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Identification of Chromosomal and Chromatid-type Alterations in Oral Cancer Patients in Tamil Nadu, India

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ABSTRACT: Oral based diseases were observed in nearly 3.5 billion people worldwide, according to the World Health Organisation. Oral Squamous Cell Carcinoma (OSCC) is one of the major oral based diseases in the Indian subcontinent. People who use chewing tobaccos and tobacco-based products are more susceptible to OSCC. The aim of the present study is to find out the chromosomal alterations in Oral cancer patients by using cytogenetic technique.

Totally, 35 blood samples were collected from cancer Hospitals. Ethical clearance was obtained from the Institutional Ethics Committee board, VMSDC & Hospital. In the present study, we analysed the Chromosomal abnormalities by using trypsin G-Banding technique.

We observed various chromosomal-type and Chromatid-type alterations in Oral Cancer patients with the chromosome locations of 2q, 3q, 21q and X using the GTG banding technique. We observed higher degree of Chromosomal abnormalities in OSCC samples compared to controls. From this we conclude that the CA in 2q, 3q and 21q shows highly significant for the clinical application of OSCC patients. Though the study method is technically sensitive, further molecular studies with large sample size would greatly aid in diagnostic support as well as oral cancer management.

The chromosome and chromatid- type abnormalities found in OSCC patients showed the significance of its use in early diagnosis and disease management in OSCC with knowledge of its pathogenesis.

Keywords: Oral squamous cell carcinoma (OSCC), Chromosomal abnormalities, trypsin G-Banding, Oncology.

INTRODUCTION

Oral squamous cell carcinoma accounts for the sixth most common cancer worldwide. The anticipated number of cancer patients in India is for the year 2021 was 26.7 million DAILYs AMI and expected to increase to 29.5 million in 2025 and reported common leading sites are lung, breast, oesophagus, mouth, stomach, liver and cervical uteri (Kulothungan *et al.*, 2022). India contributes one-third of the total burden of oral cancer cases in the world. In the last 20 years, this rate has climbed by 68%, making it the most prevalent cancer in Indian men (Borse *et al.*, 2020). The oral cancer arises from different regions of the mouth including buccal mucosa, alveolar ridge, floor of the mouth, retromolar region, and tongue (Muthu *et al.*, 2018). Tobacco and alcohol have been strongly proven etiological factors for oral cancer (Kaushal *et al.*, 2023). Field cancerization is process involved in oral squamous cell carcinoma and the carcinogenic and cancer promoting substances are likely to be tobacco and alcohol (Hriatpuii *et al.*, 2022; Balachandar *et al.*, 2008). The carcinoma arises where the exposure of these substances is maximum whereas all other exposed tissues are having the chance to become altered neoplastic phenotype.

The genetic markers play a significant part in carcinogenesis and clinical parameters of oral cancer. Since the oral cancer exhibits multistep carcinogenic process, cascade of genetic and epigenetic events happens prior to and during tumor development (Venugopal *et al.*, 2018). The identification of these markers is important as they predict the clinical outcome and the prognosis in OSCC patients. In addition, they can be considered as important tool in creating patient awareness, intervention and treatment plan decisions thereby improving the patient survival. Genetic aberrations play a crucial part in oral carcinogenesis (Shubham Pandey *et al.*, 2022). The study of chromosome structure and function is known as cytogenetics. Classical and molecular cytogenetic methods are used to identify the karyotype in OSCC

which shows different patterns of chromosomal aberrations (CA) (Vellingiri *et al.,* 2014).

