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Hypermethylation of Death-Associated Protein Kinase (*DAPK1*) and its association with oral carcinogenesis - An experimental and meta-analysis study

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ABSTRACT

Objectives: The value of abnormal DNA methylation of *DAPK1* promoter and its association with various cancers have been suggested in the literature. To establish the significance of DNA methylation of *DAPK1* promoter in oral squamous cell carcinoma (OSCC), we a) performed a case-control study, b) evaluated published data for its utility in the diagnosis and prognosis of OSCC and c) identified the association of *DAPK1* gene expression with promoter DNA methylation status.

Design: Bisulfite gene sequencing of *DAPK1* promoter region was performed on non-malignant and malignant oral samples. Further, using a systematic search, 330 publications were retrieved from PubMed, Scopus, and Google Scholar and 11 relevant articles were identified.

Results: Significant association of *DAPK1* promoter methylation with OSCC ($p < 0.0001$) was observed in the case-control study. The studies chosen for meta-analysis showed prognostic and predictive significance of *DAPK1* gene promoter, despite defined inconsistencies in few studies. Overall, we obtained a statistically significant (p-value < 0.001) association for both sensitivity and specificity of *DAPK1* DNA promoter methylation in oral cancer cases, without publication bias.

Conclusion: DNA hypermethylation of *DAPK1* gene promoter is a promising biomarker for OSCC prediction/prognostics and suggests further validation in large distinct cohorts to facilitate translation to clinics.

1. Introduction

Cancer of the oral cavity and lip is the sixth most common cancer in the world with an incidence of approximately 300,000 individuals being affected every year (Torre et al., 2015). The frequency of incidence increases according to the geographical location, as seen in the South Central Asian region, including countries such as India, Pakistan, and Sri Lanka, among others (Rao, Mejia, Roberts-Thomson, & Logan, 2013). Oral cancer is most prevalent among sections of the population who do not have access to routine screening facilities, and therefore, they are diagnosed late into the disease and have a poor prognosis and subsequently, reduced quality of living (Petti, 2009).

Predictive biomarkers for early diagnosis at the molecular level can be obtained from easily accessible sources such as blood cells (Hsiung et al., 2007), plasma (Kosaka, Iguchi, & Ochiya, 2010), serum (Chen et al., 2008), or salivary rinses (Arellano-Garcia et al., 2008; Jou et al., 2010) that can help to manage the onset of the disease, aid early diagnosis, and plan treatment modalities. Among these, aberrant DNA methylation patterns have been frequently observed in a majority of tumor types and precise genomic locations have been identified that can serve as potential biomarkers. As the sensitivity and specificity of

Abbreviations: OSCC, oral squamous cell carcinoma; TSGs, Tumor Suppressor Genes; DAPK1, Death-Associated Protein Kinase, 1; BGS, Bisulfite Genomic Sequencing; OR, odds ratio; SE, standard error; S-ROC, Summary – Receiving Operating Characteristic; TP, true positive; FP, false positive; MSP, methylation specific PCR; BST, bisulfite treatment; QMSP, quantitative methylation specific PCR; BSP, bisulfite polymerase chain reaction; N-MSP, multiplex nested methylation specific PCR; CI, confidence interval; FPR, false positive rate; T1/T2, tumor stage; HNSCC, head and neck squamous cell carcinoma

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DNA methylation markers is quite high, it can be a reliable predictor for the progression of the disease, which may require either monitoring or pre-emptive measures, such as surgery.

Hypermethylation of promoter regions of Tumor Suppressor Genes (TSG's) is a critical event in gene inactivation. Death-Associated Protein Kinase 1 (*DAPK1/DAPK*), a mediator of ɣ – interferon induced programmed cell death, is located at 9q21.33 and is a vital gene in signal transduction and apoptosis (Hupp, 2010). The enzyme is Calmodulin-dependent and phosphorylates the Serine/Threonine residues (Raveh & Kimchi, 2001). Its effect on the cytoskeleton includes loss of attachment to the matrix and blebbing of the membrane (Ivanovska, Mahadevan, & Schneider-Stock, 2014). It is also involved in neuronal cell death (Fujita & Yamashita, 2014), regulation of inflammation (Lai & Chen, 2014), cancer metastasis (Chen, Lee, & Chen, 2014), and is also considered as a therapeutic target (Schumacher, Velentza, & Watterson, 2002). *DAPK1* hypermethylation has been suggested to influence tumor progression and metastasis (Catto, 2005; Hupp, 2010; Kulkarni & Saranath, 2004). *DAPK1* hypermethylated tumors have demonstrated capabilities of forming secondary and highly aggressive tumors, especially in epithelial cells (Kulkarni & Saranath, 2004; Liu, Zhou, He, & Jiang, 2012).

