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RESEARCH ARTICLE

DETECTION OF MUTATION IN ORAL LEUKOPLAKIA AND LEUKOPLAKIA ASSOCIATED OSCC TO ASSESS THE PROGRESSION FROM PRECANCER TO CANCER

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ABSTRACT

Leukoplakia is the most potentially malignant disorder. Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. The study was undertaken with a view to assess p53 mutation in oral leukoplakia, OSCC and risk of progression from pre-cancer to cancer. A total number of 15 OL and/or OSCC patient studied. Clinical diagnosis, histopathological analysis and sanger sequencing are done. Among them 6 SNPs were detected in 5 OSCC & 3 OL patients. Two TP53 somatic mutation detected in patient sample.

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INTRODUCTION

Precancerous lesion is defined by the World Health Organization (1978) as "a morphologically altered tissue in which cancer is more likely to occur than its normal counterpart"(1). The most commonest potentially malignant disorder of the oral mucosa is Oral Leukoplakia (OL). Oral Leukoplakia was defined in 1997 by WHO as "*A predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion*"(2). Microscopically Oral Leukoplakia is characterized by hyperkeratotic surface epithelium with or without thickened spinous layer (acanthosis). The striking feature is the presence of varying degrees of epithelial dysplasia characterized by both cellular and nuclear atypia (3). Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. An oral premalignant lesion is an area of morphologically or genetically altered tissue that is more likely than no/(4). Oral Squamous Cell Carcinoma (OSCC) is the most common malignancy affecting the oral cavity and oropharynx (5). Despite the variation in the incidence rate in various parts of the world, the highest incidence is in the Indian subcontinent (6). OSCC is caused by a variety of genetic factors can lead to the development of OSCC associated with the accumulation of multiple genetic and epigenetic changes in a variety of cellular pathways. Genetic alterations in cancer may occur in the form of small intra-genic mutations, such as point mutations and insertions/deletions, or large alterations including genomic deletions, amplifications, and chromosomal rearrangements (7) .

Whole cancer exomes or genomes can be evaluated for genetic aberrations using high throughput sequencing method. The proteins produced from several of the genes associated with OSCC including *TP53*, *NOTCH1*, and *CDKN2A*, function as tumor suppressors, which means they normally keep cells from growing and dividing too rapidly or in an uncontrolled way (8). When tumor suppressors are impaired, cells can grow and divide without control, leading to tumor formation. It is likely that a series of changes in multiple genes are involved in the development and progression of OSCC (9). This study has been designed to detection the p53 mutation in normal oral mucosa, oral leukoplakia and OSCC in a patient population from Kolkata, India with following aims & objectives.

AIMS AND OBJECTIVE

- To record the cardinal features of the light microscopic histopathological images of Oral leukoplakia and squamous cell carcinoma.
- To study the mutation profiles of already reported genes in OSCC and associated leukoplakia tissues from same individual.
- To observe the progression of diseases from precancer to cancer.

MATERIALS AND METHODS

This research study was conducted in the Department of Oral and Maxillofacial Pathology, Guru Nanak Institute of Dental Sciences and

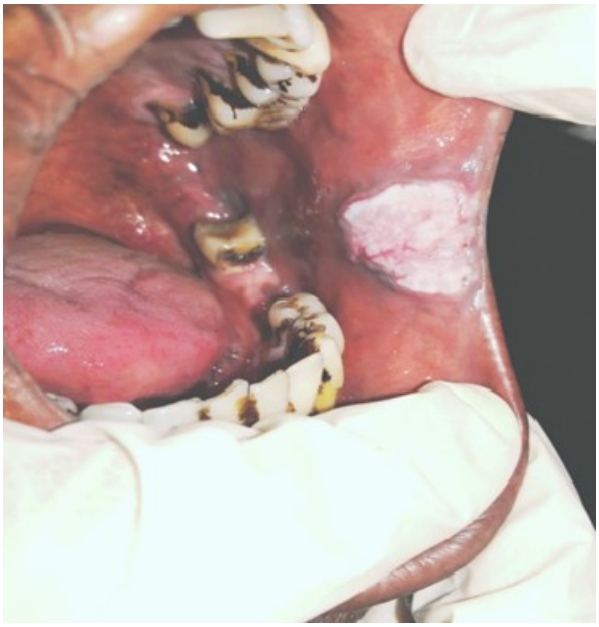

Figure 1. Clinical Photograph showing the presence of Leukoplakia associated OSCC

