ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 4, Issue – 3, June - 2021, Page No. : 321 - 329

Hypermethylation status of the promotor region of pitx1 gene in oral leukoplakia, oral submucous fibrosis

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Citation of this Article: Poongodi V, Uthara Menon, Kannan Ashokan, C.L. Krithika, Kanmani, "Hypermethylation status of the promotor region of pitx1 gene in oral leukoplakia, oral submucous fibrosis", IJDSIR- June - 2021, Vol. – 4, Issue - 3, P. No. 321 – 329.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Introduction

Oral cancer is a multifactorial disease and it accounts for approximately 30-40% of cancers in India WHO 1997. Despite recent advances in tumor surgery and multimodal treatment regimes, the prognosis of the oral carcinomas is still relatively poor. This may be attributable to the fact that, symptoms that indicate the presence of carcinoma often appear when the tumor is in advanced stage. oral cancer develops following subtle molecular genetic changes which are due to loss of molecular integrity after continued exposure to environmental risk factors or inheritance of predisposing genetic factors .Oral cancer is the sixth most common cancer reported globally with an annual incidence of over 300,000 cases of which 62% arise in developing countries and 30 % oral cancer reported in India when compared to US, where it accounts 3% of malignancies[1,2,3] and this variation in incidence is based on various risk factors such as smoking, chewing and smokeless tobacco, areca nut, pan, cigar and type of tobacco used.[4] Cancer ranks second most common morbidity and mortality after cardio vascular problems world-wide and oral squamous cell carcinoma is one of the most prevalent forms of cancer in India and majority of oral cancers are diagnosed at advanced stages and therefore, treated late. The implementation of newer screening and early detection approaches are of utmost importance which could reduce the morbidity and mortality associated with this disease. Sensitive and specific biomarkers for oral cancer are likely to be most effective for screening, diagnosis, staging and follow-up for which potentially malignant disorders undergoing malignant transformation greatly improve early diagnosis and enhance management[1,4,5,7].

Oral carcinogenesis occurs at basal cell layer under the influence of tobacco, alcohol, areca nut and/ or other carcinogens and may involve genetic aberrations. The transition of normal epithelium to invasive cancer is accompanied by promoting cellular proliferation, angiogenesis, local invasion and eventually to distant metastatic spread .The majority of the molecular/ genetic lesions will accompany histological transition from normal to cancerous epithelium[2,4,5].

Carcinogenesis is multi-step model and it is widely accepted that normal cells undergo step- wise transition to become malignant. A similar progression has been shown to occur in oral cancers arising from benign hyperplasia, to dysplasia and to carcinoma in situ, all of which have indeed shown to be accompanied by genomic alterations[2]. Potentially malignant lesion of particular relevance to oral cancer include oral leukoplakia, oral submucous fibrosis, oral lichen planus and oral erythroplakia [2,3.] Out of these erythroplakia has highest malignant potential of 90%,oral leukoplakia 3.6-17.5%, oral submucous fibrosis 0.5-6%, oral lichen planus 0.4-4% [2,3,7] and most prevalent potentially malignant disorders in India of present day scernio is oral leukoplakia [2,7] and oral submucous fibrosis [1 9,10] , .

Biomarkers are indicators of either a normal biological or pathogenic process that can be objectively measured and used as an evaluator to asses or predict the nature and behaviour of the disease. Saliva, blood, urine, tissue act as a biomarker[11]. Cancer biomarker include wide range of biochemical entities such as nucleic acid , proteins , sugars , lipids , small metabolites, cytogenic and cytokinetic parameters which can evaluate and predict genetic alteration and aberration[11,12,13]. Genetic aberrations that affect the functional expression of a gene in tumor cells include base mutation, duplications, deletions , epigenetic modification. Identification of biomarker in the past years has gained role in genetics in oral cancer as other specialization. p53, RB, DCC, caspase 8 Brush-1, BRCA-1, BRCA-2, PITX1are few biomarkers that been expressed in other cancer such as colon, bladder, lung and other cancer has also shown expression in oral squamous cell carcinoma[12,13,16,29].

Materials And Method

The study design was approved by institutional review and ethical Board. Study participants were obtained from the Department of oral medicine and radiology, SRM Dental College, Ramapuram, Chennai between the year of (Feb2013-July2015).

