

## ABSTRACT

---

**Introduction:** In the Malaysian population, the Indians were identified as having a higher oral cancer (OC) risk compared to Malays, Chinese and other ethnicities with betel quid chewing contributing the major factor among Indian female. Cancer progresses through a series of histopathological stages where the progression is thought to be driven by the accumulation of genetic alterations (Garnis et. al., 2009). Therefore, the identification of chromosomal alterations will not only enhance the understanding of the biology in this process, but will also identify important genes that might involve in oral carcinogenesis. Thus, this study utilised array Comparative Genomic Hybridization (arrayCGH) technology as a platform.

**Aims:** To identify chromosomal alterations leading to the identification of genes involved in the development of OC, and to determine the relationship between selected altered regions with sociodemographic characteristics which include gender, ethnicity and habits and selected clinicopathological parameters namely tumour nodes metastasis (TNM) stage, tumour size (T), lymph node status (N), tumour grade and tumour site.

**Methods:** A total of 20 fresh frozen tumour tissues diagnosed as OC and reference DNA from peripheral blood lymphocytes were obtained from the Malaysian Oral Cancer Data and Tissue Bank System (MOCDTBS)-Oral Cancer Research and Coordinating Centre (OCRCC), University of Malaya (UM). A series of 750 µm thick tissue sections were mounted in Optimal Cutting Temperature (OCT) compound and stained with H & E. Tissue sections containing more than 70% epithelial tumour were selected and confirmed by an oral pathologist. Analysis of arrayCGH was performed using the Human Genome CGH Microarray KIT 4x44K chip platform. Graphical

overview of chromosomal alterations was obtained by using the Genomic Workbench Standard Edition 5.0.14. Validation of *DUSP22* gene generated from arrayCGH results was then carried out at DNA (copy number) and cDNA (gene expression) levels. Statistical analysis was performed using SPSS version 19.

**Results:** In this study, the occurrence of deleted regions was slightly higher than the amplified regions. The most frequently deleted regions were detected on chromosome 19p13.3, followed by 19p13-p13.11 and 19q13.3 while amplifications were most commonly detected at 8q24.3, 8q11.1-8q11.2 and 3q26 regions. The most common amplified gene detected was *DUSP22*, followed by *KIAA0146*. For the deleted regions, the most common genes detected were *FBXO25*, *INPP5A*, *NKX6-2*, *C10orf92*, *CDH4*, *TAF4*, and *LSMI4B*. With respect to the clinicopathological parameters (tumour size (T), TNM stage, lymph node status (N), tumour grade and tumour site) and sociodemographic profile (gender, ethnicity and habits), no association was found between the most amplified (8q24.3, 8q11.1-8q11.2 and 3q26) and deleted regions (19p13.3, 19p13-p13.11 and 19q13.3). Only region 19p13-p13.11 showed significant relationship ( $p=0.044$ ) with tumour site. Validation and analysis at copy number (CN) and gene expression (GE) level for the *DUSP22* gene revealed consistent results with previous arrayCGH in which tumour cells showed over-expression.

**Conclusion:** This study showed no association between chromosomal alterations and the clinicopathological parameters and sociodemographic profile studied. Only tumour site showed correlation with chromosomal alteration. It is recommended that a bigger study be conducted using bigger sample size in order to obtain more significant association.

## ABSTRAK

---

**Pengenalan:** Di kalangan masyarakat Malaysia, etnik india merupakan golongan yang berisiko tinggi menghadapi penyakit kanser mulut berbanding etnik Melayu, Cina dan kaum lain. Ini adalah disebabkan oleh tabiat mengunyah sireh terutamanya di kalangan wanita etnik India. Perkembangan kanser dari normal ke metastasis berlaku melalui siri peringkat histopatologi di mana perebakan kanser adalah disebabkan oleh pengumpulan aberasi genetik (Garnis et. al., 2009).. Oleh itu, pengenalpastian aberasi kromosom bukan sahaja meningkatkan pemahaman mengenai proses biologi yang berlaku dalam kanser mulut bahkan dapat mengenalpasti gen-gen penting yang mungkin terlibat dalam perkembangan kanser mulut. Sehubungan dengan itu, penyelidikan berlandaskan teknologi microarray Comparative Genomic Hybridization (arrayCGH) dipilih sebagai platform utama penyelidikan ini.

