Available online at www.sciencerepository.org

Science Repository

Research Article

Black Tea Extract prevents 4-nitroquinoline 1-oxide induced oral tumorigenesis in mice by targeting Protein Tyrosine Kinases and associated biological response

***Madhumita Roy**[*] and Sutapa Mukherjee*

Department of Environmental Carcinogenesis & Toxicology, Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Kolkata-700 026, India

ARTICLE INFO

Article history:
Received: 15 January, 2019
Accepted: 8 February, 2019
Published: 13 March, 2019

Key words:
Chemoprevention
oral cancer
4NQO
black tea extract
tyrosine kinases

ABSTRACT

Oral squamous cell carcinoma of the tongue is the most common type of cancer, common site being buccal mucosa and gingiva. Despite advancement in treatment protocol, recurrence of the disease limits outcome of oral cancer; contributing factors for poor prognosis are activation, overexpression, dysregulation or mutation of many target proteins including protein tyrosine kinases (PTKs). Therefore, it is time to develop preventive measures against oral carcinogenesis, particularly by regulating tyrosine kinases. Natural compounds find a place in cancer prevention; hence, emphasis has been given to investigate the chemo-preventive efficacy of black tea extract in 4-nitroquinoline-1-oxide (4NQO)-induced tongue tumorigenesis in C57BL/6 mice. The histological diagnosis of squamous neoplasia was observed at different time intervals in mice treated with 4NQO and tea group, alone or in conjunction. Results indicate the preventive role of black tea in 4NQO-induced lesions. Expressions of different tyrosine kinases, their phosphorylated forms (EGFR, JAK-1, JAK-2, p38 MAPK) and their downstream signalling proteins (PI3K, Akt, Raf1 and Stat3) were determined by western blot analysis at different stages of oral carcinogenesis. 4NQO-induced endogenous ROS generation level and corresponding DNA damage by comet assay were followed in presence and absence of tea. An upward trend of ROS level as well as DNA damage was observed with progression of oral carcinogenesis. Administration of black tea extract inhibited tongue tumorigenesis by down-regulation of protein tyrosine kinases, reducing ROS level and counteracting DNA damage. Black tea therefore exhibited efficient chemo-preventive activity in oral carcinogenesis.

Introduction

Oral-cavity carcinoma is one of the most common human cancers in the world [1]. World Health Organization (WHO) report revealed that oral cancer ranks sixth among all malignancies worldwide [2]. Oral malignant neoplasm primarily arises on the lip and oral cavity. Histological studies revealed that 90% of cancers in the oro-dento regions are originated in the squamous cell; thus, named as Oral Squamous Cell Carcinoma (OSCC) [3]. Development of OSCC is a multistep complex process, involving genetic, epigenetic, and metabolic changes [4, 5]. Despite major advances in the treatment of OSCC, mortality rate is still very high. Predominant etiological factors in OSCC are tobacco and alcohol consumption, which are found to be responsible in 90% of cases [6]. In Indian population tobacco is used in various forms including betel quid, tobacco with lime, bidi, hookah and many more, which are major contributing factors in OSCC [7]. Apart from tobacco consumption, some other contributory factors of OSCC are infection with Human papilloma virus, poor oral hygiene, nutrient deficiency etc. [8-10]. Incidences of oral cancer is higher among people coming from poor socio-economic strata of the society due to prevalence of life style risk factors [11]. Regardless of advances in therapeutic approaches, survival rate of oral cancer is still very poor; risk factors, though known today, avoidance is not much successful [12]. Therefore, improved strategies for prevention and/or early detection of oral carcinogenesis are need of the hour. Better comprehensive remedy of oral carcinogenesis

[*]*Correspondence to: Department of Environmental Carcinogenesis & Toxicology Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Kolkata 700026, India; E-mail: mitacnci@yahoo.co.in*

