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Ethnicity and Habits in p53 Gene Mutation in Oral Squamous Cell Carcinoma

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ABSTRACT

Objectives: Our previous study demonstrated that 31% (n=58/18) of OSCC patients have p53 gene mutation. In this study, we investigate the relationship of ethnicity and habit to p53 *gene* mutation of oral squamous cell carcinoma (OSCC).

Methods: The present study used Multiplex Ligation-dependent Probe Amplification (MLPA) to examine p53 gene mutation from exon 1 to 11. DNA specimens from 58 OSCC patients and 10 healthy people (controls) were used in this study. Our results demonstrated that 31% (n=58/18) of OSCC patients have p53 gene mutation. Among them 56% (n=10/18) of mutation occurred in exon 3, followed by exon 4 which was 50% (n=9/18).

Result: The Malay, Indian, and Chinese ethnic groups each accounted for 28% of OSCC patients with p53 gene mutations. 56% of the individuals had a habit of smoking, drinking alcohol, or chewing betel quid.56% of the individuals had a habit of smoking, drinking alcohol, or chewing betel quid. Sixty percent (n=6) of Chinese and Malay patients have mutations in almost all exons, while Indian patients primarily have mutations in exon 4.

Conclusion: Our current study suggests that further investigation is needed to understand the relationship between ethnicity, habits, and their interaction with genetic and epigenetic modifiers in exon-specific p53 mutations.

1. INTRODUCTION

The p53 gene is located on chromosome 17p and consists of 11 exons (Sana et al., 2012). Mutations in this tumor suppressor gene are common in more than 50% of all human tumors. More specifically, a high

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number of p53 mutations in p53 are common in human oral squamous cell carcinoma (OSCC). The major risk factors of OSCC are chronic tobacco and alcohol use, betel quid chewing contributes to its pathogenesis (Fischer et al., 2023). Several reports have examined the relationship between p53 expression and risk habits; however, few studies have demonstrated a correlation between the dose and the duration of these habits.

Previous studies on OSCC in the western part of India reported that p53 mutations were not detected restriction site mutation, Vora et al., (2010); Jenkins et al., (2003) in the hot spots of exons 5 through 8 associated OSCC as in some form of mutated p53 proteins not detectable by some molecular techniques. Additionally, p53 mutations differ based on geographical location and ethnicity, in addition, some variants in the p53 gene are enriched in specific ethnic groups (Greenblatt et al., 1994; Raybaud-Diogène et al. 1996, Lazarus et al., 1996; Cruz et al., 2002; Wong et al., 1998; Barnoud et al., 2019).

The present study, therefore, aimed to investigate the association between exons of p53 mutation habits, and ethnicity.

2. METHODOLOGY

2.1 Sample collection

This report was the continuation of our previous report **(**Sulaiman, et al., 2018), all of the samples were obtained from patients with written informed consent, and this study was approved by the Medical Ethics Committee, Faculty of Dentistry, and University of Malaya (OI DF 1303/0041 (L).

58 frozen tissues (DNA) of OSCC samples and 10 normal samples (DNA) with the clinical information and demographic profile were taken from the Oral Cancer Research Coordinating Centre, Universiti Malaya as it is one of the tissue banks in Malaysia.

2.2 Purity and Concentration

The purity (A260/280) and concentration of the samples was measured by using Nanodrop 1000. 2 ul of the samples were used to measure purity and concentration. Only samples that have a good purity (1.75 – 2.00) were included. The concentration was standardized to 50 ng/ul. A high concentration of samples was diluted with Tris EDTA (TE) buffer and a low concentration of samples was concentrated with the concentrator machine.

2.3 Multiplex Ligation Probe-dependent Amplification (MLPA)

The MLPA protocol has five steps. There was DNA denaturation, hybridization reaction, ligation reaction, PCR reaction and fragmentation. 0.2 ml micro centrifuge tubes were labeled according to the sample and 5 µl of DNA was added to each tube. TE buffer (10mM tris HCL plus 0.1mM EDTA) was used for dilution of samples. The tubes were placed in thermal cycler and MLPA program was set and started with the denatured process for 5 minutes at 98 ºC and then cooled to 25 ºC.

