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Assessment of Hypermethylation status of the promoter region of PITX1 gene in Oral Squamous Cell Carcinoma- A cross sectional Observation

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Abstract

Management of oral cancer is challenging due to the anatomic complexities and has a greater chance of recurrence. Homeodomain 1 (PITX1) gene is a potential tumour suppressor and is essential for the differentiation of oral epithelium and first branchial arch derivatives. In view of the fact that in the recent trend, there is a need for a biomarker for determining the treatment prognosis, the aim of this study was to evaluate the hypermethylation status of the promoter region of PITX1 gene in Oral Squamous Cell Carcinoma and to compare with normal tissue samples. A cross-sectional study comprising of 56 tissue samples was designed and divided into two groups as group A (*n*=31) including clinically and histopathologically confirmed cases of well- differentiated Squamous cell carcinoma and group B (*n*=25) comprising of tissue samples taken from clinically normal appearing mucosa adjacent to surgical extraction site. Amplification of PITX1 promoter region was done by running a PCR of total Genomic DNA to confirm the presence of PITX1 gene followed by Methylation specific PCR on tumour DNA samples. Hypermethylation status between tumour DNA and control group was analysed using Pearson's Chi square analysis. The statistical results were highly significant, at p=0.002. There was a significant difference in the hypermethylation status of the promoter region of PITX1gene, which gives a few insights that they have potential clinical implication. Further research on a larger sample, is needed to analyse methylation status of PITX1 in oral cancer to validate the results.

Keywords: PITX1 gene, Hypermethylation, Oral Squamous Cell Carcinoma.

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1. Introduction

Cancer is a heterogeneous group of disorders characterized by the presence of cells that do not respond to the normal controls on division. Cancer cells divide rapidly and continuously, creating tumours that outgrow normal cells and that deprive adjacent healthy tissues of nutrients as well. The current understanding in the molecular pathogenesis of oral cancer suggests that both the genetic and epigenetic alterations are implicated in this multistep process as they are known to complement each other during successive stages of oral carcinogenesis [1]. These genetic and epigenetic changes allows cell to break free from the tight network of controls that regulate the homeostatic balance between cell proliferation and cell death [2]. Disruption of this homeostatic balance either due to hyperactivation of oncogenes or dysregulation of protective nature of tumour suppressor genes also has a potential to cause cancer. H-Ras (Rat-derived murine sarcoma viruses) is one such oncogenes which is genetically deregulated or frequently mutated in ~ 30% of the cancers including OSCC [3]. PITX1 is a transcriptional activator and regulates the H-Ras pathway by transactivating RASAL 1 (a ras-GTPase activating protein) that helps in conversion of RAS-GTP back to RAS-GDP. Methylation of promoter region of PITX1 may result in silencing of PITX1 gene expression, which may be considered as an equivalent to loss of function of PITX1 or deletion of PITX1 gene. As a result of this, RASAL 1is not expressed, and hence H-Ras remains active even long after the receptor activation has ceased [4]. The promoter hypermethylation of PITX1 gene in oesophageal squamous cell carcinoma has been reported [5].

As the status of PITX1 gene expression in OSCC is thoroughly explored, the aim of this study was to investigate the hypermethylation status of the promoter region of PITX1 gene in Oral Squamous Cell Carcinoma (OSCC).

2. Materials and methods

2.1. Sample Collection and Criteria of Inclusion:

The study was initiated after getting an ethical clearance and after obtaining written informed consent from all patients. The tissue samples of *the study group and control group* were obtained from patients visiting the outpatient department of the institute and from patients of private dental practitioners, belonging to the same geographic zone, following due protocol. The experimental samples included Incisional, fresh biopsy tissue samples from the study and control group. *Group A* constituted of patients with clinically and histopathologically confirmed cases of well-differentiated Squamous cell carcinoma or untreated cases of oral squamous cell carcinoma. The sample size in this group was 31patients. *Group B* patients had no apparent pathology, and the tissue samples were taken from clinically normal appearing mucosa adjacent to surgical extraction site. 25 patients constituted the sample size of this group. The Exclusion criteria for Group A patients included no previous history of treated or untreated malignancies of any other system and Group B patients with no habits of tobacco usage (smoked or smokeless) and alcohol consumption.

