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Aberrant Methylation of Ribonucleotide Reductase Subunit M2 Is Closely Associated with Oral Cancer

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Abstract: Gene inactivation by promoter hypermethylation is a common occurrence in the development of various human cancers including oral cancer. Previous evidences observed that DNA methylation play a vital role in the carcinogenesis of oral cancer. The aim of this study was to analyze methylation status and protein expression of RRM2 in oral cancer. Archival tissues of oral cancer and healthy mucosa tissues were retrieved for the study. The methylation status of RRM2 was examined by methylation specific polymerase chain reaction and its protein expression was measured by immunohistochemistry. In the present study, frequency of aberrant methylation of RRM2 was 80% (n=32) in tumour tissues, whereas such methylation status was negatively detected in normal mucosa. In addition, 20 % (n=8) for RRM2 immunoreactivity was revealed in tumour tissues. In tumour tissues, RRM2 promoter hypermethylation was frequently associated with its reduced protein expression. Thus, epigenetic inactivation of RRM2 through promoter hypermethylation may be important in the oral cancer progression and assessment of RRM2 methylation status in oral cancer may become a potential biomarker for early detection in oral cancer.

Key words: Oral cancer · Ribonucleotide reductase · Promoter · Methylation

INTRODUCTION

Ribonucleotide reductase (RR) is constitutively present in all dividing cells, which is involved in ribonucleotides to deoxyribonucleotides conversion, as a rate limiting step of de novo deoxyribonucleotide triphosphate (dNTP) biosynthesis [1]. RR consists of two subunits of R1 and R2 have their specific role in nucleotide biosynthesis. The larger subunit, R1 plays a role as catalytic and regulation site of gene expression, whereas, smaller R2 subunit contains two iron moieties which are used for free radicals generation in reduction of hydroxyl group of the ribose sugar [2].

In several evidences it was shown that Ribonucleotide Reductase subunit M2 (RRM2) is involved in DNA synthesis, cell growth and drug resistance of cancer cells. It was also revealed that alteration in RRM2 expression leads to tumour initiation and progression, suggesting its possible role as prognostic factor and therapeutic target for cancer [3]. Therefore, this study highlighted the methylation status and protein expression of RRM2 and clinically relevant aspects of the expression and functions of RRM2 in oral cancer were retrieved.

In year of 2008, Xu *et al.* [4] reported RRM2 overexpression results in high mutational rate in lung cancer with association of largest tumour size, greatest tumour multiplicity and higher grade of pathology stage. They also observed that the free radical of normal product of RRM2 activity may result in G to T transversion.

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A study reported that RRM2 inhibition can inhibit cancer cell growth and overcomes drug resistance, where the novel RNR inhibitor, COH29 had inhibited RRM2 expression in human cancer cell lines, ovarian cancer and leukaemia [5].

Oral cancer is the tenth most common malignancy with a worldwide incidence of 275,000 cases reported annually, two thirds of these cases occur in developing countries [6, 7]. Oral cancer remains a significant burden worldwide in terms of treating or curing the patients. Despite desirable outcomes in early diagnosis and advance treatment, most cases are still detected in late stages of disease with 5-year survival rate of less than 50% with contributing to the high cancer morbidity and mortality [8]. In addition, oral carcinogenesis is a complex biological process, which accurate molecular mechanisms regarding its pathogenesis remain to be understood. Thus of greatest advantage in molecular research would be the significant biomarkers that capable of identification in the early events of carcinogenesis.

In addition to genetic changes in molecular mechanisms, any epigenetic alteration such as DNA methylation, histone modifications, chromatin structure, microRNA and other genomic functions may make an individual susceptible to developing cancer [9, 10]. Hyper-methylation of promoter CpG islands has been recognized as one of the most important variations in the progression of the cancer [11]. Growing evidence data points to the critical role of CpG island hypermethylation in genes implicated in apoptosis, cell cycle regulation and cellular differentiation in various types of cancer [10, 12]. In respect to oral cancer development, current studies showed that epigenetic silencing of cancer-linked genes through DNA methylation plays a significant role [13_C14]. In the genomes of mammals, methylation takes place at CpG islands that are located in the promoter region of approximately half of the genes. These are heritable throughout mitosis and are copied to the new strand by DNA methylatransferase-1 during DNA replication [15]. Although the accurate function of DNA methylation in oral squamous cell carcinoma (OSCC) is not fully understood. Overall, DNA methylation represses transcription and loss of methylation restores gene activation [16]. Thus, methylation status of RRM2gene and its protein expression in patients with OSCC were revealed in this study.

