RESEARCH ARTICLE

Nuclear Anomalies, Chromosomal Aberrations and Proliferation Rates in Cultured Lymphocytes of Head and Neck Cancer Patients

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Abstract

Head and neck cancers (HNC) are extremely complex disease types and it is likely that chromosomal instability is involved in the genetic mechanisms of its genesis. However, there is little information regarding the background levels of chromosome instability in these patients. In this pilot study, we examined spontaneous chromosome instability in short-term lymphocyte cultures (72 hours) from 72 study subjects - 36 newly diagnosed HNC squamous cell carcinoma patients and 36 healthy ethnic controls. We estimated chromosome instability (CIN) using chromosomal aberration (CA) analysis and nuclear level anomalies using the Cytokinesis Block Micronucleus Cytome Assay (CBMN Cyt Assay). The proliferation rates in cultures of peripheral blood lymphocytes (PBL) were assessed by calculating the Cytokinesis Block Proliferation Index (CBPI). Our results showed a significantly higher mean level of spontaneous chromosome type aberrations (CSAs), chromatid type aberration (CTAs) dicentric chromosomes (DIC) and chromosome aneuploidy (CANEUP) in patients (CSAs, 0.0294±0.0038; CTAs, 0.0925±0.0060; DICs, 0.0213±0.0028; and CANEUPs, 0.0308±0.0035) compared to controls (CSAs, 0.0005±0.0003; CTAs, 0.0058±0.0015; DICs, 0.0005±0.0003; and CANEUPs, 0.0052±0.0013) where p<0.001. Similarly, spontaneous nuclear anomalies showed significantly higher mean level of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) among cases (MNi, 0.01867±0.00108; NPBs, 0.01561±0.00234; NBUDs, 0.00658±0.00068) compared with controls (MNi, 0.00027±0.00009; NPBs, 0.00002±0.00002; NBUDs, 0.00011±0.00007).The evaluation of CBPI supported genomic instability in the peripheral blood lymphocytes showing a significantly lower proliferation rate in HNC patients (1.525±0.005552) compared to healthy subjects (1.686±0.009520) (p<0.0001). In conclusion, our preliminary results showed that visible spontaneous genomic instability and low rate proliferation in the cultured peripheral lymphocytes of solid tumors could be biomarkers to predict malignancy in early stages

Keywords: Head and neck cancers - lymphocytes - nuclear anomalies - chromosomal aberrations

Introduction

Head and neck cancers (HNC) are placed fifth among the common cancers occurring worldwide. Overall, head and neck cancer account for more than 550,000 cases annually worldwide (Jemal et al., 2011). The present computations highlighted that in India, 1 in 10 men and 1 in 8 women would develop cancer of any form sometime after the age of 35 (Murthy et al., 2011). Over 200,000 cases of head and neck cancers occur each year in India and nearly 80,000 oral cancers are diagnosed every year. Head and neck cancer in India have distinct demographic profiles, risk factors, food habits, family and personal history (Kulkarni, 2013). HNC constitute an anatomically heterogeneous group of cancers. These arise from all mucosal sites within the head and neck, primarily the oral cavity, oropharynx, hypopharynx, larynx and nasopharynx. Over the years, clinical observations have demonstrated numerous differences in HNC from various head and neck sites. The squamous cell carcinogenesis has a progressive nature of genetic abnormalities triggered by mutational changes occurred by the risk factors or genetic predisposition in the genes regulating cell cycle progression (Weinstain, 2000). This multistep carcinogenesis is a permanent suffering and lethal if left untreated in the initial stages. Why does everyone not develop cancer? Most HNC cases and deaths are due to both individual predispositions linked to certain genetic characteristics and synergetic effect of exposure to carcinogens caused by life style behaviors: like

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tobacco use and abuse of alcohol (Kulkarni, 2013). The integrity of the genome being questioned when xenobiotic metabolism (Masood et al., 2011) and epigenetic changes (Sharma et al., 2010) along with the environmental factors (Stare and Jozefowicz, 2008) become adverse. Genomic instability, the most discussed and applied core word in the modern research world, has now become a reason for all unexplained and unveiled defectiveness of a biological system and it has been discussed and questioned from the last century, the individuality of the chromosomes, by means of chromosomal aberrations and developmental anomalies (Laubichler and Davidson, 2008) The unstable genome (or structural and numerical changes that happen to the chromosomes of a cell) an important hallmark of cancer can cause a defective cell division, programmed cell death, necrosis or cell cycle arrest. Once the cell starts defective proliferative mechanisms due to genomic instability caused, the cell becomes cancerous and makes clones uncontrollably (Hanahan and Weinberg, 2011).