http://dx.doi.org/10.31487/j.COR.2019.01.102

can aid in the invention of new biomarkers for early detection; targeting such markers with plant derived non-toxic compounds could be an efficient approach to prevent onset of the disease. Exposure of mice to a synthetic chemical carcinogen 4-nitroquinoline 1-oxide (4NQO) induces oral (tongue) carcinoma resembling the sequential advancement of different stages of human oral carcinogenesis including hyperplasia, dysplasia, severe dysplasia, papilloma and squamous cell carcinoma [13]. Furthermore, 4NQO-induced oral carcinogenesis model with distinct histopathological and molecular alterations associated with disease progression may provide an excellent opening for investigating distinct stages of OSCC. This might help in studying the efficacy of natural phytochemicals in combating against OSCC. Growth factor receptors or receptor tyrosine kinases (RTKs) are those having extracellular, transmembrane and cytoplasmic tyrosine kinase domains [14]. RTKs activated via ligand-induced dimerization leads to auto-phosphorylation at tyrosine residues and drives crucial signaling mechanism resulting into cell growth, differentiation, survival, metabolism and motility due to activation of major signaling pathways involving STAT3, Akt, MAPK (mitogen activated protein kinase). Overexpression, dysregulation or mutation of RTKs affect proteins of downstream pathways like Ras/MAP kinase and the Ras/PI3K/AKT, resulting in increased proliferation, invasion and metastasis [15, 16]. Several genetic and molecular mechanisms like gene amplification, RTK overexpression, chromosomal translocation lead to constitutively active RTKs which ultimately cause oncogenic signaling and neoplastic growth [17]. A very recent study has revealed that amplification of RTKs is an important prognostic factor for metastasis in oral squamous cell carcinoma [18]. Similar conclusion has been documented in another study where a correlation exists between aberrant expression of PTK7, a receptor tyrosine kinase and TNM (tumor node metastasis) staging and prognosis in oral tongue squamous cell carcinoma [19]. RTKs belonging to epidermal growth factor receptor (EGFR) family, comprising four distinct receptors are found to be overexpressed in majority of oral squamous cell carcinoma tumors [20]. These are associated with aggressive phenotype, poor prognosis and resistance to anticancer therapy. Overexpression of EGFR and changes in gene copy number has been observed during oral carcinogenesis [21]. Mitogen activated protein kinase (MAPK) pathway is the major downstream signaling route of EGFR pathway. c-Jun N-terminal kinases (JNKs) belonging to the family of MAPKs are thought to play both oncogenic and onco-suppressive role depending on the duration of activation [22]. A study showed that prolonged JNK activation imparts proapoptotic behavior while momentary or short-term activation enhances cell survival in oral cancer [23]. MEK/ERK, p38 and PI-3 kinase pathways are downstream cascade of EGFR signaling and are active participant in mediating epithelial mesenchymal transition (EMT) in OSCC cell lines [24]. Therefore, families of RTKs are considered as promising therapeutic earmarks for OSCC and targeting these proteins by non-toxic phytochemicals for inhibiting progression of the disease could be a more realistic translational alternative. Tea, particularly black tea is a popular beverage throughout the universe, polyphenolic content of which are known to be good scavengers of free radicals, rendering strong antioxidant effects [25, 26].

Considering the importance of black tea, the present study has been designed to investigate its role in prevention of oral squamous cell carcinoma by targeting receptor tyrosine kinases and their downstream proteins.

Materials & Methods

C57BL/6 male mice of 4-5 weeks old weighing approximately 19-20 g were procured from institutional breeding house. Ethical clearance from Institutional Animal Ethics Committee (IAEC, Reg # 1774/GO/RBi/S/14/CPCSEA) had been taken before commencement of the study. For this particular study, the Ethical Clearance number is IAEC-1.1/2015/AH/18 dated 9th March 2015. The animals were divided into several groups, with 6 animals in each. Animals were maintained in animal house in standard conditions (23±20^0 C, relative humidity 57±2 %, 12 h light/dark cycle), and were fed with standard feed and tap water *ad libitum*.

4NQO was dissolved in propylene glycol to a final concentration of 5 mg/ml. Tongue was stroked once with a paint brush dipped in 4NQO. The mice were restrained from drinking water 1 h before and after 4NQO application. This treatment was practiced thrice weekly. All mice were carefully inspected daily and weighed weekly. Mice were sacrificed at 4, 8, 12, 16, 20, 24, 32 and 40 weeks, following the guidelines of Animal Ethics Committee. Tissues from tongue were collected and divided into two parts, one part was used for histopathology and the second part was utilized for Western blot analysis and other studies.

Histologic examination

Tongue tissues were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Sections (5μm) from each specimen were cut and stained with H&E for histopathologic analysis. Briefly, tissue sections were deparaffinized in xylene followed by rehydration using descending grade of alcohols (100%, 90%, 70%). After washing in deionized water, slides were dipped in haematoxylin stain, followed by fast dip in acid-alcohol mixture. Slides were thereafter rinsed twice with deionized water. Slides dipped in eosin stain for 10 sec was further dipped in isopropanol and then in xylene followed by mounting with DPX.