SUBJECTS AND METHODS: Study population: Four healthy mucosal tissues surrounding impacted teeth and 40 OSCC tissues of archived specimens were subjected in the methylation specific polymerase chain reaction (MSPCR) and immunohistochemistry (IHC) assays. The selected normal tissues were from healthy subjects who are non-smokers, non-alcoholics, non-betel quid chewers, no clinical oral lesions and free from other diseases. All OSCC patients were new cases and had not cancer therapy treated before. The tissue samples and relevant clinical data were obtained from the Malaysia Oral Cancer Database and Tissue Bank System (MOCDTBS) coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC) of University of Malaya. This present study had obtained ethic reference no: DF OP1101/0049 (L) from the Medical Ethics Committee of the Faculty of Dentistry, University of Malaya.

DNA Extraction: DNA extraction protocol using QIAamp® DNA Extraction Mini Kit (Qiagen, Germany) based on the manufacturer's procedure was carried out. The quality and purity of the genomic DNA were analysed by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) and the quality of DNA was checked by 1% agarose gel electrophoresis.

Bisulfite-Converted DNA: EpiTect bisulfite kit (Cat. No. 59104, Qiagen, CA) was used for bisulfite-converted DNA extraction according the manufacturer's protocol. The DNA products were qualified by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) with an extinction coefficient for single stranded DNA of 33ng-cm/µl. The purified bisulfite-converted DNA was ready for down streamed analysis.

Methylation-Specific Polymerase Chain Reaction (MSPCR): The procedure was conducted according to the manufacturer's instructions (EpiTect MSP Kit, Qiagen, CA). The bisulfide-converted DNA of normal and OSCC cases were subjected to PCR machine (Eppendorf master cycler, Germany). Thermo cycling conditions were carried out using the following settings: Initial activation step: 95°C for 10 minutes; 35 cycles in the denaturation step: 94°C for 15 seconds; annealing step: Tm of primer 54°C; extension step: 72°C for 30 seconds; and final extension: 72°C for 10 minutes. Parallel with each reaction, unmethylated and methylated DNA controls (Catalog NO. D5014, ZYMO, Orange, CA, USA) were run as

negative and positive controls respectively. MSPCR was performed using the primer sequences of RRM2 listed below: The methylated reaction primer sequences were 5'TCGTTTTGTTTTGGTTGTTC3' (sense) and 5'GCGAACTCACCGTATTCTC3' (antisense), For the unmethylated reaction, the primer sequences were 5'TAGTTGTTTTGTTTTGGTTGTTT3' (sense) and 5'CCCACAAACTCACCATATTCTC 3' (antisense) and the produce size was 115 bp. Five microliters of each MSPCR products were placed on 1% agarose gels and were visualized by ethidium bromide staining and gel image was captured under Olympus BX 61 fluorescence microscope.

Immunohistochemical Staining: Normal oral mucosa and OSCC were used for the immunohistochemical detection of RRM2 protein. The confirmed OSCC cases were used as positive control on each IHC run. The protocol was performed with DAKO REAL EnVision Detection System (Dako, USA, Carpinteria, CA)

The primary antibodies applied for immunohistochemical analysis was mouse monoclonal antibody to human RRM2 (dilution factor 1:450) with 30 min. of incubation time.After process of deparaffinization and rehydration, slides were heated in a microwave for 5mins in 10 mM citrate buffer (pH 6.0) for antigen retrieval, followed by rinsing in Tris phosphate buffer (TBS) solution. After quenching endogenous peroxidase activity in 3% hydrogen peroxide in methanol for 5 min., the sections were rinsed in TBS. Later, the sections were incubated with mouse anti-human RRM2 monoclonal antibody (Dako, Glostrup, Denmark) at a dilution of 1:450 for 30 min. at room temperature in a humidified chamber. After that, slides were incubated with 10% horseradish-peroxidase complex for 30 min., followed by 10 min. incubation of diaminobenzidine substrate. The slides were then lightly counterstained with hematoxylin, dehydrated in a graded ethanol series, cleaned in xylene and mounted. To measure RRM2 protein expression, a Quantitative scoring method was applied using digital image analysis. Data are expressed as the presence of positive cells in percentages (positively brown stained divided by tumour area). The evaluation was done qualitatively by taking the definite positive brown staining at the specific cellular location of each protein [17]. In the study, RRM2 revealed cytoplasmic staining of the cells. IHC staining scoring were examined by an evaluator without prior knowledge of the methylation status of MSPCR. The percentage of median value of RRM2 immunostaining demonstrated in tumour tissues was 20.0%. Protein expression with a value of more than 20.0% as a median percentage was considered to be overexpressed.

Statistical Analysis: Data obtained from MSPCR analysis was analyzed using descriptive analysis. The quantitative data obtained as continuous abnormally distributed variables were presented as median (interquartile) for percentage of protein expressions. Association of gene hypermethylation and protein expression was conducted by Chi-square and Fisher's exact tests. All the obtained data were analysed by SPSS software, version 21 (SPSS, Chicago, USA). Statistical significance was accepted at $p < 0.05$.

