/53) among our controls. There is no association between the GSTT1 null allele and oral cancer risk. In conclusion, our study provides data to indicate that individuals who have homozygous deletion of the GSTM1 gene have increased risk for oral cancer. The risk increases further when these individuals are exposed to environmental toxicants such as chemicals in cigarette smoke, alcohol, and betel quid. These baseline data can be applied to a larger population-based study, both to verify the observation and to conduct mechanistic investigations. Environ. Mol. Mutagen. 37:111–116, 2001.

Key words: genetic susceptibility; oral cancer; cigarette smoking; metabolizing genes; GSTM1; GSTT1; betel quid chewing

INTRODUCTION

Cancer of the oral cavity is a serious health problem in Thailand. The age-standardized incidence rates (ASR) range from 2.7 to 10.0 per 100,000 population in both sexes. The incidence is highest in the southern region of the country, where oral cancer is the second leading cancer in males and the sixth in females. The ASR in the Songkhla region are 9.8 and 3.5 per 100,000 population in males and females, respectively [Deerasamee et al., 1999]. The current understanding is that poor dental hygiene is a major risk factor for oral cancer for both genders [Lamont et al., 1995; Genco, 1996; Meyer et al., 1996]. The other major risk factors are smoking and alcohol consumption that are common among males and betel chewing that is common among females in this region [Kerdpon and Sriplung, 1997]. The habit of betel chewing is different among individuals. Some combine the betel quid with smokeless tobacco and others add spices to their betel quid. The association of betel chewing and oral cancer risk was previously documented in other Asian countries, for example, India and Taiwan [Sankaranarayanan et al., 1990; Ko et al., 1995]. In India, where oral cancer is the most common malignancy, betel chewing is very common among Indian males. The chewing habit is also common among males in Taiwan but not in Thailand, where most of the chewers are females.

A review of the literature indicates that the aforementioned environmental risk factors do not account for the variations in risk for the development of oral cancer. Like lung cancer, the variations can be contributed by the exis-

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tence of susceptible genetic factors that interact with environmental factors for the development of disease [World Health Organization, 1993; Lin et al., 1994; Au et al., 1998, 1999a,b]. Most environmental carcinogens are metabolized by the phase I (activating) and phase II (detoxifying) enzymes [Guengerich et al., 1992; Hayes and Pulford, 1995; Wormhoust et al., 1999] and the genes for some of these enzymes are polymorphic. Therefore, inheritance of certain variant alleles may impose susceptibility to environmental cancer. The relationship between genetic and environmental interactions for oral cancer was not previously investigated in Thailand. Therefore, this is the focus of our investigation.

During the initial phase of the investigation, we targeted two polymorphic glutathione-*S*-transferase genes (GSTM1 and GSTT1) because the variant alleles of these two genes supposedly have high prevalence in the normal population (approximately 50 and 30%, respectively). This high prevalence would allow us to conduct reliable association studies using small population sizes [Au et al., 1998, 1999a]. GSTM1 and GSTT1 are involved in the detoxification of a wide range of environmental and tobacco-specific carcinogens. Inheritance of the null (homozygous deletion) GSTM1 and/or GSTT1 genotypes were previously shown to be associated with the development of cancer in many organ systems, particularly among cigarette smokers. Examples are lung cancer [El-Zein et al., 1997a–c], bladder cancer [Bell, 1993; Anwar et al., 1996], head and neck cancer [Jourenkova et al., 1998; Matthias et al., 1998], and oral cancer [Deakin et al., 1996; Hung et al., 1997; Park et al., 1997; Jourenkova-Mironova et al., 1999; Nair et al., 1999; Sato et al., 1999]. It should also be emphasized that the reported positive association between inheritance of susceptible genes and cancer is not universally substantiated [D'Errico et al., 1996]. Perhaps, geographical and ethnic differences in the distribution of the variant alleles are responsible for the discrepant observations. Therefore, our investigation in Thailand is needed. The evidence of positive association suggests that inheritance of the null (susceptible) genotypes can cause cigarette smokers to have increased body burden of reactive metabolites (carcinogens) from cigarette smoke to increase the risk for cancer. This suggestion is supported by the demonstration that the susceptible individuals and/or their cells had more DNA damage and chromosome aberrations than the nonsusceptible individuals [Ichiba et al., 1994; El-Zein et al., 1997; Salama et al., 1999].

