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Original Research Article

To Compare P53 Gene Aberration in Oral Leukoplakia Patients and Healthy Individuals by Fluorescence in Situ Hybridization and its Associations in Indian Population.

Kanika Rathore[1], Mohan Singh[2], Garima Khatri[3], Vikrant Shekhawat[4*]

[1]Ph.D Scholar, Department of Anatomy, S.P. Medical College, Bikaner
[2]Senior Professor, Department of Anatomy, S.P. Medical College, Bikaner
[3]Professor, Department of Anatomy, S.P. Medical College, Bikaner
[4]Assistant professor, Department of Orthopaedics, S.P. Medical College, Bikaner

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Corresponding Author: Dr. Vikrant Shekhawat.
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Abstract:

Introduction: Oral cancer is among the most prevalent medical conditions in India and the nations that collectively make up the Indian subcontinent. An innovative method for managing this malignancy is early leukoplakia detection. In the oral cavity, oral leukoplakia (OL) is thought to be the most prevalent premalignant oral lesion. Leukoplakia is one of several precancerous oral lesions that also includes erythroplakia, lichen planus, and submucousfibrosis. The primary cause of oral cancer is tobacco use.Other contributing factors to the development of oral cancer include the human papillomavirus, ethnicity, socioeconomic status, inadequate nutrition, and inadequate dental hygiene. The situation is particularly concerning for the Indian population because around 10% of all malignancies arise in this group each year. Oral squamous cell carcinoma aetiology may involve oncogene and tumour suppressor gene deactivation and aberrant expression.

Material and Methods: This study was carried out in Department of Anatomy, S.P. Medical College and Associated Group of Hospitals, Bikaner, Rajasthan. Using an informed consent form, an overall of 50 OLP individuals as well as 50 healthy people were enrolled as controls in the study. Tissue sections were submitted to FISH analysis using the locus specific identification (LSI) TP53/CEP 17 FISH Probe Kit, a publically available probe from Vysis, after protocol optimisation.

Results: Our study found that the largest number of leukoplasmic patients fell between the 40–60-year age range, and that males were more common than females in both the case and control groups. Mean age group of cases is 48.64 with SD=12.83. Mean age group of control group is 47.02 with SD=14.13. In the case group, 3 subjects (n=3) exhibited p53 gene aberrations, specifically 2 cases with gene amplification and 1 case with gene deletion while no other subjects displayed such deviations. The site of lesion in cases mainly involved was buccal mucosa, tongue and labial mucosa.

Conclusion: The chromosomal structure research field entered a new era with the development of the FISH technology. Thus, the present study has been done by the FISH technique to identify the numerical aberrations in p53 gene with the patients with oral leukoplakia and to compare the data with the control group as there are very less study on the leukoplakia in the western zone of Rajasthan.

Due to less number of cases and limited aberration there is limitation in our study to establish the correlation of type of numerical aberrations with age, gender, site, habits and grading.

Keywords: Oral leukoplakia, Fluorescence in situ hybridization (FISH), Chromosome17, p53 gene.

Introduction

A wide range of illnesses that might develop in the oral cavity are together referred to as premalignant oral disorders. The secret to getting the best results is early detection and quick management. However, medical professionals still lack a great deal of expertise in this field.[1] Therefore, there is still a great need to comprehend and identify the aetiology, presentation, and treatment of these disorders.

The oral cavity is defined as the anterior two thirds of the mouth, which include the lips, gums, buccal mucosa, gingiva/alveolar ridge, hard palate, floor of the mouth, and oral tongue. Premalignant oral lesions are often caused by abnormal growth in the stratified squamous epithelium that lines the majority of the mouth cavity. The premalignant oral lesions that are most commonly documented include erythroplakia, submucous fibrosis, lichen planus, and leukoplakia.[3-6] Tobacco, alcohol, and cigarette smoke are the most often mentioned aetiologies of premalignant oral lesions.[7,8] Chewing betel nuts has also been linked to the emergence of oral leukoplakia.[9] The pooled HPV DNA prevalence for oral premalignant illnesses was determined to be 22.5% in the most recent meta-analysis of 52 studies. This suggests that the human papillomavirus (HPV) may be a risk factor.[10]