**Objektif:** Untuk mengenalpasti aberasi kromosom dan gen-gen yang terlibat dalam perkembangan kanser mulut dan menentukan perkaitan di antara aberasi kromosom dengan ciri-ciri profil sosiodemografi serta parameter patologi klinikal tertentu termasuk peringkat TNM, saiz tumor (T) , status nodus (N), pengelasan tumor dan kawasan penyebaran tumor.

**Kaedah:** 20 tisu tumor sejukbeku didiagnosis sebagai kanser mulut disaring daripada databank MOCDTBS-OCRCC UM. 750  $\mu$ M tisu tumor sejukbeku dipotong dengan menggunakan cryostat dan diwarnakan dengan pewarnaan H & E. Diagnosis untuk sampel tisu melebihi 70% mengandungi epitelial tumor disahkan oleh pakar patologi mulut. Analisis arrayCGH dilakukan dengan menggunakan cip CGH Microarray KIT platform 4x44K. Grafik perubahan kromosom dianalisis dengan menggunakan perisian

Genomic Workbench Standard Edisi 5.0.14. Validasi gen *DUSP22* yang diperoleh dari arrayCGH dijalankan pada peringkat DNA ('copy number' (CN)) dan cDNA (pengekspresan gen (GE)). Analisis statistik dilakukan dengan menggunakan SPSS versi 19.

**Hasil:** Aberasi kromosom untuk delesi dikesan pada frekuensi yang tinggi pada kromosom 19p13.3, diikuti oleh 19p13-p13.11 dan 19q13.3 dan amplifikasi kromosom paling banyak dikesan pada kromosom 8q24.3, 8q11.1-8q11.2 dan 3q26. Sementara itu, amplifikasi gen *DUSP22* adalah yang paling kerap berlaku dalam kajian ini diikuti oleh gen *KIAA0146*. Sementara itu, untuk lokus delesi, gen yang paling banyak yang dikesan adalah *FBXO25*, *INPP5A*, *NKX6-2*, *C10orf92*, *CDH4*, *TAF4*, dan *LSM14B*. Berdasarkan kepada analisis statistik, tiada hubungan signifikan didapati di antara parameter patologi klinikal dengan aberasi kromosom yang paling kerap dikesan. Walau bagaimanapun, hanya lokus 19p13-p13.11 dikesan mempunyai hubungan yang signifikan di antara kawasan penyebaran tumor ( $p=0.044$ ). Validasi dan analisis untuk gen *DUSP22* pada peringkat CN dan GE mengesahkan bahawa sampel yang mengalami amplifikasi pada peringkat kajian arrayCGH juga mengalami over-expressed dan penggandaan kromosom.

**Kesimpulan:** Kajian ini menunjukkan bahawa tiada hubungan di antara aberasi kromosom dengan parameter patologi klinikal dan profil sosiodemografi. Hanya parameter kawasan penyebaran tumor dilihat mempunyai potensi untuk dikaitkan dengan prognosis awal kanser mulut. Kesimpulannya, adalah dicadangkan bahawa untuk kajian penyelidikan lebih lanjut, kajian terhadap aberasi kromosom harus dijalankan dalam skala saiz sampel yang besar, pengkhususan terhadap parameter

patologi klinikal yang lebih spesifik seperti lokasi tumor bagi mendapatkan hasil penyelidikan yang lebih signifikan.

## ACKNOWLEDGEMENT

Bismillahirrahmanirrahim, first of all, I would like to say Alhamdulillah, for giving me the strength and health throughout this research until its completion. This dissertation would not have been possible without the guidance and the help of several individuals who contributed and extended their valuable assistance in the preparation and completion of this study. First and foremost, I am heartily thankful to Professor Rosnah Mohd Zain and Professor Sharifah Noor Akmal Syed Husain, both my supervisors whose showed sincerity and encouragement that I will never forget. Their guidance and support from the initial to the final level enabled me to develop a better understanding of the study more thoroughly. Thanks Prof! Really appreciate it.

Not forgotten I would also like to express my utmost gratitude to my beloved husband, Abang Syahrizan Abang Slamsa and my family for their unfailing moral support and advice, who were always for me when I was having difficulties and gave me courage to finish this study. I was blessed with the advancement of internet technology, books, computers, as my sources to complete this project successfully.

