Original Article

DOI: 10.7860/JCDR/2016/19656.8637

Dentistry Section

# Clinical Utility of Serum Homocysteine and Folate as Tumor Markers in Oral Squamous Cell Carcinoma - A Cross-Sectional Study

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## ABSTRACT

**Introduction:** Oral Squamous Cell Carcinoma (OSCC) is a common malignancy involving head and neck. Identifying the markers of molecular levels or biochemical markers involving the various metabolic reactions associated with the initiation and biological behavior of individual tumors are very important in diagnosis and prognosis.

**Aim:** To measure and compare the levels of serum Homocysteine (Hcy) and serum folate in OSCC patients, smoking group and healthy subjects and also to assess the clinical utility of serum Hcy as a potential tumor marker in OSCC.

**Materials and Methods:** The study group comprised of 60 subjects, of whom 30 were classified as OSCC cases (GROUP I) and 15 were classified as smokers without OSCC (GROUP II). The control group included 15 healthy individuals without smoking habit (Group III). Hcy was measured with High Performance Liquid Chromatography (HPLC). Folate estimation was done by Chemiluminiscence Immuno Assay (CLIA). Comparison of mean Hcy and folate values among the groups was done using ANOVA with Post-Hoc Games Howell test. Gender was compared using Chi-square test. Comparison of mean age was using ANOVA with Post-Hoc Tukey's test.

**Results:** The mean serum folate level in OSCC patients was 5.34ng/mL, 7.68ng/mL in smoking group and 10.99ng/mL in control group. There was a significant difference in the mean serum folate levels among the three study groups ($p<0.001$). The mean serum Hcy in OSCC patients was 23.58µmol/L, 17.46µmol/L, in smoking group and 10.76µmol/l in controls. There was a significant difference in the mean serum Hcy levels among the three study groups ($p<0.001$).

**Conclusion:** The present study found an interesting association with serum Hcy and folate levels in OSCC which could be useful as a biochemical "Tumor Marker" and thereby providing insights into the onset and progression of the disease.

**Keywords:** Biochemical markers, Chemiluminiscence immuno assay, High performance liquid chromatography, Molecular markers, Smoking

## INTRODUCTION

Head and neck malignancy, mainly OSCC is a common human malignancy that affects about 5lacs patients worldwide, annually. Despite the improvements in surgical techniques and chemotherapy, radiotherapies, the overall 5-year survival rate for patients with Head and Neck cancers is among the lowest of the major tumor types [1]. In an attempt to improve the treatment of Oral Squamous Cell Carcinomas (OSCC), molecular abnormalities have been widely studied. Gene alterations were assessed as risk factors, although metabolic disorders, particularly biochemical alterations, have not been extensively evaluated, despite occurring frequently in cancer. Most of the biochemical markers associated with malignancies of the Head and Neck especially Oral Squamous Cell Carcinomas (OSCC) are generated from the methionine cycle as methionine is one of the major amino acid in humans and animals involving the production of various basic nutrients, which are needed for the optimal functioning of the various major and minor systems of the body like cardiovascular, skeletal and nervous systems [2]. Homocysteine (Hcy) is an intermediate cross linking metabolite of the methionine cycle which affects all methyl and sulphur group related metabolisms occurring in the body either directly or indirectly [3]. According to the author Scott JM et al., carbon transfer involving the re-methylation of homocysteine to methionine is done by folate, which is a precursor of S-adenosyl methionine. DNA methylation is an important factor in gene expression, chromosomal modifications and aberrations [3]. It is well known that the majority of patients with OSCC are smokers. Hence, the aim of the present study was to measure the levels of serum Hcy and serum folate in OSCC patients, smoking group without OSCC and healthy subjects without any habit of tobacco use and to compare the levels of serum Hcy and folate in OSCC patients with smoking group and healthy subjects and finally, to assess the clinical utility of serum Hcy as a potential tumor marker for OSCC.

## MATERIALS AND METHODS

This cross-sectional study was carried out in the Department of Oral and Maxillofacial Pathology, MNR Dental College and Hospital and Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India, for a period of two years. The study group comprised of 60 subjects, of whom 30 were classified as OSCC (GROUP I) and 15 were classified as smokers (GROUP II). The control group included 15 healthy individuals (Group III). Informed written consent from the patients and subjects was taken. Approval by the institutional ethical committee board was also taken before pursuing the research project.