The entire genome can be screened for non-random karyotype changes by means of classical cytogenetic methods. These regions would help to pinpoint the key genes involved in specific tumor or other disorders. Since, cancer is a genetic disease of somatic cell, the examination of tissue of interest through classic cytogenetic method would provide an insight to the genetic changes during the cancer progression (Yates and Campbell 2012). The recent improvements in cell culturing technique and improved technical expertise results in the successful interpretation of the chromosomal alterations of solid tumors. Furthermore, the localisation of tumor suppressor or oncogenes pertaining to the sites of molecular disruption is possible with non-random karyotyping methods. These CA would comprise the structural chromosomal changes including gross chromosome rearrangements such as amplifications and deletions of parts of chromosomes, inversion and translocations between non-homologous chromosomes. The chromatid type aberrations include gaps, breaks and dicentric (Siri *et al.,* 2021). Widespread cytogenetic analysis have revealed that chromosomes 3, 4, 7, 8, 9, 11, 14, 17, 18, 19 and 20 undergo large modifications (gains/losses) that result in distinct chromosomal instability (CIN) patterns in OSCC and are associated with an aggressive tumor nature and poor patient prognosis (Papanikolaou *et al.,* 2018).

MATERIALS AND METHODS

Patient recruitment. 35 samples of peripheral venous blood were taken from the patients reported to cancer hospital diagnosed as OSCC. This study was performed with the Human Ethical Committee approval from VMRF (DU), Salem. Informed consent was attained from all the subjects prior to sampling. The required patient data was collected from the medical records. The sampling was done prior to any kind of cancer therapy (Moorhead *et al.,* 1960).

Lymphocyte culture. The peripheral blood collected from OSCC patients and stored in Heparin vacuum containers. The collected peripheral blood of the OSCC patients was used for CA analysis using the GTG banding followed by the lymphocyte culture. Lymphocyte culture media consist of 5 ml RPMI 1640 media with 1.5 ml of antibodies, serum and phytohaemagglutinin (PHA). 0.5 ml heparinised blood was added with the complete lymphocyte culture media and maintained in 37°C for 72 hours. In the 71st hour, 130µl of 0.1% colchicine were added and incubate 1 more hour in the 37°C to arrest the cell division in the metaphase stage.

Culture harvesting. After completion of the 1 hour incubation, the culture was transferred to the centrifuge tubes and centrifuged at 1000 rpm for 5-8 minutes then the supernatant was aspirated. Consequently, 5ml of Potassium Chloride was added (KCl) and incubated for 10 minutes in the Room temperature. Centrifugation was done at 1000 rpm for 10 minutes after incubation and the supernatant was aspirated. The freshly prepared fixatives (1:3, acetic acid: methanol) were added to the cells. The fixative wash was performed until attaining the clear suspension.

Slide preparation. The slides were soaked in the soap solution and washed in the running tap water. Then it was kept in the 4°C of methanol. The harvested cells were transferred to the slides by using the Pasteur pipette and the slides were allowed to dry in 37°C.

GTG banding. The dried slides were treated with the 0.5% trypsin solution for 5-15 seconds. After that the slides were rinsed with the PBS and stained with 10% Giemsa stain for 10 minutes. The slides were allowed to rinse in distilled water to remove excess stains and kept in the room temperature for drying. Slides were then prepared for microscopy visualisation.

RESULTS

In this study we recruited 35 OC subjects comprised of 23 males and 12 females. The OSCC subjects were classified based on the age and stages of cancer which is presented in Table 1. Whereas Table 2 presents the CA based on the age, gender & stages of cancer and Table 3 shows the clinical, histopathological and cytogenetic data on 35 OSCC patients.

Based on the age we divided the subjects into Group-1(30-60) and Group-2 (60-90). The blood samples were taken and the lymphocytes were cultured and karyotyping was done with GTG banding for all subjects (Fig. 1, 2). While analysing the karyotyping results we found that 17 (48.6%) subjects have Chromosomal Abnormalities (CA). The CA was seen in chromosome location 2q,3q,21q & X in male and female subjects respectively. The listed CA was highly observed in the subjects who had the stage-III OSCC. The Group-1 males had shown the CA in the 3q&21q locations and the Group-2 Subjects had shown the higher CA in the site of 2q followed by 3q and 21q. The Females were more prominent for the 2q CA followed by the 3q and 21q alterations. While getting into the male in the Group-2 subjects had also shown high CA in the 2q site. Only single Group-2 male subject had CA in the X chromosome. While entering the risk assessments the subjects were having the history of habitual using of tobacco products and betel quid. From this we conclude that subjects were prominent for the risky habit due that the OSCC might have occurred.