Special thanks to Professor Dr Cheong Sok Ching, my colleagues and staff in Oral Cancer Research & Coordinating Centre (OCRCC), Diagnostic Laboratory of Oral Pathology, Oral Medicine and Periodontology (OPOMP), Cancer Research Initiatives Foundation (CARIF) and Molecular Cytogenetics Laboratory at Universiti Kebangsaan Malaysia Medical Centre (UKMMC), for their kind assistance, sharing ideas and guidance in the laboratory procedures and better understanding on oral cancer. Thanks all for your kind help! I also offer my regards and blessings to all of those who supported me in any respect during the completion of the study. Last but not the least,

above all of us, thanks to almighty Allah, for answering my prayers and giving me the strength to continue this study, thank you so much Dear Allah for the blessing. Alhamdulillah.

## TABLE OF CONTENTS

<b>Content</b>	<b>Page</b>
Abstract	ii
Acknowledgement	vii
Table of contents	ix
List of Tables	xiii
List of Figures	xiv
List of Appendix	xv
List of Abbreviations	xvi
 <b>CHAPTER</b>	
<b>1. INTRODUCTION</b>	<b>1</b>
<b>2. AIM AND OBJECTIVES</b>	
2.1 Aim	5
2.2 Specific Objectives	5
2.3 Rationale of Study	6
<b>3. LITERATURE REVIEW</b>	
3.1 Cancer	7
3.2 Epidemiology of Oral Cancer (OC)	8
3.2.1 OC Subsites	8
3.2.2 Epidemiological Terminologies	9
3.2.3 Prevalence and incidence of OC	9
3.2.3.1 Global Scenario	10
3.2.3.2 Malaysian Scenario	11
3.3 Clinical presentation of OC	12
3.4 Histopathology of OC	12
3.4.1 Well Differentiated	13
3.4.2 Moderately Differentiated	13
3.4.3 Poorly Differentiated	13



3.5	Clinicopathological Parameters in OC	14
3.6	Risk Habits	15
3.6.1	Betel Quid Chewing	16
3.6.2	Smoking Habits	17
3.6.3	Alcohol Consumption	18
3.6.4	Human Papilloma Virus (HPV)	19
3.6.5	Dietary Habits	20
3.6.6	Genetic Susceptibility	21
3.7	Carcinogenesis	21
3.8	Hallmarks of Cancer	24
3.8.1	Self Sufficiency in Growth Signals	24
3.8.2	Insensitivity to Antigrowth Signals	24
3.8.3	Evading Apoptosis	25
3.8.4	Limitless Replicative Potential	25
3.8.5	Sustained Angiogenesis	26
3.8.6	Tissue Invasion and Metastasis	26
3.9	Genes involved in OC	27
3.9.1	Oncogenes	27
3.9.2	Tumour Suppressor Genes (TSGs)	29
3.10	Cytogenetics Study	33
3.10.1	Human Chromosomes	33
3.10.2	Definition of Cytogenetics	33
3.10.3	Molecular Cytogenetics and Cytogenetics Techniques	34
3.10.3.1	Fluorescence <i>in situ</i> Hybridization (FISH)	34
3.10.3.2	Conventional Comparative Genomic Hybridization (CGH)	35
3.10.3.3	Array Comparative Genomic Hybridization (arrayCGH)	36
3.11	Chromosomal Alterations and OC	38
3.11.1	Studies on CGH	38
3.11.2	Studies on arrayCGH	39
3.12	Validation Technique - Real Time quantitative Polymerase Chain Reaction (RT-qPCR)	42

## 4. MATERIALS AND METHODOLOGY

4.1	Study Design	43
4.2	Sample Selection	
4.2.1	Test Samples	43
4.2.2	Reference Samples	44
4.3	Criteria	
4.3.1	Inclusion criteria	45
4.3.2	Exclusion criteria	45
4.4	Processing of Frozen Tissue Samples	46
4.5	Genomic DNA (gDNA) Extraction Procedure	46
4.6	Oligonucleotide arrayCGH Protocol	47
4.7	Validation of <i>DUSP22</i> gene using RT-qPCR	48
4.8	Statistical Analysis	50