### Diagnostic Criteria Used for Selection of Cases

#### Inclusion criteria

1. The age of the patients included in the study was between 25 to 70 years. The patients in the study group were diagnosed histopathologically during their visit. All Stage I, well differentiated to poorly differentiated OSCC were included in this study.

2. A total of 30 patients diagnosed with OSCC, having a habit of tobacco chewing (more than 10 times a day) and smoking of more

than 20 cigarettes per day and without any history of systemic diseases and/or medications were selected as the study group. Total number of males in this group was 25 and females were 5. Male: Female ratio was 5:1.

3. Of the total 15 healthy individuals, with smoking habit of more than 20 cigarettes per day, chewing tobacco (more than 10 times a day), without any complaint or history of systemic diseases and/or medication. Total males in this group were 10, females 5. Male: Female ratio was 2:1.

4. As for the control group, 15 healthy individuals with no habit of smoking and tobacco chewing and without any complaint or history of systemic diseases and/or medication were selected. Total males in this group were 10 and females were 5. Male: Female ratio is 2:1.

**Exclusion criteria:** Subjects suffering from diseases which are known to alter the serum Hcy profile such as diabetes mellitus, alcoholism, alzheimer's disease, osteoporosis, parkinson's disease, coronary artery disease, peripheral vascular disease, deep vein thrombosis etc., were excluded from the study. No patient in this study was being treated with drugs capable of altering Hcy levels.

Venous blood samples were collected in EDTA containing test tube with aseptic precautions using spirit and cotton. Only 2ml of blood was taken by using disposable syringe and without any delay the blood was transferred to clot activator tube (red top tube/vacutainer). After 2 hours of collection, sample was centrifuged at 2500 rotations per minute (rpm) for 15 min. Serum was separated and collected in polythene tube provided with tight cork. Straw colored to yellow colored serum was used for the analysis of total circulating Hcy and folic acid. Serum with pinkish red or red colour is discarded as a sign of hemolysis. Serum samples were collected from each patient before any treatment and from each healthy control subject. The collected samples were transferred to the Center for DNA Fingerprinting and Diagnosis (CDFD), HPLC analysis of Hcy. All the samples were separated and the sera were frozen and stored at –20°c until testing.

Separation was performed by reverse phase HPLC using C18 ODS Column. The HPLC-system consisted of a 1200 series Agilent with fluorescence detector. We used dl-homocysteine from sigma (Fluka), tri-n-buthylphosphine, dimethylformamide, and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (SBDF), acetonitrile from Qualigens and Ranbaxy.

A total of 250µl of serum were treated with 10% tri-n-buthylphosphine in dimethylformamide at 4°C in order to reduce thiols and to decouple them from proteins. The solution was then mixed with 250µl of 10% trichloroacetic acid solution under vigorous vortexing, followed by centrifugation at 3000rpm for 15 minutes, Then added 250µl of 0.125M borate buffer (pH = 8.0), 20µl of 1.55M NaOH, and 20µl of SBDF to 100µl of the clear supernatant. The mixture was then incubated for 60 minutes at 60°C in the dark to accomplish complete derivation of homocysteine and other thiols. After cooling, 150µl of the samples is transferred into microvials.

**Run conditions:** Injection volume 50µl, mobile phase: 0.1M of $KH_2PO_4$, pH 2.1, and flow-rate is 1.0 ml/min. Hcy concentration was then measured fluorimetrically at an excitation wavelength of 385nm and an emission wavelength of 515nm.

Folate estimation was done by Chemiluminiscence Immuno Assay (CLIA). The folate assay employs a competitive test principle using natural Folate Binding Protein (FBP) specific for folate. Folate in the sample competes with the added folate (labeled with biotin) for the binding sites on FBP (labelled with ruthenium complex).

Individual Hcy profiling in serum by High Performance Liquid Chromatography (HPLC) and folate profiling in serum by CLIA were carried out for 60 patients (30 OSCC group-Group I, 15 smoking group-Group II and 15 healthy controls-Group III).