DISCUSSION

OSCC is one of the aggressive malignancies with a poor prognosis. Karyotyping of epithelial malignancy yields knowledge about numerical /and structural chromosome defects. Chromosome segregation defects, telomere stability and the damage response of DNA contribute significantly to the development of chromosomal instability (Sowmya *et al.,* 2022). Therefore, cytogenetic analysis has specific place in the full spectrum study of OSCC. Cytogenetic analysis of OSCC have resulted the high complexity of karyotype which is reported commonly in solid tumors.

In this study, classical cytogenetic method were used to survey the non-random gross genome wide changes occurring in cultured lymphocytes harvested from 35

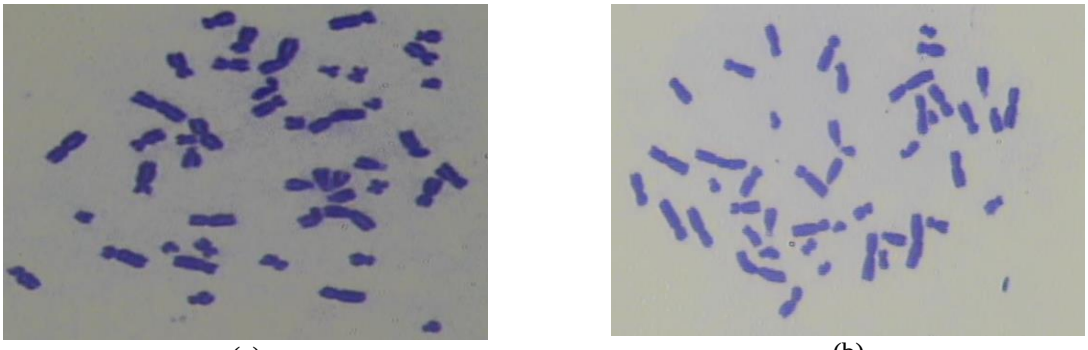
OSCC patients. Peripheral blood had been utilized commonly for sampling of OSCC karyotyping since the sample collection is feasible, higher patient acceptance and reported consistent results from peripheral lymphocyte culture. This allows for the crucial imaging of chromosomal make up and the overall assessment of the complete genome in many cells. It is frequently used for the diagnosis, prognosis, and therapeutic evaluation of cancer cells in clinical and research laboratories all over the world.

The most used banding technique for diagnosing a range of structural chromosomal abnormalities is the normal/high resolution GTG banding technique. Chromosomal instability is tracked over time to assess the stability of the human genome and cancer risk with precise understanding of molecular mechanism of cancer development and progression. In our study, 35 cases were studied among which 17 (48.6%) had shown chromosomal and chromatid abnormality whereas control group (20 subjects) had shown very low level of abnormal karyotype. This finding is consistent with previous reported studies on OSCC karyotyping. It implies that genetic instability seemed to occur in oral cancer patients.

We observed higher frequency of CA in males aged between 60-90 years. The listed CA was highly observed in the subjects who had the stage-III in the OSCC. Johanson *et al.* (1996) stated that acquired neoplastic cell features caused by adaptations to the genetic code do not exclude the importance of surrounding non-neoplastic cells. Additionally to one another, tumor cells must contend with stromal tissue in the area as well as the overall antitumor response, which includes immune surveillance. In this manner even peripheral blood can also be used as non-neoplastic tissue (Johanson *et al.,*1996; Balachandar *et al.,* 2010). According to Jin *et al*. (2006) in their study of 106 OSCC, the most common imbalances were deletion of chromosomes 18, 21, 22 and Y as well as 2q33, 3p, 4p, 6q, 8p, 10p, 11q, 13p, 14p and 15p (Jin *et al*., 2006). A study by Sreekantaiah *et al.* (1994) on Head and Neck squamous cell carcinoma exhibited consistent numerical alterations, including the loss of chromosomes 2 and 21, respectively in six and five cell lines, and the addition of chromosome 20 in five cell lines (Sreekantaiah *et al.,*1994). These results are in consistent with our reports.