Here we report, for the first time in a Thai population, the effect of GSTM1 and GSTT1 polymorphisms on the risk of cancer of the oral cavity and their relationship with some environmental risk factors.

MATERIALS AND METHODS

Study Population

Patients with cancer in the oral cavity were sequentially recruited before they had chemo- and/or radiotherapy from the Department of Radiology, Songkhlanagarind Hospital, from August 1998 to December 1999. All individuals participated in the study voluntarily and provided informed consent. Participants were personally interviewed with a questionnaire that was approved by the university ethics committee. The collected information was on each individual's demographic background; cigarette smoking, betel chewing, and alcohol consumption habits; occupational exposure; nutritional behavior; oral hygiene; as well as personal and family history of various cancers. Based on the collected information, the criteria for recruitment of controls were determined. The controls were recruited from similar locations from which the patients came (i.e., residents living in the Songkhla province and its vicinity). These locations were usually within a 1-hr drive by car from the hospital. The volunteer controls were selected based on matching with the patients according to age (±5 years), gender, and smoking or betel-chewing habits. In addition, the controls were healthy. They also lacked the following specific problems with respect to the oral cavity: (1) no oral cancer, (2) no oral ulcer, (3) no difficulties in swallowing, and (4) no oral discomfort. They were also matched with the patients based on socioeconomic conditions, that is, all of them worked on the farm (e.g., orchid workers).

Collection and Preparation of Specimens

Peripheral blood samples from the qualified participants were collected into syringes and immediately transferred into polyethylene tubes that contained sodium heparin as an anticoagulant. The collected samples were coded and maintained in cool containers for transportation to the laboratory. The time taken for the collection and delivery of blood samples to the laboratory was usually less than 3 hr. In the laboratory, each blood sample was centrifuged at 2000 rpm for 5 min to pack cells in the centrifuge tubes. The layer of white cells on top of the red blood cells was removed using a pipette and then stored frozen at −20°C. These white blood cells were used for extraction of DNA.

GSTM1 and GSTT1 Genotyping

DNA used for genetic analysis was isolated from 10 ml of frozen heparinized blood using a nonorganic DNA extraction procedure [Sambrook et al., 1989]. Amplifications of the GSTM1 and GSTT1 genotypes were obtained simultaneously in a single assay approach using a multiplex PCR method [Abdel-Rahman et al., 1996]. Briefly, each 50-μl reaction mixture contained 200 μM dNTPs, 5 μl 10× PCR buffer (10× 500 mM KCl, 100 mM Tris–HCl, pH 9.0), 1.5 mM $MgCl_2$, and 2 U Amplitaq DNA polymerase (Perkin–Elmer/Applied Biosystems, Piscataway, NJ). DNA (50 ng) was amplified together with 30 pmol of each pair of the following primers: GSTM1 primers (5′ GAA CTC CCT GAA AAG CTA AAG C 3′ and 5′ GTT GGG CTC AAA TAT ACG GTG G 3′) and GSTT1 primers (5′ TTC CTT ACT GGT CCT CAC ATC TC 3′ and 5′ TCA CCG GAT CAT GGC CAG CA 3′). CYP1A1 primers (5′ GAA CTG CCA CTT CAG C TG TCT 3′ and 5′ CAG CTG CAT TTG GAA GTG CTC 3′) were coamplified as an internal control. The PCR was performed in a Perkin–Elmer GeneAmp PCR System 2400 (Perkin–Elmer Cetus Instruments, Norwalk, CT). The reaction conditions were: initial denaturation (at 95°C, 9 min), followed by 35 cycles of melting (94°C, 1 min), annealing (59°C, 1 min), and extension (72°C, 1 min). A final elongation step (72°C, 10 min) terminated the process. The amplified products were determined by electrophoresis on ethidium bromide–stained 2% agarose gel. The presence of a band at 312 bp (corresponding to CYP1A1) indicated a successful amplification. The presence or absence of a band at 215 bp and a band at 480 bp determined normal or deletion genotypes of GSTM1 and GSTT1, respectively.