Between 1.5% and 4.5% of people worldwide have oral premalignant lesions, which preferentially affect men over women.[11,12] Of all newly diagnosed cases of cancer of the oral cavity, 17% to 35% are oral premalignant lesions. Every year, 0.7% to 2.9% of these lesions progress into malignant changes.[13-14]

The malignant transformation of OL is a serious problem. The transformation rate has been reported to fluctuate greatly, from 0.13% to 40.8%, with an estimated 1% to 3% every year.[15-17] Multiple mutations in growth regulating genes,[18] which are caused by malignancy, activate the oral mucosa's proliferative activity. It was not until the early 1970s that chromosome research, or cytogenetics, became involved in the diagnosis of cancer. Molecular cytogenetics has grown significantly in recent years and is now crucial to the detection and treatment of cancer. Fluorescence in situ hybridization (FISH), one of the most sophisticated molecular techniques, possesses an ideal blend of high specificity, sensitivity, and speed, and is frequently employed in clinical laboratories for the purpose of genetic diagnosis.[19] FISH is a more useful tool in advanced molecular cytogenetics than traditional cytogenetics (karyotyping) since it does not require in vitro culture or the preparation of the target cells for metaphase. It also allows for the analysis of cells in interphase. Generally known as "interphase cytogenetics", the fluorescence in situ hybridization (FISH or ISH) approach employs chromosomal-specific probes to enable the targeted detection of numerical chromosome abnormalities in the interphase nucleus.[21] A strong tool for the quick and accurate identification of chromosomal abnormalities is the FISH approach. Furthermore, FISH is a very suitable technique for examining individual cells and can significantly enhance our understanding of the genetic diversity present in biological specimens.[22]

Our study's objective was to compare the p53 gene aberration and its relationships in the Indian population between patients with oral leukoplakia and healthy individuals using fluorescence in situ hybridization.

Material and Methods

The present study was conducted in the department of Anatomy of S.P. Medical College and Associated Group of Hospitals in Bikaner, Rajasthan, with prior Institutional Ethical Committee's approval. A total of 100 volunteers (n=100) were chosen at random from the outpatient dental and ENT departments with their previous consent provided via a written informed consent form.

Subjects were divided in the following two groups to get the best results:

1. 50 Controls (n=50)
2. 50 Cases (oral leukoplakia Patients) (n=50).

1. Controls

Inclusion Criteria: Apparently healthy subjects without any signs and symptoms of carcinoma and oral pathology.

Exclusion Criteria: Subjects having presence of clinically proved case of any oral lesion.

2. Cases

Inclusion Criteria: Case group comprises clinically and histopathologically proved Oral leukoplakia patients.

Exclusion Criteria

1. Clinically proved cases of OSCC.
2. Clinical sign and symptoms of oral carcinoma.
3. Patient having presence of any other oral pathology except oral leukoplakia.

Fifty OLP patients and fifty healthy individuals were enrolled as controls throughout the study with prior approval using a written consent form.

The tumour tissues were ready for paraffin embedding using standard protocols. For the cases, 5 mm sections are taken from the paraffin block that contains the histological type and differentiation stage. Following the optimisation of the Abbott / Vysis manufacturer's protocol, tissue sections underwent the interphase FISH method. The locus specific identifier, LSI TP53/CEP 17 FISH Probe Kit, is a readily accessible probe designed to measure the copy number of both the CEP 17 (17p11.1-q11.1 Alpha Satellite) probe Spectrum Green Dual Colour target situated at the centromere of chromosome 17 and the LSI TP53 probe Spectrum Orange target located at chromosome 17p13.1.

Procedure

The paraffin section specimen slide is preheated to 56 degrees Celsius and submerged in xylene for 15 minutes at 40 degrees Celsius. After that, the slides were submerged in 100% EtOH to remove moisture. Next, the slides were allowed to air dry. After thirty to forty minutes at 80 degrees Celsius in 1 M NaSCN, the pre-treatment slide was immersed in the wash buffer (2 X SSC). The pre-hybridization of protease buffer is done at 37°C for seven minutes. Cleansing & dehydration in EtOH come next. Ten mm of the probe was added to each plate in the selected hybridization region after the slides were allowed to air dry.