In our study, only single subject had shown CA in X chromosome which is in contrast with the Chen et al where many of the study subjects were reported. The identified gains of chromosome X in OSCC by comparative genomic hybridization. This study includes the Asian population in whom areca nut quid chewing is common (Chen *et al*., 2004). Yamamoto *et al.* (1999) from 38 OSCC tissue samples with PCR and densitometric assessment found that three distinct tumor suppressor gene loci such as a unique deleted region on 21q11.1 in OSCC and frequent microsatellite instability on this chromosome arm may be helpful markers for the analysis of this neoplasm. They also suggested that the 21q region has possible tumor suppressor genes that may contribute to the etiology of this disease. It has been further suggested that 21q 22.1 locus harbours numerous genes such as TIAM1, AMLI, and IFNAR which are reported to be involved in development of human cancers (Yamamoto *et al.,*1999). It has been revealed that the gene PIK3CA situated in loci 3q26,32 was associated with advanced clinical stage and adverse outcome in OSCC (Ribeiro *et al.,* 2018). The genome wide analysis of chromosomal alterations in esophageal squamous cell carcinoma had shown INSIG2 candidate gene in 2q14.1 amplified region (Chattopadhyay *et al.,* 2010).

The majority of studies stated tobacco as a strongest etiological factor for OSCC development as this study also revealed majority of the subjects with CA were associated with risky habits. The limitations of OSCC cytogenetic studies include low success rate in the long-term cultures of OSCC cells, fibroblast overgrowth of OSCC cells leading to the demise of tumor cells, small numbers of mitotic cells present in the cell cultures and subsequent harvests due to slow growing tumours and unanalyzable metaphase spreads. It has been reported that no consistent results common to all OSCC. According to the numerical loss it has been generally postulated that these sites might harbour TSG as an essential mechanism in the development of OSCC. Findings from cytogenetic and cytogenomic methods can significantly advance innovative discoveries and aid in the creation of tailored therapies (Sowmya *et al*., 2022).



(a) (b)

Fig. 1(a &b). Chromosomal metaphase spread of the Oral Cancer Subjects.

