

RESEARCH ARTICLE

Screening of Differential Promoter Hypermethylated Genes in Primary Oral Squamous Cell Carcinoma

Goot Heah Khor^{1,2,3,5*}, Gabrielle Ruth Anisah Froemming², Rosnah Binti Zain³, Mannil Thomas Abraham⁴, Kwai Lin Thong^{5,6*}

Abstract

Background: Promoter hypermethylation leads to altered gene functions and may result in malignant cellular transformation. Thus, identification of biomarkers for hypermethylated genes could be useful for diagnosis, prognosis, and therapeutic treatment of oral squamous cell carcinoma (OSCC). **Objectives:** To screen hypermethylated genes with a microarray approach and to validate selected hypermethylated genes with the methylation-specific polymerase chain reaction (MSPCR). **Materials and Methods:** Genome-wide analysis of normal oral mucosa and OSCC tissues was conducted using the Illumina methylation microarray. The specified differential genes were selected and hypermethylation status was further verified with an independent cohort sample of OSCC samples. Candidate genes were screened using microarray assay and run by MSPCR analysis. **Results:** TP73, PIK3R5, and CELSR3 demonstrated high percentages of differential hypermethylation status. **Conclusions:** Our microarray screening and MSPCR approaches revealed that the signature candidates of differentially hypermethylated genes may possibly become potential biomarkers which would be useful for diagnostic, prognostic and therapeutic targets of OSCC in the near future.

Keywords: Microarray - oral squamous cell carcinoma - promoter hypermethylation - MSPCR

Asian Pac J Cancer Prev, 15 (20), 8957-8961

Introduction

Oral cancer occupies the eighth position in the worldwide cancer incidence ranking (Moore et al., 2009). Oral Squamous Cell Carcinoma (OSCC) is a predominant type of oral cancer with 263,900 new oral cancer cases being diagnosed each year and 128,000 deaths annually. GLOBOCAN in 2008 reported that 65.4% of oral cancers occur in the less developed countries including China and Asia (Jemal et al., 2011). Despite significant efforts in oral cancer research and advanced treatments in surgery, radiotherapy and chemotherapy, overall survival rate of 5-year has remained less than 50% for the last few decades, and with an unexpected rising incidence in developed countries and among younger population (Scully and Bagan, 2007; Warnakulasuriya, 2009). The prognosis of OSCC remains poor essentially due to late diagnosis; thus justifying a greater interest and effort in identifying specific molecular markers that are potentially useful for the prevention and early diagnosis of OSCC (Scully et al., 2011). As for the 5-year survival rate for oral cancer, it was found to be directly associated to pathological stage

at the time of diagnosis. Early diagnosis and immediate treatment then remain the main keys in decreasing the incidence and improving the overall survival rates of patients diagnosed with this disease (Scully, 2011).

An epigenetic phenomenon is a heritable alteration in the gene function without the sequence changes, which involved the enzymatic addition of methyl groups to deoxycytidine residues in CpG dinucleotides. Alterations in the established epigenetic patterns usually lead to changes in gene expression, which can cause transcriptional repression. The classic epigenetic mechanisms include DNA methylation, histone modification, chromatin remodelling and microRNA interference, which have been shown to be involved in post-transcriptional silencing (Herman and Baylin, 2003). DNA methylation is one of the epigenetic phenomena that occur at the molecular level involving regulation of cell of development and differentiation, aging, and diseases (Shaw, 2006; Supic, et al., 2009). Notably, most of the gene inactivation mechanism involved in cancer progression is the promoter hypermethylation of CpG islands (Kang et al., 2007; Noushmehret al., 2010). This

¹Centre of Preclinical Science Studies, ²Institute of Medical Molecular Biotechnology, Faculty of Medicine, Universiti Teknologi MARA, Selangor, ³Oral Cancer Research and Coordinating Centre, Faculty of Dentistry, ⁵Laboratory of Biomedical Science and Molecular Microbiology, UMBIO Cluster, Institute of Graduate Studies, ⁶Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, ⁴Tengku Ampuan Rahimah Hospital, Department of Oral and Maxillofacial Surgery, Ministry of Health Malaysia, Malaysia *For correspondence: gootheah@salam.uitm.edu.my; thongkl@um.edu.my

process is now widely recognized as either a causative or correlative event in carcinogenesis (Herman and Baylin, 2003; Jones, 2012).

