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FLUORESCENCE IN SITU HYBRIDIZATION TO DETERMINE THE GENOMIC CHANGES IN CHROMOSOME 17 AND P53 GENE IN ORAL LEUKOPLAKIA PATIENT IN INDIAN POPULATION: A CROSS-SECTIONAL STUDY.

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Abstract

Introduction

Oral cancer, especially squamous cell type, is common, with 274,000 instances annually. The risk of oral cancer from leukoplakia, a white patch at least 5 mm in size, has been extensively investigated. Globally, leukoplakia prevalence ranges from 2.6% to 4.1%. It usually develops after 30, peaks over 50, and is associated to tobacco, alcohol, and betel quid use. Leukoplakia-related chromosomal abnormalities can be revealed by Fluorescence in situ hybridization (FISH).

Objective

The goal of research is to clarify the genetic mechanisms behind the development of leukoplakia by examining p53 gene changes and numerical aberrations in chromosome 17 in Indian populations.

Material and methods

In this cross-sectional study, FISH was used to diagnose 50 cases of Oral leukoplakia (OLP), utilizing the " Locus Specific Identifier (LSI) TP53/Centromere Enumeration Probe (CEP) 17 FISH Probe Kit " to determine the copy number for CEP 17 (green) at the centromere of chromosome 17 and LSI TP53 (orange) at chromosome 17p13.1.

Results

Three of the fifty cases in the analysis show molecular change. In roughly 4% of instances, p53 gene amplification and chromosome 17 polysomy were present, while 2% of cases only had p53 gene deletion. In the investigation, grade 3 and grade 4 oral leukoplakia showed all aberrations, and the highest 18 subjects (n=18) out of 50 individuals (36%) had a tobacco and/or smoking addiction.

Conclusion

The research illuminates molecular abnormalities in "chromosome 17" and the "p53" gene in Indian OLP patients. The study helps bridge research and clinical practice to improve oral leukoplakia genetic diagnosis and management. This will enhance patient outcomes by preventing such events.

Recommendations

Implement routine screening for genetic alterations in chromosome 17 and the p53 gene in patients with oral leukoplakia to enhance early detection and targeted intervention strategies.

***Keywords**: Oral leukoplakia, Fluorescence in situ hybridization, Chromosome17, p53 gene.*
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Introduction

Cancer of the oral region particularly squamous cell type is widely prevalent, with an estimated 274,000 cases annually1. Despite advancements in detecting and treating various cancers, the prognosis for oral squamous cell carcinomas (OSCCs) has not significantly improved maintaining a 50% 5-year survival rate2,3,4. Recognizing the importance of early intervention, the WHO stated the term "potentially malignant disorders" to encompass conditions like oral leukoplakia (OLK) characterized by white plaque-like lesions in the oral cavity5,6. Leukoplakia, described as a white patch of at least 5 mm in size, has been extensively studied for its association with oral cancer risk.7 It often affects areas like the buccal mucosa, tongue, and alveolar sulcus7,8. The prevalence of leukoplakia varies globally, with reported figures ranging from 2.6% to 4.1%. Notably9, it tends to develop after the age of 30, peaking over 50 and is strongly linked to tobacco, alcohol, and betel quid use10. Additionally, emerging research explores the potential role of infections, particularly human papillomavirus (HPV) and Candida in leukoplakia development and progression11,12. Concerns over leukoplakia's malignant transformation underscore the need for vigilant monitoring with transformation rates of 0.13% to 40.8%13,14,15. Various influencing factors including gender, lesion size, location, and dysplasia influence this risk16.

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Histopathologically leukoplakia-associated dysplasia manifests as various cellular changes categorized into minimum, moderate, and severe indicating the severity of malignant potential17. Molecular studies have identified genetic markers associated with oral premalignant lesions, highlighting two subtypes: classical and immunological18. Alterations in genes like p53, crucial for cell cycle regulation and apoptosis play a pivotal role in leukoplakia progression19.

Methods like Fluorescence In Situ Hybridization (FISH) offer important information about chromosomal abnormalities linked to leukoplakia20. The research goal is to clarify the genetic mechanisms behind the development of leukoplakia by examining p53 gene changes and numerical aberrations in chromosome 17 in Indian populations. Comprehending these genetic alterations may offer significant perspectives on the course of the disease and enable proactive measures to be taken.

Material & method

Study design: A cross-sectional study.