TABLE I. Characteristics of Cases and Controls

	Cases (53)	Controls (53)	*P* value
Sex			1.000
Male	35	35	
Female	18	18	
Religion			0.079
Buddhist	50	53	
Muslim	3	0	
Age			0.857
Mean	67.0	67.3	
SD	10.1	10.3	
Smoking			0.677
Yes	35	37	
No	18	16	
Alcohol			0.696
Yes	30	28	
No	23	25	
Betel			0.698
Yes	25	27	
No	28	26	
Smokeless tobacco			0.567
Yes	15	11	
No	38	42	

TABLE II. Crude Odds Ratio (cOR) of GSTs and Oral Cancer

	Case no. (%)	Control no. (%)	cOR	95% CI	*P* value
GSTM1					
Wild type	23 (43.4)	37 (69.8)			
Null	30 (56.6)	16 (30.2)	3.0	1.4–6.7	0.006
GSTT1					
Wild type	35 (66.0)	28 (52.8)			
Null	18 (34.0)	25 (47.2)	0.6	0.3–1.3	0.166

TABLE III. Risk of GSTM1 and GSTT1 Genes on Oral Cancer, Adjusted for Exposure Variables

GSTM1	GSTT1	aOR[a]	95% CI	*P* value
Wild type	Wildtype	1.0		
Wild type	Null	0.7	0.2–2.3	0.508
Null	Wildtype	3.6	1.0–12.9	0.045
Null	Null	2.0	0.5–7.8	0.313
GSTM1 null or GSTT1 null		1.6	0.6–4.4	0.328
GSTM1 null and GSTT1 null		2.0	0.5–7.8	0.313

[a]aOR = adjusted odds ratio.

Statistical Analysis

The data were analyzed by using a statistical analysis software (Stata version 6.0; Stata, College Station, TX). The chi-square test was used for the comparison of proportion of characteristic variables and the Students' t-test was used to measure for the comparison of age. Stratified analysis and logistic regression methods were applied appropriately to obtain odd ratios (ORs) and their 95% confidence intervals.

RESULTS

The case-control study consisted of 53 cases of primary oral cancer (base of tongue 15, tongue 6, floor of mouth 9, palate 7, buccal 7, gum 7, and lip 2). All cases were confirmed by pathologists to be oral squamous cell carcinomas. From our studied population, there is a significant gender-specific distribution of oral cancers and our observation is consistent with that reported earlier by Kerdpon et al. [1997]: palate and tongue cancers are prevalent in male and buccal mucosal cancers are prevalent in females. The 35 male and 18 female patients (n = 53) were strictly pair-matched by age (±5 years) and gender with 53 of the 60 recruited controls without the knowledge of other information. Smoking, alcohol drinking, and betel-chewing habits were frequency matched. The mean age of the cases and controls are 67.0 ± 10.1 (SD) and 67.3 ± 10.3 (SD), respectively. A summary of the characteristics of the studied population is shown in Table I. As shown in the Table, there is no significant difference between the case and the control groups with respect to ethnicity and that based on the comparison parameters. The proportion of Muslims was marginally different ($P = 0.079$) between the two groups. However, Muslims are represented by only three individuals; therefore their inclusion probably had no influence on the result of the analysis.

A summary of the association of the GSTM1 and GSTT1 genotypes and oral cancer is shown in Table II. The GSTM1 null genotype has a significant effect on oral cancer risk (OR = 3.0, 95% CI = 1.4–6.7), whereas the GSTT1 revealed no association (OR = 0.6, 95% CI = 0.3–1.3). Adjusted for exposure variables, the GSTM1 null genotype significantly increases the risk for oral cancer (adjusted OR = 2.6, 95% CI = 1.04–6.5), whereas the GSTT1 null genotype is not independently associated with oral cancer risk (adjusted OR = 0.6, 95% CI = 0.2–1.5) (data not shown). As shown in Table III, the effect of the GST-susceptible genotypes on oral cancer risk is not increased with the combined deletion of GSTM1 and GSTT1 (OR = 2.0, 95% CI = 0.5–7.8).

The association between genetic susceptibility and environmental risk factors for oral cancer was also investigated. Table IV shows that, with respect to the tobacco smoking habit, the GSTM1 wild type and GSTM1 null genotypes have no influence on oral cancer among the nonsmokers and the occasional smokers. However, frequent smokers with the GSTM1 null have a significantly increased risk for oral cancer (OR = 4.1; 95% CI = 1.5–11.3). The GSTM1 null genotype shows a significantly increased risk among frequent alcohol drinkers (OR = 4.3; 95% CI = 1.4–13.2), but not among nondrinkers and minimal drinkers. With respect to the betel-chewing habit, the GSTM1 null demonstrates an increased risk among frequent chewers (OR = 4.0; 95% CI = 1.3–12.9). Interestingly, for individuals who chew betel without smokeless tobacco, the risk is raised to 22-fold (95% C.I = 2.2–222.0). No specific association is found

