Interaction of DNA Repair Gene XPC With Smoking and Betel Quid Chewing Behaviors of Oral Cancer

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Abstract. *Background/Aim:* Xeroderma pigmentosum complementation group C (XPC) is reported to play important roles in DNA integrity and genomic instability, however, the contribution of XPC to oral carcinogenesis is largely uncertain. Therefore, we aimed at examining the contribution of XPC genotypes to oral cancer. *Materials and Methods:* The genotypes of XPC rs2228001 and rs2228000 were examined among 958 oral cancer patients and 958 control subjects by polymerase chain reaction-restriction fragment length polymorphism methodology and corresponding DNA repair capacity was checked. *Results:* First, the percentages of XPC rs2228001 AC and CC were higher among oral cancer patients than controls. Second, no significant association was observed regarding XPC rs2228000. Third, there was a synergistic influence of smoking and betel quid chewing behaviors and XPC rs2228001 genotype on oral cancer risk. Last, functional

experiments showed DNA repair capacity was lower for AC/CC carriers than AA carriers. *Conclusion:* XPC rs2228001 C allele, which was associated with decreased DNA repair capacity, may interact with smoking and betel quid chewing behaviors on oral cancer risk.

Oral cancer is the tenth most common cancer worldwide, and Taiwan has one of the highest incidences (1). Based on the most updated annual statistics from the government, oral cancer is of the fourth death-causing cancers among Taiwanese males (2). Uniquely, betel quid chewing, in addition to cigarette smoking and alcohol drinking, has been identified as an effective environmental factor to oral cancer risk in Taiwan (3). The Taiwan government has embarked in population screen searching for the oral cancer candidates for early cure and medication to lower its incidence, however, the death rate and incidence of oral cancer were still high. Therefore, novel predictors for oral cancer risk are still needed.

There are five major DNA repair systems, consisting of more than 130 genes, and teaming up to maintain the stability and integrity of the human genome. Among them, the nucleotide excision repair (NER) system is in charge of removing DNA crosslinks, bulky adducts, alkylating DNA adducts, oxidative DNA adducts and thymidine dimers (4-6). In NER machinery, four major steps (adduct recognition, lesion DNA incision, gapped DNA fulfilling and ligation) and several core players, including xeroderma pigmentosum complementation group C (XPC)-RAD23B, play critical roles as key enzymes (4, 5) Theoretically, subtle genetic

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variation on the NER genes may change DNA repair capacity and thus interfere critically in tumorigenesis (7). However, little is revealed in the literature.

XPC gene is one of the major genes in the NER system and plays a role in very early steps of NER (8). Molecular studies have revealed that *XPC* may interact with RAD23B forming the XPC-RAD23B, and playing an important role in the DNA adduct recognition and initiation of the NER machinery (5, 8, 9). In the overall NER machinery, the DNA adduct recognition is thought to be the rate-limiting step (9). This may give us a rationale that it is towards figuring out a predictor on *XPC* gene for carcinogenesis.

Based on the National Center for Biotechnology Information website, there are more than one hundred coding-region single nucleotide polymorphisms (SNPs) for the *XPC* gene. Among all the identified SNPs of *XPC* gene, two polymorphisms Ala499Val (rs2228000) and Lys939Gln (rs2228001) have been mostly investigated. The rs2228000 located in the domain interacting with RAD23B, while the rs2228001 located in the domain interacting with TFIIF. In the literature, many studies have examined the association of rs2228001 (10-14) and/or rs2228000 (15-18) genotypes of *XPC* with the risk of cancers, but conclusions were inconsistent.