Recent computational approach into microarray technology has enabled researchers to generate large amounts of data which include approximately the entire known human genome, and has shown that many gene alterations can be involved in carcinogenesis (Bibikova et al., 2004; Nazmul-Hossain et al., 2008; Kim and Kim, 2010; Jiang et al., 2014). In this experiment, a bead array platform of microarray technology was applied for the screening of methylation status of genes (Bibikova et al., 2004), to discover the epigenetic signatures that distinguish normal from tumour tissues of OSCC. This is based on the premise that gene promoter hypermethylation process in human DNA samples can be detected by using qualitative methylation-specific PCR (MSPCR) method (Herman et al., 1996). The advancement of this MSPCR technique has demonstrated a simple, rapid and cost-effective assessment for promoter methylation status.

Materials and Methods

Study population

Genomic DNA of four snapped frozen gingival tissues surrounding impacted tooth from non-cancer patients and twenty independent OSCC snapped frozen tissues were obtained for microarray analysis. Forty independent archival tissues of OSCC in formalin fixed and paraffin embedded tissue blocks were chosen for validating significant hypermethylated genes using MSPCR. The selected healthy subjects were non-smokers, non-alcoholics as well as non-betel quid chewers, with no clinical lesions in their oral mucosa and who had not previously undergone any anticancer treatment. They were evaluated and enrolled in appropriate protocols in the joint network of the Malaysia Oral Cancer Database and Tissue Bank System (MOCDTBS) coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC) after obtaining the subjects' consent following appropriate ethical committee approval from Medical Ethics Committee of the Faculty of Dentistry, University of Malaya (Ethic reference no: DF OP1101/0049 (L)). An Oral Pathologist confirmed the histopathological findings that all tumour samples used have contained more than 70% of squamous cell carcinoma tissues and more than 70% of epithelium from non-cancer patients.

DNA extraction from tissues

QIAamp[®] DNA Extraction Mini Kit (Qiagen, Germany) was used for genomic DNA extraction of 0.1 mg snapped frozen specimen following the manufacturer's

procedure. Purity of the extracted genomic DNA was analyzed by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) and DNA intactness was checked by 1% agarose gel electrophoresis. DNA purity of the samples had an average absorbance ratio of 1.89 ± 0.10 .

Bisulfite-converted DNA

The extracted genomic DNA was bisulfite-converted by using the EpiTect Bisulfite Kit (Cat. No. 59104, Qiagen, CA) following the manufacturer's protocol. Briefly, 1 ng to 2 μ g extracted genomic DNA was chemically modified by sodium bisulfite. Twenty μ l of treated DNA was measured by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies). The qualities of products were in range of 50-100 ng/ μ l.

Microarray assay

Microarray experiments were conducted on Infinium Illumina methylation bead array. Two hundred nanogram of total bisulphite converted DNA was applied on bead-based microarray following the manufacturer's protocol (Illumine, San Diego, USA). Briefly, the bisulfite converted DNA was subjected to whole genome amplification. The amplified products were then enzymatically fragmented and applied to the chip, via allele specific annealing to either the methylation specific probe or the non-methylation probe. Hybridization step was followed by single base extension. Then, multi-layered immunohistochemical assays were performed. After staining, the chip was scanned by BeadArray Reader to show the intensities of the unmethylated and methylated bead types (Bibikova et al., 2011).

