

Research Article

Establishment of a Tongue Squamous Cell Carcinoma Cell Line from Indian Gutka Chewer

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CD cell line has been established from a poorly differentiated squamous cell carcinoma of tongue. This is a first ever cell line established from an Indian gutka chewer. Cell line was characterized for morphology, ultrastructure, doubling time, expression of epithelial markers, DNA content, karyotyping, STR markers, p53 mutations, HPV status, and tumorigenicity in SCID mice with all-*trans*-retinoic acid and cisplatin. The epithelial phenotype of the cell line was confirmed with surface markers and ultrastructure. The cell line is hyperploid with chromosomal alterations like gain of chromosomes 8q and 11q. CD cell line shows a unique pattern on STR genotyping and carries a missense mutation R273C in *TP53*. It does not show genomic integration of HPV. The cells are nontumorigenic to SCID mice and show growth inhibition upon treatment with cisplatin, and all-*trans*-retinoic acid. This cell line may be useful as an in vitro tool to understand the molecular changes associated with oral cancers.

1. Introduction

Cancer of oral cavity is the third most common cancer in India (<http://globocan.iarc.fr/Pages/online.aspx>). The important risk factors identified so far are tobacco use and alcohol consumption, which seem to have a synergistic effect. Statistics for head and neck squamous cell cancers (HNSCCs) throughout the world show these cancers to be prevalent in areas where consumption of tobacco and alcohol is high [1]. The process of oral carcinogenesis is multifactorial with interplay of various environmental factors. Though treatment modalities have shown an advancement, the survival rates of oral cancer patients have not improved markedly over the past few decades [2].

Tumor derived cell lines form a useful resource as model systems with reflections of the original tumors [3]. Therefore, development of cell lines from tumor tissues will aid in understanding the events associated with development of

cancer. Moreover, cell lines are required to study the effects of various known and novel drug formulations and help in studying future treatment strategies. In spite of these advantages, there are very few cell lines developed from Indian oral cancer patients [4–6].

We have established an oral cancer cell line CD from a young gutka chewer. Gutka is a dry mixture of areca nut, catechu, and slaked lime with tobacco. Due to its easy availability and low cost, it has become popular chewing substitute in young Indian tobacco habitués. Use of gutka has been strongly implicated in increased incidence of oral submucous fibrosis, a precancerous lesion, which has a high rate of malignant transformation, even after a short period of use [7–9]. In this report, we describe the establishment and characterization of oral cancer cell line derived from gutka user. We have characterized the in vitro growth and genetic characteristics of this cell line for authentication. This cell line may serve as useful tool to unravel the regulatory pathways in

oral carcinogenesis and may help in better understanding of oral cancer biology.

2. Materials and Methods

2.1. Tumor Specimen Collection and Establishment of Cell Line. Surgically resected fresh tongue tumor sample was obtained from a 34-year-old male gutka chewer through the Bio-Repository, Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Navi Mumbai. The tumor tissue was collected aseptically in plain Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) containing double strength antibiotic mixture of gentamycin 50 $\mu\text{g}/\text{mL}$, streptomycin 125 $\mu\text{g}/\text{mL}$, and antifungal antibiotic forcan 150 $\mu\text{g}/\text{mL}$. The tumor tissue was washed thoroughly in PBS with double strength antibiotic mixture and cut into smaller pieces which were incubated in trypsin (0.025%) for 30 min at 37°C. The tumor tissue was freed of blood vessels and adhering tissue. Explant cultures were set up in DMEM containing 10% fetal bovine serum (Invitrogen, USA) and double strength antibiotic mixture. The medium was supplemented with epidermal growth factor (EGF) 5 ng/mL, insulin 5 $\mu\text{g}/\text{mL}$, hydrocortisone 0.4 $\mu\text{g}/\text{mL}$, sodium selenite 5 ng/mL, and transferrin 10 $\mu\text{g}/\text{mL}$. Medium was changed after 48 h and the epithelial pool of cells was enriched by differential trypsinization and subsequent removal of fibroblasts. The cell line was established by subculturing further and enriching the epithelial cells.