In 2006, Kietthubthaw and colleagues firstly investigated the contribution of genotypes of *XPC* to oral squamous cell carcinoma in a population of 106 cases and 164 controls (19). They found that *XPC* rs2228001 may not contribute to oral cancer susceptibility, and no joint effect with environmental factors including smoking, alcohol drinking or betel quid chewing (19). In 2007, Wang and colleagues provided evidence showing that *XPC* rs2228000 genotypes have conferred a protective effect on oral cancer susceptibility, evident in older individuals, women, ever smokers, and never drinkers (20). In 2019, Senghore and colleagues found that the TT genotype at *XPC* rs2228000 increased the risk of poor overall survival at borderline significance compared to the CC+CT genotypes (HR=1.86, 95% CI=0.97-3.56), while *XPC* rs2228000 or *XPC* rs2228001 genotypes could not serve as good predictors for oral cancer susceptibility (21). Based on the limited literature on *XPC* and oral cancer, we are keen to assess whether rs2228000 and rs2228001 polymorphisms of *XPC* are associated with the risk of oral cancer in Taiwan. Additionally, we aimed to investigate the joint effect of betel quid chewing behaviors and *XPC* genotypes on oral cancer susceptibility. Furthermore, the genotype-phenotype pilot study investigating the DNA repair capacity will be firstly conducted.

Materials and Methods

Recruited Taiwanese oral cancer cases and controls. In brief, 958 oral cancer patients had been recruited at the China Medical University Hospital (22-24). The frequencies of their demographic characteristics

Table I. Demographic characteristics of the 958 oral cancer patients and 958 non-cancer healthy controls.

Characteristics	Controls (n=958)	Cases (n=958)	p-Value
Age (years)	56.8±8.7	56.4±7.5	0.3755 ^a
Gender, n (%)			1.0000 ^b
Male	728 (76.0%)	728 (76.0%)	
Female	230 (24.0%)	230 (24.0%)	
Personal behaviors, n (%)			
Cigarette smokers	668 (69.7%)	718 (74.9%)	0.0107^b
Alcohol drinkers	642 (67.0%)	684 (71.4%)	0.0377^b
Betel quid chewers	508 (53.0%)	773 (80.7%)	<0.0001^b
Primary tumor site, n (%)			
Tongue		397 (41.4%)	
Buccal mucosa		356 (37.2%)	
Mouth floor		39 (4.1%)	
Retromolar trigone		33 (3.4%)	
Alveolar ridge		29 (3.0%)	
Palate		27 (2.8%)	
Lip		39 (4.1%)	
Other		38 (4.0%)	

SD: Standard deviation; ^aBased on Student's *t*-test; ^bBased on Chi-square test. Significant *p*-Values (*p*<0.05) are shown in bold.

including age, gender, personal behaviors and tumor sites were summarized in Table I. The study had been approved and supervised under the Institutional Review Board (DMR101-IRB1-306).

Oral cancer *XPC* genotyping methodology. DNA from all participants was processed in typical polymerase chain reaction (PCR) processes as in our previous papers (25-27). The sequences of designed forward and reverse primers, corresponding restriction enzymes (New England BioLabs, Ipswich, MA, USA) and sizes of PCR products after enzyme digestion for oral cancer *XPC* genotyping identification are shown in Table II.

***XPC* mRNA expression pattern.** Thirty-five surgically removed oral cancer tissue samples obtained from tumor sites were collected. The patients were all non-smokers, non-alcohol-drinkers, and non-betel quid chewers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primers for *XPC* mRNA were forward 5'-GACAAGCAGGAGAAGGCAAC-3' and reverse 5'-GGTTCGGAATCCTCATCAGA-3', respectively. The primers for GAPDH were forward 5'-GAAATCCCATCACCATCTTCCAGG-3' and reverse 5'-GAGCCCCAGCCTTCTCCATG-3', respectively. Fold changes were normalized using GAPDH, and each experiment was carried out at least thrice (28-30).

***XPC* protein expression pattern.** As mentioned above, the tissue specimens from oral cancer were spontaneously prepared for western blotting. Briefly, after 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferring to a nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA), the membrane was blocked with mouse monoclonal anti-human *XPC* antibody (1:1,000; Thermo Fisher Scientific, Waltham, MA, USA), and then with horseradish

Table II. Sequences of the designed primers, corresponding endonucleases and fragments identifications for genotyping of XPC rs2228000 and rs2228001.