## STATISTICAL ANALYSIS

All the statistical analysis was done using Statistical Product and Service Solutions (SPSS) version 14. A p-value of <0.05 was considered statistically significant. Comparison of mean Hcy and folate values among the groups was done using ANOVA (Analysis of Variance) with Post-Hoc Games Howell test. Gender was compared using Chi-square test. Comparison of mean age was using ANOVA with Post-Hoc Tukey's test.

## RESULTS

The mean folate level in OSCC patients was 5.34ng/ml. The mean serum folate level was 7.68ng/mL in smoking group, 10.99ng/ml in control group. There was a significant difference in the mean serum folate levels among the three study groups (p<0.001). The mean serum Hcy in OSCC patients was 23.58µmol/l. The mean serum Hcy level was 17.46µmol/l in smoking group and 10.76µmol/l in controls. There was a significant difference in the mean serum Hcy levels among the three study groups (p<0.001) [Table/Fig-1,2].

The mean Hcy was significantly higher in OSCC patients (Group I) than controls (Group III). Statistically significant difference was seen between OSCC (Group1) and controls (Group III), (p<0.001) [Table/Fig-2].

Inter-group comparison by Post-Hoc Games-Howell test for serum folate and Hcy is presented in [Table/Fig-3].

| GROUP I | | GROUP II | | GROUP III | |
|---|---|---|---|---|---|
| Hcy (µmol/L) | FOLATE (ng/mL) | Hcy (µmol/L) | FOLATE (ng/mL) | Hcy (µmol/L) | FOLATE (ng/mL) |
| 32.4 | 3.8 | 20.7 | 5.8 | 12.4 | 11 |
| 28 | 4.5 | 7.6 | 9.4 | 21 | 5.9 |
| 29.5 | 4.1 | 28 | 4.2 | 9 | 12.0 |
| 13.8 | 6.6 | 26.4 | 5.5 | 23 | 5.2 |
| 10.4 | 8.5 | 4 | 13.1 | 8 | 11.9 |
| 8.76 | 9.2 | 11.6 | 9.4 | 5 | 13.9 |
| 19.9 | 5.9 | 19.3 | 5.2 | 10 | 12.1 |
| 18.4 | 5.8 | 17.6 | 4.1 | 13.6 | 11.4 |
| 26.7 | 5.0 | 8.8 | 11.2 | 11 | 11.3 |
| 28.5 | 4.3 | 8.9 | 11 | 1.8 | 14.2 |
| 14 | 6.8 | 12.8 | 9.0 | 15 | 8.9 |
| 39.1 | 2.4 | 13.4 | 9.2 | 8 | 11.0 |
| 26.1 | 4.9 | 20.8 | 5.6 | 6.3 | 13 |
| 25.4 | 4.8 | 12 | 10.4 | 10.5 | 9.6 |
| 18 | 5.4 | 50 | 2.1 | 6.8 | 13.4 |
| 27.6 | 4.6 | | | | |
| 12 | 7.4 | | | | |
| 30.8 | 3.4 | | | | |
| 28.8 | 4.2 | | | | |
| 45.3 | 2.1 | | | | |
| 39.6 | 2.5 | | | | |
| 25.2 | 5.1 | | | | |
| 11.5 | 8.2 | | | | |
| 10.8 | 8.4 | | | | |
| 6.24 | 10.1 | | | | |
| 27.6 | 4.4 | | | | |
| 18.6 | 5.2 | | | | |
| 18 | 5.1 | | | | |
| 41.6 | 2.3 | | | | |
| 24.82 | 5.2 | | | | |

**[Table/Fig-1]:** Showing the Hcy and folate values and comparison among the groups.

| | Group | | | | | | p-value |
|---|---|---|---|---|---|---|---|
| | OSCC | | Smokers | | Controls | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| Hcy | 23.58 | 10.24 | 17.46 | 11.35 | 10.76 | 5.68 | <0.001 |
| Folate | 5.34 | 2.06 | 7.68 | 3.22 | 10.99 | 2.64 | <0.001 |