2.2. Sample storage

The excised tissue sample was preserved in sterile microfuge tubes containing 1.0ml of RNA Save reagent and transferred to the refrigerator and 24h later the samples were transferred to deep freezer (-80^0C) and stored until transported to the laboratory.

2.3. DNA extraction & Analysis

DNA extraction was done using the chloroform method. This was followed by analysis of DNA quality by running the aliquot of the stock on 0.8% agarose gel. The quantity of DNA was determined by reading the samples in QUBIT fluorometer (Invitrogen, USA). Amplification of PITX1 promoter region was confirmed by running a PCR of total Genomic DNA to confirm the presence of PITX1 gene. Methylation Sensitive restriction digestion of tumour DNA samples was done using HpaII enzyme which was followed by Methylation specific PCR on tumour DNA samples. After the Gel electrophoresis, the data was interpreted using an UV camera. The results were statistically evaluated using Pearson's chi square analysis.

2.4. Statistical Analysis:

Statistical analysis was performed with SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY:IBM Corp. Released 2019. Hypermethylation status between tumour DNA and control group was analysed using Pearson's Chi square analysis. The level of significance was set at $p < 0.005$.

3. Results and Discussions

Gel analysis after Methylation specific PCR on HpaII digested tumour DNA showed that 10 out of 31 tumour DNA samples were amplifiable by PCR, which indicated hypermethylation of the promoter region of PITX1 in these samples (figure 1). Gel analysis showed that all the 25 normal tissue samples showed no amplified bands by PCR, which indicated absence of hypermethylation of the promoter region of PITX1 in these samples (figure 2). Hypermethylation status between tumour DNA and control group was analysed using Pearson's Chi square analysis. The statistical results were highly significant, at $p=0.002$. Contemporary literature informs that, various genetic markers, such as point mutations, Loss of Heterozygosity, Micro Satellite Instability have been extensively used in head and neck cancer for early tumour detection, prognostic prediction and elucidation of the genetic progression model; however, promoter hypermethylation is an alternative, attractive epigenetic marker that has recently gained in popularity. An epigenetic marker has advantages over a genetic markers as it is much more common than genetic alteration in cancer and this occurs in the same well-defined region of any given gene across all forms of cancer, so promoter hypermethylation analysis represents a much more efficient and cost-effective tumour detection approach [6]. Although many important genes and gene products have been identified through DNA methylation changes, no single unifying pathway has been identified that accounts for all OSCC [7].

PITX1 belongs to the homeobox family of genes and as stated these genes play a crucial role during development and when dysregulated can cause oncogenesis in adult tissues. PITX1 gene regulates the H-Ras pathway and thereby tumorigenesis and could be good prognostic markers [8]. *PITX1* expression is also decreased in several malignant tumours like gastric, colon and bladder cancer as well as HNSCC [4,9]. Reduced *PITX1* expression is associated with treatment response in oral cancer [8,10,11]. The molecular mechanisms of the PITX1 in tumour tissue need more clinical investigations and might be the result of epigenetic regulation. Aberrant PITX1 methylation has been found in salivary gland adenoid cystic carcinoma and is associated with survival in clear cell renal cell carcinomas [12,13].

H-Ras and K-Ras mutations may be present in majority of the tumours in Asian population [14]. Signal transduction from activated transmembrane receptors like EGFR depends on a variety of downstream mediators that are frequently altered in various cancer types [15]. Downstream components of the signal transduction cascade, like Raf and ERK and other MAP kinases, have received relatively less attention and are less well studied [16]. Much less attention has been shown on analyzing the transactivation factors for *Ras* in the upstream molecules. Literature search has revealed that there are only a few studies evaluating the dysregulation of PITX1 in OSCC.