Polymorphic site	5' to 3' primer sequences	Endonucleases	Allelic subtypes and product size (bp)
rs2228000	GTGCCCCGTATCTGTTGGTCT TAATATCGGGGCTCACCTG	<i>Pvu</i> II	C: 121+21 T: 142
rs2228001	GGAGGTGGACTCTCTTCTGA TAGATCCCAGCAGATGACC	<i>Pvu</i> II	A: 765 C: 585+180

Table III. Distribution of XPC rs2228000 and rs2228001 genotypes among the 958 oral cancer patients and 958 non-cancer healthy controls.

Genotype	Cases		Controls		OR (95%CI)	<i>p</i> -Value ^a
	n	%	n	%		
rs2228000						
CC	383	40.0%	389	40.6%	1.00 (reference)	
CT	450	47.0%	454	47.4%	1.01 (0.83-1.22)	0.9455
TT	125	13.0%	115	12.0%	1.10 (0.83-1.48)	0.5035
CT+TT	575	60.0%	569	59.4%	1.03 (0.86-1.23)	0.7799
<i>P</i> _{trend}						0.7862
<i>P</i> _{HWE}						0.3182
rs2228001						
AA	381	39.8%	406	42.4%	1.00 (reference)	
AC	453	47.3%	448	46.8%	1.08 (0.89-1.30)	0.4444
CC	124	12.9%	104	10.8%	1.27 (0.95-1.71)	0.1121
AC+CC	577	60.2%	552	57.6%	1.11 (0.93-1.34)	0.2457
<i>P</i> _{trend}						0.2758
<i>P</i> _{HWE}						0.2336

n: Number; OR: odds ratio; *P*_{trend}: *p*-Value for trend analysis; *P*_{HWE}: *p*-Value for Hardy–Weinberg equilibrium. ^aBased on Chi-square without Yate's correction test.

peroxidase-conjugated goat anti-mouse IgG secondary antibody (Chemicon, Temecula, CA, USA). The ECL density was quantified using a computer-assisted imaging analysis system (GeneTools Match software; Syngene) (31).

DNA repair capacity measurement. About ten milliliters of peripheral venous blood was collected from oral cancer patients into heparinized tubes, mixed 1:1 with RPMI 1640 medium (Sigma-Aldrich) and centrifuged at 1200 rpm for 30 min. Isolated peripheral blood mononuclear cells were counted and their viability was checked for their viability (higher than 95%) and ready for UVC-irradiated comet assay (32). Cells were irradiated with UVC 40 J/m² with UV light crosslinker (Spectrolinker XL-1000. Spectronics Co., Westburg, NY, USA) at a dose-rate of 0.5 W/m² and the Comet assay was performed immediately (R0) and 6 h later (R6). UV endonuclease V was used for the removal of cyclobutane pyrimidine dimers and 50 randomly selected cells from two parallel slides per person. For each person, (the average comet moment at R0 - the average comet moment R6)/the average comet moment at R0 * 100%=individual repair capacity. The standard 100% was set as the average of those carrying AA genotype of XPC rs2228001.

Statistical analysis. The *Student's t*-test was applied to compare the distribution of ages between the case and control groups. Pearson's chi-square test had been used to compare the distribution of the XPC rs2228000 and rs2228001 genotypes among the subgroups, and also to evaluated the possible interaction among the smokers, non-smokers, alcohol drinkers, non-alcohol drinkers, betel quid chewers, non-betel quid chewers. The association between the XPC rs2228000 and rs2228001 genotypes and oral cancer risk had been investigated using odds ratios (ORs) and their corresponding 95% confidence intervals (CIs). Any difference with *p*<0.05 was taken as statistically significant.

Results

The demographic characteristics for the 1,916 Taiwan participants (958 oral cancer cases and 958 non-cancer healthy controls) are summarized in Table I. First, since we matched the cases and control by age and gender, there is no difference in respect to these aspects (both *p*>0.05). Second, as for smoking, alcohol drinking and betel quid chewing,

Table IV. Distributions of *XPC* rs2228000 and rs2228001 genotypes among the 958 oral cancer patients and 958 non-cancer healthy controls.