Microarray data analysis

The raw data were analysed by GenomeStudio Data Analysis Software (Illumine, San Diego, USA), and the fluorescence intensity ratios between the two bead types were calculated. A ratio value of 0 equals a non-methylation of the locus; a ratio of 1 equals to total methylation. The array signals were uploaded to the Illumina's Genome Studio software for background normalization and filtered by β values for methylation levels (Bibikova et al., 2011). Methylation level was defined as follows: Hypermethylated and hypomethylated alleles as those having an average β value of more 0.6 and below 0.4 of the overall mean for all samples respectively. Differences in average β values of differentially methylated probes between normal subjects and patients were identified. Only selected differentially hypermethylated probes in OSCC patients that passed the filtration criteria were then subjected to Partek Genomics Suite 6.5 analysis (Partek Inc., USA) for gene selection. Three differential genes

Table 1. Details of Primers Used in MSPCR with Primer Sequence, Annealing Temperature and Amplicon Size

Genes	Methylated		Unmethylated		Annealing temp (°C)	Amplicon size (bp)
	Forward primer	Reverse primer	Forward primer	Reverse primer		
CELSR3	5'TAGATTAGG CGTTCGGTTTC3'	5'AAAAAATAACC TCGACGAACC3'	5'GAGTAGATTAGG TGTTTGGTTTT3'	5'CCAAAAAATAA CCTCAACAAACC3'	50	136
PIKCR5	5'GTAGTTGGG' ATTATAGGCGC3	5'ACGAATCACG AAATCAAAA3'	5'GGGTAGTTGGG ATTATAGGTGT3'	5'AAACAAATCACAA AATCAAAA3'	52	157
TP73	5'TGAAGATGTG CGAGTTAGTC3'	5'TCTTAAAAA TCGCGTCAAT3'	5'TTATGAAGATGT GTGAGTTAGTT3'	5'ACATCTTAAAAA ATCACATCAAT3'	55	125

of TP73, PIK3R5, and CELSR3 were selected from the gene list based on the selection criteria from our previous experiment (Khor et al., 2013).

Methylation-specific polymerase chain reaction

The protocol was performed according to the manufacturer's instructions (EpiTect MSP Kit, Qiagen, CA). Unmethylated and methylated DNA controls (Catalog NO. D5014, ZYMO, Orange, CA, USA) were used as negative and positive controls respectively, and double distilled water (ddH₂O) was used as a blank control in all the experiments. Primers were designed using Methyl Primer Express Software v1.0 (Applied Biosystems, USA). The primer sequence, annealing temperature and amplicon size are shown in Table 1.

The bisulfite-converted DNA was subjected to PCR amplification using Eppendorf Mastercycler Gradient PCR (Germany) following these cycling conditions: Initial activation step was conducted at 95°C for 10 mins; 35 cycles of PCR, each with a denaturation step at 94°C for 15 sec; annealing step at the T_m of primer, extension step at 72°C for 30 sec and the final extension was at 72°C for 10 mins. 5 µl of PCR products were loaded into 1% agarose gels, electrophoresed and visualized under the image analyser (Typhoon 9410 variable mode imager, Amersham Biosciences; Baied'Urfe, Quebec, Canada) after staining with SYBR Safe DNA gel stain (Invitrogen, USA).

Statistical analysis

The statistical analysis was analysed by descriptive test and one sample T-tests by SPSS software, version 17 (SPSS, Chicago, USA). The statistical difference was regarded as significant when $p < 0.05$.

Results

Microarray analysis

In this study, mean methylation value differences between normal tissues in different stages of OSCC cases were obtained (Figure 1). The data obtained were further analysed by Partek Genomic Suite software. The data set was clustered using Principle Component Analyses (PCA). The cluster set of normal and tumour samples (stage I, II, III and IV) were clearly segregated in the PCA (Figure 2).

MSPCR analysis

The selected genes of CELSR3, PIK3R5 and TP73 were assessed using MSPCR in independent FFPE



Figure 1. Mean Methylation Value Difference Between Normal Tissues with Different Stages of OSCC Cases (Stage 1, 2, 3, 4). CpG sites have increased methylation in OSCC ($\beta > 0.6$) compared with normal tissues ($\beta < 0.4$).