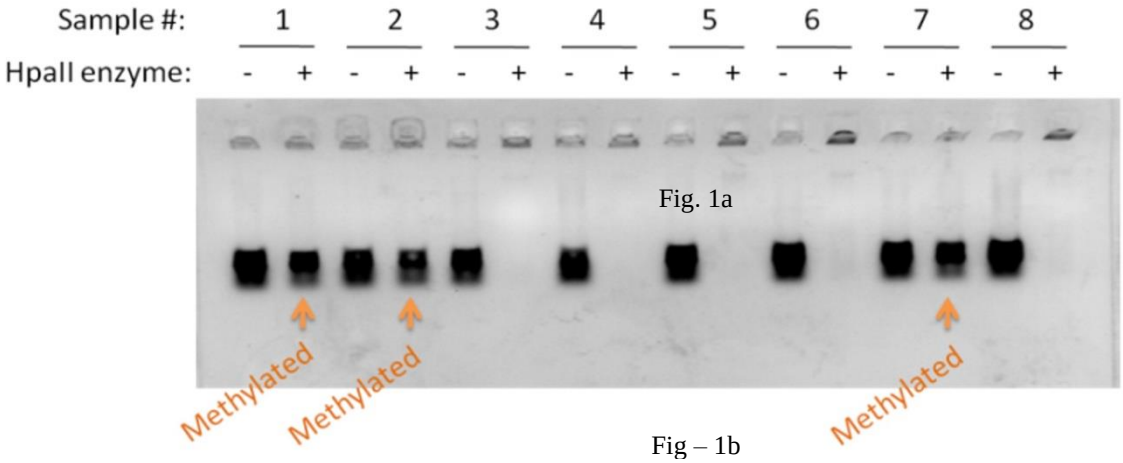

Fig. 1a

Fig – 1b

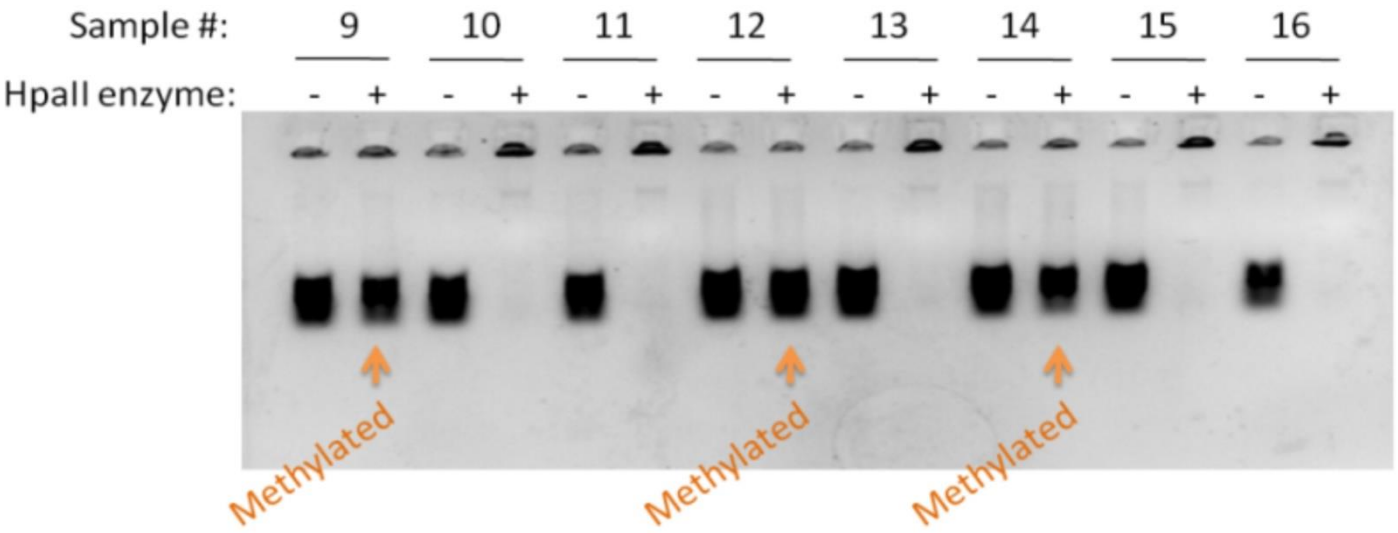

Fig. 1b

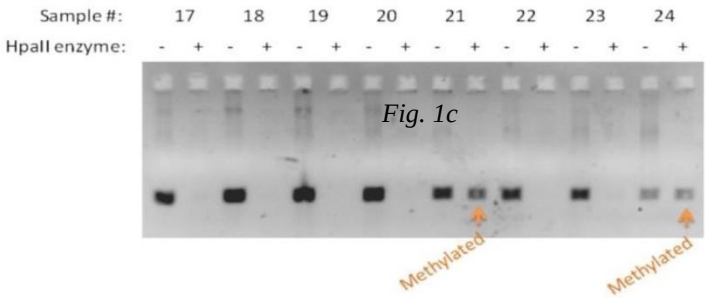

Fig. 1c

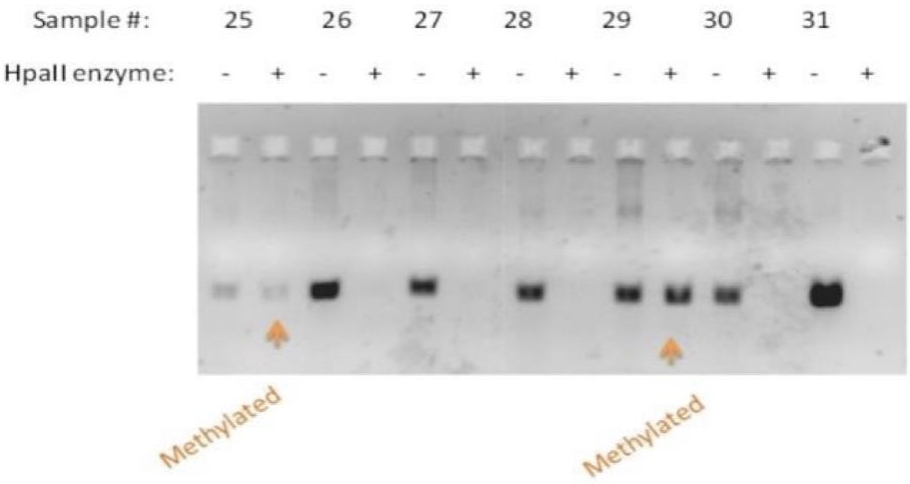

Fig. 1d

Figure 1(a,b,c & d): *PCR amplification of HpaII digested tumor DNA sample for the promoter region of PITX1.Gene amplification seen in samples 1, 2, 7, 9,12,14,21,24,25,29. (Indicated by orange arrow), suggestive of hypermethylation*