Allele	Cases	%	Controls	%	OR (95%CI)	p-Value ^a
rs2228000						
Allele C	1216	63.5%	1232	64.3%	1.00 (reference)	
Allele T	700	36.5%	684	35.7%	1.04 (0.91-1.18)	0.5905
rs2228001						
Allele A	1215	63.4%	1260	65.8%	1.00 (reference)	
Allele C	701	36.6%	656	34.2%	1.11 (0.97-1.27)	0.1285

n: Number; OR: odds ratio; CI: confidence interval; ^aBased on Chi-square without Yate's correction test.

there are different distributions between the oral cancer and control cohorts, respectively (all $p < 0.05$, Table I).

In Table III, we summarize the results of the distributions of genotypic frequencies of the two *XPC* SNPs, rs2228000 and rs2228001. First, the allelic frequencies in *XPC* rs2228000 and rs2228001 of the control group fitted well with the Hardy-Weinberg equilibrium (both $p > 0.05$). Second, in *XPC* rs2228000, there was no significant difference between the case and control groups (Table III, top panel, p for trend > 0.05). Last, concerning *XPC* rs2228001, although there was no significant difference between the case and control groups with regards to the frequency of the AA, AC and CC genotypes (Table III, bottom panel, p for trend > 0.05), it seemed that both AC and CC genotypes were higher in the case group (47.3 and 12.9%) than the control group (46.8 and 10.8%), respectively (Table III, bottom panel). Overall, it can be seen that not only the *XPC* rs2228000 polymorphism, but also *XPC* rs2228001 failed to serve as a predictor of oral cancer risk in Taiwan.

We also examined the distribution of the allelic frequencies for *XPC* rs2228000 and rs2228001, and results are presented in Table IV. The variant T allele in *XPC* rs2228000 was not to be associated with an increased oral cancer risk (OR=1.04, 95%CI=0.91-1.18, $p=0.5905$) (Table IV, top panel). At the same time, the variant C allele of *XPC* rs2228001 was not associated with and increased risk of oral cancer either (Table IV, bottom panel).

Since oral cancer has been found to be closely related with the consumption of cigarette, alcohol and betel quid in Taiwan, it is important to investigate the interaction between *XPC* rs2228000 and rs2228001 genotypes with these risk behaviors and whether such an interaction poses an even higher risk for getting oral cancer. Interestingly, there is a significant joint effect of *XPC* rs2228001 with smoking habits on oral cancer, which is shown in Table V ($p=0.0283$). At the same time, there was no higher risk for non-smokers (Table V, bottom panel). However, there was no significant difference for *XPC* rs2228000 among smokers or non-smokers (data not shown). Next, we stratified the age- and gender-matched oral cancer patients and controls according

to their alcohol drinking behaviors, and results are shown in Table VI. We found that there was no higher risk for either drinkers or non-drinkers for *XPC* rs2228001 (Table VI) and *XPC* rs2228000 (data not shown). Last, we also stratified the age- and gender-matched oral cancer patients and controls according to their betel quid chewing behaviors and analyzed their joint effect (Table VII). We found that there is a significant joint effect of *XPC* rs2228001 with a personal habit of betel quid chewing on oral cancer ($p=0.0300$, Table VII top panel). However, there was no altered oral cancer risk for non-betel quid chewers with the variant AC or CC genotypes in *XPC* rs2228001 ($p=0.1480$) (Table VII, bottom panel). There was no higher risk for either betel quid chewers or non-chewers as for *XPC* rs2228000 (data not shown).

We examined the expression levels of *XPC* mRNA and protein according to their *XPC* rs2228001 genotypes and the results are presented in Figures 1 and 2. There is a slight trend that CC genotypes at *XPC* rs2228001 have lower expression of *XPC* at protein and mRNA levels, while it did not reach a statistical significant level ($p=0.0919$, Figure 1A). After the combination of AC with CC, it still did not reach a statistical significance compared with AA genotype ($p=0.1355$, Figure 1B). Interestingly, compared to those with wild-type AA genotype, oral cancer patients with variant CC genotype was of lower protein level ($p=0.0106$, Figure 2B). After the combination of AC with CC, it still reached a statistical significance compared with AA genotype ($p=0.0376$, Figure 2C).