TABLE IV. Risk of GSTM1 Null Genotype for Oral Cancer by Cigarette Smoking, Alcohol Consumption, and Betel Chewing

	Case		Control					
	Null	Normal	Null	Normal	cOR[a]	95% CI	aOR[a]	95% CI
Tobacco smoking								
No or minimal	11	9	7	10	1.7	0.5–6.4	2.0	0.4–10.2
Frequent	19	14	9	27	4.1	1.5–11.3	4.0	1.2–13.7*
Alcohol								
No or minimal	13	13	8	17	2.1	0.7–6.6	1.5	0.4–6.2
Frequent	15	10	8	20	4.3	1.4–13.2	7.2	1.5–33.8*
Betel chewing								
No or minimal	13	15	7	20	2.5	0.8–7.7	2.6	0.6–11.9
Frequent	17	8	9	17	4.0	1.3–12.9	4.4	1.1–17.8*
With ST[a]	6	7	4	7	1.5	0.3–7.8	2.3	0.2–23.4
Without ST	11	1	5	10	22.0	2.2–222.0	26.9	2.0–339.8*

[a]cOR and aOR = crude and adjusted odd ratios, respectively; ST = smokeless tobacco.
*$P < 0.05$.

TABLE V. Risk of GSTT1 Null Genotype for Oral Cancer by Cigarette Smoking, Alcohol Consumption, and Betel Chewing

	Case		Control					
	Null	Normal	Null	Normal	cOR[a]	95% CI	aOR[a]	95% CI
Tobacco smoking								
No or minimal	7	13	8	9	0.6	0.2–2.3	0.5	0.1–2.9
Frequent	11	22	17	19	0.6	0.2–1.5	0.8	0.2–2.8
Alcohol								
No or minimal	9	17	12	13	0.5	0.2–1.5	0.5	0.1–2.1
Frequent	9	18	12	16	0.7	0.2–2.0	1.3	0.3–5.3
Betel chewing								
No or minimal	7	21	12	15	0.4	0.1–1.3	0.7	0.1–2.9
Frequent	11	14	13	13	0.8	0.3–2.4	0.7	0.2–2.9
With ST[a]	4	9	6	5	0.4	0.1–2.0	0.4	0.0–4.1
Without ST	7	5	7	8	1.6	0.3–7.4	3.1	0.4–25.1

[a]cOR and aOR = crude and adjusted odd ratios, respectively; ST = smokeless tobacco.

with the consumption of beans. After the adjustment for all other variables (i.e., smoking, drinking, betel chewing, local beans consumption), the effect of the GSTM1 null genotype on oral cancer development is more prominent among drinkers and chewers (OR = 7.2, 95% CI = 1.5–33.8; OR = 4.4, 95% CI = 1.1–17.8, respectively). A strikingly increased trend is noted among chewers, who inherited susceptible GSTM1 genotype and those who do not use smokeless tobacco (OR = 26.9, 95% CI = 2.1–339.8). Too few individuals had all three exposure conditions (cigarette smoke, alcohol, and betel) for us to investigate their interactions for risk to develop oral cancer, with or without the GSTM1 gene. From multivariate analyses, the interaction between any of the two exposure conditions is not found. This observation may be the result of a true lack of interaction or to the effect of too-small sample sizes.

The interaction of the GSTT1 genotype with cigarette smoking, betel chewing, alcohol consumption, and consumption of three local beans for oral cancer is demonstrated in Table V. There is no significant influence on the GSTT1 null allele and oral cancer among smokers and nonsmokers, drinkers and nondrinkers, chewers and nonchewers, and on bean consumption. This indicates there is no association between the GSTT1 genotypes and the risk for oral cancer development with respect to the major lifestyle risk factors examined in this study.

DISCUSSION

Polymorphism of GSTM1 and GSTT1 genes and their effect on oral cancer were studied recently in several countries [Hung et al., 1997; Park et al., 1997; Jourenkova-Mironova et al., 1999; Nair et al., 1999; Sato et al., 1999]. We report the first such investigation in a Thai population. The frequencies of GSTM1 and GSTT1 null among our noncancer population are 30.2 and 47.2%, respectively (n = 53). The results show that individuals with a susceptible version of GSTM1 genotype (the null genotype) have a 2.6-fold increased risk for oral cancer independent of exposure to environmental risks. There is no increased risk when this genotype is combined with the GSTT1 null genotype. However, the estimate of the risk in this latter case may not be precise because of the limited sample sizes. In addition,

we could not find an association of GSTT1 null allele and oral cancer risk.