RESULTS

Study Population: Fixed formalin paraffin embedded (FFPE) tissue samples of 40 OSCC and 4 normal tissues were collected over a time period of five years from 2005 to 2010 for the assay.

Methylation-Specific Polymerase Chain Reaction: Methylation-specific polymerase chain reaction was applied for RRM2 methylation status. The methylation status and frequency of BCL2 hypermethylation revealed 80% (n=32). Whereas, RRM2 shown 20% (n=8) for unmethylation status. Representative agarose gel image is shown in Fig. 1.

Immunohistochemical Analysis: To detect the protein expression levels of RRM2, IHC analysis was performed on normal oral mucosa and OSCC tissues. Negative immunostaining of OSCC tissue was observed in negative control sections of RRM2 (Fig. 2). RRM2 demonstrated cytoplasmic immunostaining (Fig 3) in OSCC tissue. A low percentage of cases of positive immunostaining was demonstrated in tumour tissues for RRM2 with 22.5% (9/40).

Association Between Gene Methylation Levels and Protein Expressions of RRM2: The Chi-square and Fisher's exact tests were conducted to investigate the association between gene hypermethylation and protein expressions. The results showed an association between gene hypermethylation level with protein expression of RRM2 (p=0.001).

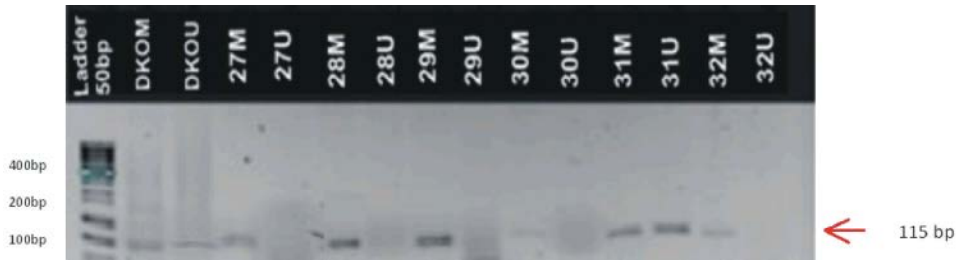

Fig. 1: Representative agarose gel electrophoretic image of methylation status for gene RRM2 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Samples no. 27, 28, 29, 30, 31 and 32 show methylated status.

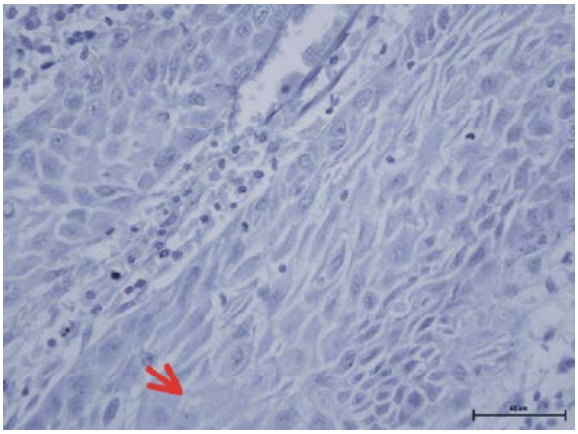

Fig. 2: Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against RRM2. Negative RRM2 cytoplasmic staining (with red arrow) shows in the tumour cells of the negative control section (40X magnification).

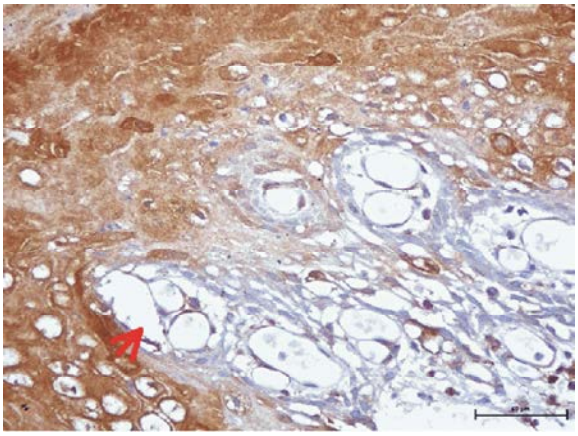

Fig. 3: Section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against RRM2. Positive RRM2 cytoplasmic staining (with red arrow) was detected in the tumour cells (40X magnification).