We examined the DNA repair capacity of oral cancer patients with different *XPC* genotypes and results are shown in Figure 3. Thirty- oral cancer patients were collected. They were all non-smokers, non-alcohol drinkers and non-betel quid chewers. According to their genotyping results, there is 15, 14 and 6 individuals carrying AA, AC and CC genotypes at *XPC* rs2228001. Based on these pilot results, those with CC genotype at *XPC* rs2228001 were of lower DNA repair capacity than those of wild-type AA genotype ($p=0.0306$, Figure 3A). After the combination of AC with CC, the group was still statistically lower than AA genotype ($p=0.0279$, Figure 3B).

Table V. Distribution of *XPC* rs2228001 genotypes among the 958 oral cancer patients and 958 non-cancer healthy controls after stratification by smoking status.

Smoking status	<i>XPC</i> rs2228001 genotype			<i>p</i> -Value ^a
	AA (%)	AC (%)	CC (%)	
Smokers				
Controls	287 (43.0%)	312 (46.7%)	69 (10.3%)	0.0283*
Patients	276 (38.4%)	336 (46.8%)	106 (14.8%)	
Non-smokers				
Controls	119 (41.0%)	136 (46.9%)	35 (12.1%)	0.2160
Patients	105 (43.7%)	117 (48.8%)	18 (7.5%)	

^aBased on Chi-square without Yate's correction test; the significant *p*-Value and odds ratio are bolded and marked with a star (*).

Table VI. Distribution of *XPC* rs2228001 genotypes among the 958 oral cancer patients and 958 non-cancer healthy controls after stratification by alcohol drinking status.

Drinking status	<i>XPC</i> rs2228001 genotype			<i>p</i> -Value ^a
	AA (%)	AC (%)	CC (%)	
Drinkers				
Controls	274 (42.7%)	299 (46.6%)	69 (10.7%)	0.3870
Patients	273 (39.9%)	323 (47.2%)	88 (12.9%)	
Non-drinkers				
Controls	132 (41.8%)	149 (47.1%)	35 (11.1%)	0.6970
Patients	108 (39.4%)	130 (47.5%)	36 (13.1%)	

^aBased on Chi-square without Yate's correction test.

Table VII. Distribution of *XPC* rs2228001 genotypes among the 958 oral cancer patients and 958 non-cancer healthy controls after stratification by betel quid chewing status.

BQ status	<i>XPC</i> rs2228001 genotype			<i>p</i> -Value ^a
	AA (%)	AC (%)	CC (%)	
Chewers				
Controls	223 (43.9%)	236 (46.5%)	49 (9.6%)	0.0300*
Patients	304 (39.3%)	358 (46.3%)	111 (14.4%)	
Non-chewers				
Controls	183 (40.7%)	212 (47.1%)	55 (12.2%)	0.1480
Patients	77 (41.6%)	95 (51.4%)	13 (7.0%)	

BQ: Betel quid. ^aBased on Chi-square without Yate's correction test; the significant *p*-Value and odds ratio are bolded and marked with a star (*).

Discussion

Certain points of the study must be underlined. First, we examined the contribution of *XPC* genotypes to elevated oral cancer risk among an extremely large population of

Taiwanese, containing 958 oral cancer patients and 958 age, gender-matched healthy controls. The results showed that *XPC* rs2228001 C allele carriers were of a non-significant higher risk for oral cancer, while this significance was not found for the *XPC* rs2228000 genotypes (Table IV). Second,

Figure 1. Analysis of XPC mRNA expression levels among oral cancer patients. (A) Quantitative RT-PCR of oral cancer tissue samples for the three genotypes of XPC rs2228001 was performed. GAPDH was used as an internal control. Fold changes were normalized using the levels of GAPDH expression, and each assay was performed at least in triplicate. (B) The AC and CC groups were combined and compared with the AA group.

Figure 2. The expression levels of XPC in oral cancer tissues from patients with different XPC rs2228001 genotypes. (A) Western blot analysis of XPC expression in tumor tissues from cases with AA, AC, and CC XPC rs2228001 genotypes. (B) Quantification of the western blot data from (A). β -actin was used as the loading control. Data were averaged from at least three repeat analyses of the tissues of each group, with 15 μ g total sample protein for each lane. (C) The AC and CC groups were combined and compared with the AA group.

