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ORIGINAL LITERARY WORK DECLARATION

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Title of Thesis ("this work"): Methylation Profiling in Oral Squamous Cell Carcinoma

Field of study: Molecular Biology

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ABSTRACT

Introduction: DNA methylation is an epigenetic phenomenon at molecular level that involves gene expression regulation of cell development and differentiation, and diseases. DNA hypermethylation in a gene promoter region shows dramatic effects on gene expression and is a common phenomenon in initiation and progression of many solid tumours that includes Oral Squamous Cell Carcinoma (OSCC). Silencing of hypermethylated genes in promoter regions is a frequent phenomenon in different types of cancer and has achieved increasing diagnostic and therapeutic importance since the changes are reversible. Thus, methylation analysis may provide promising clinical applications that include the development of biomarkers, assessment of prognosis and prediction of the therapeutic response in oral malignancy. For years, OSCC has been amongst the leading cancers in developing countries. Despite considerable efforts in research studies and cancer treatments, a 5-year survival rate for OSCC has not shown any significant improvement. To improve on this situation, it is therefore necessary to understand the fundamental biological processes and to identify appropriate prognostic factors of OSCC on DNA hypermethylation-mediated silencing that leads to cancer progression.

Objectives: OSCC methylation profiling was investigated by microarray analysis followed by identification and verification of significantly hypermethylated genes and their protein products. To achieve this, methylation specific polymerase chain reaction (MSPCR) and immunohistochemical (IHC) analysis were used. Both analyses were conducted on selected promoter hypermethylation markers used for detecting the epigenetic alterations associated with OSCC. In this study, the significant pathways of selected hypermethylated genes that are involved in oral carcinogenesis were also elucidated. Finally, relations of demographic and

clinicopathological characteristics along with these signature genes were conducted for prognostic purposes of OSCC.

Materials and methods: Genome-wide analysis of 4 normal oral mucosa and 20 OSCC tissues were conducted using Illumina methylation microarray. The specified differential genes were selected from a gene list and their methylation statuses and protein expressions were further verified by an independent cohort sample of 40 OSCC samples. Lastly, statistical analysis conducted on demographic, clinicopathological data and gene hypermethylations for OSCC prognostication.

Results: Unsupervised hierarchical clustering of methylation data revealed distinct methylation patterns between the normal and the tumour tissues. For tumour tissues, high frequencies of promoter hypermethylation were found in p16, DDAH2, DUSP1, PIKCR3, TP73, MEF2D, RRM2 and CELSR3 genes in the MSPCR analysis; whereas low positive immunostaining of DDAH2, DUSP1, MEF2D and RRM2 were demonstrated in the IHC analysis. Notably, an inverse correlation was observed between hypermethylations and protein expressions of DDAH2, DUSP1, MEF2D and RRM2. In addition to that, significant association was found between p16 and TP73 hypermethylation with patients' tumour site, and CELSR3 and TP73 hypermethylation with patients' invasive stages. Furthermore, DDAH2 and CELSR3 hypermethylation, and RRM2 expression were correlated significantly with patients' age. Finally, gender showed a significant difference in the survival rate with 24.2% for males and 46.5% for females.

Conclusions: Multiple candidate genes were identified using computational and gene-specific validation approaches in this study. The results provide a new insight into the molecular basis of promoter hypermethylation and prognostic values of OSCC. Nevertheless, the identified candidate genes revealed from the present research are worth making further investigations on oral carcinogenesis.

Abstrak

Pengenalan: Metilasi DNA adalah suatu fenomena epigenetik di peringkat molekul yang melibatkan ekspresi gen dalam pembangunan dan pembahagian sel-sel, dan penyakit. Hipermetilasi DNA dalam penganjur gen telah menunjukkan kesan dramatik ke atas gen regulasi dan merupakan fenomena biasa dalam perkembangan banyak tumor pepejal termasuk karsinoma sel skuamus mulut (OSCC). Penyenyapan dalam proses hipermetilasi adalah satu mekanisme yang kerap berlaku dalam pelbagai jenis kanser dan menjadi semakin penting dalam bidang diagnostik dan terapeutik kerana metilasi merupakan proses tindakbalas berbalik. Oleh itu, analisis metilasi boleh menyediakan aplikasi klinikal yang menjamin, termasuk penemuan penanda-penanda baru, penilaian prognosis dan pemberian terapeutik pada kanser mulut. Sejak beberapa tahun yang dulu, OSCC telah menjadi salah satu kanser utama di negara-negara yang sedang berkembang. Walaupun pelbagai usaha dalam penyelidikan dan pengubatan barah berkembang telah dibuat , kadar kelangsungan hidup 5 tahun untuk pesakit-pesakit OSCC masih tidak bertambah baik. Bagi memperbaiki keadaan ini, adalah perlu untuk memahami proses biologi asas pada DNA hipermetilasi dalam penganjur gene yang merupakan punca kanser.

Objektif: Penyiasatan profil metilasi OSCC dijalankan oleh analisis microarray, diikuti dengan pengenalan dan pengesahan status perbezaan gen metilasi yang signifikan dan ekspresi protein menggunakan metilasi khusus “polymerase chain reaction” (MSPCR) dan immunohistokimia (IHC) analisis. Kedua-dua analisis telah dijalankan ke atas gen-gen terpilih untuk mengesan perubahan epigenetik yang berkaitan dengan OSCC. Dalam kajian ini, laluan sel-sel penting untuk gen hipermetilasi yang terlibat dalam karsinogenesis mulut juga dijelaskan. Akhir sekali,

hubungan ciri-ciri demografi dan klinikopathologikal dengan gen-gen penanda telah diadakan untuk tujuan ramalan OSCC.