**[Table/Fig-2]:** Comparison of three groups statistically.
*Analysis method - ANOVA with Post-Hoc Tukey's test

| | Group | Mean Difference | p-value |
|---|---|---|---|
| Hcy | OSCC vs Smokers | 6.12 | 0.203 |
| | Smokers vs Controls | -6.70 | 0.127 |
| | OSCC vs Controls | -12.82 | <0.001 |
| Folate | OSCC vs Smokers | -2.34 | 0.047 |
| | Smokers vs Controls | 3.31 | 0.013 |
| | OSCC vs Controls | 5.65 | <0.001 |

**[Table/Fig-3]:** Inter-group comparison.
*Analysis method-Post-Hoc Games Howell test

| | Group | | | | | | p-value |
|---|---|---|---|---|---|---|---|
| | OSCC | | Smokers | | Controls | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| Age | 49.13 | 12.97 | 48.47 | 10.34 | 37.47 | 10.25 | 0.007 Smokers, OSCC> Controls |

**[Table/Fig-4]:** Comparison of mean age between three groups.
*Analysis method- ANOVA with Post-Hoc Tukey's test

Mean age comparison was done by ANOVA with Post-Hoc Tukey'test: The mean age was 37.47 years in controls 48.47 years in smoking group. The mean age level in OSCC patients was 49.13. There was a significant difference in the mean age levels among the three study groups (p<0.007) [Table/Fig-4].

## DISCUSSION

Carcinoma of the Head and Neck region is placed at 5th position among the cancers most common histological subtype is squamous cell carcinoma [4,5].

In the Head and Neck region of squamous cell carcinoma, predominantly involves the oral cavity, pharynx and larynx [6-8]. Squamous cell carcinoma of the oral cavity is the commonest. Early diagnosis plays a crucial role in the treatment and prognosis of the disease. Despite advances in all area of diagnosis and treatment, the prognosis of patients with OSCC has remained unchanged during the last two decades and efforts toward early detection and prevention have not been entirely successful [9-11].

According to review of literature, various authors reported that molecular abnormalities in oral carcinogenesis are mainly due to genes involved in the control and regulation of the cell cycle, which results in a growth advantage in the altered cell population. Carcinogenesis is also associated with metabolic derangements, though non-specific might promote or derive from tumoral progression [11]. Thus, analysis of metabolic disorders forms a valuable approach in understanding the biochemistry of tumors and also provides a pathway to identify new targets for therapy.

The methionine cycle is the major metabolic process associated with carcinogenesis associated with OSCC and diseases associated with smoking and smokeless tobacco. Hcy is an important intermediate product of methionine metabolism and usually metabolized by two pathways: the re-methylation pathway, which regenerates methionine, and the trans-sulphuration pathway, which initially converts Hcy into cysteine and finally into taurine. In essence, the intermediate metabolite Hcy is located at a critical metabolic crossroad and, therefore, both directly and indirectly, affects all methyl and sulphur group metabolism occurring in the body [3].

Hcy is an amino acid with a free sulfhydryl group which does not occur in the natural human diet but is an essential part of the metabolism of methionine and it is produced as a result of methylation reactions. The other metabolic functions of the Hcy include: recycling of tissue folates and being a precursor for cystathionine, cysteine and further metabolites, as a part of choline metabolism. Hyperhomocysteinemia is increased concentration of Hcy in blood and it can be due to various internal and external factors [12]. The most important internal factors are the genetic polymorphisms coding for the important enzymes involved in Hcy metabolism such as C677T transition which codes for Methylene Tetrahydrofolate Reductase (MTHFR), ultimately resulting in a heat sensitive variant of the enzyme. The most significant exogenous factors are connected with the composition of the diet, e.g., dietary deficiency of folate. Hyperhomocysteinemia is considered to be an independent risk factor in cardiovascular diseases and high Hcy concentration has also been shown to be present in patients with cerebrovascular diseases, like brain stroke or dementia [12].