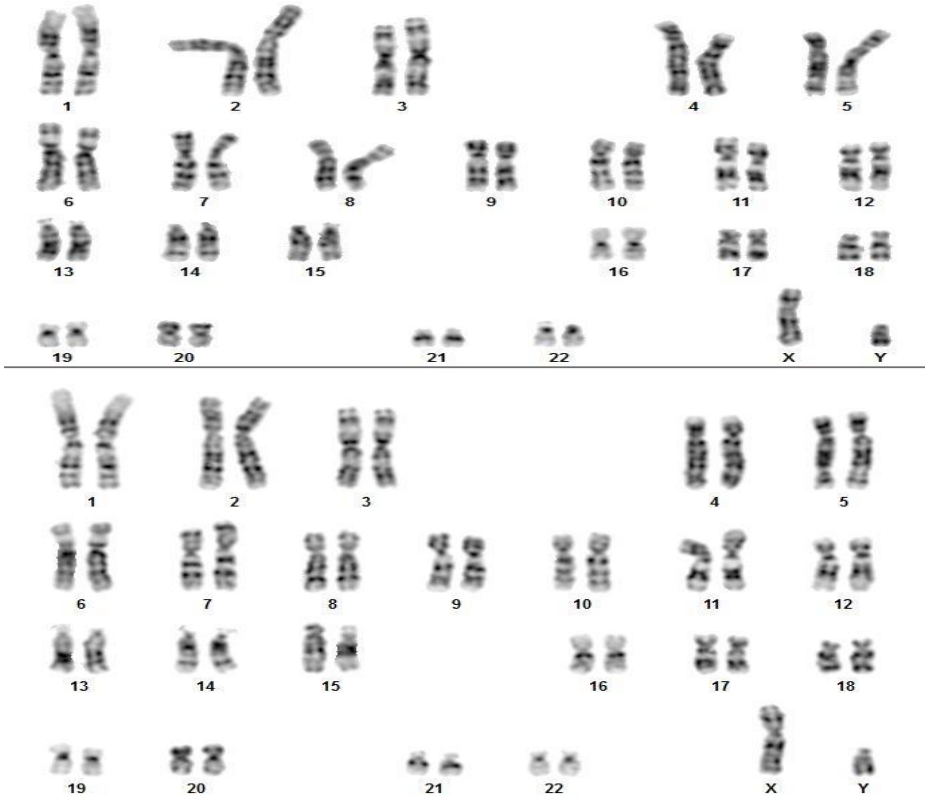


Fig. 2(a&b). Karyotype derived from Oral Cancer Subjects.

Table 1: General characteristics of Oral Cancer patients.

Sr. No.	Group	Gender	Total (%)	Stage		
				II (%) (Mean±SD)	III (%)(Mean±SD)	IVa (%)(Mean±SD)
1.	Group-1	Male	7 (20%)	3 (42.9) (43.7±12.3)	4 (57.1) (53.25±6.7)	-
		Female	3 (8.6%)	-	2 (66.7) (51±7.07)	1 (33.3)
2.	Group-2	Male	16 (45.7%)	3 (18.7) (66.3±6.02)	11 (68.8) (69.2±5.7)	2 (12.5) (71±1.4)
		Female	9 (25.7%)	2 (25) (75.5±16.3)	7 (75) (69.57±7.4)	-

Table 2: Chromosomal Alterations (CA) based on the age, gender & stages of cancer.

Sr. No.	Age group	Gender	Total (%)	Stage	CA 2q (%)	CA 3q (%)	CA 21q (%)	CA X (%)
1.	Group-1	Male	7 (20%)	II	-	**1 (14.3)**	**2 (28.6)**	-
				III	-	-	**4 (57.1)**	-
				IVa	-	-	-	-
		Female	3 (8.6%)	II	-	-	-	-
				III	-	-	-	-
				IVa	-	-	-	-
2.	Group-2	Male	16 (45.7%)	II	-	-	-	-
				III	**3 (18.8)**	**1 (6.3)**	**1 (6.3)**	**1 (6.3)**
				IVa	-	**1 (6.3)**	**1 (6.3)**	-
		Female	9 (25.7%)	II	**1 (11.1)**	-	-	-
				III	**4 (44.4)**	**2 (22.2)**	**2 (22.2)**	-
				IVa	-	-	-	-

Table 3: Clinical, Histopathological and cytogenetic data on 35 OSCC patients.