2.2. Light and Electron Microscopy. Using inverted phase contrast microscope, cell cultures were photographed at 20x magnification. The cells were grown as monolayer in petri dishes and processed for ultrastructure analysis as reported earlier [6]. Briefly, the cells were washed with 3% glutaraldehyde in 0.1M sodium cacodylate buffer and were scraped with rubber policeman. After 1 h of fixation, the cells were washed with the same buffer and postfixed with 1% osmium tetroxide. The cells were dehydrated through a series of graded alcohol, embedded in araldite, and polymerized at 60°C for 45 h. Ultrathin sections of 60–70 nm thickness were cut, contrasted with uranyl acetate and lead citrate. The sections were examined under transmission electron microscope (Tecnai G2) at 80 kV.

2.3. Growth Characteristics. The cells were collected and seeded at a concentration of 1×10^4 cells per well in 6-well plates. Cell count of three random wells was taken every day up to 5 days using trypan blue method. The doubling time was calculated according to the formula $T_d = T \times \log 2 / \log (N/N_0)$, where T_d is the doubling time; T is the time interval; N is the final cell number; and N_0 is the initial cell number [10].

2.4. Determination of Tumorigenicity. The in vivo tumorigenicity of the cell line was evaluated by injecting 1×10^7 cells subcutaneously into dorsal flank of the SCID mice. Mice were observed for development of tumors for four months.

2.5. Soft Agar Colony Formation Assay. Anchorage-independent growth of the cell line was monitored by soft agar assay. Different number of cells (1×10^3 , 5×10^3 , and 1×10^4) were suspended in 0.3% (w/v) agar layered over a 0.6% (w/v) base agar. Agar was supplemented with DMEM, 10% FBS, and growth supplements. The plates were incubated at 37°C in CO₂ incubator for a month and observed for colony formation.

2.6. Chromosome Analysis and DNA Flow Cytometry. Karyotyping of the cell line was carried out by culturing 5×10^5 cells in 25 cm² flasks. After 24 h, the cells were arrested in metaphases by incubating the cultures with colchicine at 1 $\mu\text{g}/\text{mL}$ for 2–3 h at 37°C. The cultures were harvested, incubated in hypotonic (0.075 M) KCl for 30 min at 37°C, fixed in fresh, cold methanol acetic acid fixative, and stored at 4°C. The slides were prepared by dropping the fixed cells on clean glass slides and G-banded using standard protocol. A minimum of 50 metaphase spreads was counted for model number and at least five G-banded karyotypes were made for the cell line. The DNA content of the cell line was measured by flow cytometry over a 3-day period by seeding 5×10^5 cells per plate in triplicate for 3 time points of 24 h, 48 h, and 72 h as reported earlier [5]. Ten thousand events were acquired on Becton-Dickinson FACS SCAN and the data was analyzed using Modfit software. Human peripheral blood lymphocytes were used as control.

2.7. Fluorescence In Situ Hybridization (FISH). The results of karyotyping were validated by interphase FISH (I-FISH) using 8q24.3, 11q13 locus specific probes; centromere-specific probe for the respective chromosome served as a hybridization control for all FISH experiments. A dual color procedure was followed as previously described [11, 12].

2.8. Immunocytochemical Studies. The cells grown on coverslips were fixed either in ice cold methanol followed by 0.3% TritonX 100 for probing with anticytokeratin antibodies AE1 (Invitrogen), CK8, and CK 18 (Sigma) or in 1% paraformaldehyde for anti-epithelial membrane antigen (Invitrogen), anti-EGFR (Invitrogen), and antibasic fibroblast growth factor antibodies (Abcam). Goat anti-mouse IgG labeled with FITC was used as secondary antibodies (Sigma). The nuclear staining was carried out by ethidium bromide or Hoechst 33258 staining.

2.9. TP53 Mutation Analysis. DNA was isolated from CD cells by using commercially available DNA purification column (Qiagen Catalogue Number 51104) following the manufacturer's instructions. The primer sequences to amplify exons 1–11 of TP53 gene were synthesized and used as described [13] with minor modifications.