Determination of level of intracellular ROS

Endogenous ROS was detected by using a small non-polar, cell membrane permeable oxidation sensitive probe (DCFH-DA), following the lab protocol (Sinha and Roy, 2010). Briefly, liver tissue homogenate was suspended in HEPES buffered saline (HBS, pH 7.4 containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 1mM $MgCl_2$, 10 mM glucose). 10 μM DCFH-DA was added to the cell suspension; volume was made upto 3 ml and incubated at 37^{0}C for 30 min in dark. DCFH-DA diffuses into the cells, deacetylated by intracellular esterases into a non-fluorescent compound DCFH, which is further oxidized by ROS to a fluorescent compound 2,7-dichlorofluorescein (DCF). ROS levels were measured using spectrofluorimeter (Varian Cary Eclipse). Excitation-emission wave lengths were set at 485 nm and 530 nm respectively.

Measurement of comet assay

4NQO-induced DNA damage (single strand breaks) was assessed by single cell gel electrophoresis (SCGE) or comet assay. Peripheral whole blood was collected from the tail vein of Swiss albino mice using heparinized needle, suspended in 0.6% (w/v) low melting agarose and was layered over a frosted microscopic slide previously coated with a layer of 0.75% normal melting agarose. Slides were kept in lysis buffer (pH 10.0) overnight at 4^0C. Next day, slides were transferred into a horizontal gel electrophoresis chamber containing alkaline electrophoresis buffer, pH 13.0 (NaOH 300 mM and Na_2EDTA 1 mM). Slides were presoaked in electrophoresis buffer for 20 min in order to unwind DNA. Electrophoresis was performed at 20 V, 300 mA for 20 minutes. Slides were washed thrice with neutralizing buffer (Tris buffer 0.4 M, pH 7.5), followed by staining with ethidium bromide with a final concentration of 40 µg/ml. Slides were visualized under a fluorescence microscope, comet tail length and Olive tail moment were noted employing a software.

Western Blot Analysis

Mice were sacrificed by cervical dislocation at different intervals of treatment and tongue tissue was isolated, collected and washed in ice cold saline. Tissue extracts were prepared by homogenizing tongue tissues in ice cold RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) for 60 sec with an interval of 15-20 sec. Homogenized samples were kept on ice for 30 mins followed by sonication for 5 sec to shear the DNA. Samples were then centrifuged at 10,000 rpm for 20 mins at 4^0C. Supernatant was collected in a fresh tube for western blot analysis. Immunoblotting was performed to study the expressions of receptor tyrosine kinases and their downstream effector proteins. Briefly, proteins were resolved in SDS-PAGE gels and blotted onto nitrocellulose membranes. The membranes were then blocked in BSA and probed with primary antibodies (anti-EGFR, anti-JAK-1, anti-p38, anti-MAPK, anti-PI3K, anti-Akt, anti-Raf1 and anti-Stat3 antibodies) overnight at 4^0C with constant shaking. The blots were washed 4 times with TBS containing Tween-20 (TBST). Alkaline phosphatase conjugated anti-mouse IgG (1:1000 dilutions in TBS) was added to the membrane at room temperature. Membranes were washed properly and BCIP/NBT was added to visualize the protein. Western Blot bands were scanned and quantified by using IMAGE MASTER Software (Amersham Pharmacia Biosciences, NJ, USA).

Results

Black tea extract reduced the rate of oral tumors in mice

To elucidate the role of black tea on the development of tongue tumors induced by 4NQO, mice were divided into 5 groups.

Group 1 was the control, receiving normal diet and water *adlibitum.* Group 2 was the vehicle control group where mice were painted with propylene glycol only. Mice belonging to Group 3 were treated with 4NQO only. Mice in Group 4 were painted with 4NQO and black tea was given by oral gavage. Group 5 mice were provided with black tea only. Ten mice were taken in each group, divided in two cages. 8 weeks after treatment, leucoplakia was developed in one mouse in Group 3, six mice showed similar symptoms after 12 weeks and the rest were doing fine during that time. When the treatment continued till 20 and 32 weeks, red patches (erythroplakia) became visible in few of the mice who had leukoplakia in early weeks. Development of multifocal lesions indicates that different sites of the tongue have been affected.

Of the 10 mice in group 3, 1 mouse died at 30^{th} week. Overall a roughened granular surface on the tongue mucosa with varying degrees of erythema and occasionally white plaque-like lesions were observed in 9 mice of Group 3, when treatment was continued till 32 weeks. Two such cases out of ten were observed in Group 4. Average body weight of each group was measured on a weekly basis. Group 5 receiving tea only did not show any change in body weight during the treatment period. Mice belonging to Group 3 showed a significant decrease in body weight, 12 weeks onwards compared to control and vehicle control group. However, water and diet consumption profiles were checked weekly and no significant change was observed among different groups during the study period.