After that, the hybridization master mix was prepared by adding 1.5 µl of MLPA buffer and 1.5 µl of probemix for each reaction. The solution was mixed well by pipetting up and down or by gently vortex. 3 µl of the hybridization master mix was added to the DNA samples for denaturing. The hybridization master mix and the DNA were mixed well. The MLPA program was continued with incubation for one min at 95 ºC and followed by other incubation for 16 to 20 hours at 60 ºC.

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After the incubation, the ligase master mix was prepared by adding 3 µl of Ligase-65 buffer A, 3 µl Ligase-65 buffer B and 25 µl of distilled water for each sample. The solution was mixed well by pipetting up and down or gently vortex. Then, 1 µl of Ligase-65 was added to each ligase master mix reaction and mixed well. The MLPA program was continued by pause at 54 ºC. 32 µl of the ligase master mix was added to each sample to hybridize the DNA sample. The sample was mixed well and continued the MLPA program with ligate for 15 minutes at 54 ºC, heat inactivates for 5 minutes at 98 ºC and pause at 15 ºC.

The new PCR tubes were labeled according to the sample for PCR reactions. The PCR buffer mix was prepared by adding 4 µl SALSA PCR buffer and 26 µl of distilled water for each sample and mixed well. 30 µl of the PCR buffer mix was added to each new tube and then 10 µl of each ligation product was transferred to a corresponding PCR tube. The polymerase master mix was prepared by adding 2 µl SALSA PCR-primes, 2 µl SALSA Enzyme dilution buffer, 5.5 µl of distilled water and 0.5 µl SALSA Polymerase for each sample. The solution was mixed well and stored in ice until used. The MLPA program was continued by pausing at 60 ºC. The PCR tubes were placed in thermal cycler and 10 µl of polymerase master mix was added to the ligated DNA and mixed well. The MLPA program was continued immediately, which were 30 seconds at 95 ºC, 30 seconds at 60 ºC, and 60 seconds at 72 ºC for 35 cycles. It was ended with 20 minutes of incubation at 72 ºC and pause at 15 ºC.

MLPA methods provided internal control as a reference sample. DNA samples obtained from the same type of tissue of the healthy patients were used as reference samples.

Finally, the MLPA products were analyzed using a Genetic Analyser (Applied Biosystem, USA; Model: 3730). The results were displayed as a ratio between the reference and experimental samples. According to the manufacturer, a ratio between 0.75 to 1.3 was considered normal, < 0.75 was considered as deletion/loss, and >1.3 was considered as amplification/gain.

2.4 Statistical analysis

Statistical analysis was performed using Fisher-exact test using SPSS 20.0 to associate TMD development to stress with a significance level set at $P \leq 0.05$.

3. RESULT

Out of 58 patients, our study detected 31% (n=58/18) of OSCC patients with p53 gene mutation at different exons. Most of the mutations were observed in exon 3 and exon 4. A 56% (n=10/18) mutation was detected at exon 3 and 50% (n=9/18) at exon 4. The mutations were detected either in deletion or amplification form in all exons of the p53 gene. 59 % (n=11) of the patients with p53 gene mutation were females. **(**Sulaiman et al., 2018).

The mean age of these patients is 63 years old. This study showed a similar percentage of OSCC patients with p53 gene mutation across the three major ethnic groups. The Malay, Indian and Chinese ethnic groups each accounted for 28% of the OSCC patients with p53 gene mutation the remaining 16% were made up of other ethnic groups (Figure 4.11). 56% of them had a habit of either smoking, drinking alcohol, betel quid chewing or a combination of them (Figure 4.12). Both betel quid chewing (30%), and a combination of smoking and drinking made up 30%. Smoking (20%), combination of smoking and betel quid chewing accounted for 20% (Figure 1).