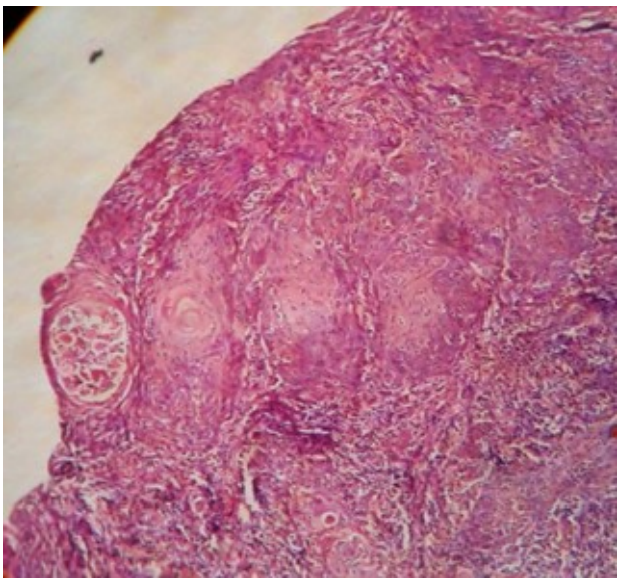

Figure 2. Photomicrograph showing severe degree dysplasia associated with OSCC

Figure 3. Dna Isolation Steps

Research (GNIDSR), Kolkata in collaboration with Human Genetics Unit, Indian Statistical Institute, Kolkata during the period of December 2015 to July 2017. The study was accorded approval by Institutional Ethical Committee (GNIDSR).

SNP: Single Nucleotide Polymorphism; rsID: SNP id provided by dbSNP database; Hg19: Human genome reference 19

Figure 3. Electropherogram showing the somatic mutation in p53gene; Intron 7, 7577424 A>T

SELECTION OF STUDY SUBJECTS

For selection of the study subjects, the patients attending the outpatient department of GNIDSR, Kolkata, were screened thoroughly with a view to detect the presence of Oral Squamous Cell Carcinoma (OSCC) and Oral Leukoplakia (OL) as per clinical criterion laid down by Neville (2009) and Marx & Stern *et al*(2003). Initially, 26 patients of OSCC & Oral Leukoplakia were selected and the following inclusion and exclusion criteria were laid down:

Inclusion criteria

- Patients of 40-80 years of age associated with the history of tobacco usage (smoking and/or chewing).
- Individuals without systemic disorders.
- Confirmed cases of Oral Leukoplakia and OSCC.
- Exclusion criteria –
- Medically compromised patients.

RECORDING OF CLINICAL DATA

Then the details of subjective and objective features of all the study subjects were recorded in the especially prepared clinical case sheets as per guidelines laid down by Neville *et al*, 2009. Clinical photographs were taken from appropriate sites. Information on age, sex, ethnicity, occupation, economical status (per capita income in India 8,583INR per month by CSO(10), food habit, alcohol consumption, types of tobacco habit, daily frequency of tobacco usage, duration of tobacco habit was collected from the patients in person. Written informed consent was taken from each patient before collecting samples. However five of the study subjects did not extend their written consent. Finally 15 patients were included in this study.

COLLECTION OF BLOOD AND TISSUE SAMPLES

2-3 ml. of intravenous blood was collected from each patient by venepuncture method. Incisional biopsy was performed from representative sites of the lesion after achieving local anaesthesia using lignocaine hydrochloride with adrenaline (1:80000). Proper care was taken so that the specimens were having sufficient depth and without any laceration. Adjacent clinically normal tissue samples were also collected.

PRESERVATION OF SAMPLES

After collection, each tissue sample it is divided into two parts. One part is kept in 10% neutral buffered formalin for histopathological evaluation and the other part (10-30mg) is stored in "RNA later" solution for mutation detection. Collected blood samples were stored at -20˚C temperature while the tissue samples were stored at -800˚C temperature for mutation detection.