Histopathological Examinations

Histopathological examination revealed that 8 weeks after 4NQO–treatment, mice developed lesions, ranging from hyperplasia to malignant squamous-cell carcinomas, in later weeks. Initial treatment up to 8 weeks with 4NQO indicated hyperplasia as evident from abnormal increase in the number of cells. Characteristic lesions with histopathologic alterations like enlargement of cells and nuclei with large and prominent nucleoli, increased nuclear to cytoplasm ratio and many others are indicators of dysplasia. Treatment with 4NQO up to 16 weeks was mostly found to be dysplasia, mild dysplastic changes were noted at 12 weeks (photograph not given). Severe dysplasia was observed at 24 weeks onwards. Continuation of treatment till 32 weeks showed development of papilloma and carcinoma *in situ*. Frequency of incidences of hyperplasia, dysplasia and papilloma were very less in mice group receiving both 4NQO and black tea. The histopathologic results clearly indicated that black tea strongly inhibited 4NQO-induced pre-neoplastic lesions and efficiently attenuated the carcinogenesis process. The results have been depicted in (Figure 1).

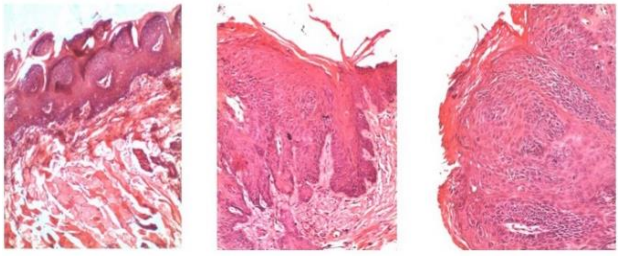

Figure 1: Histopathology of tongue tissues by staining with Haematoxylin Eosin stain at different stages of oral carcinogenesis. 8 weeks after 4NQO treatment, lesions were developed. Microscopic observations reveal hyperplastic changes as evident in (a). 24 weeks after (b), dysplastic changes were observed. Severe dysplasia was evident at this week of treatment. Continuation of treatment beyond 32 weeks showed features of carcinoma in situ (c). (400 original magnification, H & E stain)

ROS Determination

To assess whether any oxidative stress was developed during 4NQO induced oral carcinogenesis, level of reactive oxygen species (ROS) was analyzed in liver tissue homogenates stained with fluorescent dye DCFH-DA employing a spectrofluorometer. The result (Figure 2) showed that with increased treatment duration of 4NQO the level of ROS generation was also enhanced over the background level found in control cells. ROS level further increased till development of carcinoma in tongue, i.e. up to 32 weeks (data not shown). However, this increased ROS generation was effectively quenched by black tea extract as revealed in the mice group receiving both 4NQO and tea simultaneously. (Figure 2) shows the increased level of ROS by 4NQO and its quenching by black tea after 24 weeks of treatment. Control group, vehicle control group, as well as mice group receiving black tea only did not generate ROS as revealed from the spectrofluorimetric results.

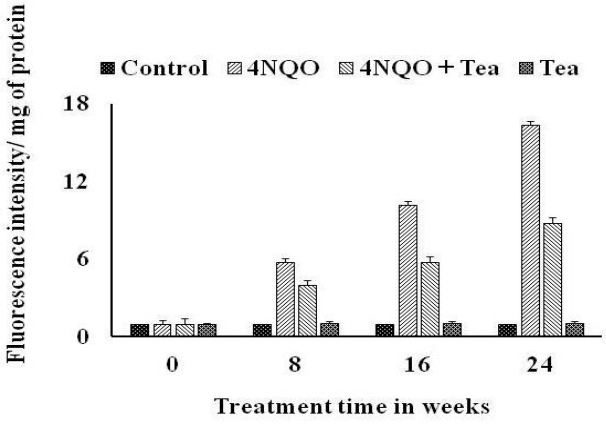

Figure 2: Generation of ROS in 4NQO treated mice at different time point.

During development of carcinogenesis, ROS is generated. Generated ROS has been efficiently quenched by administration of black tea. Black tea alone does not generate ROS at the experimental condition.

Assessment of DNA Damage by Comet Assay

Treatment of mice with 4NQO for different time intervals showed a gradual increase in tail moment with time. Results showed a significant increase in comet formation (as evident from comet tail moment) from untreated to 4NQO treated mice with gradual increase in treatment time range of 0 week, 8 weeks, 12 weeks, 20 weeks and 24 weeks. Severe damage with no head DNA (type 4 comet pattern) was observed when treatment continued for 32 weeks. The extent of DNA damage induced by increased treatment duration of 4NQO was considerably reduced in the mice group receiving tea along with 4NQO. Comet tail moments have been represented in Figure 3.

