RESEARCH COMMUNICATION

Influence of Regular Black Tea Consumption on Tobacco Associated DNA Damage and HPV Prevalence in Human Oral Mucosa

Debolina Pal, Sarmistha Banerjee[1], Dipanjana Indra, Shyamsundar Mandal, Anirudha Dum, Anup Bhowmik, Chinmay Kr Panda, Sukta Das*

Abstract

Black tea is more widely consumed than green tea worldwide, particularly in India. Therefore, it is necessary to focus attention on black tea with respect to its health promoting and anti-cancer actions. In order to establish the concept that black tea is a potential candidate for cancer prevention, it is important to provide epidemiological evidence derived from investigations of human populations. In view of this, the objective of the present study was to determine the correlation between nature of black tea consumption and DNA damage in normal subjects with or without tobacco habit and oral cancer patients, taking the latter as positive controls. Much experimental evidence points to associations between tobacco habit and HPV 16 and HPV 18 (Human Papilloma virus) infection. But no studies have taken into account the possible confounding effect of black tea consumption on DNA damage along with HPV infection. A pilot study was therefore undertaken. Comet assay was used to evaluate the DNA damage among normal subjects including tobacco users (n = 86), non-tobacco users (n = 45) and Oral cancer patients (n = 37). Percentage of damaged cells was scored in the buccal squamous cells of all subjects mentioned above. HPV analysis was performed on 79 samples (including 37 oral cancer patients). The evaluation of various confounding factors like age, tenure of tobacco habit and tea habit showed significant associations with DNA damage. The observations strongly indicate that regular intake of black tea at least above four cups can reduce tobacco associated DNA damage among normal tobacco users. HPV prevalence was not seen to be associated with age, tenure of tobacco habit or the tea drinking habit.

Key Words: Black tea - protection - smoking-associated DNA damage - HPV prevalence

Asian Pacific J Cancer Prev, **8**, 263-266

Introduction

Multiple pathways are involved in development of genomic instability (Limoli et al., 1997). Gene mutation, altered DNA repair capacity, chromosomal aberrations and cellular transformation are features of genomic instability (Morgan et al., 1996). Aberrantly expressed mutated genes may provide impetus towards cellular transformation leading to development of malignant growth. These pre-clinical genomic abnormalities are useful biomarkers for detecting pre-neoplasia. Single cell gel electrophoresis (comet assay) has been introduced to human bio-monitoring as a useful test for assessment of genetic damage in exposed population (Heepchantree et al., 2006; Valvarde et al., 1999). Various kinds of DNA alterations can be detected by Comet assay such as double strand breaks, single strand breaks, alkali-labile sites, incomplete repair sites and cross-links (Tice et al., 2000). This method is sensitive, simple, and economic, can be applied to proliferating cells, such as nasal and buccal tissues that are exposed directly to the mutagenic and carcinogenic substances (Heepchantree et al., 2006).

Tea (*Camellia sinensis*)- a widely consumed beverage worldwide has been shown to possess many health protective properties including chemoprevention of cancer. This is attributed largely to the presence of the polyphenolic compounds in tea (Dreosti et al., 1997). Tea consumption is associated with health beneficial effect due to its antioxidative property (Wiseman et al., 1997; Rice-Evans, 1999). Our previous studies had explored the influence of black tea and its principal components viz. EGCG, ECG and Theaflavin in experimental carcinogenesis models. It was revealed that they could protect from or restrict progression of carcinogenesis on skin, colon and lung in mouse and rat (Saha and Das, 2002; Sengupta et al., 2003; Banerjee et al., 2006).

The present report furnishes our preliminary observations on the effect of tea drinking on tobacco associated DNA damage of buccal squamous epithelial cells in normal subjects. The role of tea habit on HPV prevalence in human buccal mucosa which is associated with oral cancer was also assessed.

*Dept. of Oncogene Regulation, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700026, India, [1]H. Lee Moffitt Cancer Center and Research Institute. Tampa, Florida, USA. *Corresponding author : E- mail: suk_tadas@yahoo.com*

Materials and Methods

Studied population

The population studied comprised 168 samples (tobacco users 86, non-tobacco users 45 and oral cancer patients 37). Prior to the study all subjects gave informed consent in project participation. Oral cancer patients who had medical treatment or radiotherapy were excluded . Studied subjects were interviewed using a questionnaire to survey possible confounding factors.

Sample collection

Buccal squamous cells were collected from subjects by oral brushing. Prior to brushing subjects were made to wash their mouth with normal saline (0.9% NaCl solution) to avoid the interference of mucus. Collected samples were taken in cold phosphate buffer saline (PBS) and cells were allowed to pellet down the cells were resuspended in 300µl PBS and 50µl of cell suspension were taken for comet assay. Rest of the cell suspension was spun down and the pellet was stored at -20°C for HPV detection.