Seventy-five study participants are recruited based on inclusion and exclusion criteria. All the Study participants were explained about the study details and informed consent form was obtained from all patients before entering into the study Annexure (1,2). 75 patients with mean age group of 40 years with (64) males,(11) females respectively. Out of which 25 control samples where obtained from patients who underwent extraction for impaction of mandibular teeth and are clinically stated as otherwise normal patients and so, biopsy of the control tissue sample is not performed (In 17cases tissue obtained from the right mandibular region and 8 cases from the left mandibular region) and of approximately 3mm tissue sample is obtained uniformly from all the subjects collected in 1ml of RNA save reagent and stored in -20C until DNA extraction) and fifty study samples consist of clinically diagnosed as oral potentially malignant disorder (Out Of which 25-oral leukoplakia and 25-oral sub mucous fibrosis are included). These study participants were subjected to biopsy for histopathological confirmation. Tissue sample thickness of 6mm is incised and divided into two equal halves.one part of tissue sent to histopathological confirmation in 10% formalin & another part of tissue sample is uniformly collected in 2ml clean microfuge tubes containing 1ml of RNA save reagent and stored at -20C until the further use for DNA extraction. Histopathological evaluation of oral potentially malignant lesion was given by experienced oral pathologist based on architectural and cytological changes of cells and interpreted as (mild/ moderate/ severe) are included in the

study and after histopathological confirmation of dysplastic changes. Samples are further evaluated for DNA extraction to investigate the hypermethylation status in the promoter region of PITX1 gene.

Maximum storage capacity of tissue sample in RNA save reagent is 2 years without risk of degradation and reliable in gene expression and gene- profiling data.but, present study samples are processed less than a month after sample collection. Stored samples were uniformly transported in ice box with freeze gel packs to the laboratory.Minimum of 10 samples were carried to the lab at each time after sample collection.Twelve samples were processed at a time to extract DNA ,QUANTIFY, PCR and Gel Documentation.

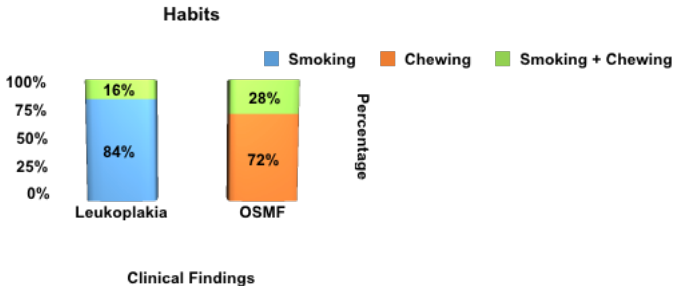
Results

To investigate whether methylation status is present in the promoter region of PITX1 gene in oral leukoplakia and oral sub mucous fibrosis and to associate the methylation status of PITX1 gene promoter region with the presence of leukoplakia and oral sub mucous fibrosis in relation to normal tissue samples and PITX1 gene role in potentially malignant lesion.In present study screening for promoter region of PITX1 gene in all samples, PCR amplification and agarose gel images are documented.

The study group comprised 50 cases of potentially malignant lesion and 25 normal patients with age ranging from 16 to 60 years with a mean age of 27.44 +/- 5.49years.

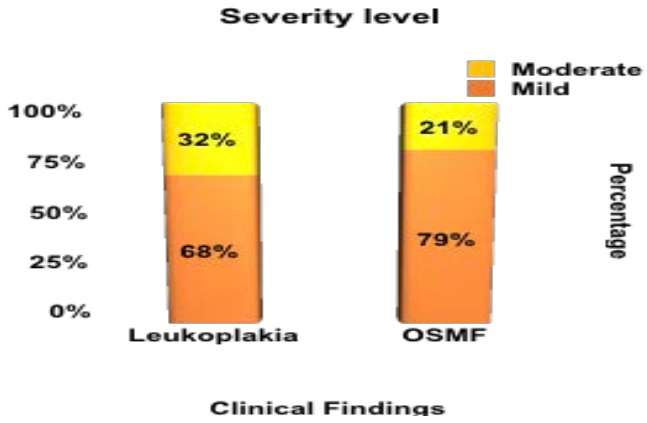
The study and normal group showed male predominance with 64 males(85.3%) and 11females(14.7%).

Most prevalent habits in the study group is smoking(42%) - cigarette (34%); tobacco chewing(36.0%) - pan masala (30%); smoking + tobacco chewing in (22.0%) - cigarette + pan masala 4% (chart-1).



Graph 1: Mean duration of habits in leukoplakia is 11.04% and osmf is 6.54%.