Effect of black tea extract on receptor tyrosine kinases in 4NQO-treated tongue tissue

To understand the underlying molecular mechanism for 4-NQO-induced oral carcinogenesis, expressions of some important protein tyrosine kinases (EGFR, JAK-1, JAK-2, p38 MAPK and their phosphorylated forms) were examined by western blotting using specific antibodies. It was analyzed whether black tea extracts could contribute its chemo preventive efficacy by regulating the expressions of these kinases. It was observed that expressions of these proteins were low in tongue tissues of untreated group, vehicle control group as well as black tea group. 4NQO treatment resulted in increase in the expressions of these protein tyrosine kinases. 4NQO plus black tea group showed diminished expressions of protein levels in tongue epithelia compared to only 4NQO treated group (Figure 4a). Expressions of certain downstream target proteins of protein tyrosine kinases (PI3K, Akt, Raf1 and Stat3) and their phosphorylated forms were also observed in all the groups. Western blot (Figure 4b) results clearly indicated that increased expressions of these proteins were efficiently lessened by black tea as revealed from the western blot bands obtained from 4NQO and black tea group. Collectively, these results suggested that black tea extract efficiently counteracted oral carcinogenesis process in mice induced by 4NQO, thereby contributing its chemo preventive efficacy.

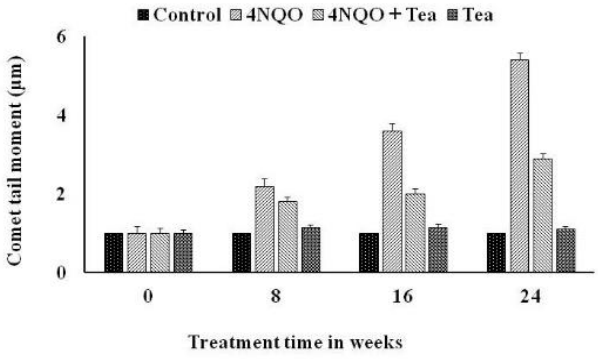

Figure 3: Time dependent increase in comet tail moment.

Treatment with 4NQO induces DNA damage, as revealed by formation of comet. Tail moment, as estimated using COMET software has been found to increase with time. Simultaneous intervention with black tea reduces comet tail moment, indicating its inhibitory effect on DNA damage.

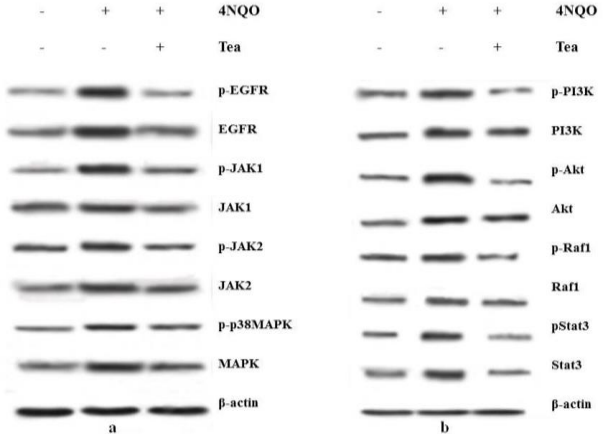

Figure 4: Expression of different protein tyrosine kinases in the development of oral carcinogenesis and its modulation by black tea.

Protein isolated from tongue tissue has been subjected to western blot analysis. (a) shows the modulatory effect black tea of protein tyrosine

kinases, indicating the inhibitory effect of tea. Western blot analysis reveal that downstream target proteins and their phosphorylated forms are downregulated by black tea. Samples from three animals in each group were analysed and representative data are given.

Table 1

	Control	Tea	4NQO	4NQO+Tea
Catalase	100	102±4.2	30±1.2	78±3.6
SOD	100	100.4±2.8	16±2.2	55±5.2
GPx	100	100±1.4	32±1.8	58±3.4
GST	100	101±3.6	34±1.6	46±2.8
GR	100	100±2.9	14±1.2	26±2.2
GSH	100	102±4.4	45±3.6	82±5.6

