

Exon 3 of p53 gene is the hot spot region for Oral Squamous Cell Carcinoma**

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Abstract

p53 gene mutations observed at about 50 to 60 % in oral squamous cell carcinoma (OSCC). Until now, most of the studies were focused on exon 5 to 8 of p53 gene instead of the whole exons and it becomes a trend for p53 gene mutation study. However, the site of p53 gene mutation is still in controversy as available techniques showed different results. More importantly, the reports with the same technique demonstrated inconsistency mutation sites. The objective of the present study is to examine the status of p53 gene mutation in all exons (1 to 11). Recently, Multiplex Ligation-dependent Probe Amplification (MLPA) technique has proven to be reliable and efficient for the detection of mutation. Therefore, 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 peoples (controls) were used in this study. Our results demonstrated that 31% (n=58/18) of OSCC patient 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). Our study strongly indicated that exon 3 and 4 could be reliable and positive markers of OSCC development and progression. To our knowledge, this study for the first time showed that exon 3 is the hot spot region for p53 gene mutation in OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the worldwide¹ that are arising in the head and neck anatomical region which are arising from the lips, tongue, salivary glands, gingival, floor of the mouth, oropharynx, buccal surfaces and other oral locations¹⁻⁵. There are about 300,000 to 400,000 individuals worldwide are diagnosed annually with oral cancer and nearly 90% of them are diagnosed with OSCC⁶⁻¹¹. In Malaysia, it becomes one of the emerging cancer¹² where it is ranked at 20th most common cancer for females and 28th for males of overall cancer incidence per 100,000 populations in the year of

2006¹³. It is estimated that 21,773 Malaysians being diagnosed with cancer and about 10,000 unregistered cases per year and this means that one over four will develop cancer by 75 years old¹³.

There are many causes that lead to OSCC which are smoking, drinking, betel quid chewing, dietary factors, virus infection like HPV (Human Papilloma Virus) and also gene alteration^{14,15}. This study focused on one of the OSCC causes, which is gene alteration. Several genes that can cause OSCC were p53, p16, FAM5B, TIPARP, PIK3CA, THRAP3 and CTTNBP2NL in OSCC^{16,17}. Among these genes, p53 gene is the most significantly involved in carcinogenesis and its mutation indicates tumor progression. P53 positivity also indicates high risk of tumor recurrence and it may be associated with poor prognosis in OSCC.

Present study focusing on p53 gene mutation as p53 gene mutation occur about 50 to 60 % in all human cancers including OSCC¹⁸. In OSCC, alteration in p53 gene seems to be an early event in developing the carcinoma¹⁵.

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P53 gene plays a major role in preventing tumor development and it has been called as tumor suppressor gene and guardian of the genome¹⁹. This gene encompasses 16-20 kb of DNA on the short arm of human chromosome 17 and contains 11 exons²⁰. Most of the studies focused on p53 gene mutation of OSCC on exon 5 to 8 and it became a trend for p53 gene mutation study²⁰⁻²². It is noteworthy to mention that, p53 gene mutation in exons still in controversy. There were several reports observed p53 gene mutation not only confined to exon 5 to 8²³⁻²⁵. Moreover, there were no studies that focused on the all exons (1 to 11) of p53 gene mutation in OSCC cases.

Different technical approaches have been used to detect p53 gene mutation such as sequencing, PCR-SSCP, DNA micro-array, microsatellite, Fluorescent in situ hybridization (FISH), array-based comparative genomic hybridization (aCGH) and others^{17,21-26}. However, studies reported different mutation sites of p53 gene in OSCC. Notably, using same method in detecting mutational sites detection of the exons of the p53 gene in OSCC demonstrated inconsistency results. It might be due to different technical approach or the sensitivity of the techniques. As for example, several studies that used Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) showed different in mutational sites. PCR-SSCP as a tool to detect mutation, Acha-Sagredo et al., 2009 detected p53 gene mutation at only exon 6. However this result was not in line with Astori & Arzese, 2001, Popovic et al., 2009 and Thongsuksai et al., 2003. Astori & Arzese, 2001 those observed p53 gene mutation in exon 8 and 5 while Popovic et al., 2009 and Thongsuksai et al., 2003 demonstrated at exon 5, 6, 7, and 8^{21,27-29}. In addition, in previous studies, exon 4 to 9 have been reported as a hot spot region for p53 gene mutation³⁶. Therefore, above reports clearly indicated that a reliable sensitive technique was the cause of failure in detecting exact mutational profile of exons for p53 gene in OSCC. Reports emphasized that p53 mutational study for OSCC to incorporate with the all exons⁴².