Material dan cara:

Untuk mengenal pasti kelainan metilasi DNA yang berkaitan dengan OSCC, kita menyiasat analisis keseluruhan genom keatas 4 mukosa mulut biasa dan 20 tisu OSCC, dengan menggunakan Illumina metilasi microarray. Status metilasi dan ekspresi protein untuk calon-calon gen telah disahkan oleh sampel kohort berbeza daripada 40 sampel tumor. Akhir sekali, analisis secara statistik juga dijalankan keatas data demografi dan klinikopathologika, dan tanda-tanda hipermetilasi untuk nilai prognostik bagi pesakit OSCC.

Keputusan: Kelompok hierarki tanpa pengawasan data metilasi mendedahkan pola metilasi berbeza antara tisu normal dan tisu tumor. Dalam kajian tisu tumor, frekuensi tinggi dalam penganjur hipermetilasi ditemui pada gen-gen P16, DDAH2, DUSP1, PIKCR3, TP73, MEF2D, RRM2, BCL2 dan CELSR3 dalam MSPCR analisis, begitu juga perwarnaan imuno dalam IHC analisis yang rendah bagi DDAH2, DUSP1, MEF2D dan RRM2 telah ditunjukkan. Hubungan songsang antara hipermetilasi dan imunoreaktiviti untuk DDAH2, DUSP1, MEF2D dan RRM2 telah dikesan dalam kajian ini. Tambahan pula, hubungan yang ketara ditemui antara hipermetilasi P16 dan TP73 dengan lokasi tumor, hipermetilasi CELSR3 dan TP73 dengan pesakit peringkat invasif. Tambahan pula, hipermetilasi bagi DDAH2 dan CELSR3, and RRM2 ekspresi adalah berkait rapat dengan ketara dengan usia pesakit. Akhir sekali, jantina menunjukkan perbezaan yang signifikan dalam kadar jangkaan hidup dengan 24.2% bagi lelaki dan 46.5% untuk wanita.

Kesimpulan: Pelbagai calon gen telah dikenal pasti menggunakan pendekatan pengesahan pengiraan dan gen khusus dalam kajian ini. Keputusan ini juga menyediakan wawasan baru ke dalam asas molekul hIpermetilasi penganjur dan nilai prognostik OSCC. Lagipun, calon-calon gen yang dikenal pasti dari kajian ini adalah bernilai untuk siasatan lanjut mengenai karsinogenesis mulut.

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LIST OF ABBREVIATIONS

5-MCs	5-Methylcytosines
A	Adenine
ANOVA	One-way analysis of variance
ASR	Age standard ratio
BCL2	B-cell lymphoma 2
C	Cytosine
Calmodulin	Calcium-bound calmodulin 2
CaMK	Calcium/calmodulin-dependent kinase
CELSR3	Cadherin EGF LAG seven-pass G-type receptor 3
CH3-	Methyl
COBRA	Combined bisulfite restriction analysis
CpG	Cytosine-Guanine
DAB	Diaminobenzidine
DAPK	Death associated protein kinase
dd	Dideoxynucleotides
DDAH2	Dimethylarginine dimethylaminohydrolase 2
ddH2O	Double distilled water
ddNTPs	Dideoxynucleotide triphosphate
DNMTs	DNA nucleotide methyltransferases
DNP	2,4-dinitrophenol
dNTPs	Deoxynucleoside triphosphates

dRNs	Deoxyribonucleotides
DUSP	Dual specificity phosphatase
EBV	Epstein–Barr virus
EGCG	Epigallocatechin-3–gallate
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
G	Guanine
GSTP1	Glutathione S-transferase gene
H&E	Haematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HDACs	Histone deacetylases
HNSCC	Head and neck squamous cell carcinoma
HP1	Heterochromatin protein 1
HRP	Horseradish peroxidase
lincRNAs	Large intergenic non- coding RNAs
MAPKs	Mitogen-activated protein kinase
MCs	Methylcytosines
MEF2D	Myocyte Enhancer Factor 2D
MGMT	Methylguanine-DNA methyltransferase
Mins	Minutes
ml	Mililiter
mM	Mili Molar

MOCDTBS	Malaysia Oral Cancer Database and Tissue Bank System
MS-HRM	Methylation-sensitive high-resolution melting
MSPCR	Methylation-specific polymerase chain reaction
MS-SNuPE	Methylation-sensitive single nucleotide primer extension
nc RNAs	Non-coding RNAs
NCR	National Cancer Registry
ng	Nanogram
NORs	Nucleolar organizing regions
NTP	Nucleoside triphosphate
OCRCC	Oral Cancer Research and Coordinating Centre
OSCC	Oral Squamous Cell Carcinoma
p101	PI3K regulation class IB
PCA	Principle Component Analyses
PCP	Planar cell polarity
Pik3r5	Phosphoinositide-3-kinase, regulatory subunit 5
piRNAs	PIWI-interacting RNAs
RefSeq	Reference sequence
RR	Ribonucleotide reductase
RRM2	Ribonucleotide reductase small subunit M2
RT-PCR	Real-time polymerase chain reaction
SAM	S-Adenosyl-L-Methionin
SCC	Squamous cell carcinoma

snoRNAs	Small nucleolar RNAs
SPSS	Statistical Package for Social Sciences
T	Thymine
TBS	Tris phosphate buffer
TP 73	Tumour protein 73
TSS	Transcription start site
T-UCRs	Transcribed ultra-conserved regions
U	Uracil
ul	Microliter
µg	Microgram
µm	Micrometer
USA	United States of America
VEGF	Vascular endothelial growth factor
WHO	World Health Organization