It has recently been found that hyperhomocysteinemia is linked with cancer, which leads to the suggestion that Hcy can also be regarded as a tumour marker. Some of the co-factors such as pyridoxine and/or cobalamin are used by the enzymes of Hcy metabolism; this clearly explains that vitamins level is often an important factor to be considered in cancer/Hcy research and treatment even though vitamins cannot be directly usually treated as strict determinants of Hcy. Since, Hcy levels are elevated in several diseases; a lot of research has been directed at methods of its measurement. Hcy exists in blood in three forms — protein-bound (about 80%), oxidised (homocysteine or homocysteine–cysteine, about 18%) or free (about 1%) [12].

According to the authors, particularly according to the Scott JM et al., [3], folate plays an essential role in transferring carbon atom involving the re-methylation of Hcy to methionine, which is a precursor of S-adenosylmethionine, the primary methyl donor group for most biological methylations. DNA methylation is an important factor in gene expression, chromosomal modifications and aberrations. Currently, the most popular hypothesis is the promoter hypermethylation of key tumor suppressor genes [3]. DNA methylation is a regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. Alterations in DNA methylation are common in a variety of tumors as well as in development. Of all epigenetic modifications, hypermethylation, which represses transcription of the promoter regions of tumor suppressor genes leading to gene silencing, has been most extensively studied. However, global hypomethylation has also been recognized as a cause of oncogenesis. New information concerning the mechanism of methylation and its control has led to the discovery of many regulatory proteins and enzymes. The contribution of dietary folate and MTHFR polymorphisms to methylation patterns in normal and cancer tissues is under intense investigation [3].

Cigarette smoke which contains many poisonous substances can lead to alterations in the metabolic pathway of Hcy and folic acid. A modification in the methionine cycle eventually results in an increase in Hcy levels in the blood due to the various poisonous products generated during smoking [13]. Sobczak AJ et al., reported that cigarette smoking influences the levels of Hcy, folate in the blood because of the alterations that take place in the methionine cycle and the fact that smokers generally consume less fruits and vegetables, the main source of folate [14].

Repeated respiratory epithelium insults due to exposure to cigarette smoke will result in folate, pyridoxine and cobalamine derived coenzymes, making the cells less immune to the carcinogens present in tobacco smoke [15]. Vitamin B-12 and folate play a role in converting Hcy to methionine in Hcy metabolism. Vitamin

B-12 acts as a co-enzyme and folate acts as a co-factor in Hcy metabolism. The basic biochemical function of vitamin B-12 is to support the synthesis of DNA for cell division by regenerating folate levels following folate consumption [15].

Cyanides, one of the poisonous substances in cigarette adversely affect cobalamine nutritional status. The cyanides readily combine with hydroxy cobalamin; a form of cobalamine to form cyanocobalamin, which lacks co-enzyme function [15]. Hydrogen sulphide which also exists in gaseous phase of cigarette smoke has great affinity for the cobalt atom of cobalamine, thus resulting in inactive sulphur containing derivative (sulf-cobalmin) [15].

Organic nitrites present in the cigarette smoke, inactivate methylcobalamin by cleaving the methyl-cobalamin bond, resulting in the formation of nitro-cobalamin [15]. Nitrous oxide present in cigarette smoke is known to inactive methylcobalamin through oxidation of the cobalt atom. Nitrous oxide irreversibly inactivates the only methyl cobalamin requiring enzyme, methionine synthase [15]. Methyl cobalamin is essential for the removal of the methyl group from 5-methyl tetra hydrofolate in the homocysteine –methionine trans-methylation reaction catalyzed by methionine synthase. As a consequence vitamin B-12 deficiency or inactivation results in most of the folates being trapped in a form that cannot be utilized. Folate deficiency secondary to the “methyl-folate trap” could therefore develop in the vitamin B-12 depleted tissues [15].

Various direct reactions of smoke components with tetrahydrofolates could result in folate deficiency in tissues affected by cigarette smoke. The reaction of tetrahydrofolates with cyanates to form a biologically inactive derivative and the reaction of methyltetrahydrofolates with organic nitrites leading to decomposition of the co-enzymes, suggests lower circulating folate concentration in smokers as compared with non-smokers [15].