DISCUSSIONS

Ribonucleotide reductase small subunit M2 (RRM2) (chromosome 2p25.1) is the main component in modulating enzymatic activity of RR, which is composed of RRM1 and RRM2. RRM1/RRM2 complex is the major source of deoxynucleoside triphosphates for DNA replication during S phases [18]. It is a rate-limiting enzyme in DNA synthesis and thus plays a pivotal role in cell growth. Increased RR activity has been shown by increasing DNA synthesis rate to serve the proliferative activity of cancer cells. Thus, aberration of the RR is dramatically associated with malignant transformation and tumour cell growth [19]. These important roles have made RRM2 to be an attractive target for chemotherapeutic development [20]. Methylation of CpG promoter regions of RRM2 was rare, when analysed by direct sequencing of bisulphate-modified DNA in primary renal cell carcinoma [21]. However, Hsu *et al*. [22] has correlated its overexpression with tumour malignancy and progression in the early stages of lung cancer. Rahman *et al.* [23] also observed that RRM2 regulates BCL2 protein stability. The RRM2 gene suppression leads to increased Bcl-2 degradation in head and neck and lung cancer, where RRM2 represses expression of the antiapoptotic protein BCL2 resulting in induction of mitochondria-mediated intrinsic apoptosis. The repression effect of RRM2 on BCL2 was observed in our unpublished data, where BCL2 demonstrated 47.3% negativity in protein expression. In addition, Zhang *et al*. [24] found that

RRM2 is involved in tumour angiogenesis and growth through regulation of the expression of antiangiogenic thrombospondin-1 and proangiogenic vascular endothelial growth factor.

RRM2, the DNA damage repair-related nucleotide metabolism enzyme was predominantly activated in pathways of dCTP/dUTP metabolism, dATP/dITP metabolism and ATP/ITP metabolism as observed in the study [25]. RR was a catalyst in the conversion of ribonucleotides to 2'-deoxyribonucleotides which are the precursors for DNA synthesis and repair. Endogenous ribonucleotides and deoxyribonucleotides are essential metabolites that play a critical role in cell function and determination of their levels is of fundamental importance in understanding key cellular processes involved in energy metabolism and molecular as well as biochemical signalling pathways [26]. An increase of RRs activity was observed in transforming tumour cells as the DNA synthesis rate has increased to serve the proliferative activities of tumour cells. Thus, aberration of the RR is dramatically associated with malignant transformation and tumour cell growth [27]. These important facts have made the RRM2 gene to become an attractive target for chemotherapeutic treatment.

A protein expression of the RRM2 was examined by IHC. The protein expression was 100% in normal mucosa tissues in this study. In the tumour tissues, the IHC results revealed a low immunostaining frequency in RRM2. Xie *et al*. [28] revealed that RRM2 protein expression does not show any prognostic value even though it was associated with pancreatic adenocarcinoma. The fact reflected that protein expression of RRM2 is observed previously in other cancers as in our OSCC cases. Thus RRM2 protein expression may be used as diagnostic and prognostic biomarkers for OSCC identification.

In this study, there was an association observed between gene hypermethylation levels and protein expression of RRM2 (P=0.001). Few studies reported that the gene hypermethylation was significantly associated with decreased protein expression in various carcinomas such as ovarian cancer [29] and gastric cancer [30]. Similar finding was reported in the present study. In addition, Xie *et al.* [28] showed that RRM2 protein expression does not show any prognostic value even though it was associated with pancreatic adenocarcinoma. The observed inverse correlations of the hypermethylation level of RRM2 with their protein expression was evident in OSCC samples of this study, suggesting that the protein expressions of RRM2 was silenced by promoter hypermethylation in OSCC, as promoter hypermethylation is an epigenetic change which acts as one of the pathways that leads to oral carcinogenesis [13]. However, Xu *et al*. [31] stated that no inverse relationship was observed between methylation statuses with expression level. This means no statistically significant association between the gene hypermethylation levels with protein expression and this might be attributed to small tumour sample size and also type of samples selection. This may also be explained by the multiplicity of molecular mechanisms regulating gene expression by many other mechanisms including histone and MicroRNA modifications as these modifications are known to impact the transcription regulation [32, 33]. To the best of my knowledge, presently there are no other OSCC studies conducted on the association of RRM2 hypermethylation with the protein expression.

CONCLUSIONS

In summary, the potential signature candidate of RRM2 in OSCC is revealed for cancer identification and prognostication purposes. Protein expression of RRM2 gene was reduced by their promoter hypermethylation in OSCC, as promoter hypermethylation is the predominant mechanism in RRM2 deregulation, which seems to play an important role in oral carcinogenesis. The inverse correlation between gene hypermethylation and protein expression of RRM2 in the study suggests that the hypermethylation mechanism is an important event in silencing expression RRM2 in the OSCC cases. Furthermore, protein expression of RRM2 is newly explored in the OSCC cases, which may encourage deeper molecular understanding of OSCC progression. Hence, this biomarker can become a helpful tool for OSCC prognostication and diagnosis. Last but not least, integrated available clinical data for discovering novel prognostic marker of RRM2 may indicate poor prognosis of OSCC as patient's age increase.

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