## 5. RESULTS

5.1	Socio-demographic Characteristics of the Study	53
5.2	Characterizing Chromosomal Alterations and Gene Identification in OC	54
5.2.1	Chromosomal Alterations	54
5.2.2	Genes Identified	58
5.3	Relationship between Chromosomal Alterations and Selected Clinicopathological Parameters and Socio-demographic Data	60
5.4	Validation of results using RT-qPCR of <i>DUSP22</i> gene	
5.4.1	Melt Curve for RT-qPCR analysis of <i>DUSP22</i> gene and Internal Control	70
5.4.2	The distribution of copy number (gDNA) for <i>DUSP22</i>	71
5.4.3	The distribution of gene expression (cDNA) for <i>DUSP22</i>	72
5.4.4	Similarity between arrayCGH, copy number (CN) and gene expression (GE) study	73

<b>6.</b>	<b>DISCUSSION</b>	
6.1	Limitations of Study	77
6.2	Chromosomal Alterations	78
6.2.1	Most Amplified Regions Detected (8q24.3, 8q11.1-11.2 and 3q26)	80
6.2.2	Most Deleted Regions Detected (19p13.3, 19p13-13.11 and 19q13.3)	82
6.2.3	Other Alterations in OC and Comparison with previous Studies.	85
6.3	Chromosomal Alterations and Its Association with Clinicopathological Parameters	87
6.3.1	Socio-demographic Profile	87
6.3.2	Clinicopathological Parameters	
	6.3.2.1 Primary Tumour Site	89
	6.3.2.2 Tumour Size & Thickness (T)	90
	6.3.2.3 Lymph Node status (N)	91
	6.3.2.4 TNM staging	92
	6.3.2.4 Tumour grading	93
6.4	Genes Identified in arrayCGH	95
<b>7.</b>	<b>CONCLUSION</b>	<b>98</b>
	<b>RECOMMENDATIONS</b>	<b>99</b>
<b>8.</b>	<b>REFERENCES</b>	<b>101</b>
<b>9.</b>	<b>APPENDIX</b>	<b>123</b>

## LIST OF TABLES

<b>Table No.</b>		<b>Page</b>
3.1	Summary of oncogenes and TSGs involved in oral carcinogenesis reported in multiple studies	32
3.3	Summary of most frequent chromosomal alterations involved in OC reported in multiple studies	41
4.1	Primer sequences for internal control and <i>DUSP22</i> gene	50
5.1	Socio-demographic profile of 20 OC cases	53
5.2	44 deleted regions and 41 amplified regions detected in OSCC cases (n=20)	55
5.3	Frequency of amplified and deleted genes in OSCC on arrayCGH analysis (n=20)	59
5.4	Relationship between deleted region 19p13.3 and clinicopathological parameters and socio-demographic characteristics in OSCC cases (n=20)	62
5.5	Summary of relationship between selected chromosomal alterations and clinicopathological parameters and socio-demographic characteristics in OSCC cases (n=20) (Deleted regions)	65
5.6	Summary of relationship between selected chromosomal alterations and clinicopathological parameters and socio-demographic characteristics in OSCC cases (n=20) (Amplified regions)	68
5.7	Validation of arrayCGH results through CN and GE	73
5.8	Details of RQ values of <i>DUSP22</i> gene for copy number (gDNA) and gene expression (cDNA)	75
6.1	Summary of common chromosome alterations in OC (Tsantoulis et. al., 2007)	86

## LIST OF FIGURES

<b>Figure No.</b>		<b>Page</b>
3.1	Theoretical model of carcinogenesis in the oral cavity based on multistep carcinogenesis (Adapted from Tsantoulis et. al., 2007 and Choi & Myers, 2008).	23
3.2	Possible oncogenes and TSGs involved in OC as the tumour progress from normal to squamous cell carcinoma.	31
4.1	Flow chart of Research Methodology	52
5.1	An arrayCGH genome view for Sample A01	56
5.2	Patterns and distribution of chromosomal alterations in arrayCGH result (n=20) for selected altered chromosome 3, 8 and 19	57
5.3	Melt curve for the <i>DUSP22</i> gene and internal control primers	70
5.4	Distribution of copy number fold changes in 32 samples for <i>DUSP22</i> gene (gDNA samples) compared to normal patients	71
5.5	Distribution of gene expression (GE) in 30 tumour samples for <i>DUSP22</i> gene (cDNA samples)	72
5.6	The similarity between copy number and gene expression study for 7 samples that were amplified in arrayCGH	74