Hypermethylation of *DAPK1* in oral cancer has been validated by several research groups in different populations. Consolidation of all the available studies will help to present a conclusive result regarding hypermethylation of *DAPK1* and its association with prediction of outcome in patients. Here, we have sought to perform a meta-analysis, to obtain a scientifically valid conclusion by combining multiple heterogeneous populations and several individual studies, to remove potential bias, as well as to summarize and analyse the results. The purpose of this study was to perform a meta-analysis on the diagnostic and prognostic utility of *DAPK1* methylation in malignant samples for oral cancer detection. The resulting data provides an accurate outcome and could serve as a useful analytical tool in guiding further research.

2. Methods

2.1. Case-control study

2.1.1. Study population and ethics statement

The study involved sixty age and gender-matched cases and thirty controls of south Indian origin, recruited from Kasturba Hospital, Manipal, India. The study was approved by the institutional review board, Kasturba Hospital Ethical Committee of Manipal University, and written informed consent was obtained from all the study participants. The cases and controls were recruited on the basis of defined inclusion and exclusion criteria. For cases: a) subjects above 18 years of age, b) newly diagnosed as oral malignant cases (diagnosed with oral squamous cell carcinoma), c) with no history of treatment for oral cancer, d) belonging to south Indian origin. For the 60 patients recruited, the mean age was 57 years (42 males and 18 females). The inclusion criteria for controls were a) subjects above 18 years of age, b) histopathologically classified as having normal/non-malignant tissue, c) belonging to south Indian origin. The clinical status of the samples were confirmed by histopathological examination.

2.1.2. DNA Extraction and Bisulfite Genomic Sequencing (BGS)

DNA was isolated from tissue biopsies (confirmed as non-malignant and malignant by histopathological examination), by standard phenol-chloroform extraction and ethanol precipitation method. Genomic DNA (1.5 μg) was used for bisulfite treatment using EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Primers were designed using Methyl Primer Express V.1 (Applied Biosystems, USA) for +836 to +1234 bp, with respect to TSS of *DAPK1* (Table 1) (Bhat et al., 2017). The PCR amplicons were purified and directly sequenced in ABI 3130 Genetic Analyzer according to manufacturer's instructions, using BigDye Terminator Cycle

Table 1
Selected Studies (12) with the Primers and Methylation Percentages.

Study (Author)	Method Used	Forward Primer	Reverse Primer	Amplicon Size (bp)	Controls (Methylation%)	Cases (Methylation%)
Present Study (2017)	BST-BSP	5′-GGCAGCTTAGCAATGTGTCACAGGTGG-3′	5′-ACTCGCAGCGGGCAGGGTCT-3′	391	1	73.3
Arantes et al. (2015)	BST-QMSP	5′-GGATAGTCGGATCGAGTTAACGTC-3′	5′-CCCTCCCAAACGCCGA-3′	98	7.5	80
Rettori et al. (2013)	BST-QMSP	5′-GGATAGTCGGATCGAGTTAACGTC-3′	5′-CCCTCCCAAACGCCGA-3′	106	2.6	8.2
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCG-3′	98		
Nagata et al. (2012)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	25	55.88
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		
Supic et al. (2011)	BST-QMSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	20.5	30
		m 5′-ATAGTCGGATCGAGTTAACGTC-3′	m 5′-AAAACTAACCGAAACGACGACG-3′	153		
Su et al. (2010)	BST-QMSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	57.5	42.4
		m 5′-ATAGTCGGATCGAGTTAACGTC-3′	m 5′-AAAACTAACCGAAACGACGACG-3′	153		
Laytragoon-Lewin et al. (2010)	BST-MSP	5′-CGGTAGGGTTTGGGGTCG-3′	5′-AAACCTCCCAACTTCGATCG-3′	227	11	39
Steinmann et al. (2011)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	33	67
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		
De Schutter et al. (2009)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	40	11.4
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		
Righini et al. (2007)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	15	23
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		
Maruya (2004)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	13	19
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		
Rosas et al. (2001)	BST-MSP	u 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′	u 5′-CAAATCCCTCCCAAACACCAA-3′	106	60	33
		m 5′-GGATAGTCGGATCGAGTTAACGTC-3′	m 5′-CCCTCCCAAACGCCGA-3′	98		

Abbreviations: BST – Bisulfite Treated, MSP – Methylation Specific Polymerase Chain Reaction, BSP – Bisulfite Polymerase Chain Reaction, QMSP - Quantitative Methylation Specific PCR, N/A – Not Available, m – methylated, u – unmethylated.