Study setting

The current study was conducted in the anatomy department of S.P. Medical College and the Associated Group of Hospitals in Bikaner, Rajasthan.

Participants

A total of 50 OLP patients who were randomly selected from the outpatient departments of the dental and ENT departments were included in the study.

Inclusion criteria

Cases clinically and histopathologically proved Oral leukoplakia patients.

Exclusion criteria

1. Clinically or histopathologically proved cases of OSCC.
2. Clinical signs and symptoms of oral carcinoma.
3. The patient has the presence of any other oral pathology except oral leukoplakia.

Bias

Potential bias in this study may arise from the small sample size and the specific population selected, which may not represent the broader Indian population.

Sample size

To calculate the sample size for this study, the following formula was used for estimating a proportion of a population:

$$n = \frac{Z^2 \times p \times (1-p)}{E^2}$$

Where:
- n = sample size
- Z = Z-score corresponding to the desired level of confidence
- p = estimated proportion in the population
- E = margin of error

Methodology

Standard procedures were used to prepare the tumor samples for paraffin embedding. Sections of 5 mm from the paraffin blocks showing the histopathological type and differentiation stage were used for each participant. After the manufacturer's protocol, Abbott/Vysis was optimized, and tissue sections were subjected to the interphase FISH technique. The identification of "LSI TP53/CEP 17 FISH Probe Kit". According to the protocol set by the manufacturer, this kit aims to determine the copy number for the "CEP 17" which is green in color as per the probe spectrum situated at the centromere of "chromosome 17" and the "LSI TP53" orange color as per the probe spectrum 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. For pretreatment, the slide was dipped in NaSCN at 80 degrees Celsius for thirty to forty minutes, and then it was submerged in the buffer solution. Protease buffer is used for a 7-minute pre-hybridization process at 37 degrees Celsius. Then the slide is washed and dehydrated using ethanol. After letting the slides air dry, 10 milliliters of the probe were applied to each plate in the chosen hybridization region. The smears were sealed, incubated at 78 degrees Celsius for 10 minutes, and then enclosed with a coverslip measuring 22 by 22 mm. 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 degrees "SSC/0.1% NP40 for a minute".

Following air drying and dehydration, "40,6-Diamidino-2-phenylindole (DAPI II)" was applied to the slides for counterstaining. An x100 magnification fluorescence microscope fitted using DAPI filter sets was used to

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examine the slides. A digital camera was used to take the pictures. Hybridized signals were recognized in the 200 interphase nuclei for every patient. The study examined the amount of "chromosome 17" aberrations and "p53 gene" deletions/amplifications in OLP samples that were immersed in paraffin using in situ hybridization.

Ethical considerations

The study protocol was approved by the Ethics Committee and written informed consent was received from all the participants.

Results

Hybridization was performed in situ on 50 paraffin-embedded OLP samples to identify the disparities in numbers of "chromosome 17" and "p53 gene" removal/amplification. For each distinct histological location from the tumor, 200 nuclei were observed at 100 using a fluorescence microscope and an oil immersion objective. Every nucleus was then analyzed for its copy number.

"Chromosome 17's" numerical abnormalities differed amongst individuals. In particular, paired impulses were evaluated as single occurrences, and only different isolated nuclei were counted. If each subject's mean amount of signals in the examined cells was less than two, the study classified it as monosomy 17. The percentage of cells that show three or more signals in each nucleus was dubbed chromosome polysomy.

The majority of the premalignant cells in the study did not have a significant amount of aneuploidies found in them. Of the 50 cases, 02 cases (4%) had molecular alterations (p53 gene deletion and chromosomal polysomy), while the remaining cases had normal configuration or no aberrations.

In the study total no. of 38 subjects (n=38) out of 50 (76%) were males and a total no. of 12 subjects (n=12) out of 50 subjects (24%) were females. It was observed that in the studied group, males were in higher frequency than females.

In the studied population according to the addiction habits there were highest 18 subjects (n=18) out of 50 subjects (36%) were addicted to tobacco and smoking, while a total of 15 subjects (n=15) out of 50 subjects (30%) were addicted to areca nut and betel nut and 7 (14%) and 6 (12%) subjects had habits of smoking and tobacco respectively, 2 subjects had the habit of alcohol consumption.

In this studied group out of 50, 16 cases (n=16) (32%) were in grade 1, while 15 cases (n=15) (30%) belonged to grade 2 of OLP, Total of 8 (n=12) subjects (24%) were suffering from grade 3 and 7 (n=7) subjects (10%) from grade 4 of oral leukoplakia.