This study found that 39% (n=7) of the cases involved single mutations, while 61% (n=11) had multiple mutations. These results indicate that multiple mutations in oral squamous cell carcinoma (OSCC) are more prevalent than single mutations. In cases with multiple mutations, the four most commonly affected exons were exons 3, 4, 6, and 9 (Figure 1 and 2; Table 1-5).

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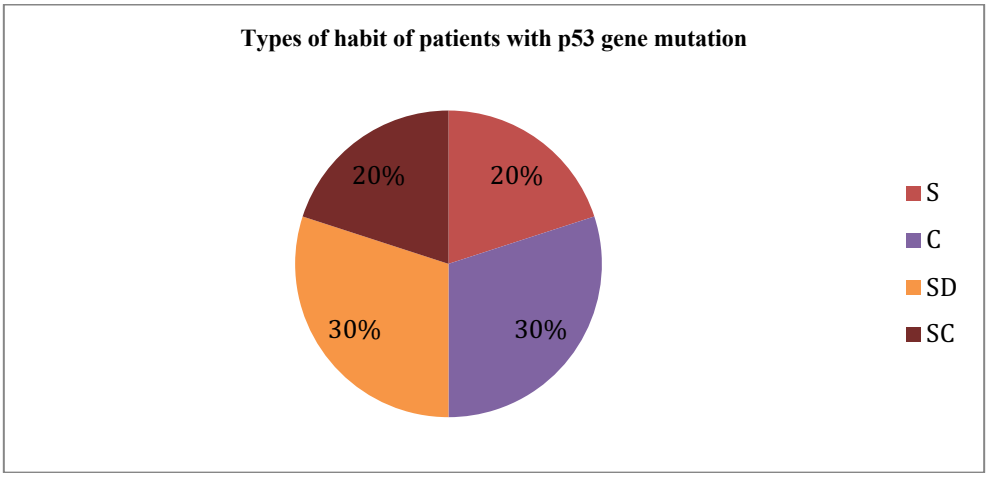

Fig 1. Types of habit of patients with p53 gene mutation. S: Smoking; C: betel quid Chewing; SD: Smoking and Drinking; SC: Smoking and betel quid Chewing.

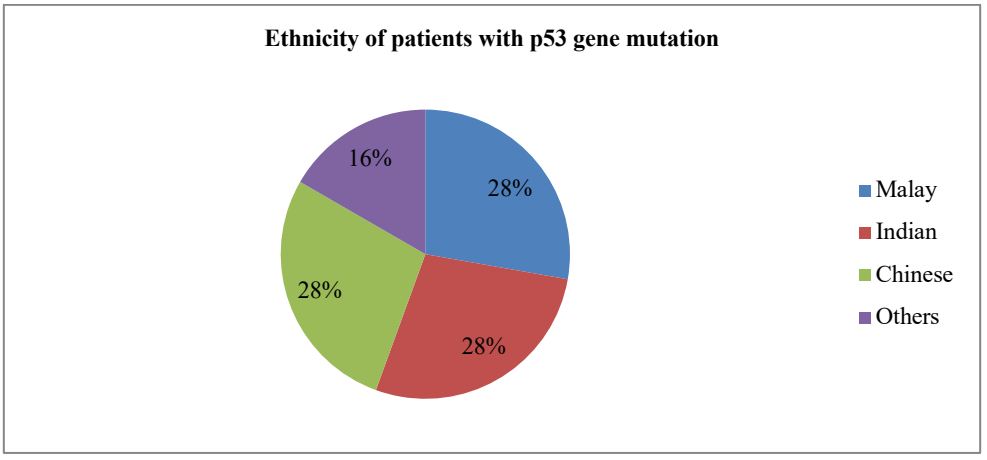

Fig. 2. Distribution of OSCC patients with p53 gene mutation according to ethnicity.