PROCESSSING, SECTIONING AND STAINING OF TISSUE SAMPLES: After 24 hours of the tissue fixation done in 10% buffered formalin. These sections were then stained with Haematoxylin and Eosin in accordance to the protocol laid down by Bancroft & Gamble (11).

EVALUATION OF H & E STAINED SECTION: All the aforesaid stained sections were carefully evaluated under light microscope (Olympus CH20i) and accordingly histopathological diagnosis of OL and OSCC were confirmed and findings were recorded. After histopathological confirmation was made, all the tissues preserved in RNA Later solution were used for subsequent genetic mutation study.

ISOLATION OF DNA FROM COLLECTED SAMPLES: DNA isolation from blood and tissue samples were done by QIAGEN kit-based method. The prescribed protocols of the kit manufacturers were followed during isolation procedure.

Isolation of DNA from blood samples: QIAamp DNA mini kit was used for isolation of DNA from blood samples (200 µl volume of whole blood).

- Thawing
- Lysis
- Binding
- Washing
- Elution

Isolation of DNA from tissue samples: Qiagen All Prep DNA/RNA mini kit was used for purification of DNA from oral tissue samples.

Sample thawing

1)Tissue samples stored in *RNA-Later* solution were thawed from -80°C to room temperature.

Homogenization and lysis

DNA eluting

2)The All Prep DNA spin column was placed in a new 1.5 ml. collection tube. 100 µl buffer EB was directly added to the spin column membrane and lid was closed. It was incubated at room temperature (15-25°C) for 1 minute and then centrifuged for 1 minute at ≥ 8000g (≥ 10000 rpm) to elute DNA. Once the DNA was isolated it was kept at -20°C for future use.

Quantification of isolated DNA samples: DNA samples were quantified and checked for quality by Nanodrop 2000 instrument. Sam

PCR: PCR amplification of DNA samples with primer pairs was performed to obtain the desired amplicon (Table2). The PCR amplicon is visualized in agarose gel electrophoresis method.

Sequencing PCR: Sequencing PCR is performed using the following protocol. Sequencing buffer (5X) – 1.5 µl , BigDye Terminator – 1.0 µl, Forward/ Reverse primer – 0.3 µl, PCR product – 1.5 µl, DNase-free water – 5.7 µl

DNA sequencing by Sanger method: Sequencing PCR products are washed for removing excess PCR reagents from the reaction mixture. The reaction mixture was loaded to the ABI 3100 Genetic Analyzer instrument for capillary electrophoresis. An optical detection device detects the fluorescent labels of DNA fragments and converts the fluorescent signals to digital data. The DNA sequence data is obtained as a chromatogram.

Sequencing Data Analysis: DNA sequences of tumor and blood samples were obtained after Sanger sequencing. Sequences of blood and disease tissue DNA of the same patient were compared with the human genome reference sequence (Hg19) for variant calling with the help of alignment software BioEdit. Somatic mutation was called when the disease and blood/normal DNA sequences of same patient were different in a nucleotide position. SNP was called when the disease and blood/normal sequences of the same patient were same but differed from the reference (Hg19) sequence.

RESULTS

This study conducted at the city of Kolkata revealed that all the selected patients lived in the city of Kolkata or its suburbs being located at the eastern part of India. Most of the patients belonged to low-income group and had similar nutritional status. None of these patients were exposed to specific occupational or environmental carcinogens other than tobacco usage.