Alkaline single cell gel electrophoresis (Comet assay)

Comet assay was performed under alkaline conditions by using a standard protocol (Singh et al., 1988) with some modifications. Cells were embedded in low melting point agarose on glass slide precoated with 1% normal agarose. After solidification of gel the slide was submerged into cool lysis solution [2.5M NaCl, 100mM EDTA, 10mM Tris (pH 10.0), 1% LSS lauryl sarcosine sodium salt to which 10% DMSO, 1% Triton X-100 were freshly added] and kept overnight at 4°C. The slides were then placed on the horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min and then subjected to electrophoresis at 25V/300mA for 40 min. After electrophoresis the slides were neutralized for ~60 min in 0.4 M Tris/HCl, pH 7.5 on ice, followed by staining in ethidium bromide (stock concentration 25µg/ml in distilled water) and mounting on glycerol. All steps were performed on ice to prevent the removal of thin agarose gel layer from the slide. DNA damage was scored using fluorescence microscopy (see Figure 1).

DNA damage analysis

Slides were examined at 100x and 400x magnification using a fluorescence microscope (Nikon) using 515-560 nm excitation filters (figure). DNA damage is represented as percentage data [(Number of damaged cells/total number of cells) x100].

DNA Isolation for HPV Detection

High molecular weight genomic DNA was extracted from squamous cell pellets (previously obtained) by proteinase K digestion followed by phenol-chloroform extraction using standard protocol (Sambrook et al., 1989). The presence of HPV in the DNA samples was detected by PCR using primers from the consensus L1 region. Typing of HPV (16/18) in the L1 positive samples was done by PCR using specific primers from the E6 region of HPV 16 and the E7 region of HPV18. The PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, visualized under ultraviolet light. For final confirmation of the HPV types, after gel electrophoresis the PCR products were transferred on to nylon membrane for Southern hybridization with [32P] labeled HPV type specific probes. DNA from HPV type specific plasmids was used as positive control.

Statistical Analysis

Statistical analyses performed in the present study include T-test of unequal variance, chi-square test and multivariate analysis.

Results

Sample collection by oral brushing usually produces heterogeneous mixtures of cells, including squamous cells and leukocytes (Osswald et al. 2003; Nicole et al. 2006). In this study, particular emphasis was given to buccal squamous cells. Buccal cells are large size, and have non-granular cytoplasm, centerline located nucleus and a large cytoplasm-to-nucleus ratio. Patient samples were usually loaded with blood lymphocytes. These cells were also scored with squamous cells. The preliminary information revealed from this study was, significant differences in DNA damage were noted in normal subjects with or without tobacco habit.

Table 1. General Characteristics of the Subjects

		Tobacco Users	Non-tobacco Users	Cancer Patients
Total		86	45	37
Age	< 40 yrs	32	23	1
	≥ 40 yrs.	54	22	36
Sex	Male	84	31	31
	Female	2	14	6
Religion	Hindu	85	45	33
	Muslim	1	-	4
Occupation	Student	10	15	-
	Service	43	10	1
	Business	14	4	1
	Daily labour	7	1	10
	Cultivation	-	-	11
	Sweeper	3	1	-
	Retired	9	8	10
	Housewife	-	6	3
	Bus Conductor	-	-	1
Tobacco	Non-smoker	-	45	5
	Betel nut	3	-	4
	Occasional	5	-	-
	< 5 cigs/day	38	-	1
	> 10 cigs/day	21	-	17
	Bidi/cigarettes	21	-	9
	Chewing Tobacco	12	-	-
	Ex-Smoker	6	-	1
Period	< 30 yrs.	41	-	7
	≥ 30 yrs.	45	-	30
Tea habit	No tea habit	7	14	11
	< 4 cups/day	32	17	16
	≥ 4 cups/day	47	14	10
HPV*	HPV Positive	5	1	12
	HPV Negative	31	5	25

* Total evaluated was 42??

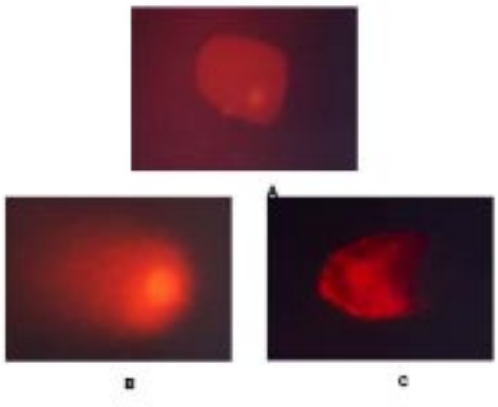

Figure 1.Comet Assay Results A. Normal buccal squamous epithelial cell with intact nucleus and prominent cell architecture. B and C, Fragmented DNA of squamous epithelial cells by Comet assay from oral cancer patient and normal tobacco user respectively