Most characteristic cytological and architectural changes in the tissue sample of study group with mean value showed mild dysplasia in 35(70%) and moderate dysplasia in 15(30%) samples. Methylation status in potentially malignant lesion (leukoplakia is of 16 % and in osmf and normal samples is of 0.0%).



Graph 2

Discussion

Methylation is a major epigenetic modification in humans and changes in methylation patterns play an important role in tumorigenesis.[39] Robertson et al stated from his study on DNA methylation that regions are frequently targeted for hypermethylation events are CpG islands. CpG islands are GpC- and CpG-rich regions of approximately 1 kb that are usually associated with the promoter or 5 end of genes.[40] Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner to mutations and deletions and act as one of the "hits" in Knudson's 2-hit hypothesis for tumor

generation.[40] Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation.[40]

Aberrant promoter hypermethylation of tumor-associated genes leading to their inactivation is a common event in many cancer types. Using a sensitive restriction-multiplex PCR method. Earlier studies have identified that promoter hypermethylation profile of the p16, p15, hMLH1, MGMT and E-cad genes in oral squamous cell carcinoma (OSCC) of Indians population.In their study total of 51 samples for the p15 tumor-suppressor gene and 99 samples for each of the remaining genes indicate an incidence of promoter hypermethylation of 23% each for p16 and p15, 8% for hMLH1, 41% for MGMT and 35% for E-cad. They observed aberrant hypermethylation of the promoter region of at least 1 of these genes in 74.5% of cases (n=51) for which all the 5 genes were studied. Abnormal methylation was detected in tumors irrespective of stage and location in the oral cavity, whereas no abnormal methylation was detectable in normal oral squamous tissues obtained from 25 OSCC patients. Detection of aberrant hypermethylation patterns of cancer-associated genes listed above is therefore suitable for diagnosis of OSCC in individuals at high risk for this disease.[41]

PITX1 Homeodomain transcription factors play fundamental roles in directing cellular proliferation, differentiation, and in determining cell fates,selected homeobox genes have been implicated in neoplastic development.[42] Jinyoung Yoo et al studied that tumorigenicity by downregulation of the RAS pathway through RASAL1, and low expression of PITX1 was found in colon cancer cell lines containing wild-type RAS.[43] Decreased PITX1 expression was also documented in lung cancer and Barrett's associated adenocarcinoma and was associated with tumor progression.Absence of PITX1 in cells may result in absence of RAS-GTPase and hence the activated RAS remains active even after the cessation of membrane borne signals. This scenario is equivalent to activating point mutations within H-ras gene that are known to cause the H-RAS protein to be constitutively active (i.e, active even in the absence of proliferation signals from the membrane). Activating point mutations within H- ras gene has been observed in several types of cancers including oral squamous cell carcinomas (OSCC). However, a significant numbers of OSCCs tumors still carry wild type H- ras gene, which suggests for the possible occurrence of genetic aberrations in other regulatory genes.

Recent investigations have identified a reduced level of expression of PITX1 in colon, bladder and prostate gland carcinomas. In addition, loss of expression of PITX1 due to promoter hypermethylation has been observed in salivary gland tumors. As promoter hypermethylation is known to silence the associated gene, we propose to investigate whether such hypermethylation is present within the promoter of PITX1 gene, in biopsy samples of two oral potentially malignant disorder namely, leukoplakia and oral submucous fibrosis. To the best of our knowledge, it is for the first time that the genetic status of PITX1 gene in the above two lesions is being investigated.

In our present study we have included samples of potentially malignant disorders consisting twenty five in each of oral leukoplakia and oral submucous fibrosis and.We arrived at sample size conclusion based on our study type and limitations of financial contribution and where this type of initial study may give us a clue for an extensive study in context to hypermethylation of PITX1 in potentially malignant disorders.

In current study, control samples were chosen from normal healthy individuals, instead of selecting the tissues from contra-lateral side of the lesions tissue. Due to ethical consideration incisional biopsy form a clinically uninvolved contra- lateral site could not be obtained in the study group hence,twenty five normal control tissue samples are obtained from the individuals who underwent treatment for impaction and who were willing to participate in the study with signed consent form.