Discussions

Incidence and mortality rates of oral squamous cell carcinoma have been increasing rapidly in developing countries, especially among young males [27]. Understanding the mechanism of OSCC initiation and progression is essential and identification of molecular targets for development of preventive and therapeutic strategies is compelling [28]. Chronic exposure to a synthetic chemical carcinogen 4NQO in the tongue of the mice is an established method for oral tumorigenesis, bringing about carcinomatous changes in similar fashion as in humans [29]. Employing this mouse model, present study exhibited strong chemo preventive efficacy of black tea extract against 4NQO-induced oral tumorigenesis. The underlying chemo preventive mechanism is by regulating the expressions of protein tyrosine kinases and associated biological responses. Tea particularly black tea containing theaflavin, a polyphenol is a very popular aromatic beverage among Indians. Several studies indicated tea as a protective factor during developmental process of oral squamous cell carcinoma [30-32]. Histopathological studies revealed that black tea extract efficiently inhibited 4NQO-induced hyperplasia and pre-neoplastic lesions (dysplasia) and inhibited the growth of papilloma, indicating ability of tea in inhibiting tumor promotion and progression at various stages of oral cancer. Accumulation of excessive reactive oxygen species due to carcinogen exposure contributes to oral carcinogenesis by causing oxidative modifications of DNA, proteins and lipids [33]. Reports indicated that 4NQO induce oxidative stress either directly through generating ROS or indirectly through depleting GSH, thereby generating oxidative DNA damages [34]. In agreement with previous reports, present study clearly indicated that level of ROS increases during developmental process of 4NQO mediated oral carcinogenesis. Black tea efficiently counteracted 4NQO mediated ROS generation by quenching the level of ROS, suggesting its antioxidant activity. It is known nowadays that function of ROS is oncogenic as it imparts DNA damage, genetic instability, epigenetic alteration and metabolic reprogramming [35]. DNA damage was measured by comet assay or single cell gel electrophoresis assay. Reduction of comet tail moment by treatment with black tea extract ascertains antagonistic role of tea on 4NQO induced DNA damage. Effect of black tea extract on protein tyrosine kinases during the developmental stages of oral carcinogenesis was examined. Results demonstrated that phosphorylated forms of several PTKs like EGFR, JAK-1, JAK-2 and p38MAPK were efficiently downregulated in presence of black tea. Consequently, diminished expressions of the phosphorylated forms of their downstream target proteins like PI3K, Akt, Raf1 and Stat3 were observed. Decrease is phosphorylated forms by black tea are more pronounced than the total proteins. Some of the constituents of black tea are catechins, flavanol glycosides, theaflavins and thearubigins, of which theaflavins are considered to be most effective against carcinogenesis. Laboratory research indicated that black tea extract by reversing epithelial to mesenchymal transition could inhibit metastasis in oral cancer cells [36]. Theaflavins are reported to induce apoptosis in gingival carcinoma cells, but not in normal fibroblasts [37]. Previous study indicated inhibition of activation of EGFR by theaflavin in chronic airway inflammation [38]. Blockade of NF-κB and MAPK signaling pathways in bone marrow-derived macrophages by theaflavin has been documented in one study [39]. In another study, DNA damage and ROS level was assessed in exfoliated buccal cells collected from healthy individuals of all age groups and showed that DNA damage and ROS level were much lower among subjects having regular habit of tea intake [40]. Our laboratory study showed antigenotoxic, anticlastogenic, antioxidant potential of tea in *in vitro* and *in vivo* systems [25, 41-43].

Conclusion

Black tea suppresses oral carcinogenesis and proliferation by inhibiting the expressions of protein tyrosine kinases and their targets. These findings might support consumption of black tea or its extracts for prevention of carcinogenesis process.

Acknowledgement

Authors are indebted to Dept. of Biotechnology, Govt. of India for financial support.

Authors are also indebted to Director, Chittaranjan National Cancer Institute for providing infrastructural facilities.

References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. *CA Cancer J Clin* 63: 11-30. [Crossref]
2. Selvamani M, Yamunadevi A, Basandi PS, Madhushankari GS (2015) Prevalence of oral squamous cell carcinoma of tongue in and around Davangere, Karnataka, India: A retrospective study over 13 years. *J Pharm Bioallied Sci* 7(Suppl 2): S491-S494. [Crossref]
3. Lingen MW, Kalmar JR, Karrison T, Speight PM (2008) Critical evaluation of diagnostic aids for the detection of oral cancer. *Oral Oncol* 44: 10-22. [Crossref]
4. Fukuda M, Ohmori Y, Sakashita H (2012) The Role of Tumor Microenvironment in Oral Cancer. In: Biswas S, editors. *Tumor*

Microenvironment and Myelomonocytic Cells.