Demographic details of samples: Tissue and blood samples were collected from a total of fifteen patients of which seven patients were diagnosed as sufferng from oral squamous cell carcinoma), four patients each of oral leukoplakia and leukoplakia associated OSCC . The age of the patients ranged from 40-80 years. Twelve patients were males and the rest were females. All of the patients had the habit of either smoking or chewing or mixed habit of both smoking and using smokeless tobacco. Six exons and the introns within these exons of TP53 gene were selected for searching somatic mutations and SNPs. Four primer pairs were used to amplify the desired regions of the gene. A total 8 variations were identified in 8 patient samples. Six SNPs were detected in 5 OSCC and 3 leukoplakia patients . Two TP53 somatic mutations were detected in GNDC-48 and GNDC-66 patient samples. Among the 6 SNPs (chr17:7577407 T>G, chr17: 7577427 C>T, chr17: 7579472 C>G, chr17: 7579642 G>A, chr17: 7579619 C>A and chr17: 7576963 G>A), 5 SNPs are located in intronic region (chr17:7577407 T>G and chr17: 7577427 C>T in intron 7, chr17: 7579642 G>A and chr17: 7579619 C>A in intron 3, chr17: 7576963 G>A in intron 8) and one SNP is located in exonic region (chr17: 7579472 C>G in exon 4). Two SNPs (chr17:7577407 T>G, chr17: 7577427 C>T) are present in both patients. The SNP chr17: 7579472 C>G is present in 5 patients. SNPs (chr17: 7579642 G>A and chr17: 7576963 G>A) are present in two patient samples. SNP, chr17: 7579619 C>A, is present in 2 patient samples. Among the 6 SNPs, only chr17: 7579472 C>G is a non-synonymous mutation (P33R). Two somatic mutations are identified in the disease tissue samples compared to the blood samples of the respective patient. GNDC-48 has a somatic non-synonymous mutation in exon 7 (chr17: 7577551 G>A; G205S). The second somatic mutation is at chr17: 7577424 A>T in intron 7 in patient sample GNDC-66 (Table 5.2).
Mutations in p53 gene

DISCUSSION

The aims & objectives of the present study are to detect the variations in the gene TP53 with a view to predict the possible contribution of these variations in the patient suffering from Oral Leukoplakia, OSCC or both. The present study revealed that maximum number of patient affected with OL were in the age group of 40-60 years while the OSCC cases primarily belonged to the age group of 40-80 years. Considering the above facts it can be suggested that, individuals belonging to older age group are at higher risk of developing precancerous lesions and/or malignancy. Gender predilection as assessed through the present study showed that both OL (85%) and OSCC (81%) affects men more than woman. Several researchers reported that 55-87% of OL were diagnosed in male (Mehta *et al.*1969, Roed-Petereson and Pindborg 1973; Bhonsle *et al.*1976; Salem *et al.*1984) while Saxena *et al.*1965, Wahi *et al.*1971 and

Fahmy *et al* 1983, Sanghvi *et al*.1986, SEER 2012 also reported higher incidence rates of oral cancer among male. Thus the present study upholds the opinion of the previous researchers regarding sex predilection. Hence the above facts strengthen the observations made by the earlier researchers. Thus the entire gamut of clinico-epidemiological evaluation of the study subjects have had justified their inclusion for further genomic assay - the nuclear aim and objective of the present study. Thereafter, the study subjects suffering from OL or OSCC or both were subjected to analysis of genetic variations relating to the gene TP53. Six exons and the introns within these exons of TP53 gene were targeted for PCR amplification and sequencing for variant detection. DNA sequencing was performed from blood/normal tissue and disease tissue of 15 patients (7 patients with OSCC, 4 patients with Leukoplakia and 4 patients with both OSCC and Leukoplakia). Six SNPs and 2 somatic mutations were identified in 8 patient samples. One SNP and one somatic mutation were exonic and non-synonymous, whereas all other variations were located in the introns. TP53 gene plays an essential role in the cellular response to stresses and in maintaining genomic integrity of the cell. Thus it also has important functions in carcinogenesis and has been known as a tumour-suppressor gene for a long time. TP53 protein has been found to be involved in multiple pathways of cancer and is believed to have context-specific roles. In absence of TP53 activating signals, the cellular expression of TP53 is maintained at a comparatively low level with stress inductions like oncogenic expression, DNA damage and metabolic dysfunction, TP53 protein is accumulated and activated. Activated TP53 protein acts as a sequence-specific transcription factor. TP53 mediated transcriptional regulation varies with the type of stress stimuli and the type of cells. The tumour suppressor functions of TP53 also include apoptosis, senescence, cell cycle arrest, DNA damage repair etc. It has been suspected that TP53 might also have some other unknown functions to operate tumour suppression (A Petitjean *et al*.2007). Functional inefficiency of TP53 is mostly due to mutations and can contribute in initiation or progression of cancer. TP53 gene is one of the most frequently mutated genes identified in different cancers. Nearly 50% of the cancer patients are reported to carry somatic mutations in TP53 gene (Pierre Hainaut1 *et al* 2016 (12).Somatic mutations in genes are generally considered as the signatures of cancer, but genetic polymorphisms in TP53 as well as in some other genes are also known to increase the susceptibility to cancer. Most of the TP53 mutations are non-synonymous mutations causing change in amino acid. The non-synonymous SNP (chr17: 7579472 C>G; P33R) and somatic mutation (chr17: 7577551 G>A; G205S), found in this study, will cause amino acid change which may lead to structural change of the protein and also alter the function of TP53. However the functional effect of the intronic SNPs and somatic mutation could not be predicted through this work although there are reports of intronic TP53 mutation resulting in alteration in splicing (Takashi Takahashi *et al*.1990 (13). So, mutation study in a large number of samples might give a better insight regarding the role of these polymorphisms and somatic mutations in the function of TP53 protein.