## LIST OF APPENDIX

<b>Appendix No.</b>		<b>Page</b>
A	Detailed protocol on DNA extraction from fresh frozen tissue	123
B	GenomePlex Complete Whole Genome Amplification (WGA) Kit procedure	124
C	Sigma GenElute PCR Clean-Up Kit protocol	126
D	Procedure for arrayCGH from labelling to washing chip	127
E	Details on arrayCGH analysis procedure	131
F	Socio-demographic profile of 20 patients with OSCC	134
G	Other genes identified in all 20 cases of arrayCGH	135
H	Clinicopathological parameters for all OSCC cases (n=20) (arrayCGH)	136
I	Statistical analysis of chromosomal alteration	137
J	Summary of results for RT-PCR (cDNA Level) on 30 samples (independent samples)	161
K	Clinicopathological measurement for parameters studied	163

## LIST OF ABBREVIATIONS

---

WHO	World Health Organization
ICD	International Statistic Classification of Disease
NCR	National Cancer Registry
NIH	National Institutes for Health
OSCC	Oral Squamous Cell Carcinoma
FISH	Fluorescence in-situ Hybridization
CGH	Comparative Genomic Hybridization
arrayCGH	array Comparative Genomic Hybridization
GE	Gene Expression
SNP	Single Nucleotide Polymorphism
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
gDNA	genomic Deoxyribonucleic acid
NCI	National Cancer Institute
ASR	Age Standardize Rate
CR	Crude Incidence Rate
GLOBOCAN	Estimation of the Burden of Cancer
IARC	International Agency for Research on Cancer
TNM	Tumour-Node-Metastasis
UICC	The International Union Against Cancer
AJCC	American Joint Committee on Cancer
UM	University of Malaya
HPV	Human Papilloma Virus
OC	Oral Cancer
BQ	Betel Quid
TSNA	Tobacco Specific Nitrosamines
ROS	Reactive Oxygen Species
USA	United States of America
mg/g	miligram/gram
$\beta$	Beta
ACS	American Cancer Society
<i>GSTM1</i>	Glutathione S-Transferase MU1

<i>GSTT1</i>	Glutathione S-Transferase Theta1
<i>CYP1A1</i>	Cytochrome P450, family 1, subfamily A, polypeptide 1
M	Mitosis
G1	Growth One
G2	Growth Two
S	Synthesis
RNA	Ribonucleic acid
TSG	Tumour Suppressor Gene
ONCO	Oncogene
GS	Growth Signal
bp	base pair
<i>EGFR</i>	Epidermal growth factor receptor
<i>c-myc</i>	Myelocytomatosis viral oncogene homolog
<i>int-2</i>	Integrator complex subunit 2
<i>hst-1</i>	Heparin-binding secretory transforming factor 1
<i>PRAD-1/CCND1/ bcl-1</i>	Cyclin D1
<i>VEGF</i>	Vascular endothelial growth factor
<i>MMP</i>	Matrix metalloproteinase
<i>TGF</i>	Transforming growth factor
<i>Her-2</i>	Verb erythroblastic leukemia viral oncogene homolog 2
Rb	Retinoblastoma
<i>RUNX3</i>	Runt-related transcription factor 3
<i>PRTFDC1</i>	Phosphoribosyl transferase domain containing 1
<i>p53</i>	p53 gene
LOH	Loss of heterozygosity
<i>CDKN2A/p16</i>	Cyclin-dependent kinase inhibitor 2A
<i>FHIT</i>	Fragile histidine triad
HGD	High Grade Dysplasia
CIS	Carcinoma in situ
MOCDBS	Malaysian Oral Cancer Database and Tumour Bank System
OCRCC	Oral Cancer Research and Coordinating Centre



$\mu\text{m}$	micrometer
ml	milliliter
OCT	Optimal Cutting Temperature
$^{\circ}\text{C}$	Celcius
rpm	revolutions per minute
$\mu\text{l}$	micro-liter
mg/mL	milligram per mililiter
mg/g	milligram per gram
TE	Tris EDTA
ng/ $\mu\text{l}$	nanogram per microliter