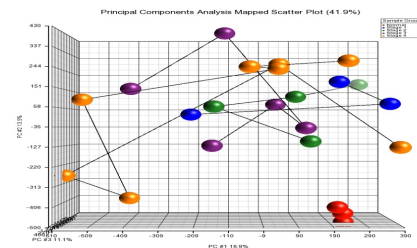


Figure 2. Principle Component Analyses Segregated the Normal Samples (Red Colour) from Tumour Samples (Stage I: Blue; Stage II: Green; Stage III: Purple; and Stage IV: Orange)

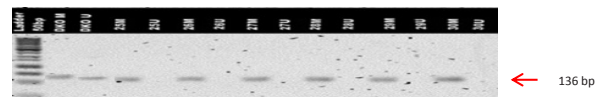


Figure 3. Representative Agarose Gel Electrophoretic Images of Methylation Status for Gene of CELSR3 in Methylation Control and Tumour Samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. bp represents of base pairs

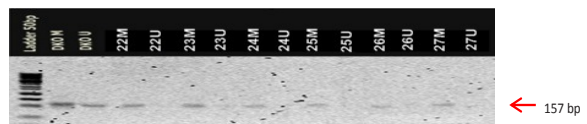


Figure 4. Representative Agarose Gel Electrophoretic Images of Methylation Status for Gene of PIK3R5 in Methylation Control and Tumour Samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. bp represents of base pairs

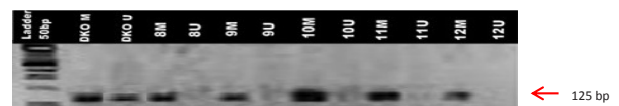


Figure 5. Representative Agarose Gel Electrophoretic Images of Methylation Status for Gene of TP73 in Methylation Control and Tumour Samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. bp represents of base pairs

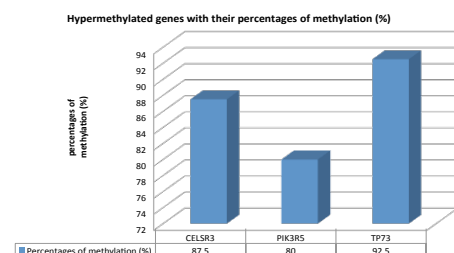


Figure 6. Percentage of Methylation for Hypermethylated Genes of CELSR3, PIK3R5 and TP73

samples. Methylation status of each gene is shown in Figure 3, 4 and 5. There is statistically significant for hypermethylation of CELSR3 ($p=0.000$), PIK3R5 ($p=0.003$) and TP73 ($p=0.000$) respectively. The percentage of hypermethylation levels for CELSR3, PIK3R5, and TP73 genes were 87.5% ($n=35$), 80% ($n=32$), and 92.5% ($n=37$) respectively (Figure 6). These results

correspond to the microarray data.

Discussion

The results demonstrated the demarcation between the 20 tumour and 3 normal samples using PCA analysis. It is concordant with results reported elsewhere (Bibikova et al., 2006; Nazmul-Hossain et al., 2007; Killian et al., 2012). This microarray approach provided a very comprehensive and high throughput data in genome wide study, which covered 99% of RefSeq genes. More than 450,000 methylation sites including promoter region of CpG islands and shores per sample at single nucleotide resolution are provided by this platform (Bibikova et al., 2011; Cheng et al., 2014). The recent advances in high-throughput microarray technologies have enabled investigators to study site-specific DNA methylation events on a much broader scale (O'Sullivan and Goggins, 2013). Many hypermethylation events in CpG islands near known genes transcriptional start sites, which correlated with reductions in gene expression have been identified. These approaches have led to the discovery of hundreds of affected genes previously not identified, which potentially provide prognostic DNA methylation in cancer events (Noushmehr et al., 2010; Oster et al., 2011).

Verification of microarray results by MSPCR in the study showed that these results were reliable indicating methylation array analysis is very specific and useful in identifying the signature hypermethylated genes and helpful in understanding the roles of hypermethylated genes that are involved in oral carcinogenesis (Bibikova et al., 2006).