5. Rivera C (2015) Essentials of oral cancer. *Int J Clin Exp Patho* 8: 11884-11894. [Crossref]
6. Dissanayaka WL, Pitiyage G, Kumarasiri PV, Liyanage RL, Dias KD, et al. (2012) Clinical and histopathologic parameters in survival of oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol* 113: 518-525. [Crossref]
7. Singh MP, Kumar V, Agarwal A, Kumar R, Bhatt MLB, et al. (2016) Clinico-epidemiological study of oral squamous cell carcinoma: A tertiary care centre study in North India. *J Oral Biol Craniofac Res* 6: 31-34. [Crossref]
8. Joseph AW, D'Souza G (2012) Epidemiology of human papillomavirus-related head and neck cancer. *Otolaryngol Clin North Am* 45: 739-764. [Crossref]
9. Oji C, Chukwuneke F (2012) poor oral hygiene may be the sole cause of oral cancer. *J Maxillofac Oral Surg* 11: 379-383. [Crossref]
10. Scardina GA, Messina P (2012) Good oral health and diet. *J Biomed Biotechnol*. [Crossref]
11. Warnakulasuriya S (2009) Significant oral cancer risk associated with low socioeconomic status. *Evid Based Dent* 10: 4-5. [Crossref]
12. El-Bayoumy K, Chen KM, Zhang SM, Sun YW, Amin S, et al. (2017) Carcinogenesis of the Oral Cavity: Environmental Causes and Potential Prevention by Black Raspberry. *Chem Res Toxicol* 30: 126-144. [Crossref]
13. Shrotriya S, Tyagi A, Deep G, Orlicky DJ, Wisell J, et al. (2015) Grape seed extract and resveratrol prevent 4-nitroquinoline 1-oxide induced oral tumorigenesis in mice by modulating AMPK activation and associated biological responses. *Mol Carcinog* 54: 291-300. [Crossref]
14. Takeuchi K, Ito F (2011) Receptor tyrosine kinases and targeted cancer therapeutics. *Biol Pharm Bull* 34: 1774-1780. [Crossref]
15. Regad T (2015) Targeting RTK Signaling Pathways in Cancer. *Cancers (Basel)* 7: 1758-1784. [Crossref]
16. Kim M, Baek M, Kim DJ (2017) Protein Tyrosine signaling and its potential therapeutic implications in carcinogenesis. *Curr Pharm Des* 23: 4226-4246. [Crossref]
17. Elferink LA, Resto VA (2011) Receptor-Tyrosine-Kinase-Targeted Therapies for Head and Neck Cancer. *J Signal Transduct*. [Crossref]
18. Oikawa Y, Morita KI, Kayamori K, Tanimoto K, Sakamoto K, et al. (2017) Receptor tyrosine kinase amplification is predictive of distant metastasis in patients with oral squamous cell carcinoma. *Cancer Sci* 108: 256-266. [Crossref]
19. Dong Y, Chen X, Li H, Ni Y, Han W, et al. (2017) PTK7 is a molecular marker for metastasis, TNM stage, and prognosis in oral tongue squamous cell carcinoma. *Pol J Pathol* 68: 49-54. [Crossref]
20. Ribeiro FA, Noguti J, Oshima CT, Ribeiro DA (2014) Effective targeting of the epidermal growth factor receptor (EGFR) for treating oral cancer: a promising approach. *Anticancer Res* 34: 1547-1552. [Crossref]
21. Bates T, Kennedy M, Diajil A, Goodson M, Thomson P, et al. (2016). Changes in Epidermal Growth Factor Receptor Gene Copy Number during Oral Carcinogenesis. *Cancer Epidemiol Biomarkers Prev* 25: 927-935. [Crossref]
22. Gkouveris I, Nikitakis NG (2017) Role of JNK signaling in oral cancer: A mini review. *Tumour Biol*. [Crossref]
23. Ventura JJ, Hübner A, Zhang C, Flavell RA, Shokat KM, et al. (2006) Chemical genetic analysis of the time course of signal transduction by JNK. *Mol Cell* 21: 701-710. [Crossref]
24. Brusevold IJ, Aasrum M, Bryne M, Christoffersen T (2012). Migration induced by epidermal and hepatocyte growth factors in oral squamous carcinoma cells in vitro: role of MEK/ERK, p38 and PI-3 kinase/Akt. *J Oral Pathol Med* 41: 547-558. [Crossref]
25. Sinha D, Roy M, Siddiqi M, Bhattacharya R K (2005) Modulation of arsenic induced DNA damage by tea as measured by comet assay (single cell gel electrophoresis). *Int J Cancer Prev* 2: 143-154.
26. Sinha D, Dey S, Siddiqi M, Bhattacharya RK, Roy M (2007) In vitro mitigation of arsenic toxicity by tea polyphenols in human lymphocytes: Antioxidant function and repair inducing activity. *Journal of Env Pathol Toxicol Oncol* 26: 207-220. [Crossref]
27. Majchrzak E, Szybiak B, Wegner A, Pienkowski P, Pazdrowski J, et al. (2014) Oral cavity and oropharyngeal squamous cell carcinoma in young adults: a review of the literature. *Radiol Oncol* 48: 1-10. [Crossref]
28. Ishida K, Tomita H, Nakashima T, Hirata A, Tanaka T, et al. (2017) Current mouse models of oral squamous cell carcinoma: Genetic and chemically induced models. *Oral Oncology* 73: 16-20. [Crossref]
29. Vered M, Yarom N, Dayan D (2005) 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. *Oral Oncol* 41: 337-339. [Crossref]
30. Sur S, Pal D, Roy R, Barua A, Roy A, et al. (2016) Tea polyphenols EGCG and TF restrict tongue and liver carcinogenesis simultaneously induced by N-nitrosodiethylamine in mice. *Toxicol Appl Pharmacol* 300: 34-46. [Crossref]
31. Ramirez-Mares MV, Kobayashi H, de Mejia EG (2016) Inhibitory effect of Camellia sinensis, Ilex paraguariensis and Ardisia compressa tea extracts on the proliferation of human head and neck squamous carcinoma cells. *Toxicol Rep* 1: 269-278. [Crossref]
32. Katiyar SK (2016) Emerging Phytochemicals for the Prevention andTreatment of Head and Neck Cancer. *Molecules*. [Crossref]
33. Hanafi R, Anestopoulos I, Voulgaridou GP, Franco R, Georgakilas AG, et al. (2012) Oxidative stress based-biomarkers in oral carcinogenesis: How far have we gone? *Curr Mol Med* 12: 698-703. [Crossref]
34. Arima Y, Nishigori C, Takeuchi T, Oka S, Morimoto K, et al. (2006) 4-Nitroquinoline 1-oxide forms 8-hydroxydeoxyguanosine in human fibroblasts through reactive oxygen species. *Toxicol Sci* 91: 382-392. [Crossref]
35. Wang L, Wise JT, Zhang Z, Shi X (2016) Progress and prospects of reactive oxygen species in metal carcinogenesis. *Curr Pharmacol Rep* 2: 178-186. [Crossref]
36. Chang YC, Chen PN, Chu SC, Lin CY, Kuo WH (2012) Black tea polyphenols reverse epithelial-to-mesenchymal transition and suppress cancer invasion and proteases in human oral cancer cells. *J Agric Food Chem* 60: 8395-8403. [Crossref]
37. Babich H, Gottesman RT, Liebling EJ, Schuck AG (2008) Theaflavin-3-gallate and theaflavin-3′-gallate, polyphenols in black tea with prooxidant properties. *Basic Clin Pharmacol Toxicol* 103: 66-74. [Crossref]