The results of the present study are consistent with the previous studies done by Almadori et al., who reported markedly higher levels of serum concentrations of Hcy and lower levels of folate concentrations in head and neck squamous cell carcinoma patients when compared with smokers and healthy individuals. They reported that Vitamin B-12 and folate deficiencies can cause chromosomal damage in buccal mucosa in tobacco users [11].

Another study, done by Eleftheriadou et al., reported high levels of serum Hcy, low levels of serum folate in head and neck squamous cell carcinoma patients, when compared with smoking group and healthy patients [16]. These results are consistent with the present study results.

The majority of patients with OSCC are smokers. In present study folate deficiency and hyperhomocysteinemia were observed in a large number of smokers confirming previous studies [14,16].

Similarly the results of the present study are consistent with the previous studies done by Francesco Nicola Bartuli et al., who reported increased levels of Hcy, decreased levels of folate in OSCC patients when compared with non-smoking healthy individuals [17]. The higher levels of Hcy and lower levels of folate in OSCC are improved by supplementation of folate for 6 months.

Warnecke PM et al., described alterations in DNA methylation, and in particular a global hypomethylation and a regional hypermethylation, especially of promoters of tumor suppressor genes in human tumors [18]. Sanchez-Cespedes M et al., described promoter hypermethylation of key genes in critical pathways is common in Head and Neck carcinoma [19].

Our study also is in correlation with the meta-analysis study Donghong Zhang et al., where a meta-analysis of studies involving 15,046 cases and 20,712 controls was done to examine the association of serum levels of Hcy as well as its metabolizing factors on overall risk of cancer [20]. High serum level of Hcy and folate deficiency was consistently associated with risk of cancer, with little effect by type of cancer or ethnicity. However, only MTHFR C677T homogeneity/wild-type (TT/CC) polymorphism was positively associated with overall risk of cancer. This particular study highlights the role of Hcy metabolism in carcinogenesis. Indeed, Hcy and folate have a critical role in maintaining DNA stability by donating one-carbon moieties [21,22]. Multiple studies have shown that folate deficiency induces epigenetic changes, which leads to global DNA hypomethylation, DNA repair, chromosomal instability, proto-oncogene activation, uracil misincorporation, DNA strand breakage, chromosomal breakage and malignant transformation. Moreover, hyperhomocysteinemia may promote inflammatory processes via oxidative stress [23,24], by increased levels of cell adhesion molecules, cytokines (interleukin 6 and tumor necrosis factor-α) and chemokines (high-sensitivity C-reactive protein), which may contribute to the biology of cancer. Thus, as for cardiovascular disease, supplementing folate to lower serum level of Hcy and repair DNA synthesis and methylation to prevent carcinogenesis might be reasonable. Recent findings from several large-scale human observational or placebo-controlled intervention trials indicated that supplementing synthetic folic acid decreased the risk of cancer at several sites, including the breast [25], colon [26], lung [27] and prostate [28].

## LIMITATION

There was discrepancy in the sample size in the groups of the study and also the sample size was small. Moreover, the study design was that of a cross-sectional analysis. Further prospective studies can be conducted in future to substantiate the present study results.

## CONCLUSION

The use of serum-based analysis may serve as a biomarker of early detection of malignancies. This study investigated comprehensive serum Hcy profile in OSCC patients by using HPLC and serum folate profile by using CLIA method. In the present study, increased serum Hcy levels and decreased serum folate levels was observed in OSCC when compared with smokers and controls. The profile of these two metabolites revealed a valuable approach to understand the biochemistry of OSCC and provided a means for identifying new targets for therapy. The present study concludes by finding an interesting association with serum Hcy and folate levels in OSCC which could be useful as a biochemical “Tumor Marker” and thereby providing insights into the onset and progression of the disease. Hypofolatemia which is a risk factor for oral carcinogenesis might implicate folate as a novel chemo preventive agent, both in patients with potentially malignant disorders and in patients with treated OSCC at risk for locoregional recurrence and second primary tumors.

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**FINANCIAL OR OTHER COMPETING INTERESTS:** None.

Date of Submission: **Feb 20, 2016**
Date of Peer Review: **Apr 21, 2016**
Date of Acceptance: **Jun 11, 2016**
Date of Publishing: **Oct 01, 2016**