Table 1: Arranging population as per the changes in the chromosome

Type of Numerical aberrations	Cases (n=50)	
	Number(n)	Percentage %
Multiple chromosome and amplification of the gene	2	4%
Single chromosome and removal of the gene	0	0
Multiple chromosome	0	0
Removal of the gene	1	2%
Multiple chromosome and removal of a gene	0	0
No changes in the structure of chromosome	47	94%
Total	50	100%

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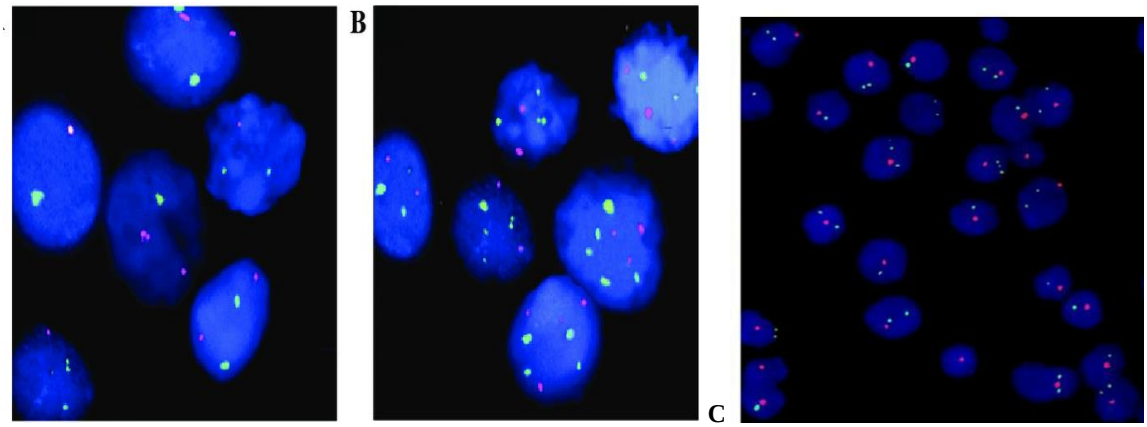

Fig.*1* *A.showing two green signals (normal chromosome17) and two orange signal (p53 gene normal). B shows "chromosome 17" polysomy and "p53 gene" amplification. C shows p53 gene deletion without chromosomal 17 abberation.*

Discussion

In the past, there have also been numerous reports of chromosomal aberrations with HNSCC. There are several techniques for detecting them, such as loss of heterozygosity (LOH)22 and "classical and molecular cytogenetics" (CGH and interphase FISH) 21. The level of genomic instability & aneuploidy in oral 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 levels23.

The majority of malignancies have tumor cells that have gained or lost complete or significant portions of human chromosomes24. This has been suggested as a key factor influencing how quickly particular genomic hits accumulate in several human cancers25.

FISH technique marked the beginning of a new era for the study of chromosome structure20 thus the present study has been done by the FISH technique to identify the numerical aberrations in the p53 gene within the patients with oral leukoplakia as there are very less studies on the leukoplakia in the western zone of Rajasthan.

The present study incorporated a total of 50 subjects (n=50). All the subjects under consideration were previously informed about the purpose of the study and their consent was taken in the form of the informed consent form. The study inspired by Christian F. Lenz's s26 seminal work in 1998, explores the potential of Fluorescence In Situ Hybridization (FISH) in identifying oral leukoplakias at heightened risk of malignant transformation. The study highlights the prevalence of numerical aberrations within the cases, with 2 out of 50 subjects exhibiting chromosomal polysomy & gene amplification and 1 case with gene deletion while the remaining showed no such deviations.

Although the study did not delve into specific chromosomal alterations, it aligns with Lenz's findings, emphasizing the presence of chromosomal abnormalities in leukoplakia lesions. The maximum samples were collected from buccal mucosa i.e. 20 (40%) cases and then from tongue i.e. 12(24%) and labial vestibule i.e. 5 (10%). 5 samples were collected from the gingival and vestibule. 8 (16%) samples were collected from the floor of the mouth.

In the study, all aberrations were detected in grade 3 & grade 4. Chromosomal polysomy and gene amplification are both aberrations seen in grade 3 and grade 4 oral leukoplakia cases and, 1 gene deletion aberration in grade 4 oral leukoplakia case.