38. Wu H, Li Q, Zhou X, Kolosov VP, Perelman JM (2012) Theaflavins extracted from black tea inhibit airway mucous hypersecretion induced by cigarette smoke in rats. *Inflammation* 35: 271-279. [Crossref]
39. Kim S, Joo YE (2011) Theaflavin inhibits LPS-induced IL-6, MCP-1, and ICAM-1 expression in bone marrow-derived macrophages through the blockade of NF-κB and MAPK signaling pathways. *Chonnam Med J* 47: 104-110. [Crossref]
40. Pal D, Sur S, Mandal S, Das S, Panda CK (2012) Regular black tea habit could reduce tobacco associated ROS generation and DNA damage in oral mucosa of normal population. *Food Chem Toxicol* 50: 2996-3003. [Crossref]
41. Sinha D, Bhattacharya R K, Siddiqi M, Roy M (2005) Amelioration of sodium arsenite induced clastogenicity by tea extracts in Chinese hamster V79 cells. *J Environ Pathol Toxicol Oncol* 24: 77-89. [Crossref]
42. Sinha D, Dey S, Siddiqi M, Bhattacharya RK, Roy M (2007) Mitigation of arsenic toxicity by tea in human lymphocytes: Antioxidant functions and repair inducing activity. *J Environmental Pathol Toxicol Oncol* 26: 207-220.
43. Sinha D, Roy S, Roy M (2010) Antioxidant potential of tea reduces arsenite induced oxidative stress in Swiss albino mice. *Food Chem Toxicol* 48: 1032-1039. [Crossref]