**Table of Contents**

**Original Articles**

**Obesity and High Risk Pathological Features of Papillary Thyroid Carcinoma: A Retrospective Analysis of a University Hospital in Pakistan** ..........................................................06
Shakeel Uz Zaman, Mohammad Sohail Awan, Mohammad Ahsan Sulaiman

**Quantification of circulating plasma cell free DNA fragments in patients with oral cancer and precancer** ..........................................................11
Ami Desai, Shreenivas Kallianpur, Abin Mani, Manisha S. Tijare, Samar Khan, Megha Jain, Vidhi Mathur, Rinky Ahuja, Vijay Saxena

**Clinical and microbiological profile of infections during induction phase of acute myeloid leukemia** ..........................................................18
Sonia Parikh, Parijat Goswami, Asha Anand, Harsha Panchal, Apurva Patel, Rahul Kulkarni, Bhadresh Shastri

**Breast Cancer Risk factor awareness and utilization of screening program: A cross-sectional study among women in the Northern Emirates** ..........................................................24
Prashanth Hegde, Jyothi Pande, Hanaa Hosny Adly, Padma V. Shetty, Jayakumari

**BRCA1 and BRCA2 Germline Mutation Screening in Western Algeria using High Resolution Melting Analysis (HRM)** ..........................................................31
Amina Chami Sidi Boulenouar, Florence Coulet, Farida Mesli Taleb Bendib, Fatima Zohra Boudinar, Rachid Senhadji

**Colon Cancer in Patients below Age of 50 Years: Kuwait Cancer Control Center Experience** ..........................................................38
Mohamed Salah Fayaz, Gerges Attia Demian, Heba El–Sayed Elsa, Sadeq Abu–Zlouf

**Awareness, understanding, attitude, and barriers toward prescribing modern cancer immunotherapies in the Arabian Gulf countries** ..........................................................45

**The Need for Regulatory Reforms in the Use of Opioids for Pain Management and Palliative Care in the Middle East** ..........................................................52
Bassim Jaffar Al Bahrani and Itrat Mehdi

**Sporadic colon cancer in Lebanon: A clinicopathological study** ..........................................................60
William A. Nehmeh, Marc Rassy, Claude Ghorra, Pamela Abdayem, Cyril Tohmè.

**Case Reports**

**Malignant Phyllodes tumor in a young female: Report of a rare case** ..........................................................64
Priyanka Anand, Namrata Sarin, Anmul K. Butti, Sompal Singh

**Cutaneous Metastasis of Sigmoid Adenocarcinoma to Face and Scalp at Initial Diagnosis: Case Report** ..........................................................70
Mariam Aloitaib, Jaroslav Nemec

**Cervical metastasis of testicular cancer: Case report and review of literature** ..........................................................73
Guhan Kumarasamy, Anusha Balasubramanian, Baharudin Abdullah

**Metachronous Testicular Seminoma After Testicular Tumor** ..........................................................78
Xh. Çuni, I. Haxhiu, Sh. Telegrafi, M. Berisha, N. Rexha, M. Myftari, P. Nuraj, S. Mehmeti, A. Fetahu, R. Dervishi, S. Manoxhuka, F. Kurshumliu

**Conference Highlights/Scientific Contributions**

- **Highlights of the International Conference on Genitourinary and Gynecological Cancers, Kuwait Conference (GUG–KC): Recent Updates, 14–16 April 2018, State of Kuwait** ..........................................................82

- **News Notes** ...........................................................................................................................................................................87

- **Advertisements** ........................................................................................................................................................................91

- **Scientific events in the GCC and the Arab World for 2018** ........................................................................................................92
Abstract

**Purpose:** Study was aimed to quantify plasma level of total, short and long fragmented cell-free DNA (cfDNA) along with DNA integrity in patients with oral cancer, oral precancer and tobacco users without lesions and normal controls. In addition, study evaluated the correlation of cfDNA with clinicopathologic parameters of oral cancer.