2.10. DNA Profiling. Genomic DNA extracted as above was used for DNA profiling. DNA was amplified by PCR using short tandem repeats (STRs) markers. Cell line was genotyped using 3100 Avant Genetic Analyzer (Applied Biosystems, USA) for 8 autosomal STRs (CSF1PO, D5S818, D7S820,

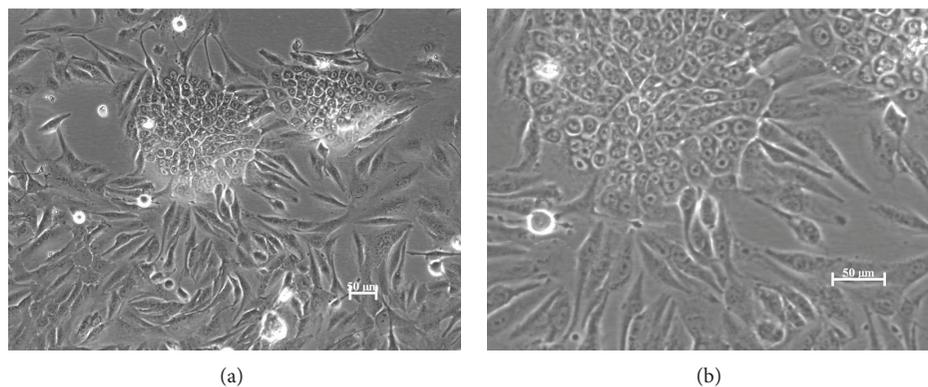


FIGURE 1: Epithelial colonies and fibroblast-like cells in primary culture at (a) (DIC $\times 200$) and (b) (DIC $\times 400$).

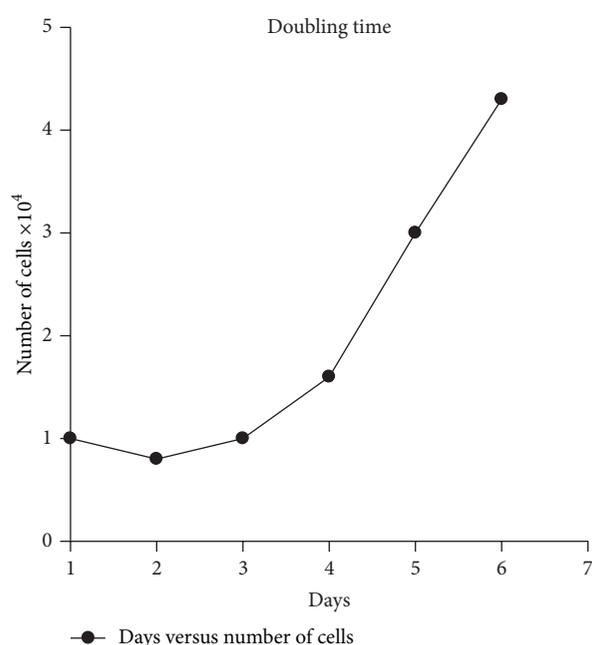


FIGURE 2: Growth curve of CD cell line.

D13S317, D16S539, THO1, TPOX, and vWA) and the gender determining amelogenin marker.

2.11. Cytotoxicity Assay. The cells at a density of 2×10^3 per well were seeded in 96-well plates and allowed to grow for 24 h and thereafter the cells in quadruplets were treated with all-*trans*-retinoic acid (ATRA) (Sigma) and cisplatin (Cipla) for different time points from 1 to 4 days. ATRA was used at various concentrations ranging from $0.0005 \mu\text{M}$ to $0.5 \mu\text{M}$ while cisplatin was used at $0.0005 \mu\text{g}$ to $10 \mu\text{g}/\text{mL}$. The effect on growth was observed using the MTT assay [14].

3. Results

3.1. Establishment of Cell Line. The cell line CD (Cancer Research Institute-developed) was established from a poorly differentiated squamous cell carcinoma excised from tongue. Initially, within 8–10 days of explant cultures, the culture

showed two different populations of cells with epithelial and fibroblastic morphology. Small patches of epithelial cells (Figures 1(a) and 1(b)) were enriched by removing fibroblasts using the method of differential trypsinization. The resulting cultures were pooled and expanded further. Over subsequent passages, fibroblastic cell population was removed selectively by differential trypsinization and the epithelial population was enriched. The cell line exhibited a doubling time of about 31 ± 1 h (Figure 2).