This report supports the evidence found in India where the null GSTM1 genotype had a 22-fold (95% CI = 9.2–57.0) estimated risk for oral leukoplakia, a precancerous lesion [Nair et al., 1999]. It also showed concordance with a study in the Japanese, which showed that deletion of the GSTM1 gene increased oral cancer risk by 2.2-fold (95% CI = 1.4–3.6) [Sato et al., 1999]. Our study did not support the results from the study in English Caucasians [Deakin et al., 1996], which found no risk associated with GSTM1 null. Another study in Swiss Caucasians showed a 2.4-fold increased risk with GSTT1 null (95% CI = 1.0–5.5) but no association with the GSTM1 null [Jourenkova-Mironova et al., 1999]. A study in the United States detected no association of the GSTM1 and oral cancer risk [Park et al., 1997]. One possible explanation for the disagreements is the ethnic differences in allelic frequency of the GSTM1 and GSTT1 polymorphisms. In this regard, we carefully compared our result with data from the previous reports. Interestingly, we found that the baseline frequency of GSTM1 null from controls has a strong influence on oral cancer risk, that is, when the frequencies were high (approaching or above 50%), their effect on oral cancer risk was low or minimal. Examples can be found in the reports from Deakin et al. [1996], Park et al. [1997], Jourenkova-Mironova et al. [1999], and Hung et al. [1997], in which the GSTM1 null frequencies are 55, 51, 52, and 58%, respectively. In contrast, when the frequencies of the genotypes were low (17% as observed by Nair et al. [1999] and 30.2% in our study), their influence on the risk was strikingly high. Another possible explanation of the discrepancy is the difference in environmental and lifestyle risk factors. As described earlier, the betel quid may have significantly different mixtures from various countries. The higher cancer risk among betel quid chewers without smokeless tobacco compared with those who consumed both is very interesting and may suggest some antagonistic effects between them. This observation requires further investigation.

Tobacco smoking, alcohol drinking, and betel chewing are known risk factors of oral cancer in southern Thailand [Kerdpol and Sriplung, 1997]. When the interaction between GSTM1 null and the lifestyle risk factors is analyzed, our data demonstrate that each of these environmental factors enhances the risk from GSTM1 null (OR = 4.0, 7.2, 4.4 for habitual tobacco smokers, alcohol drinkers, and betel chewers, respectively). In multivariate analyses, interactions between any two of the environmental risk factors are not found. This may be the result of the true lack of interactions or to the effect of too-small sample sizes. We found no difference in the age of onset on the oral cancer, whether the patients inherited susceptible or normal versions of the GSTs genes.

We compared our data with those from the study on the Taiwanese [Hung et al., 1997], whose ethnicity and exposures are similar to ours. The influence of the GSTM1 null on oral cancer is stronger based on our study when the association is conducted separately for GSTM1 and GSTT1 (OR = 3.6 to 1.3). However, when the two genes were combined in the analysis, we could not detect the association (OR = 2.0; 95% CI = 0.5–7.8), whereas the Taiwanese detected an increased risk (OR = 4.9). The age of onset for our patients was 67 years old; for the Taiwanese cases, 54.1 years old. The observed discrepancies can possibly be explained by the difference in tobacco smoking and betel-chewing habits. Our oral cancer patients who smoked also drank alcohol, but rarely chewed betel, whereas most of the Taiwanese chewers in the study by Hung et al. [1997] were smokers. In addition, betel-chewing style and ingredients may also be different.

Although we collected oral hygiene information from our patient and control groups, the information is not adequate for us to conduct an association analysis. This is because the majority of them had many types of oral problems and about half of the patients lost all their teeth.

In conclusion, we found that there is a gene–environment interaction for the risk of oral cancer in Thailand: GSTM1 null with cigarette smoking, alcohol consumption, and betel chewing. In that the development of oral cancer, like other cancers, is based on multifactorial contributions, additional environmental and genetic factors should be explored. For this reason, a study with a larger sample size is ongoing in our laboratory for further exploration of gene–gene and gene–environment interactions on the development of oral cancer.

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