Studied population

General characteristics of studied samples are summarized in Table 1. The age ranges were 15-80 years in both tobacco user and non-tobacco user groups with an average (mean ± SD) of 43.9 ± 13.21 and 40.6 ± 16.21 yr, respectively. All the 12 cases (comet assay performed on 12 patient subjects) were above 40 years. Among tobacco users, non-tobacco users and patients 98%, 69% and 84% of the subjects were male respectively. All except four subjects in the three groups were Hindu. Samples were collected from subjects with different occupations. In case of healthy tobacco users 50% of the subjects were serviceman. Among non-tobacco (normal) users almost 50% were students, whereas cancer patients were mainly farmers and daily laborers.

According to smoking habit of collected subjects, 44% (maximum) of the healthy tobacco users smoked less than 5 cigarettes per day. In case of cancer patients, 46% (maximum) smoked more than 10 bidis per day. Tenure of tobacco habit was more than 30 years in 81% of patients. Among healthy tobacco users the number of subjects was almost equal in both groups of tobacco habit i.e. above and below 30 years of tobacco use. Among normal subjects, 55% of tobacco users and 31 % of non-tobacco users consumed more than 4 cups of tea per day. In case of oral cancer patients only 27 % take more than 4 cups of tea per day. About 32% of the oral cancer patients were HPV positive. This number was 14% in case of normal subjects.

Association of DNA damage with different factors

The average (mean ± SD) percentages of DNA damage of all three groups are summarized in Table 2. There was significant difference (p value 0.0002) in DNA damage between tobacco users and non-tobacco users. As shown in (Table 3A) DNA damage among nonsmoker subjects in the ≥ 40 yrs age group was significantly high. No

Table 2. Percentage of Cases with DNA Damage

Tobacco Users	Non-tobacco Users	Oral cancer patients
7.10 ± 3.65	4.56 ± 2.68	19.1 ± 9.14

Date are mean ± SD values

Table 3A. Effect of Different Factors on DNA Damage in Normal Subjects

Factors	Tobacco Users		Non-tobacco Users	
	DNA damage	p Value	DNA damage	p Value
Age				
< 40 Yrs	6.23 ± 4.15	0.11	3.35 ± 2.71	0.005
≥ 40 Yrs	7.61 ± 3.25		5.65 ± 2.18	
Period of tobacco use				
< 30 Yrs	6.44 ± 3.84	0.11	-	-
≥ 30 Yrs	7.70 ± 3.40		-	
Tea habit in <40 yrs age group				
<4cups/day	7.52 ± 4.89	0.09	3.28 ± 2.82	0.647
≥4cups/day	4.95 ± 2.87		3.99 ± 1.61	
Tea habit in ≥ 40 yrs age group				
<4cups/day	9.52 ± 3.34	0.0002	5.55 ± 2.97	0.811
≥4cups/day	6.20 ± 2.78		5.76 ± 1.06	

Table 3B. P-Value Results of Multivariate Analysis using SPSS Software

Independent variables	All subjects	Normal subjects only
Age	0.009 *	0.279 *
Period of tobacco use	0.025 *	0.001 *
Tea habit	0.001 **	0.001 **

Dependent variable: Percentage DNA damage.* Positively correlated ** Negatively correlated

significant association was noted in between DNA damage in < 30 yrs and ≥ 30 years of tenure of tobacco habit. It is interesting to note that high tea intake (≥ 4 cups per day) was significantly associated with lower DNA damage among tobacco users, in ≥ 40 yrs. age group.

In case of oral cancer patients comparatively high frequency of DNA damage (8 - 40%) was observed. No association was seen between tea intake and DNA damage among cancer patients. Multivariate analysis of samples (normal and patients) for DNA damage by comet assay revealed that the increase in DNA damage was significantly associated with increase in age and tenure of tobacco habit. Reduced DNA damage was found to be significantly associated with increase in tea intake (Table 3B). Multivariate analysis of only normal subjects showed significant association between increase in DNA damage and tenure of tobacco habit (Table 3B) and inverse correlation between tea habit and DNA damage.

Association of HPV detection with different factors among normal population

The population studied comprised of total 79 samples (including 37 oral cancer patients). It was evident that about 14% (6/42) of the normal subjects were HPV positive compared to the 32% (11/37) of the cancer patients (Table 1). No significant correlation was seen between HPV prevalence and DNA damage. Furthermore, HPV prevalence was not seen to be associated with age, tenure of tobacco habit or tea habit (data not shown).