3.2. Analysis of Epithelial Markers. The epithelial nature of the culture was ascertained by immunostaining with antibodies against epithelial membrane antigen and EGFR (Figure 3(a)). The removal of fibroblasts was confirmed by negative staining with fibroblast surface protein specific antibody (Figure 3(b)). The ultrastructure analysis further supported the epithelial nature of the cell line. The transmission electron micrographs of cell sections demonstrated irregular

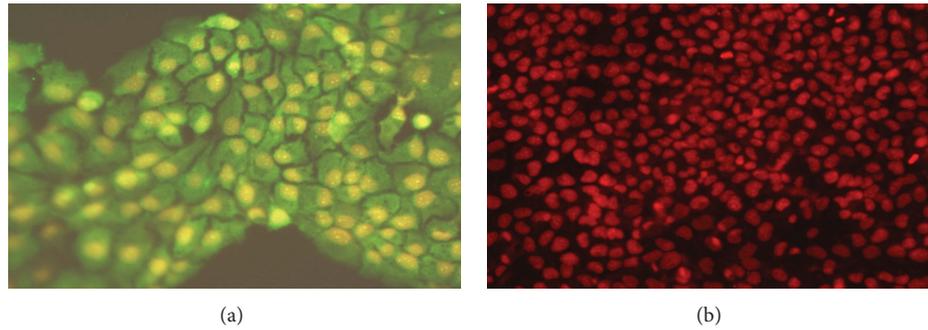


FIGURE 3: Immunofluorescent staining of CD cells for the presence of (a) epithelial membrane antigen and (b) absence of fibroblast specific antigen.

nuclear envelope. It also showed presence of bundles of intermediate filament like structures in cytoplasm and microvilli on the cell surface (Figures 4(a) and 4(b)), indicative of the epithelial origin of the cells. Confocal imaging of the enriched epithelial population devoid of fibroblasts showed staining for the epithelial markers with pan cytokeratin antibody (Figure 5(a)), cytokeratin 8 (Figure 5(b)), and epidermal growth factor receptor (Figure 5(c)). This confirmed that the cell line is derived from epithelial cells.

3.3. In Vivo Tumorigenicity and Soft Agar Assay. An in vivo tumorigenicity test showed that CD cells were nontumorigenic in SCID mice when injected subcutaneously. Soft agar assay showed that CD cells formed colonies in soft agar after about a month showing anchorage-independent growth.

3.4. Chromosome, Flow Cytometry, and FISH Analysis. Chromosome analysis of CD cell line showed human male karyotype. The chromosome number ranged from 68 to 74 and displayed aneuploidy. Most of the metaphases showed one marker chromosome (Figure 6). Flow cytometric analysis revealed the hyperploid nature of the cell line (Figure 7), thus confirming the results of karyotyping. Further, the locus specific FISH analysis validated the results of karyotype (Figure 8).

3.5. HPV Infection and TP53 Analysis. High risk HPV infection has been implicated in OSCCs and many studies have examined the relationship between HPV infections and head and neck SCCs. CD cells were analyzed for the presence of HPV DNA by PCR amplification and the cells did not show genomic integration of HPV (Figure 9). Mutations in TP53 gene were detected in CD cell line. A well-documented 16-base-pair homozygous deletion in TP53 gene was found in the cell line. This 16 bp deletion (rs17878362) has been reported as a polymorphism in the TP53 database of the International Agency of Research in Cancer (IARC). A known deleterious homozygous mutation R273C (CGT to TGT at codon 273 changing an arginine residue to a cysteine) was also detected in this cell line (Figure 10).

3.6. DNA Profiles. In order to authenticate the cell line, CD cells were genotyped for 8 STR markers and one gender

TABLE 1: Analysis of STR markers in the CD cell line.

Locus	Observed alleles
CSF1PO	9,3, 12
D5S818	11, 13
D7S820	10
D13S317	8, 13
D16S539	10, 12
THO1	7
TPOX	9, 11
vWA	14, 19,2
AMEL	X, Y

determining marker (Table 1). STR profile confirmed that the cell line has a unique genotype.

3.7. Cytotoxicity Analysis. The effect of retinoic acid, a differentiation inducer, and cisplatin was studied for growth inhibition using the MTT assay. As seen in Figure 11(b), cisplatin showed a dose dependent growth inhibition of the cells as compared to control over a 4-day period. At 10 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ concentrations, on day 4, only 29 and 32% cells were surviving, respectively, as compared to 100% in controls (Figure 11(b)). However, retinoic acid showed a marginal growth inhibition, even at a concentration as high as 0.5 μM , of 81% and 71% surviving cells by days 3 and 4, respectively (Figure 11(a)).