3.1 Correlation between types of habit and mutated exon in p53 gene mutation:

The correlation between smoking habits and mutated exons in the p53 gene mutation in this study showed that 61% (n=11) of patients had no smoking habit, whereas the remaining 39% (n=7) did. Analysis of exons 3 and 6 showed an equal number of total mutations in smokers and non-smokers: 5 patients for exon 3 and 3 patients for exon 6. There are eight patients with mutations in exon 4 who do not have a history of smoking, while only one patient with a history of smoking has mutations in the same exon. This suggests that smoking has a minimal impact on mutations in exon 4 of the p53 gene. (Table 1).

In the study, 83% of the patients (15 individuals) reported that they did not have any drinking habits, while 17% (3 individuals) did engage in drinking. Notably, the highest number of patients without drinking habits—nine in total—had mutations in exon 4. In contrast, none of the patients who engaged in drinking exhibited mutations in exon 4 (Table 2). Seventy-two percent (n=13) of patients did not chew betel quid, while the remaining twenty-eight percent (n=5) had a chewing habit (Table 3). The highest prevalence of mutations in exon 3 was observed among patients who did not chew betel quid, with eight patients

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presenting mutations. In contrast, only two patients who chewed betel quid exhibited mutations in exon 3. (Table 1- 5).

Table 1: Smoking habit vs exon mutated

	Patient	Mutated Exon	Habit	Patient	Mutated Exon
Smoking	1	3,10	No Smoking	2	2,3,9
	3	3,6,7,9		7	3,4,6,9
	4	1,2,4,6,9,10,11		8	4
	5	2,3,9		9	3
	6	1,3,6,9		10	1,4
	13	5		11	4
	15	3		12	4
				14	2,3,4,6,7,10
				16	1,4
				17	10
				18	1,2,3,4,5,6,7,8,10,11
Total	7 (39%)		Total	11 (61%)	

Table 2. Drinking habit vs exon mutated

	Patient	Mutated Exon	Habit	Patient	Mutated Exon
Drinking	1	3,10	No Drinking	2	2,3,
	6	2,3,9		3	3,6,7,9
	15	3		4	1,2,4,6,9,10,11
				5	2,3,9
				7	3,4,6,9
				8	4
				9	3
				10	1,4
				11	4
				12	4
				13	5
				14	2,3,4,6,7,10
				16	1,4
				17	10
				18	1,2,3,4,5,6,7,8,10,11
Total	3 (17%)		Total	15 (83%)	

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Table 3: Chewing habit vs exon mutated

	Patient	Mutated Exon	Habit	Patient	Mutated Exon
Chewing	3	3,6,7,9	No chewing	1	3,10
	4	1,2,4,6,9,10,11		2	2,3,9
	8	4		5	2,3,9
	14	2,3,4,6,7,10		6	1,3,6,9
	16	1,4		7	3,4,6,9
				9	3
				10	1,4
				11	4
				12	4
				13	5
				15	3
				17	0
				18	1,2,3,4,5,6,7,8,10,11
Total	5 (28%)		Total	`13 (72%)	

Table 4. Habit and the total of mutation of each exon

Exon / Habit	1	2	3	4	5	6	7	8	9	10	11
Smoking	2	2	5	1	1	3	1	0	4	2	1
No Smoking	3	3	5	8	1	3	2	1	2	3	1
Drinking	0	1	3	0	0	0	0	0	1	1	0
No Drinking	4	5	7	9	2	5	3	1	5	4	2
Chewing	2	2	2	4	0	3	2	0	2	2	1
No Chewing	3	3	8	5	2	3	1	1	4	3	1

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Table 5. Ethnicity vs site vs exon