In the case group, there were highest 18 subjects (n=18) out of 50 subjects (36%) were addicted to tobacco and smoking, while a total of 15 subjects (n=15) out of 50 subjects (30%) were addicted to areca nut and betel nut and 7 (14%) and 6 (12%) subjects had habits of smoking and tobacco respectively, 2 subjects had the habit of alcohol consumption. 2 case with chromosomal polysomy and gene amplification aberration has addiction of tobacco and smoking and 1 case with gene deletion has addiction of areca nut & betel nut.

Lenz's study identified specific chromosomal aberrations in dysplastic oral leukoplakia biopsies, including trisomy of

"chromosomes 1, 7, and 17", and monosomy of "chromosome 9", underscoring the potential clinical significance of these aberrations. While the study corroborates the presence of chromosomal abnormalities, it also underscores the need for further research to confirm the findings and explore the potential utility of FISH as a non-invasive diagnostic tool for stratifying patients based on their risk of malignant transformation. By bridging the gap between research and clinical practice, the study contributes to the ongoing efforts to enhance the early detection and management of oral leukoplakia, ultimately improving patient outcomes in the realm of oral cancer prevention and treatment.

The tumor suppressor "gene p53" plays a role in cell metabolism, and it also causes a G1 arrest. Mutations in the "p53 gene" result in an ineffective checkpoint mechanism that prevents mutant cells from being repaired or destroyed. This causes instability23 table delineates the distribution of the study population based on types of numerical aberrations. In the case group, two subjects (n=2) exhibited numerical aberrations, specifically chromosome polysomy and gene amplification and one case showed p53 gene deletion, while no other subjects displayed such deviations. The scarcity of published data on p53 mutagenesis in Oral Leukoplakia (OLP) using the FISH technique complicates comparisons with the findings. Nonetheless, Suwasini's (2018)27 study further supports this, observing p53 protein expression in all oral leukoplakia samples, emphasizing its potential for early PMD detection and risk assessment for OSCC development. The study thus underscores the importance of exploring molecular markers like p53 in OLP progression, contributing valuable insights for its clinical management and risk assessment.

Generalizability

While the study provides valuable insights into the genetic changes associated with oral leukoplakia in a specific population, these results could be extrapolated to a larger population through broader research, enhanced screening programs, and targeted public health initiatives. This would ultimately contribute to better prevention, early detection, and management of oral leukoplakia and its progression to cancer on a wider scale.

Conclusion

In summary, the research clarifies the molecular alterations in "chromosome 17" and the "p53 gene" seen in patients with oral leukoplakia (OLP) in the Indian community. The findings indicate that 4% of OLP patients exhibited molecular changes, with polysomy of "chromosome 17" and amplification of the "p53 gene" and 2% of OLP patients exhibited gene P53 deletion being the most prevalent alterations observed.

Importantly highlighting the specificity of these alterations to OLP patients. Furthermore, the analysis is not feasible to determine any correlation between the type of numerical aberrations and factors such as age, gender, site, habits, and grading.

The importance of the p53 gene's function in controlling apoptosis, DNA repair, the cell cycle, and cell-cycle regulation following DNA damage highlights the significance of molecular changes in this gene seen in OLP patients. The application of Fluorescence In Situ Hybridization (FISH) methodology yielded vital insights into the genetic variability of biological samples by enabling the quick and sensitive detection of chromosome aberrations. Notably, the literature on the correlation between OLP and the p53 gene using the FISH technique is limited, emphasizing the novelty and importance of the findings. Moving forward, further research in this area is warranted to validate the observations and explore the clinical implications of these molecular changes in OLP progression and oral cancer risk assessment.

Limitations

The limitations of this study include a small sample population who were included in this study. Furthermore, the lack of a comparison group also poses a limitation for this study's findings.

Recommendation

Implement routine screening for genetic alterations in chromosome 17 and the p53 gene in patients with oral leukoplakia to enhance early detection and targeted intervention strategies.

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List of abbreviations

FISH - Fluorescence in Situ Hybridization
OLP - Oral Leukoplakia
LSI - Locus Specific Identifier
CEP - Centromere Enumeration Probe
OSCC - Oral Squamous Cell Carcinoma
DAPI - 4′,6-Diamidino-2-Phenylindole

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The study was not funded.

Conflict of interest

The authors report no conflicts of interest in this work.

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