It is noteworthy to mention that, each method demonstrated its own advantages and limitations those lead to the variation of the results³⁰. However, recent technology developments which is multiplex ligation-

dependent probe amplification (MLPA) have been shown to be the reliable and efficient technique for the detection of mutation¹⁷. This method showed consistent results with the cytogenetic method which is FISH (Fluorescence in situ hybridization)²⁶. Thus MLPA has become an established method to detect mutation³¹. Advantages of MLPA method includes minimum working concentration for DNA analysis¹⁶, allows detection of various kind of specimens for assay including the cell lines, tissues and also saliva^{16,32}. Other advantages observed of this detection assay were easy to handle, cost-effective and also time saving compared to the array-based such as DNA microarrays which is costly and time consuming³³.

Hence, the aim of this study was to investigate the p53 gene mutation at all exons (1 to 11) using MLPA technique within OSCC patients and to confirm and reveal the exact status of the exons of the p53 gene mutation of OSCC.

Materials and methods

Sample collection. 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 was taken from Oral Cancer Research Coordinating Centre, Universiti Malaya as it is one of the tissue banks in Malaysia.

Purity and Concentration. The purity (A260/280) and concentration of the samples was measured by using Nanodrop 1000. 2 ul of the samples was used to measure purity and concentration. Only samples that have a good purity (1.75–2.00) was 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.

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 °C and then cooled to 25 °C.

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 °C and followed by other incubation for 16 to 20 hours at 60 °C.

After the incubation, the ligase master mix was prepared by adding 3 µl of Ligase-65 buffer A, 3 µl Ligase-65 buffers 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 °C. 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 °C, heat inactivates for 5 minutes at 98 °C and pause at 15 °C.

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-primers, 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 pause at 60 °C.

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 °C, 30

seconds at 60 °C, and 60 seconds at 72 °C for 35 cycles. It was ended with 20 minutes of incubation at 72 °C and pause at 15 °C.

MLPA methods provided internal control as 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 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 in between 0.75 to 1.3 was considered as normal, < 0.75 was considered as deletion/loss and >1.3 was considered as amplification/gain.

Results

A total of 58 patients were enrolled in this study. Our study detected 31% (n=58/18) of OSCC patients with p53 gene mutation at different exons (Table 1). Most of the mutations observed in exon 3 and exon 4. 56% (n=10/18) mutation detected at exon 3 and 50% (n=9/18) at exon 4 (Table 2) in our study. The mutations detected in this study were either amplified or deleted for each exon.

Sample	Exon										
	1	2	3	4	5	6	7	8	9	10	11
1			Amp								Amp
2		Amp	Amp							Del	
3			Amp			Amp	Amp		Amp		
4	Del	Amp		Del		Amp			Del	Amp	Amp
5		Del	Amp						Amp		
6	Amp		Amp			Amp			Amp		
7			Amp	Amp		Amp			Amp		
8				Amp							
9			Amp								
10	Amp			Amp							
11				Amp							
12				Amp							
13					Amp						
14		Del	Del	Del		Del	Del			Del	
15			Amp								
16	Del			Amp							
17											Amp
18	Amp	Amp	Amp	Amp	Amp	Amp	Amp	Amp	Amp	Amp	Amp

Table 1. Showing that mutation detected either in deletion or amplification form in all exons of p53 gene. Amp: Amplification, Del: Deletion

Exon	Mutation Status		
	Mutation(n=Patient)	No mutation(n=Patient)	Mutation (%)
1	5	13	27
2	5	13	27
3	10	8	56
4	9	9	50
5	2	16	11
6	6	12	33
7	3	15	17
8	1	17	6
9	6	12	33
10	5	13	27
11	2	16	11

Table 2. showing 56% mutation detected in exon 3 and 50% mutation detected in exon 4.