Discussion

The anti-carcinogenic action of tea and its components has been repeatedly demonstrated by many laboratories, studies including our own. Most of the earlier studies were focused on green tea. However it was reported that the total amount of tea produced and consumed in the world, 78% is black tea which is primarily consumed in our country and many European countries (Katiyar and Mukhtar, 1966). Hence our group has concentrated on understanding the anti-carcinogenic action and cancer chemopreventive role of black tea.

The present pilot study was an attempt to explore the association between black tea consumption with tobacco habit and HPV prevalence- both identified as etiological factors in many human cancers. DNA damage in cells collected by oral brushing in normal subjects (131 cases) were determined by single cell gel electrophoresis (comet assay), which forms a useful biomarker, among men and women with or without tobacco habit. Selected samples were analyzed for presence of HPV 16 and 18. The possible confounding effect of black tea consumption on DNA damage and presence of HPV were assessed. A few oral cancer patients (37 cases) were also included in the study for comparison.

The frequency of DNA damage and HPV infection was comparatively high in oral cancer patients than the normal subjects. Significant association has been seen between the extent of DNA damage and age of the subjects as well as tenure of tobacco habit. It is interesting to note a significant inverse correlation between DNA damage and tea habit, indicating a protective role of black tea. No correlation was observed between HPV infection and tea habit. More samples should be analyzed in this regard. The significantly lower percent of DNA damage in subjects with tobacco habit consuming over 4 cups of tea per day than those recorded with lower tea intake; imply tea drinking may protect tobacco users from associated DNA damage that may eventually lead to development of cancer. This finding support in the report of Weisburger (1999) who had suggested the health protective role of tea drinking. Only further studies will reveal whether black tea can be recommended for prevention of human cancers.

Acknowledgements

We are indebted to the volunteers of the study for providing the mouth brushing samples. Grateful acknowledgement is made to National Tea Research Foundation (NTRF), for financial support. Thanks are also due all members of the Department of Oncogene Regulation for their help and encouragement.

References

Banerjee S, Manna S, Mhukherjee S, et al (2006). Black tea polyphenols restrict benzopyrene-induced mouse lung cancer progression through inhibition of Cox-2 and induction of Caspase-3 expression. *Asian Pac J Cancer Prev*, **7**, 661-6.

Dreosti IE, Wargovich MJ, Yang CS (1997). Inhibition of carcinogenesis by tea: the evidence from experimental studies. *Crit Rev Food Sci Nutr*, **37**, 761-70.

Heepchantree W, Paratasilpin T, Kangwanpong D (2006). A biological evaluation of DNA damage detected by comet assay in healthy populations residing in areas that differ in lung cancer incidence. *J Toxicol Environ Health*, **69**, 1071-82.

Katiyar SK, Mukhter H (1966). Tea in chemoprevention of cancer: Epidemiological and experimental studies. *Int J Oncol*, **8,** 221-38.

Limoli C L, Kaplan M L, Philips J W, et al (1997). Differential induction of chromosomal instability by DNA strand-breaking agents. *Cancer Res*, **57**, 4048-56.

Morgan W F, Day J P, Kaplan M L, et al (1996). Genomic instability induced by ionizing radiation. *Radiat Res*, **146**, 247-58.

Nicole K P, Geraldine M P, Maureen S N, et al (2006). Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer- A Review. *Cancer Epidemiol Biomarkers Prev*, **15**, 1061-77.

Osswald K, Mittas A, Glei M, et al (2003). New revival of old biomarker: characterization of buccal cells and determination of genetic damage in the isolated raction of viable leukocytes. *Mutat Res*, **544**, 321-9.

Rice-Evans C (1999). Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans. Proc. Soc. Exp. Biol. *Med*, **220**, 262-6.

Saha P, Das S (2002). Elimination of deleterious effects of free radicals in murine skin carcinogenesis by black tea infusion, theaflavin and epigallocatechin gallate. *Asian Pac J Cancer Prev*, **3**, 225-30.

Sambrook J, Fritsch E F, Maniatis T, in Molecular Cloning(1989): A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 .

Sengupta A, Ghosh S, Das S (2003). Tea can protect against aberrant crypt foci formation during azoxymethane induced rat colon carcinogenesis. *J Exp Clin Cancer Res*, **22**, 421-7.

Singh NP, McCoy MT, Tice RR,et al (1988). A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res*. **175**, 184-91.

Tice R R, Agrurell E, Anderson D, et al. (2000). Single cell gel/ comet assay: Guideline for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen*, **35**, 206-21.

Valvarde M,Ostrosky-Wegman P, Rojas E (1999). The application of single gel electrophoresis or comet assay to human biomonitoring studies. *Salud Publica Mex 41*, 5109-13.

Wiseman S A , Balentine D A , Frei B (1997). Antioxidants in tea. *Crit Rev Food Sci Nutr*, **37**, 705-18.

Weisburger JH (1999). Tea and health - a historical perspective. *Cancer Lett*. **114**, 315-7.