Classical cytogenetic methods used in peripheral blood lymphocytes (PBLs) have been applied for over 30 years in occupational, environmental and medical settings as a biomarker for the detection of genotoxic effects (Obe et al., 2002). Chromosome instability and genome damage is a key initiating factor in numerous conditions including cancers, chromosome instability syndrome, infertility, neurodegenerative diseases, and accelerated aging syndromes. Large prospective studies conducted over several countries demonstrated that increased chromosomal aberrations in peripheral blood lymphocytes (PBL) is a predictor of future cancer risk (Bonassi et al., 2007). These studies investigated the incidence of chromosome type aberrations (CSAs), Chromatid type aberrations (CTAs) and they focused specifically on cancer risks, while correcting for exposure to potential carcinogenesis such as smoking or workplace hazards (Rossner et al., 2005; Bonassi et al., 2007). Results suggested that both DNA double strand breaks and other initial DNA lesions responsible for CSAs and CTAs associated with cancer risk (Hagmar et al., 2004).

The Cytokinesis block micronucleus cytome (CBMN Cyt) assay is known to be an effective and comprehensive tool to measure cytogenetic damage (Fenech, 2007). It can be an alternative method to analyze the stability of genome by blocking the cytokinesis in the culture and observing them microscopically. Chromosomal instability may be one of the primary causes of tumor initiation and progression. Understanding of biological basis of chromosomal instability is critical for effective diagnostic and prognostic evaluation and therapeutic intervention of cancer. The aim of present study is to analyze the spontaneous DNA damage and chromosome changes by using conventional CA analysis and CBMN cyt assay simultaneously to detect the spontaneous genetic changes in peripheral lymphocytes of Head and Neck Cancer patients.

Materials and Methods

Cytogenetic analysis was carried out on 36 patients with newly diagnosed Head and Neck cancers (patients of the Department of ENT, Govt. Vellore Medical College Hospital (GVMCH), India) and 36 controls of similar age, sex and socioeconomical background (recruited by the help of physicians from the same department of GVMCH). The participating subjects were properly informed and signed a written consent and the approval for cytogenetic analysis in accordance with the Helsinki declaration obtained. The design of the study was approved by the University Human Ethical Committee (UHEC).

Clinical and pathological observations

The selection of patients, clinical tests and diagnosis were done with the help of physicians and histopathologists (GVMCH). Clinical and epidemiological data were recorded in a standard proforma.

Cytogenetic studies

Peripheral blood samples were processed immediately on sampling and cultured. Chromosome preparations were made according to the standard procedure (Hungerford et al., 1965). Cultures were set up by mixing 0.5 ml of whole blood with 6 ml of RPMI (Hi Media), supplemented with 1.2 ml of fetal bovine serum (Hi Media) and 0.3 ml of phytohemagglutinin (GIBCO).

Analysis of chromosomal aberrations

Lymphocyte cultures were harvested after 72 hours from the time of initiation. They were arrested at metaphase with colchicine (0.6μg/ml final concentration Sigma). The cells were treated hypotonically for 11 minutes with 0.075 M KCl (Merck), followed by fixation with methanol:acetic acid (3:1). Fixed cells were dropped onto clean microscopic slides, air dried and stained with Giemsa solution (4%). For each sample, 100 well spread metaphases were scored for all types of chromosomal aberrations.

CBMN CYT assay

The cytokinesis-block micronucleus (CBMN) assay is an established cytogenetic method for the measurement of chromosome breakage and loss in nucleated cells (Fenech, 2000) For this assay, lymphocyte cultures were set up as described above. Cells were blocked in cytokinesis by the addition of Cytochalasin B (6μg/ml final concentration, Sigma) at the 44th hour. Harvesting was carried out at the end of 72 hours. The cells were treated hypotonically with cold 0.075 M KCl for 8 minutes, followed by fixation with methanol-acetic acid (3:1). Subsequently, slides were prepared and stained with Giemsa solution (4%). For each sample, 1000 binucleated cells were scored for abnormalities following the criteria specified by (Fenech et al., 2003; 2007).