Figure 3. The DNA repair capacity of oral cancer patient cells with different XPC rs2228001 genotypes. (A) DNA repair capacity from cases with AA, AC, and CC XPC rs2228001 genotypes. (B) The AC and CC groups were combined and compared to the AA group.

the variant genotypes of *XPC* rs2228001 were associated with an elevated oral cancer risk in the group of smokers and betel quid chewers, but not in the non-smoker or non-chewer groups (Tables V and VII). Third, the mRNA and protein levels were first investigated using the samples from oral cancer patients (Figures 1 and 2). Last but not the least, we found that the DNA repair capacity was lower in cells from patients carrying variant genotypes at *XPC* rs2228001 than those carrying wild-type (Figure 3).

The novel findings showed that the variant AC and CC genotypes at *XPC* rs2228001 cannot serve as a good biomarker for oral cancer risk prediction in Taiwan. On the contrary, these results can help us predict whether a smoker or betel quid chewer has a higher risk for oral cancer. An increased risk has also been seen in several types of cancers, including breast (33, 34), lung (35), bladder (11, 36), colorectal (37), prostate (38), gastric cancer (39) in various populations. Notably, the role of *XPC* in oral cancer is still lacking, and we are the first to investigate its expression levels of mRNA and protein. Most of all, we have the access to measure the DNA repair capacity in oral cancer patients. Interestingly, in a meta-analysis, *XPC* rs2228001 was significantly associated with an increased overall cancer risk, especially in Asian populations (40). However, in that study, the OR were not obvious, and 95%CI were all near 1.00 (40). Thus, in the current study, although we examined up to about 1,900 cases, *XPC* rs2228001 seemed not to serve as a biomarker alone. It should interact with smoking or betel quid chewing, to be significant. It is of interest to investigate whether *XPC* rs2228001 can serve as a significant biomarker for these types of cancers in Taiwan in the near future.

It is frequently a difficult mission for SNP studies about the genotype-phenotype correlation, since the phenotypic samples are not easily available. Thus, the SNP studies seldom provide data from the angles of mRNA or protein levels. However, it is very helpful in revealing the biological meanings of these SNPs, extending our understanding of the personalized etiology of each oral cancer patient. In this study, with 35 samples collected from the oral cancer patients, we determined their expression of XPC at the mRNA and protein levels. The protein patterns showed that oral cancer patients with CC genotypes at *XPC* rs2228001 were of significant lower level than those with wild-type AA genotype (Figure 2B). Furthermore, a combination of AC plus CC genotypes at *XPC* rs2228001 was also higher than AA genotype (Figure 2C). Very possibly, a lower expression of protein was caused by the limited number of examined samples. Most valuably, we investigated the DNA repair capacity according to the various *XPC* rs2228001 genotypes. The results showed that people carrying AC and CC genotypes at *XPC* rs2228001 are of lower DNA repair capacity and thus may have higher risk in oral cancer (Figure 3). However, the samples we collected were from non-smoking, non-alcohol and non-betel quid oral cancer patients. In the future, not only oral cancer patients, but also healthy individuals should be collected. Also, more samples as well as those collected from smokers, alcohol drinkers, and betel quid chewers and corresponding measurements are valuable to fully understanding the genotype-behavior correlation.

In conclusion, the study provides evidence that the CC genotype of *XPC* rs2228001 is associated with decreased

DNA repair capacity, contribute to higher risk of oral cancer in Taiwan. Genotype prediction is useful for those smokers and betel quid chewers. Furthermore, genotype-phenotype studies would be very valuable to reveal the relationship between XPC and oral etiology.

Conflicts of Interest

All the Authors declare no conflicts of interest regarding this study with any company or any person.

Authors' Contributions

Research design was done by WCN and CWS. Patient and questionnaire summaries were provided by SLC and HTC. Experimental work was done by CWS, WYC, and YCC. Statistical analysis was done by LHT, WZH, and MMC. TCW and BDT wrote the manuscript, whereas BDT reviewed it and are responsible for the revision.

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