4. Discussion

Difficulties and low success rates have been well reported in establishing cell lines from oral tumor tissue [15–17]. Here, we describe the establishment and characterization of CD cell line from a poorly differentiated primary tongue tumor. There are very few reports regarding the development of cell lines from smokeless tobacco users from Asian countries including India [4, 5, 18]. It is interesting to note that the patient was a young tobacco habitué and this is the first cell line developed from an Indian gutka chewer. Cancers of head and neck arise as a result of accumulation of genetic changes. The most important etiological factor associated with the development of these cancers is use of tobacco for smoking or in various

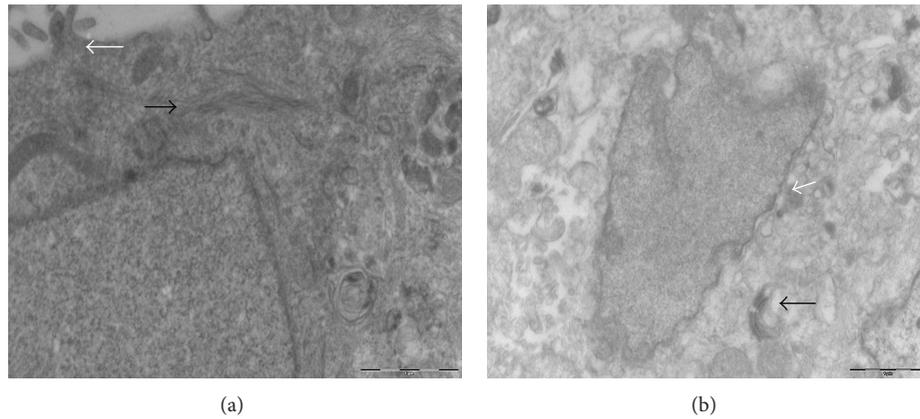


FIGURE 4: Transmission electron microscopy images of the CD cells exhibiting (a) cytoplasmic filaments (black arrow) and microvilli (white arrow) and (b) rough nuclear membrane (white arrow) and desmosomes (black arrow).

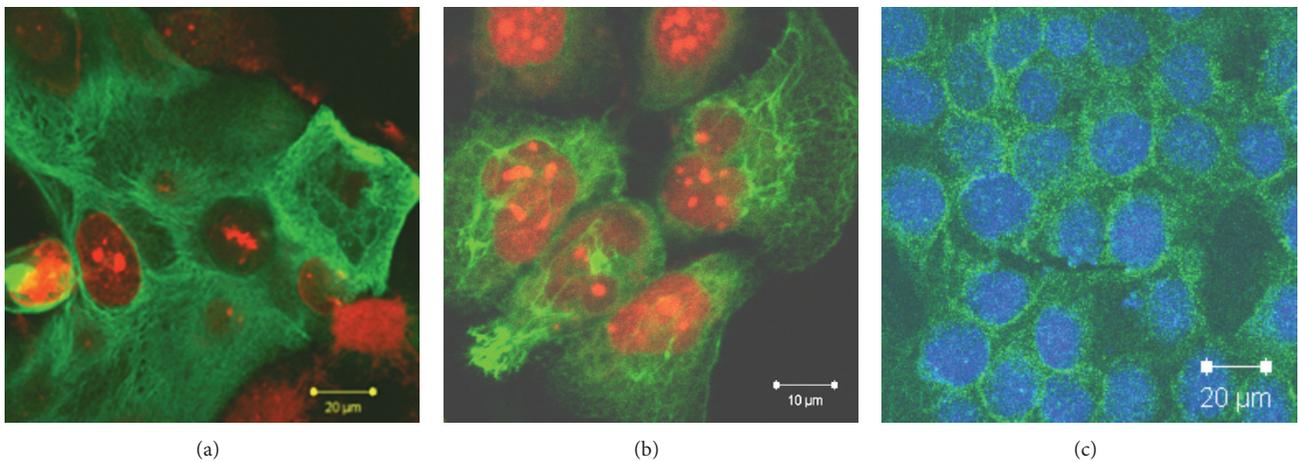


FIGURE 5: Expression of (a) pan cytokeratin, (b) cytokeratin 8, and (c) epidermal growth factor receptor on CD cells.