Cytokinesis block proliferation index (CBPI)

For each slide, the proportion of mono-nucleated, bi-nucleated, tri-nucleated and tetra nucleated cells per 500 cells scored was assessed (Kirsch-Volders et al., 2003).

$CBPI=(M1+2M2+3(M3+M4)/N$ (Eastmond et al., 1989). Where M1- mono nucleate Cells; M2- Binucleate cells; M3- Tri nucleate cells; M4- Tetra nucleate and; N the total number of cells analyzed (N=500).

Statistical analysis

A comparison between the data obtained from the patient groups and the control group was analyzed using Student's t-test. A p value of <0.001 with confidence limit of 95% was defined as statistically significant. Numerical data are presented as Mean±SE/ Mean±SD. Graph pad prism 5 is used for statistical analysis.

Results

The study population included 36 HNC patients and same number of healthy, age and sex matched ethnic control subjects.The patients consisted of 31 men and 5 women (mean age of 59.64±2.167 years, N=36), there were 8 non-smokers and 28 smokers. The control individuals exhibited a mean age of 43.11±2.142 years N=36; there were 29 men and 7 women and there were 32 non-smokers and 4 smokers. Average age of male study subjects in HNC patient group (n=30) was 61.23 and the healthy control samples (n=29) were 53.79. The average age of HNC (n=6) and Control (n=7) female study subjects were 53.33 and 50.14 respectively. The mean exposure of tobacco smoking in HNC male subjects were 31.82±12.2 (M±SD n=29) and control males were 18.09±10.2 (M±SD n=23), where female groups of the study did not show any exposure to smoking habit. The alcohol exposure in HNC male subjects were 33.09±12.19 (M±SD n=21) and control males were 20.231±8.62 (M±SD n=21) where female groups of the study did not show any alcohol exposure. In cancer patients group both males and females had a history of tobacco chewing with an average exposure of 20±15.41 (M±SD n=5) years and 28.33±20.3 (M±SD n=3) years respectively where the control group didn't have a habit of tobacco chewing. The observations show

Table 1. The Frequency of Different Cancer Sites in HNC Patients

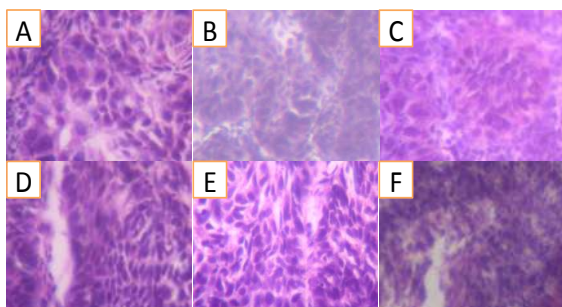| Tumor Site	No. of affected males	No. of affected females
Oropharynx	9 (30%)	0 (0%)
Hypopharynx	1 (3%)	1 (16.7 %)
Pharynx	3 (10%)	1 (16.7 %)
Larynx	6 (20%)	1(16.67%)
Buccal cavity	7 (24%)	2 (33.3%)
Other	4 (13%)	1 (16.7 %)

Figure 1. Biopsy Images from Different Cancer Sites in HNC Patients Squamous Cell Epithelium with Malignant Transformation Composed of Polygonal Cells with Hyperchromatic Pleomorphic Nuclei. A) oropharynx (soft palate), B) hypo pharynx, C) pharynx D) larynx (supraglottis), E) buccal cavity (tongue) and F) nasopharynx

that the cancer patients had a long time exposure to the risk factors than the control group. The consanguinity and the family history are non significant in this study. In the earlier studies in various cancers by researchers show the risk factor and exposure time trigger the cancer initiation and development (El-zein et al., 2005; Basu et al., 2008; Sudha et al., 2009; Hashim et al., 2012; Siddique et al., 2012; Mokoto et al., 2013). In this study the exposure to tobacco smoking and alcohol consumption caught high compared to healthy subjects. The consumption of tobacco in various forms such as tobacco smoking and chewing along with alcohol consumption is dose-dependent (Basu et al., 2008) and considered as major risk factors.

Clinical observations

Clinical observations showed that the anatomical sites affected are important for functions such as speech, swallowing, taste, and smell. The primary cancer sites vary as given in Table 1. The most affected sites were oropharynx and oral cavity (Table 1). Biopsy reports showed hyper chromatic pleomorphic nuclei which differentiate neoplastic malignant cells from normal cells. Biopsy results of moderately differentiated squamous cell carcinomas are shown in Figure 1.