Case No.	Age/sex (years)	Risky habits	Site/clinical type	Differentiation	TNM stage	CA (2q, 3q, 21q & X)
OSCC1	80/F	Chewing betel quid	Right buccal mucosa	Well differentiated	III	2q
OSCC2	72/F	Chewing betel quid	Lower alveolus	Moderately differentiated	III	2q & 21q
OSCC3	59/M	Pan masala	Right buccal mucosa	Moderately differentiated	III	21q
OSCC4	47/M	Smoking, alcohol	oropharynx	Well differentiated	II	21q
OSCC5	65/M	Nil	Right lateral tongue	Well differentiated	III	2q
OSCC6	72/M	Smoking, alcohol	Tongue	Moderately differentiated	III	-
OSCC7	81/M	Smoking, alcohol	Upper alveolus	Poorly differentiated	III	-
OSCC8	67/M	nil	Tongue	Poorly differentiated	III	2q & X
OSCC9	65/M	Smoking, alcohol	Tongue	Moderately differentiated	III	21q
OSCC10	70/F	Betel nut	Buccal mucosa	Moderately differentiated	III	2q
OSCC11	72/M	Smoking	Floor of the mouth	Well to moderately differentiated	IVa	3q & 21q
OSCC12	70/M	Smoking	Lower alveolus	Well to moderately differentiated	IVa	11q
OSCC13	60/F	Betel nut	Buccal mucosa	Verrucous carcinoma	III	3q
OSCC14	46/F	Betel nut	Buccal mucosa	Well differentiated	III	-
OSCC15	74/M	Betel nut	Buccal mucosa	Moderately differentiated	III	12p
OSCC16	53/M	Betel nut	Buccal mucosa	Moderately differentiated	III	21q
OSCC17	56/F	Betel nut	Buccal mucosa	Well differentiated	III	-
OSCC18	61/M	Nil	Tongue	Moderately differentiated	III	-
OSCC19	67/M	Betel nut, Alcohol	Buccal mucosa	Adeno squamous carcinoma	II	-
OSCC20	75/F	Betel nut	Buccal mucosa	Moderately differentiated	III	-
OSCC21	70/F	Nil	Oropharynx	Moderately differentiated	III	21q & 3q
OSCC22	45/F	Betel nut	Buccal mucosa	Moderately differentiated	IVa	12p
OSCC23	67/M	Smoking, alcohol	Oropharynx	Moderately differentiated	III	-
OSCC24	57/M	Betel nut, smoking	Buccal mucosa	Moderately differentiated	III	21q & 3q
OSCC25	54/M	Betel nut, smoking, Alcohol	Oro pharynx	Moderately differentiated	II	-
OSCC26	64/F	Betel nut	Buccal mucosa	Well differentiated	II	-
OSCC27	72/M	Smoking, alcohol	Tongue	Moderately differentiated	II	11q
OSCC28	87/F	Betel nut	Buccal mucosa	Moderately differentiated	II	-
OSCC29	65/M	Smoking	Hard palate, soft palate	Well to Moderately differentiated	III	-
OSCC30	70/M	Betel nut, smoking	Buccal mucosa	Well differentiated	III	-
OSCC31	44/M	Betel nut, smoking	Tongue	Moderately to well differentiated	III	21q
OSCC32	74/M	Smoking	Oropharynx	Poorly differentiated	III	2q
OSCC33	60/F	Nil	Oropharynx	Poorly differentiated	III	-
OSCC34	30/M	Smoking, alcohol	Floor of the mouth	Moderately differentiated	II	21q & 3q
OSCC35	60/M	Smoking ,alcohol	Soft palate	Moderately differentiated	II	-

CONCLUSIONS

The chromosomal instability is hallmark of cancer and involved both in carcinogenesis and therapeutic response. They act as useful biomarkers and therapeutic target for novel and more effective cancer therapy. The CIN is also responsible for tumor evolution and act as a genomic source between the tumor and its microenvironment in the course of immune editing and evasion. The complex karyotype observed in OSCC might be due to the presence of genetic alterations associated with the development of immortal phenotype, together with spindle checkpoint and chromosome defects. The transformation of oral oncogenesis also includes promoter methylations and miRNA deregulations. Our study demonstrated higher degree of Chromosomal abnormalities in OSCC samples compared to controls. We conclude that the CA in 2q, 3q, and 21q shows highly significant for the clinical application of OSCC patients. Additional molecular studies are needed to confirm or elucidate these karyotype findings. Patients with OSCC are defined by unique genomic profiles that give rise to specific signatures based on this and similar precise genetic abnormalities, which may allow for personalized targeted treatment care. The association of CA with age, gender and risk habits substantiates the strongest association of tobacco in oral cancer progression. The incorporation of clinical findings, microscopic examination and molecular diagnostics will progress the ability to risk stratify people for the development of OSCC and change towards reducing the consequence of this aggressive disease.

FUTURE SCOPE

The knowledge regarding the genetic and epigenetic root of cancer is important to understand the pathophysiology, disease diagnosis, disease prevention and therapeutic applications. The classical cytogenetic analysis provides global information of a genome in a single assay. Further molecular cytogenetic and epigenetic analysis of specific chromosomal alterations might be paving a pathway in identifying gene level abnormality. These can be used as unique/combined biomarkers for different clinical applications in cancer

patients. Our work further demonstrated that tobacco use raises the risk for mouth cancer by identifying particular chromosomal changes in oral cancer patients. These chromosomal alterations may also help with proper counseling for long-term smokers. Thus the cytogenetic technique can be considered as an indispensable diagnostic tool in cancer management and treatment.

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Conflict of Interest. None.

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