SUMMARY AND CONCLUSION

The study was undertaken with a view to assess the p53 mutation in OL, OSCC and risk of progression from pre-cancer to cancer in patients from Kolkata. The study was conducted in the Department of Oral and Maxillofacial Pathology, Guru Nanak Institute of Dental Sciences and Research, Kolkata and Human Genetics Unit, Indian Statistical Institute (ISI), Kolkata. A total number of 15 OL and/or OSCC patients were included from the patients attending OPD of Guru Nanak Institute of Dental Sciences & Research, Kolkata. Biopsies were performed from representative sites and adjacent normal of the lesion with due consent. Biopsy specimens taken from diseased oral mucosa were divided into two halves. One half was used for subsequent microscopic evaluation to confirm the clinical diagnosis while the other half was used for mutation detection. P53 mutation,SNP were detected in DNA isolated from blood as well as tissues of the OL and/or OSCC patients using procedures like PCR and DNA sequencing. The findings thereafter were recorded carefully, analysed, compare and corroborated in reference to the aims and objectives of this study and following observations were drawn.

- Six SNPs were detected in 5 OSCC patients and 3 OL patients.
- Two TP53 somatic mutation were detected in patient samples. Among the 6 SNPs (chr17:7577407 T>G, chr17: 7577427 C>T, chr17: 7579472 C>G, chr17: 7579642 G>A, chr17: 7579619 C>A and chr17: 7576963 G>A), 5 SNPs are located in intronic region (chr17:7577407 T>G and chr17: 7577427 C>T in intron 7, chr17: 7579642 G>A and chr17: 7579619 C>A in intron 3, chr17: 7576963 G>A in intron 8 and one SNP is located in exonic region (chr17: 7579472 C>G in exon 4). Two SNPs (chr17:7577407 T>G, chr17: 7577427 C>T) are present in both patients, GNDC-48 and GNDC-67. The SNPs chr17: 7579472 C>G is the most commonly present among in 5 patients (GNDC-48, GNDC-49, GNDC-66, GNDC-67, GNDC-71). SNPs chr17: 7579642 G>A and chr17: 7576963 G>A SNPs are present in each of the patient samples, GNDC-51 and GNDC-78 respectively. SNP, chr17: 7579619 C>A, is present in 2 patient samples, GNDC-51 and GNDC-56. Among the 6 SNPs, only chr17: 7579472 C>G is a non-synonymous mutation (P33R). Two somatic mutations are identified in the disease tissue samples compared to the blood samples of the respective patient. GNDC-48 has a somatic non-synonymous mutation in exon 7 (chr17: 7577551 G>A; G205S). The second somatic mutation is at chr17: 7577424 A>T in intron 7 in patient sample GNDC-66. However the functional effect of the intronic SNPs and somatic mutation could not be predicted through this work although there are reports of intronic TP53 mutation resulting in alteration in splicing. However, keeping in view the complex biology involved in the conversion OL to OSCC and role of mutation, further in-depth studies involving more number of study samples are required to authenticate the present research study.

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