Cytogenetic study

It is observed from the results that the HNC patients have a higher frequency of chromosomal and nuclear (Figure 2) abnormalities than the healthy control subjects. The end points of genomic instability observed in this study are CSAs, CTAs, DIC, CANEUP (≥47 chromosomes), MNi, NPBs and NBUDs. The proliferation rates in the cultures of PBL were analyzed by calculating the CBPI. To determine the statistical significance of the genomic instability in HNC, two tailed T-test (α=0.05) were performed where a p value <0.001% at confidence limit of 95%

Chromosomal aberration (CA) analysis

The frequencies of the chromosomal aberration types are significantly high (Table 2) when compared to control study subjects. The damage to the human genome incurs on the order of 1,000-1,000,000 DNA lesions/cell/day (Lodish, 2000). It is said most of the lesions are caused by endogenous sources, including reactive oxygen and nitrogen species (ROS, RNS) that can oxidize cellular macromolecules including lipid, protein and nucleic acid where one or the other takes part in the integrity of genome and DNA damage signals. Here in this study most observed are chromatid type and chromosome type breaks (0.0925±0.0060 and 0.0294±0.0038; p<0.001)
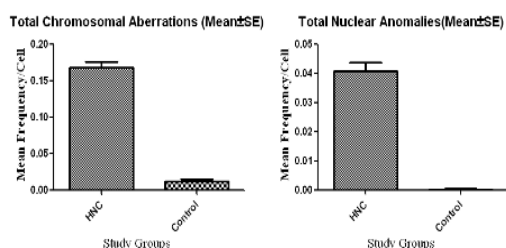

Figure 2. Frequency of CA and Nuclear Anomalies

which may arise from recombinational exchanges that occurs between sister chromatids (Rogers-Bald et al., 2000). The higher rate of ploidization (0.0308±0.0035; p<0.001) and dicentric chromosomes (0.0213±0.0028; p<0.001) studied in the patient group support the higher nucleoplasmic bridge formation in CBMN Cyt Assay, as chromatid bridges are known to originate from dicentric chromosomes at anaphase and these bridges can cause ploidy and improper cytokinesis (Pampalona et al., 2012) The aberrations found in the patients are given in the Figure 3. The high frequency of spontaneous CSAs/CTAs shows that there is a high level DNA loss happens in the lymphocytes of newly diagnosed HNC patients. The elevated frequency in the dicentric chromosomes is rarely identified in constitutional genetics. However, in cancer cells the formation of dicentric chromosomes is a well recognized event, which may contribute to the malignant phenotype and clonal evolution. In this study the frequency of dicentric chromosomes in the cultured lymphocytes have a higher frequency than the normal control subjects, which can be an early detector of malignancy (Gascoigne et al., 2013).

Cytokinesis block micronucleus Cytome Assay (CBMN Cyt Assay)

The cytokinesis blocked binucleated cells were analyzed for the presence of micronucleus and other nuclear anomalies (NPBs and NBUDs). Analysis results of HNC subjects showed significantly higher frequency (Table 2) of DNA damages than control in CBMN Cyt Assay. The higher frequency of micronuclei (0.01244±0.0008423; p<0.001) could be a result of the acentric fragments or whole lagging chromosome during mitosis. The other end points (NPBs-0.00168±0.0001947 and NBUDs-0.00112±0.0001776; p<0.001) also showed
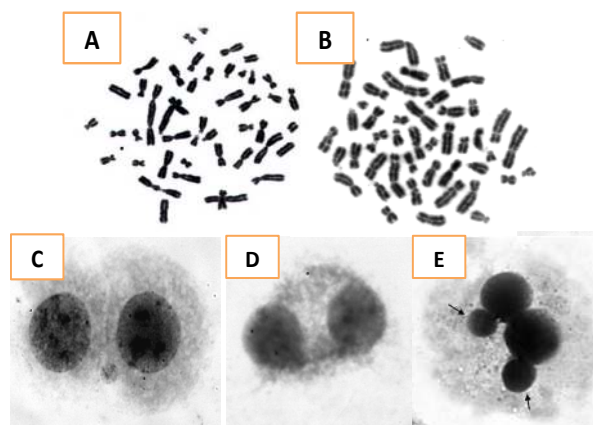