The smears were sealed, placed on a 22 x 22 mm coverslip, and incubated for 10 minutes at 78 degrees Celsius and then for 18 hours at 37 degrees Celsius. Following hybridization, two washes were carried out using the following washing solutions: 0.4 x SSC/0.3% NP40 for two minutes at 73 degrees Celsius, and 2 degree SSC/0.1% NP40 for one minute.

40,6-Diamidino-2-phenylindole (DAPI II) has been added to the slides for counterstaining after they had been air dried and dehydrated. The slides were examined using an x100 magnification fluorescent microscope equipped with DAPI filter sets. The images were captured with a digital camera. For each patient, hybridised signal have been found in 200 interphase nuclei. Using in situ hybridization, we counted the number of p53 gene deletions/amplifications and chromosome 17 abnormalities in 50 paraffin fixed OLP samples.

Results

The subjects under study were made to fill a questionnaire regarding age, weight, height, their socio demographic details, addiction habits and consent.

In our study, 38 (76%) males and 12 (24%) females were observed in the case group while 35 (70%) males and 15 (30%) females were there in the control group. It was observed that in the case group and control group males were in high frequency than of females**.**

In our study case group, 7(14%) belonged to the 31-40 years of age group and 5 (10%) belonged to 21-30 years of age group and 17 (34%) belonged to 41-50 years of age group,13 subjects (26%) were belonged to 51 to 60 years of age group, 61 to 70 years age group had 7 (14%)subjects and more than 70 years of age groups had 1(2%) subject in case group. Mean age group of cases is 48.64 with SD=12.83.

In control group, 13 subjects (n=13) out of 50 subjects (26%) belonged to 31-40 years of age group and total no. of 7 subjects (n=7) out of 50 subjects (14%) were in the age group of 21-30 years. While total of 13 subjects (n=13) out of total 50 subjects (26%) belonged to age group of 41-50 years. Total no. of 12 subjects (n=12) out of 50 subjects (24%) were in age group of 51-60 years along with 6 subjects (n=6) out of our total 50 subjects (12%) were in age group of 61-70. In the age group of more than 70 years, only 2 subject (n=2) subject (4%) was present. There were no subjects in case as well as control group between the age group of 11-20 years of age. Mean age group of control group is 47.02 with SD=14.13.

In our study, all cases and controls were assessed for osteoporosis by BMD (bone mineral density) and Z-score was calculated. Total 12 cases and controls were detected to have osteoporosis, 22 subjects were found to be osteopenic and 66 subjects had normal bone density. One case with p53 gene amplification could be categorize as having osteoporosis, one case with gene amplification was observed to be osteopenic and gene deletion case had normal bone density.

Table 1: Distribution of leukoplakia patients according to site of lesion

Case		
Site	**Number**	**Percentage%**
Tongue	12	24%
Buccal vestibule	20	40%
Labial vestibule	5	10%
Gingiva and vestibule	5	10%
Floor of mouth	8	16%

Table 2: Distribution of study population according to Grading in OLP cases

Grade	Cases(n=50)	Percentage %
Grade 1	16	32%
Grade 2	15	30%
Grade 3	12	24%
Grade 4	7	14%
Total	50	100%

Table 3: Distribution of study population based on the many kinds of numerical aberrations

Type of Numerical aberrations	Cases (n=50)		Control(n=50)	
	Number(n)	Percentage%	Number(n)	Percentage%
Gene Amplification	2	4%	0	0
Chromosome Monosomy and Gene Deletion	0	0	0	0
Chromosome Polysomy only	0	0	0	0
Gene Deletion only	1	2%	0	0
Chromosome Polysomy and Gene Deletion	0	0	0	0
No Aberrations	47	94%	50	100%
Total	50	100%	50	100%

Table 4: Distribution of study population between Type of Numerical Aberrations and Grading

Type of numerical aberrations	Grade 1	Grade2	Grade3	Grade 4	Total
Gene Amplification	0	0	1	1	2
Chromosome Monosomy and Gene Deletion	0	0	0	0	0
Chromosome Polysomy only	0	0	0	0	0
Gene only Deletion	0	0	0	1	0
Chromosome Polysomy and Gene Deletion	0	0	0	0	0
No Aberrations	16	15	11	5	47
Total	16	15	12	7	50