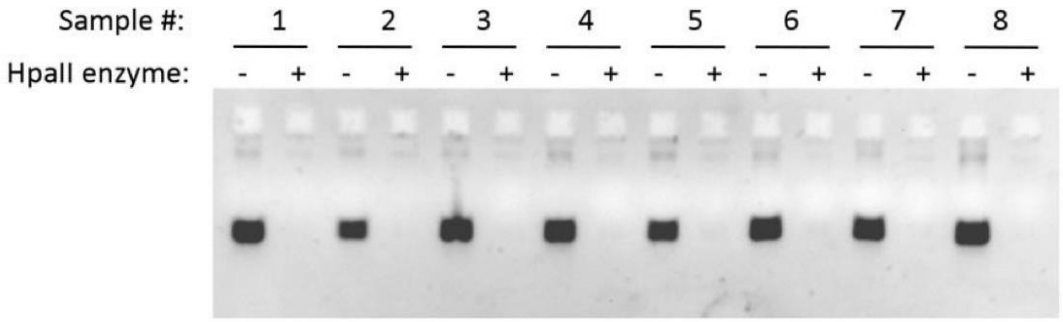

Fig. 2a

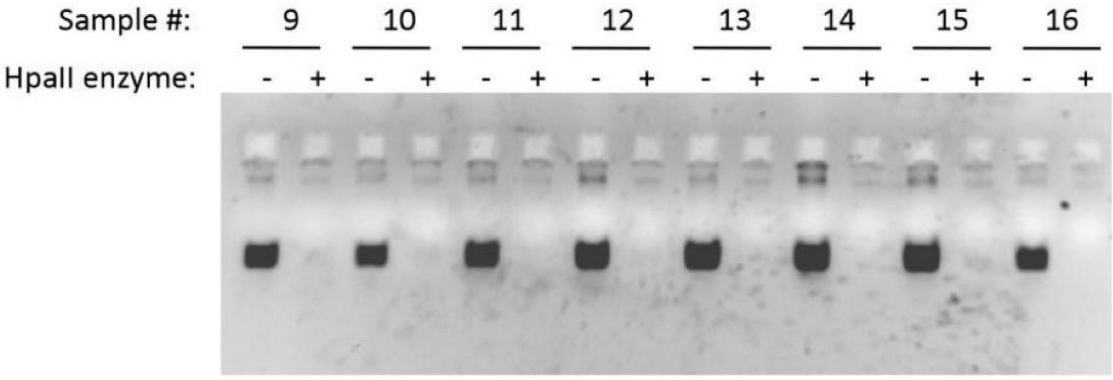

Fig. 2b

Figure 2 (a&b): *PCR amplification of representative HpaII digested control sample for the promoter region of PITX1 gene. No amplification seen*

Exclusion criteria were followed up to a reasonable level of certainty by not selecting individuals with any known conditions that may be influenced by dysregulation of PITX1 gene. The clinical history evaluation ensured that the patients did not have a past or current history of any coexisting malignancies (treated or untreated) of any system. It is an established fact that PITX1 dysregulation is consistently observed in malignancies of bladder and prostate where PITX1 protein expression levels were low compared with normal tissues [10,12]. Studies have also found link between PITX1 hypermethylation associated with adenoid cystic carcinoma of salivary gland [13]. The present study revealed methylation of PITX1 genes in 10 out of the 31 samples (32%) and statistically significant with Pearson's Chi square analysis at p=0.002. This result was higher than the work done on p16 gene [17], E-Cadherin [18,19]**,** p1448 and DAP kinase [20]. It is therefore imperative to analyse the results of 10 out of 31 patients of OSCC demonstrating hypermethylation with keen interest. Even though other researchers have shown methylation of genes such as MGMT [21], RARB [22], p16 [23] in oral samples to be greater than the present study, PITX1 gene is vital in DNA methylation and tumour-infiltrating immune cell regulation [24]. Hence the results of the current study reaffirms the understanding that it should be a part of the gene panel for screening of early OSCC.

4. Conclusions

The result of the present study predictably gives a few insights that have potential clinical implications. The pattern of hypermethylation of PITX1 offers several advantages and it can represent a chemically and biologically stable marker system that can be readily detected. It could serve as an efficient independent prognostic marker in management of oral cancers and has diagnostic/screening utility. The road ahead involves establishing the presence of hypermethylation of PITX1 in oral potentially malignant disorders as well as in tissues adjacent to the tumour site. Further research is need of the hour to analyse methylation status of PITX1 post operatively after therapeutic intervention as epigenetic changes are known to be potentially reversible.

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