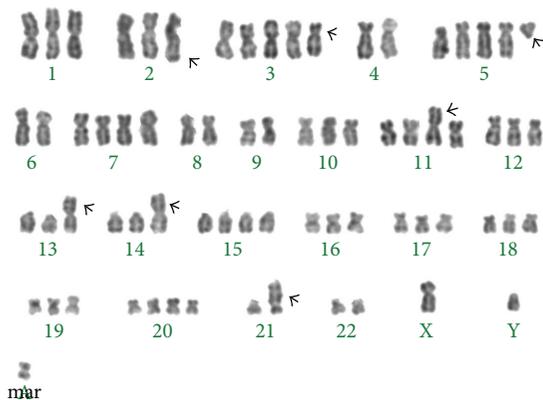


FIGURE 6: Karyotype of CD cells showing multiple chromosomal alterations.

smokeless forms [1, 19]. White et al. have reported that the majority of tumors that yielded a cell line were from patients who were smokers while few cell lines developed from tumors of nonsmokers [3]. Smoking has stronger causative links

than smokeless tobacco use which is directly related to the duration and dose [1, 20]. However, tobacco use was not found to be significantly associated with the formation of cell line because accounting for exposure levels based on type of tobacco use is difficult [3]. Hence, the cell line developed from the tumor of a smokeless tobacco chewer may be a valuable research tool.

The cell line is epithelial in nature and shows expression of epithelial membrane antigen and EGFR. Negative staining by antifibroblast antibody negated the contamination of fibroblast cells. Further, the ultrathin sections of CD cells show presence of cytoplasmic filaments, microvilli, and desmosomes confirming their epithelial origin. The cells express cytokeratins which belong to class of intermediate filament proteins and are involved with regulation of cell proliferation, differentiation, and cell death. Cytokeratins are known to form an extensive network with desmosomes on plasma membranes of epithelial cells. Apart from staining positive to pan keratin antibodies, the cells are immunopositive to CK8 antibodies, confirming their epithelial nature. Downregulation of CK8 has been reported to decrease the tumorigenic potential, alterations in cell motility, and organization of

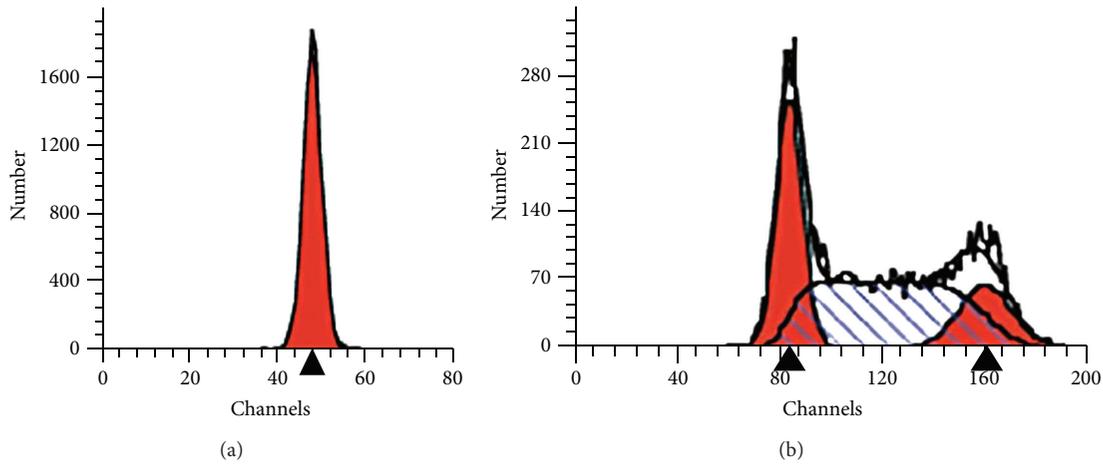


FIGURE 7: Flow cytometric analysis of the CD cell line. (a) Normal lymphocytes with diploid peak and (b) CD cells with hyperploidy.

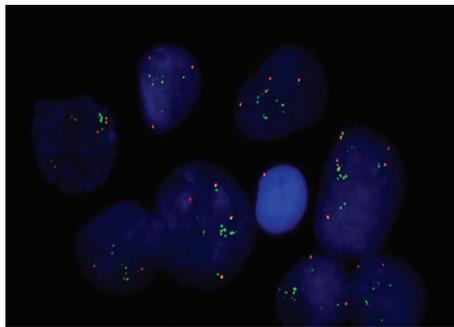


FIGURE 8: FISH analysis of CD cells with red signal indicating centromeres and green signal indicating 11q13 locus.