No	Case	Gender	Age	Ethnic	Smoking	Alcohol	Quid chewing	Site	Stage	Mutated Exon
1	1	M	75	Chinese	+	+	-	Gum	I	3,10
2	2	M	70	Malay	-	-	-	Buccal	I I	2,3,
3	3	F	48	Malay	+	-	+	Buccal	I I I	3,6,7,9
4	5	F	49	Malay	+	-	+	Buccal	I I I	1,2,4,6,9,10,11
5	6	M	34	Chinese	+	-	-	Tongue	I I I	2,3,9
6	7	M	73	Chinese	+	+	-	Gum	IV	2,3,9
7	8	F	71	Malay	-	-	-	Buccal	IV	3,4,6,9
8	10	F	56	Indian	-	-	+	Floor of mouth	I I I	4
9	13	M	70	Chinese	-	-	-	Palate	IV	3
10	23	F	61	Malay	-	-	-	Lip, buccal, gingiva	I I I	1,4
11	24	F	44	Indian	-	-	-	Floor of mouth	IV	4
12	25	M	74	Indian	-	-	-	Buccal	IV	4
13	36	F	67	Iban	+	-	-	Retromolar	IV	5
14	38	F	77	Others	-	-	+	Gingiva, hard palate	IV	2,3,4,6,7,10
15	43	F	60	Others	+	+	-	Tongue	IV	3
16	46	F	69	Indian	-	-	+	Maxilla	IV	1,4
17	51	F	77	Indian	-	-	-	Buccal	I I	10
18	53	M	66	Chinese	-	-	-	Gingiva	IV	1,2,3,4,5,6,7,8,10,11

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Table 6: Demographic and clinical profile of most common exons affected by mutation of p53 gene in OSCC cases.

Exon	3	4	6	9
No of patients (n)	10 (32%)	9 (30%)	6 (19%)	6 (19%)
Parameters				
Sex				
Male	6 (60%)	2 (22%)	2 (33%)	3 (50%)
Female	4 (40%)	7 (78%)	4 (67%)	3 (50%)
Age				
Below 30	0	0	0	0
31-49	2 (20%)	2 (22%)	2 (33%)	3 (50%)
50-69	2 (20%)	4 (44%)	1 (17%)	0
70 and above	6 (60%)	3 (34%)	3 (50%)	3 (50%)
Ethnicity				
Malay	3 (30%)	3 (33%)	3 (50%)	4 (67%)
Indian	0	4 (44%)	0	0
Chinese	5 (50%)	1 (4%)	2 (33%)	2 (33%)
Others	2 (20%)	1 (4%)	1 (17%)	0

4. DISCUSSION

In Malaysia, the three major ethnic groups are Malay, Indian and Chinese. Other ethnics include Iban, Kadazan and Bidayuh. Most of these minor ethnics are from the East of Malaysia (Sabah and Sarawak). In this study, the Indian population showed the highest incidence of OSCC at 34%; n=20), followed by Malay (25%; n= 15), Chinese (24%; n=14) and other ethnics (16%; n= 9). It is noteworthy to mention that in Malaysia, OSCC is recognized as the 19th most common cancer. Further, OSCC is ranked 8th and 4th most common malignancy among Indian males and females (Azizah et al., 2016; Chan et al., 2023; Zain et al., 1997; Zain et al., 2001). The Indian population has a higher tendency to chew betel quid. The combination of betel quid chewing and smoking habits is found in 13% of the patients. Further studies with more sample sizes for each ethnicity are essential (Fig. 1 and 2).

In this study, we compared the mutational sites of the p53 gene across different ethnicities. The findings revealed that Indian patients (n=5/18) predominantly had mutations at exon 4 (n = 4), while exons 1 and 10 each had one mutation. In contrast, Malay patients (n=5/18) displayed mutations in multiple exons, specifically exons 1, 2, 3, 4, 5, 6, 7, and 9. Chinese patients (n=5/18) also exhibited multiple exon mutations, with alterations found in exons 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, specifically, exon 3 and exon 9 showed 4 and 9 mutations, respectively (Table 1- Table 6).