Methylation-specific analysis

DNA methylation plays an essential role in maintaining cellular function, and methylation aberrations may lead to carcinogenesis. Thus, several methods have been developed recently to analyse genes' methylation status in human cells, depending on their detection strengths and weaknesses (O'Sullivan and Goggins, 2013). One of the methods is the MSPCR which applies specifically designed methylated and unmethylated primers for detecting the methylation level (Herman et al., 1996). It can be applied for distinguishing unmethylated from the methylated cytosine residues. As few as 1 to 10 tumour cells among 104 normal cells in clinical samples could be detected (Cottrell and Laird, 2003). It is a sensitive but not quantitative technique for the methylation level (Herman et al., 1996; Towle et al., 2012).

Further verification of candidate genes on a separate cohort of healthy and oral cancer population by MSPCR analysis has confirmed the hypermethylation in promoter region of existing biomarker of TP73, as reported by others (Jha et al., 2012) and new biomarkers of PIK3R5 and CELSR3. Confirmation of the gene methylation statuses using methylation specific technique, MSPCR in TP73, PIK3R5 and CELSR3 genes, which provides a reliability of the microarray data that shows 100% concordance (Weber et al., 2005).

The methylation frequency of promoter regions of TP73 is high in OSCC and has also been well documented

in other cancer studies (Jha et al., 2012). The variation of gene expression frequencies in individual studies may be due to the different geographical origin, which suggests population differences (Jha et al., 2012; Syeed et al., 2012; Patel et al., 2013). In fact, frequency variation of gene hypermethylation can also be due to cancer heterogeneity in promoter methylation, such methylation alterations were generally not correlated with gene expression (Aryee et al., 2013).

However, direct comparison of OSCC to normal tissues by larger clinical samples in microarray assay will allow research to identify more hypermethylated-based biomarkers in OSCC in the near future. In addition, network analysis of protein-protein interaction may help to rule out dysregulation of transcriptional responses in oral carcinogenesis (Liu et al., 2013). This will provide critical insight into the differences found in oral malignancy and may also provide a new strategy in cancer prevention and develop a molecular targeted therapy.

In conclusion, TP73, PIK3R5 and CELSR3 hypermethylations were identified using microarray screening and gene-specific validation approaches. These genes may possibly become potential epigenetic biomarkers for OSCC as diagnostic, prognostic and therapeutic targets in the near future.

Acknowledgements

I would like to acknowledge the research grants, PS001/2010A and PV046/2011B provided by the University of Malaya, 600-RMI/DANA/5/3/RIF (633/2012) by the Universiti Teknologi MARA (UiTM) and the Fundamental Research Grant Scheme (FRGS) of the Ministry of Higher Education, Malaysia. We thank for the Oral Cancer Research and Coordinating Centre (OCRCC), the University of Malaya for providing the tissues and data from the Malaysia Oral Cancer Database and Tissue bank System (MOCDTBS). We are grateful to Dr Mariam Mohamad of Population Health and Preventive Medicine Department, Medical Faculty, UiTM for her invaluable advice in the statistical analysis. We acknowledge the Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, UiTM, Malaysia for providing laboratory facilities.

References

- Aryee MJ, Liu W, Engelmann JC, et al (2013). DNA methylation alterations exhibit intraindividual stability and interindividual heterogeneity in prostate cancer metastases. *Sci Transl Med*, **5**, 169ra110.
- Bibikova M, Yeakley, JM, Chudin E, et al (2004). Gene expression profiles in formalin-fixed, paraffin-embedded tissues obtained with a novel assay for microarray analysis. *Clin Chem*, **50**, 2384-6.
- Bibikova M, Lin ZW, Zhou, L.X, et al (2006). High-throughput DNA methylation profiling using universal bead arrays. *Genome Res*, **16**, 383-93.
- Bibikova M, Barnes B, Tsan C, et al (2011). High density DNA methylation array with single CpG site resolution. *Genomics*, **98**, 288-95.
- Cheng Y, Yan Z, Liu Y, et al (2014). Analysis of DNA methylation