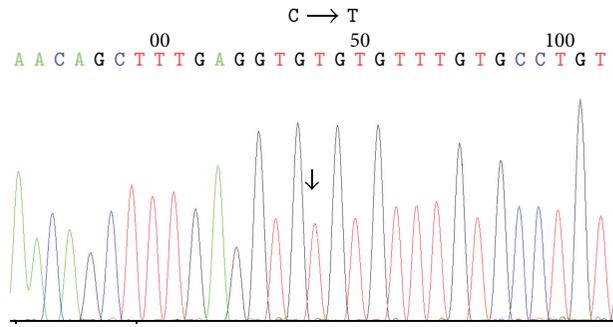


FIGURE 10: Chromatogram depicting a homozygous missense somatic mutation R273C in *TP53* in CD cells.

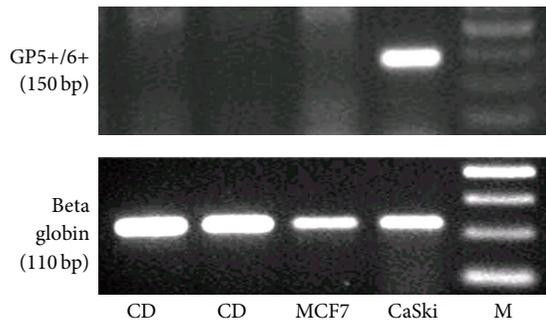


FIGURE 9: Screening of CD cells for HPV DNA. HPV 16 was not detected in CD cells. Controls used CaSki positive control for HPV 16 and MCF7 as negative control. M: 50 bp marker.

cytoskeleton in a tongue cancer cell line [21, 22]. Although CD cells show expression of CK8, they are nontumorigenic in SCID mice but formed colonies in semisolid agar, suggestive of transformed phenotype which is similar to the earlier reports [4, 17].

The cells are hyperploidy in nature and show chromosomal alterations peculiar to HNSCC indicating chromosomal instability as reported earlier in oral cancer cell lines

[23, 24]. In fact, correlation of gain/amplification of 11q13 band has been reported in about 45% of oral and head and neck squamous cell carcinomas [24]. FISH analysis also depicts the gain of 11q13 locus in our cell line which is consistent with the karyotype data.

Although the etiology of oral cancer is not very clear, there is substantial evidence that the disease development underlies the activation of protooncogenes and the inactivation of tumor suppressor genes. To understand the mechanism underlying the development of oral cancer, the status of HPV infection, protooncogene (EGFR), and tumor suppressor (*TP53*) was investigated. Human papillomavirus (HPV) infection is associated with a subset of head and neck squamous cell carcinoma (HNSCC). HPV-infected oropharyngeal tumors comprise a distinct molecular, clinical, and pathological disease entity with distinct genetic alterations and better prognosis when treated with radiation or chemoradiation [25–29]. At a molecular level, HPV-positive OPSCC is associated with increased expression of wild-type p53, reduced expression of the epidermal growth factor receptor (EGFR), and downregulation of cyclin D and pRb [2]. CD cell line derived from chronic tobacco gutka user does not show genomic integration of HPV which is in agreement with earlier reports of other cell lines [30, 31]. Further, it has also