Earlier studies have detected that hypermethylation have shown influence of tumour site because methylation status is not static in all sites but, in our study to avoid this differences in site, samples were selected only from the origin of buccal mucosa and this may also have interference in the outcome of results but,to prove the findings from the present study is not practically possible with smaller sample size but this can definitely can be taken as a one of the factor in further research.Present study result was agreed with Reibel. j et al has reported that most common sites for PMDs in India are buccal mucosa followed by tongue, palate and floor of the mouth. Location of PMDs differs from distribution of OSCC, for which the tongue, alveolar ridge and floor of mouth are the most common sites[44].

In present study demographic data of age group in study and control were mean of 27.44 years this data was agreed with study of antony george et al that PMDs affect the younger age group of 30 years. This may be due to the fact that various extrinsic and intrinsic etiological factors are now more prevalent in today's younger population[45].

Among genders present study revealed that male predominance of 85.3 % and female of 14.7% with was agreed with previous study PMDs have traditionally shown a predilection for males [45]. But recent studies showed that 1:1 male to female ratio. This could be due to the increased habitual use of tobacco and alcohol among women and it was contradictory to our present study.

In India, the main risk factors for potentially malignant disorders and OSCC are tobacco, bidi, pan masala, alcohol consumption and unhygienic conditions with poor nourishment.[46, 47,7] Vikram Bhatia et al reported that high promoter methylation frequency of MGMT AND p16 genes were observed in patients with premalignant lesions and OSCC who were tobacco chewers and bidi smokers indicating contribution of these risk factors to gene silencing by promoter hypermethylation has been studied in previous studies.[48] Our study showed most prevalent habits in the study group is smoking(42%) - cigarette (34%); tobacco chewing(36.0%) - pan masala (30%); smoking + tobacco chewing in (22.0%) - cigarette + pan masala 4%. Mean duration of habits in leukoplakia is 11.04% and osmf is 6.54% and this result was consensus with our study of prabha balaram[49]

The strength of our study is the well- characterized population of potentially malignant disorders,Which has gold standard baseline characteristic cytological and architectural changes in the tissue sample of study group with mean value showed mild dysplasia in 35(70%) and moderate dysplasia in 15(30%) samples. Methylation status in potentially malignant lesion (leukoplakia is of 16 % and in osmf and normal samples is of 0.0%) and there is no severe dysplasia in the study group.

The present study results have showed that hypermethylation and unmethylated DNA samples of PITX1 gene in study and control groups. Pertinently, in our study, we observed significant promoter methylation in 16% of samples with oral leukoplakia and on correlating these samples with histopathological findings they are diagnosed as mild dysplasia. Which gives us a break through results of such hypermethylation in

potentially malignant disorders and study results also showed that the absence of hypermethylation status/ unmethylated region in oral submucous fibrosis and control samples.The proportion of present study between control and study samples. Leukoplakia samples are statistically significant (P<0.03) when compared to oral submucous fibrosis and control samples.

This findings can attribute because of prevalence of malignant transformation of potentially malignant disorders (i.e) oral leukoplakia transformation rate multiple studies over the years have shown a malignant transformation rate of 3.6-17.5%, while few Indian studies have shown a transformation rate as low as 0.3-0.5%. and for oral submucous fibrosis is 0.5-6%[50,51,45] hence, the variance in result could have occurred and to prove this occurrence of result and to apply in day to day clinical practice larger sample size with same ethnic background are required.

Hypermethylation status of promoter region of PITX1 has been reported colon, bladder, prostate gland, salivary gland carcinomas. As of now there is absolutely no data available on the prevalence of PITX1 gene with potentially malignant disorder and early diagnosis of cancer with higher transformation rates of potentially malignant disorder and with their likely occurrence change of rate to oral squamous cell carcinoma can be prevented by identifying biomarkers and were the genotype identification are the end point in preventing and reducing the morbidity of cancers. Our study have may be considered as the fit one to address the new findings in the field of oral cancers.

Conclusion

Genetic and epigenetic changes are shown to influence virtually in the tumour development. A better understanding of this perspective will influence and guide us in preparing diagnostic protocol and determine the good prognosis of the cases.

Hypermethylation in promoter region of PITX1 gene was evident in four of our study may have effect on oral leukoplakia, but a substantial evidence need to be obtained by doing an explorative with larger sample size.

The result of the present study predictably gives a few insights about potential clinical implication.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 would be pertinent to point out that use of this gene in panels of genes would have diagnostic/ screening utility.

Further research is need of the hour to analyse methylation status of PITX1 gene in larger population with long - time follow up are proposed from the present study hence, we can explore a new area in regard with the genetic analysis in potentially malignant disorders of Indian population.

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