Figure 3. Chromosomal Aberrations and Nuclear Anomalies. A) metaphase with dicentric chromosome; B) metaphase with chromatid and chromosome type breaks C) micronucleus in a binucleated cell D) nucleoplasmic bridge E) nuclear buds

significantly elevated frequency of genomic instability. The higher frequency of MNi in the lymphocytes of HNC patients in this study shows the increased loss of genetic material and function loss during cell division. The Measurement of NPBs/anaphase bridges in the CBMN assay adds important information on asymmetrical chromosome rearrangement that is otherwise only achievable using metaphase analysis of dicentric and ring chromosomes (Fenech et al., 2011). The higher mean frequency of NPBs analysed support the data achieved from the CA analysis and confirms the chromosome rearrangements and dicentric formation (Thomas et al., 2003) in the lymphocytes of HNC patients. The end points studied and their higher frequency or visibility concludes that the lymphocytes of HNC patients have a lesser DNA repair capacity. One of the studies conducted a decade back, the lymphocytes of HNC patients showed higher DNA damage, slower repair, and higher residual un-repaired damage than those of healthy donors (palyvoda et al., 2003). Another end point presented and studied in CBMN assay is NBUDs, which can be a result of nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division or from excess DNA that is being extruded from the nucleus. The NBUD are characterised by having the same morphology as an MN with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Fenech et al., 2011).

Table 2. The Frequency of CAs and Nuclear Anomalies Studies in Lymphocytes of HNC and Control Subjects

Cytogenetic parameters: Types	No of CA Observed inHNC Patients	Mean Frequency M±SE (Frequency/cell)	
		HNC (N=36)	Control (N=36)
CA:			
Chromosome Breaks	17	0.0294±0.0038	0.0005±0.0003
Dicentric chromosomes	12	0.0213±0.0028	0.0005±0.0003
Aneuploid Cells	18	0.0308±0.0035	0.0052±0.0013
Chromatid Breaks	53	0.0925±0.0060	0.0058±0.0015
Total Chromosomal Aberrations		0.1681±0.0076	0.0119±0.0031
CBMN:			
Micronuclei(Mni)	46	0.01867±0.00108	0.00027±0.00009
Nucleoplasmic Bridges(NPBs)	38	0.01561±0.00234	0.00002±0.00002
Nuclear Buds(NBUDs)	16	0.00658±0.00068	0.00011±0.00007
Total nuclear anomalies		0.04086±0.00285	0.00038±0.00013

*p value< 0.001 (CA) & <0.0001 (CBMN)

Table 3. Cytokinesis Block Proliferation Index in HNC Patients and Control Subjects

Sl.No	Parameters	HNC Patients (Mean±SD)	Control Subjects (Mean±SD)
1	Frequency of Mono Nucleate Cells	257.13±13.86	162.47±28.51
2	Frequency of Bi Nucleate Cell	229.88±12.59	333.47±28.67
3	Frequency of Tri Nucleate Cell	5.88±1.99	2.75±1.57
4	Frequency of Tetra Nucleate cell	6.86±2.34	1.31±0.52
5	Frequency of CBPI	1.52±0.03	1.68±0.05

Cytokinesis blocked proliferation index

It is very important to check the proliferation rate to understand the nature of cell or the viability of cell population towards the DNA damage in the cancer patients. In cytokinesis blocked proliferation index the proportion of Mono-, Bi-, Tri- and tetra nucleated cells was studied to understand the nature of proliferation. HNC patients showed a lower frequency (1.52±0.03), higher frequency (257.13±13.86) of mono nucleated cells and lower frequency (229.88±12.59) of Bi nucleated cells as compared to the control subjects (Table 3).

Discussion

The mechanisms of genomic instability are complex and meagerly known, which are influenced to a different extent by intrinsic single base DNA mutation to extrinsic environmental factors. It has been reported since early in 19th century the qualitative and quantitative changes in genetic material by means of observing errors during mitotic phases & metaphase analysis.

In conclusion this study was a conventional cytogenetic observation of spontaneous nuclear anomalies, chromosomal aberrations and proliferation rate in cultured lymphocytes of HNC patients to understand the basic cytology. These cytogenetic endpoints and the statistically significant variations are likely to be useful tools for distinguishing differences in behavior and response to therapy in HNC. However, before using chromosomal aberration or nuclear anomalies as a marker of cancer risk, it is essential to establish not only the presence of an association with exposure but also the link with cancer occurrence.

Acknowledgements

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