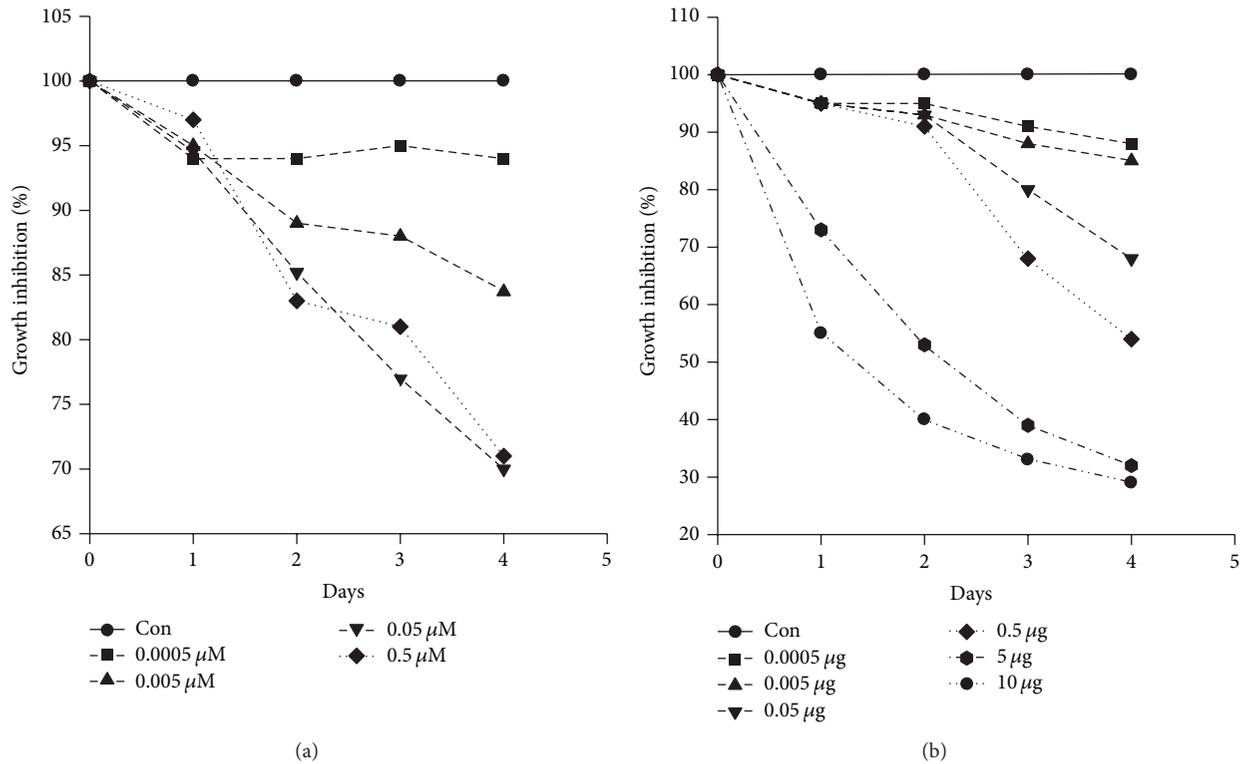


FIGURE 11: Effect of (a) retinoic acid and (b) cisplatin on CD cells using cell viability MTT assay.

been reported that HPV infections are detected in tumors with intact *TP53* pathway [32]. However, absence of HPV infection in the cells suggests that HPV is not associated with the development of oral cancer in this particular individual, indicating that mutation in *TP53* protein may have given growth advantage to this cell line. Therefore, we examined CD cell line for the mutation in the *TP53* genes. Genetic characterization showed a 16-base-pair homozygous deletion and an amino acid change at codon 273. The 16 bp deletion has been reported as a polymorphism in the *TP53* IARC database (rs17878362) and the mutation at codon 273 has previously been reported in OSCC cell lines [33]. To understand the role of protooncogene, we examined the expression of EGFR in the cell line. EGFR is known to be overexpressed in several epithelial malignancies and also in HNSCCs [34]. Our results were also in accordance with the reports mentioned. It has been reported that a mutant P53 protein may transactivate the promoter of the gene expressing human EGFR, resulting in more aggressive growth of the cells [35].

Role of ATRA in the management of tobacco related oral cancers has been suggested earlier [4]. CD cells did not show any morphological changes upon treatment of ATRA although growth inhibition was observed at higher ATRA concentrations. Cisplatin based combinatorial chemotherapy is often used for the treatment of oral cancers although its use is limited due to acquired resistance [36]. However, CD cells are sensitive to the treatment of cisplatin. Thus, this cell line can be used as an in vitro model system to study tobacco induced oral cancers and the effect of various chemotherapeutic drugs used in treatment. The establishment of cell lines

for research purposes is crucial but is biased towards more aggressive tumors, which are likely the result of interactions between multiple relevant exposures and consequent genetic alterations. Hence, it is critical that information about the tumor and the patient from which a cell line was derived is well documented especially when it is used for translational research [3].

In summary, this cell line developed from a gutka chewer provides an in vitro model system established from smokeless tobacco induced oral squamous carcinoma. The cell line with its hyperdiploid nature and drug sensitivity will be very useful for screening of anticancer drugs and new drug formulations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

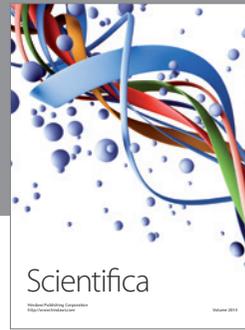
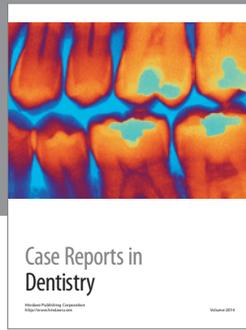
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