- patterns associated with the gastric cancer genome. *Oncol Lett*, **7**, 1021-6.
- Cottrell S, Laird PW (2003). Sensitive detection of DNA methylation. *Ann N Y Acad Sci*, **983**, 120-30.
- Herman JG, Graff JR, Myohanen SBDN, et al (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Nat Acad Sci USA*, **93**, 9821-6.
- Herman JG, Baylin SB (2003). Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*, **349**, 2042-54.
- Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA: A Cancer J Clin*, **61**, 69-90.
- Jha, AK, Nikbakht M, Jain V, et al (2012). Promoter hypermethylation of p73 and p53 genes in cervical cancer patients among north Indian population. *Mol Biol Rep*, **39**, 9145-57.
- Jiang Q, Yu YC, Ding XJ, Luo Y, Ruan H (2014). Bioinformatics analysis reveals significant genes and pathways to target for oral squamous cell carcinoma. *Asian Pac J Cancer Prev*, **15**, 2273-8.
- Jones PA (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*, **12**, 484-92.
- Kang GH, Lee S, Cho NY, et al (2007). DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. *Lab Invest*, **88**, 161-70.
- Khor GH, Froemming GRA, Zain RB, et al (2013). DNA Methylation Profiling Revealed Promoter Hypermethylation-induced Silencing of p16, DDAH2 and DUSP1 in Primary oral squamous cell carcinoma. *Int J Med Sci*, **10**, 1727-39.
- Killian JK, Walker RL, Bilke S, et al (2012). Genome-wide methylation profiling in archival formalin-fixed paraffin-embedded tissue samples. *Methods Mol Biol*, **823**, 107-18.
- Kim JC, Kim, TW (2010). Promoter methylation of specific genes is associated with the phenotype and progression of colorectal adenocarcinomas. *Ann Surg Oncol*, **17**, 1767-76.
- Liu Y, Liu CX, Wu ZT, Ge L, Zhou HM (2013). Mining proteins associated with oral squamous cell carcinoma in complex networks. *Asian Pac J Cancer Prev*, **14**, 4621-5.
- Moore MA, Ariyaratne Y, Badar F, et al (2009). Cancer epidemiology in South Asia - past, present and future. *Asian Pac J Cancer Prev*, **11**, 49-66.
- Nazmul-Hossain ANM, Patel KJ, Rhodus NL, et al (2008). Microarrays: applications in dental research. *Oral Dis*, **14**, 25-9.
- Noushmehr H, Weisenberger DJ, Diefs K, et al (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*, **17**, 510-22.
- O'Sullivan E, Goggins M (2013). DNA methylation analysis in human cancer. pancreatic cancer (pp. 131-156): Springer.
- Oster B, Thorsen K, Lamy P, et al (2011). Identification and validation of highly frequent CpG island hypermethylation in colorectal adenomas and carcinomas. *Int J Cancer*, **129**, 2855-66.
- Patel KR, Valaria BN, Begum R, et al (2013). Association between p53 variants and oral cancer susceptibility in population from Gujarat, West India. *Asian Pac J Cancer Prev*, **14**, 1093-100.
- Scully C (2011). Oral cancer aetiopathogenesis; past, present and future aspects. *Med Oral Patol Oral Cir Bucal*, 306-11.
- Shaw R (2006). The epigenetics of oral cancer. *Int J Oral Maxillofac Surg*, **35**, 101-8.
- Supic G, Kozomara R, Brankovic-Magic M, et al (2009). Gene hypermethylation in tumor tissue of advanced oral squamous cell carcinoma patients. *Oral Oncol*, **45**, 1051-7.
- Towle R, Truong D, Hogg K, et al (2013). Global analysis of DNA methylation changes during progression of oral cancer. *Oral Oncol*, **49**, 1033-42.
- Warnakulasuriya S (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol*, **45**, 309-16.
- Weber M, Davies JJ, Wittig D, et al (2005). Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet*, **37**, 853-62.