Exon 3	Mutation Status		p value
	Yes	No	
Yes	10 (56%)*	0	< 0.001
No	8 (44%)**	40 (100%)	
Total	18	40	

Table 3. Showing a Fisher's Exact Test was applied to test the associations between exon 3 and mutation status for OSCC patients. A very significant association was observed, p value < 0.001.

*Number of OSCC patients with mutation at exon 3.

**Number of OSCC patients with no mutation at exon 3.

Exon 4	Mutation Status		p value
	Yes	No	
Yes	9 (50%)*	0	< 0.001
No	9(50%)**	40 (100%)	
Total	18	40	

Table 4. A Fisher's Exact Test was applied to test the association between exon 4 and mutation status for OSCC patients. A very significant association was observed, p value < 0.001.

*Number of OSCC patients with mutation at exon 4.

**Number of OSCC patients with no mutation at exon 4.

Discussion

Reports showed that in oral cancer, p53 gene mutation was about 25% to 69%³⁴. Our present study detected 31% (n=18/58) of p53 gene mutation among OSCC patients in our sample size (n=58) using MLPA method. The results of this study were in agreement

with the results of Chang et al., 2014 that showed that 32.91% of p53 gene mutation in OSCC patients³⁵.

Present study demonstrated that in general p53 gene mutation in OSCC occurred in all exons (Table 1). Most striking finding in this study was that the majority of the mutation observed at exon 3 (56%, n=10/18, Table 3) and 4 (50%, n=9/18; Table 4) with strong predominance of the mutation in exon 3. Both exon 3 and 4 showed significance with p value <0.001.

Hwang et al., 2012, using YD39OSCC cell line demonstrated a 33-bp nucleotide deletion by DNA sequencing (between codons 80 and 112) in exons 3–4 which indicated the existence of possible mutation in exon 3²⁴. The difference between our present study and above study are that the above study was performed using OSCC cell line by DNA sequencing method whereas, in our study OSCC tissues was examined by MLPA detection method. However, at least this report supported our hypothesis that exons of the p53 gene mutation in OSCC might not confine to 5-8 or 4-9. Therefore, further study is warranted to confirm this controversy. It is noteworthy to mention that, studies reported that abnormalities in p53 gene might have diagnostic, prognostic, and therapeutic implication³⁷. Sukhija et al., 2015 using PCR detection method demonstrated that exon 4 could be a potential molecular diagnostic marker for OSCC and demanded that exon 4 might be a probable hot-spots for the p53 gene mutation in OSCC³⁸. However our present study observed that exon 3 (56%, p < 0.001) was more noticeable than exon 4 (50%, p < 0.001). Most importantly, exon 1 to 3 were not included for the detection of mutation in that study and only focused on exon 4 for the investigation³⁸. Previous study demonstrated that, the polymerases that used in PCR were subjected to error rates from 1/10,000 to more than 1/500 base pairs³⁹.

Previous studies showed the inter- and intra-lesional mutational heterogeneity in head and neck squamous cell carcinoma. In our present study we also observed that even in a same sample there was mutational heterogeneity⁴³.

Our present study detected only one patient with mutation at exon 8. In contrast, previous report claimed that the hot spot region for p53 gene mutation that was observed between exon 5 to 8^{15,28}. In support to our result, Acha-Sagredo et al., 2009 reported that no alterations was found at exon 8²¹ in OSCC. Analysis of the above studies indicated that mutation in exon 8 might be rare in OSCC. However, further study is required to confirm these findings.

Reports showed that in oral cancer, p53 gene mutation was about 25% to 69%³⁴. Our present study detected 31% (n=18/58) of p53 gene mutation among OSCC patients in our sample size (n=58) using MLPA method. The results of this study were in agreement with the results of Chang et al., 2014 that showed that 32.91% of p53 gene mutation in OSCC patients³⁵.

Conclusion

Previous study showed that introduction of mutant p53 into OSCC cells promotes resistance to standard chemotherapeutics and radiation⁴⁰. The exact exon for mutational spectrum of p53 is an urgent demand. Some suggestive links could be made between specific types of exon's mutations and OSCC. Such as, histological grading of OSCC and correlate with its molecular events of development and progression^{41,42}.

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