Table 5: Distribution of study population between Type of Numerical Aberrations and Addiction Habits

Type of numerical aberration	Areca nut & betel leaves	Tobacco only	Smoking only	Alcohol consumption only	Tobacco and smoking	No habits
Gene Amplification	0	0	0	0	2	0
Chromosome Monosomy and Gene Deletion	0	0	0	0	0	0
Chromosome Polysomy only	0	0	0	0	0	0
Gene Deletion	1	0	0	0	0	0
Chromosome Polysomy and Gene Deletion	0	0	0	0	0	0
No Aberrations	14	7	6	2	16	2
Total	15	7	6	2	18	2

Table 6: Comparison of Bone mineral density (Z score) in cases and controls.

Z- Score	Cases		Control		Total
	Male	Female	Male	Female	Total
< -1	23	7	27	9	66
-1 to -2.5	10	3	5	4	22
> -2.5	5	2	3	2	18
Total	38	12	35	15	100

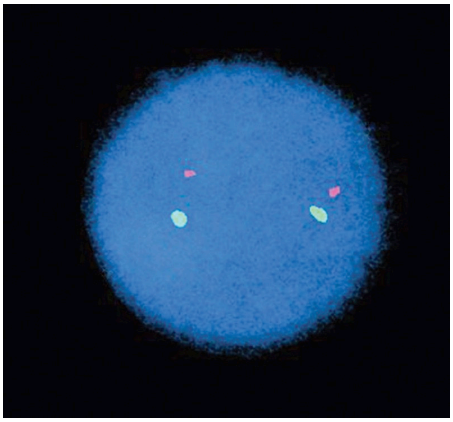
Discussion

According to a study by Xiong, Yang, et al.[21] patients with osteoporosis have higher blood p53 levels, and p53 knockdown partially restored in vitro and in vivo BMD declines. They propose that p53 might have a role in the osteoporosis aetiology.

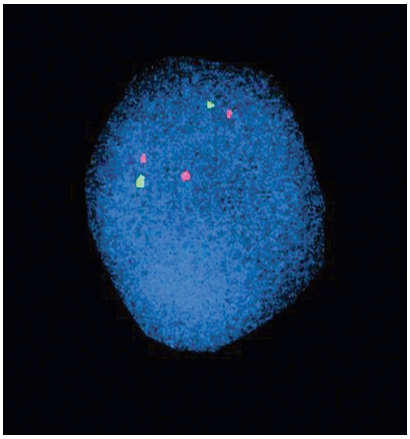
Future research can examine the potential interactions between p53 and osteoporosis, as we were unable to remark on the relationship between the two conditions due to the small number of p53 gene abnormalities in our sample.

In the past, (HNSCC) has also been linked to a considerable number of chromosomal abnormalities. Numerous methods, including losing heterozygosity (LOH), classical among molecular cytogenetics (CGH and interphase FISH), among others,[22] can be used to identify them.[22] The degree of genomic instability and aneuploidy in different oral premalignant lesions can be assessed using the FISH method.

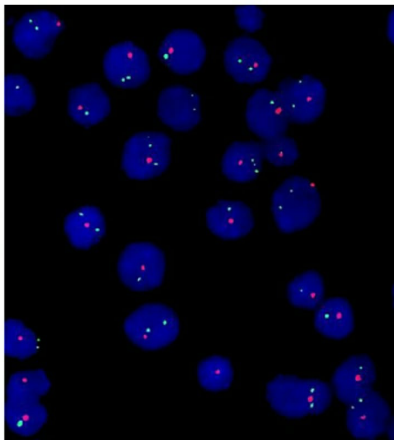
The multistep carcinogenesis process responsible for the majority of malignancies is thought to entail genetic instability. According to available data, genomic instability can happen at two different levels: chromosomal and nucleotide levels.[23]



Normal FISH Interphase Showing two signals for each probe



FISH showing three red signals and two green signals in interphase cell i.e



FISH showing one red signals and two green signals in interphase cell i.e deletion

The majority of malignancies have tumour cells that have gained or lost complete or significant portions of human chromosomes.[24] This has been suggested as one of the main factors influencing how quickly particular genetic hits accumulate in a variety of human cancers.[25]

Observations of present research can be discussed under following headings:

1. **Comparison of study population according to the P53 aberrations:**

The tumour suppressor gene p53 is involved in DNA repair and apoptosis, and it also causes a G1 arrest. Increased genomic instability is the

outcome of p53 gene abnormalities, which also lead to an ineffective checkpoint mechanism for the repair and elimination of mutant cells.[26] The distribution of the study population according to the different types of numerical aberrations is shown in Table 4. Three participants (n = 3) in the case group showed p53 gene aberrations, two of which had gene amplification and one of which had gene deletion; no other subjects showed these abnormalities. Comparisons with our results are complicated by the paucity of published data on p53 mutagenesis in oral leukoplakia (OLP) using the FISH technique.

This is further corroborated by Suwasini's (2018)[26] study, which found that all oral leukoplakia samples expressed the p53 protein. This highlights the protein's potential for early PMD identification and risk assessment for the development of OSCC. Thus, our research highlights the significance of investigating molecular markers such as p53 in the course of OLP, offering important new information for clinical management and risk assessment.

2. Comparison of OLP prevalence according to age group.

Table no. 2 and fig. no 2 shows the distribution of study population according to the age groups. In present study, we found that the age group of 41-50 has the higher number of OLP cases than in any other age group. After that, age group of 51-60 showed second higher number of cases, thus these two age groups have higher prevalence of OLP.

Comparable to our research, K. T. Ashwini et al[27] retrospective observational study found that oral premalignant leukoplakia has a strong predilection towards the age group of 50–60 years old (29.71%), which is the most commonly affected when compared to the other age groups. This study involved 239 leukoplakia cases that were reported to a private dental hospital located in Chennai.

2. Study population according to addiction habits

In the present study, we concluded that 36% of cases were indulged in the habit of chewing tobacco and smoking, 12% were indulged in smoking and 14% were indulged in tobacco chewing 30% indulged in areca and betal nut consumption In their studies, such as those conducted by Harris C.K et al. in 2004 and Silvermen S et al. in 1974, numerous researchers have suggested the significance of tobacco smoking in the aetiology of leukoplakia. 42.5% of the 4886 individuals with oral leukoplakia in the retrospective study by Chaturvedi et al.[30] had a history of smoking, and 5.2% had abused alcohol.

Table no.5 shows that the subjects in case group who had the habit of tobacco chewing and smoking had the numerical aberrations which was only 3 subjects (n=3) out of total 50 subjects. As compared to the other addiction habits, no other subject showed numerical aberrations.

4. Comparison of study population according to grade of OLP.

In our study 32% of subjects were suffering from grade 1(mild dysplasia), 30% subjects were suffering from grade 2(moderate dysplasia) of OLP, 24% subjects were suffering from grade 3 (severe dysplasia) of OLP and only 14% of subjects were suffering from grade 4 (carcinoma in situ) of OLP. A 2014 study by Dr. Praveen Birur et al.[31] revealed that mild dysplasia was present in 46 percent of patients with homogeneous leukoplakia, moderate dysplasia in 26.2%, severe dysplasia in 13.5%, and microinvasive squamous cell carcinoma in 3.2% of patients.

All our numerical aberration are observed in grade 3 and 4histopatological grading as shown in Table no. 4 but we can't determine any association between grading of leukoplakia and p53 numerical aberration because of limited no. of cases and limited aberration detected.

Conclusion

In the present research, there were 76% males and 24% females and maximum leukoplakic patient was in age group 40-60 year.

Three subjects (n=3) exhibited p53 gene aberrations, specifically 2 cases with gene amplification and 1 case with p53 gene deletion. 30% of cases were indulged in the habit of

chewing betal nut, 12% were indulged in smoking and 36% were indulged in both, tobacco chewing and smoking. The site of lesion mainly involved was buccal mucosa, tongue and labial mucosa.

The advent of the FISH technology signaled a new era in the research of chromosomal structure.[66] Thus the present study has been done by the FISH technique to identify the numerical aberrations in p53 gene with the patients with oral leukoplakia and to compare the data with the control group as there are very less study on the leukoplakia in the western zone of Rajasthan.

Limitation: Due to less number of cases and limited aberration there is limitation in our study to establish the correlation of type of numerical aberrations with age, gender, site, habits and grading.

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