We compared the most common mutational sites of the p53 gene detected in our study (exon 3, exon 4, exon 6, and exon 9, p-value < 0.001) across different habits, In exon 3, both smoking and non-smoking patients showed an equal number of mutation, with five patients in each group, in exon 4 smoking detected mutation in only one patient and non-smoking showed mutation in eight patients, in exon 6 both smoking and non-smoking patients showed an equal number of mutation, with three patients in each group, in exon 9 smoking detected mutation in four patients and non-smoking showed mutation in two patients.

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In exon 3, drinking detected a mutation in three patients while non-drinking showed a mutation in five patients, in exon 4 only non-drinking showed a mutation in nine patients, in exon 6 only non-drinking showed a mutation in five patients, in exon 9 drinking detected mutation in one patient and non-drinking showed a mutation in five patients.

In exon 3, betel quid chewing detected mutation in two patients while non-chewing showed mutation in eight patients, in exon 4 betel quid chewing detected mutation in four patients and non-chewing showed mutation in five patients, in exon 6 mutation both betel quid chewing and non-chewing groups detected mutation equally in three patients, in exon 9 betel quid chewing detected mutation in two patients while non-chewing showed mutation in four patients (Table 1- Table 6).

Previous studies on p53 mutations in OSCC reported that the detection of mutations depends on molecular techniques applied, geographical location, ethnicity, and genetic and epigenetic modifications of (Greenblatt et al., 1994; Raybaud-Diogène et al. 1996, Lazarus et al., 1996; Cruz et al., 2002; Wong et al., 1998; Barnoud et al., 2019).

An increasing number of studies have identified specific lifestyle factors, such as smoking, betel quid chewing, and alcohol consumption, that may influence epigenetic modulation in carcinogenesis. However, the role of p53 in maintaining epigenetic stability in oral squamous cell carcinoma (OSCC) is still poorly understood. The interaction between the mutational status of p53 and its stability, along with the crosstalk between genetics and epigenetics, represents a significant area of investigation. Understanding this relationship is crucial for unraveling the underlying mechanisms of cancer and for developing more effective treatments and preventive strategies (Wang et al., 2017).

More importantly, recent studies indicate that advancements in high-throughput assays, CRISPR technology, patient-specific diagnostic tests, and AI-based methods can offer deeper insights into cellular transformation of OSCC. These developments may enable the creation of clinically applicable tests for personalized and quantifiable cancer risk assessments, as well as predictive markers. The advancement of more accurate and personalized cancer risk assessment tools is essential for uncovering the specific molecular mechanisms of p53 involved in the development and progression of OSCC. Additionally, it is important to understand the genetic and epigenetic risk factors linked to cancer susceptibility across different ethnic groups and lifestyle habits. Developing more accurate and personalized cancer risk assessment tools is crucial to uncovering the precise underlying molecular mechanisms of p53 in the development and progression of OSCC and understanding the genetic epigenetic risk factors associated with cancer susceptibility across ethnicities and habits (Redman-Rivera et. al., 2021; Erwood et. al., 2022; Raad et. al., 2021; Noguchi et. al., 2022; Ma et. al., 2022; Ben-Cohen et. al., 2022; Fischer et al., 2023).

5. CONCLUSION

The results of this study emphasize the significant interaction between certain lifestyle factors, such as smoking, betel quid chewing, and alcohol consumption, and the mutational status and stability of p53, indicating a crucial area for further research. The development of more accurate and personalized tools for cancer risk assessment is crucial for revealing the specific molecular mechanisms of p53 that contribute to the onset and progression of OSCC.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest related to the contents of this article.

AUTHORS' CONTRIBUTIONS

Fatimah Suriati Sulaiman is involved as a Master's student in this Research Project. **Jamil Ahsan Kazi** is conceptualized the central research idea, developed the theoretical framework, conducted data analysis, drafted and revised the manuscript, and approved the submission of the article. Main supervisor of this Master project.

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7. APPENDIX

A. ABOUT THE AUTHORS

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