**Methodology:** Plasma samples were collected preoperatively from 44 patients with oral cancer, 40 patients with oral precancer, 40 tobacco users without any oral lesion and 40 healthy controls without any tobacco habit. cfDNA extraction was carried out from the plasma followed by quantitative and qualitative assessment of extracted DNA. Quantity of short and long fragmented DNA was assessed by using PCR with two different primer sets for the beta-actin gene, amplifying short (102 bp) and long (253 bp) products. The DNA integrity index was measured by calculating the ratio of quantity of long fragmented to short fragmented DNA. All quantitative cfDNA parameters were statistically analyzed to verify their correlation with clinicopathologic parameters.

**Results:** Results showed that total cfDNA level, short and long fragmented cfDNA concentration and DNA integrity was significantly higher in oral cancer group as compare to other (p=0.0001). Study demonstrated that there is no correlation total, short and long cfDNA and DNA integrity with tumor size and histologic type or grading. But positive correlation of total cfDNA was found with nodal metastasis (p=0.001) and clinical stages (p=0.006).

**Conclusion:** Quantitative analysis of total cfDNA may be applied as a screening marker for early detection of precancer and cancer as well as for prognostication of oral cancer. Additionally, plasma levels of short and long fragmented cfDNA and DNA integrity index can be applied for early detection of oral cancer.

**Keywords:** Oral squamous cell carcinoma (OSCC), Oral precancerous lesion, Cell-free DNA, Polymerase chain reaction (PCR), DNA integrity index, Beta-actin gene

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common cancers occurring in India due to tobacco use, with the age adjusted incidence rate ranging from 3.4 to 13.8 per 100,000 as per cancer registries (7). Despite therapeutic advances, survival rates for patients with oral OSCC remain low and have not improved over several decades due to failure to diagnose and treat oral cancer at an early stage. Improving diagnosis and treatment of precancerous changes and early cancers is imperative in increasing survival rates and improve functional outcome for persons at risk. Clinical and histopathologic investigations remain the mainstay in diagnosis of oral precancer and cancer, but these features may not always predict whether premalignant disorders of the oral mucosa remain stable, regress or progress to malignancy.
and nor do they predict progression of oral carcinoma or effectiveness of the treatment. Molecular markers which can assess disease progression and treatment effectiveness are therefore necessary to improve the management of premalignant lesions and oral cancer.

Studies have focused on measuring the circulating cell–free DNA (cfDNA) concentration as a possible new biomarker since increased amounts of cfDNA are detected in various body fluids during the process of tumorigenesis and metastasis formation. Moreover, cfDNA levels decline as a consequence of anti–tumor treatment and increase during follow–up associated with relapse in several cancers; e.g. lung cancer, breast cancer and colorectal cancer. Several studies have assessed the diagnostic and prognostic usefulness of quantitative and qualitative tumor–specific alterations of cfDNA in cancer patients, such as total cfDNA concentration, short and long fragmented cfDNA quantity, DNA strand integrity, frequency of mutations and abnormalities of microsatellites and methylation of genes. Among all these, quantification of cfDNA is relatively effortless and inexpensive method as it can be extracted using simple purification procedures from blood, which in turn is easily procured from patients. Moreover, cfDNA appears to be stable for several years in stored samples of plasma or serum enabling short term as well as long term evaluation.

cfDNA quantification has been utilized as a diagnostic, prognostic and monitoring marker in other cancers, however there is lack of information in oral precancer and cancer especially with regard to short and long cfDNA fragments and DNA integrity index. We have therefore, aimed this study to quantify the level of total, short and long fragmented cfDNA along with DNA integrity in patients with oral cancer, oral precancer and tobacco users without lesions. In addition, our study evaluated the correlation of cfDNA quantification with various clinicopathologic parameters of oral cancer.

Materials and Method

Sample selection

Ethical clearance was obtained from the institutional ethics committee for the study and written informed consent was taken from all participants before blood collection. Blood samples were collected from the 40 subjects with history of tobacco usage of minimum 2 years, but without any oral lesion; 40 patients with oral precancerous lesion; 44 patients with oral cancer and 40 healthy controls without any tobacco habit (Table 1). Subjects with history of recent severe trauma, cardiac diseases, autoimmune disorders, liver cirrhosis, hepatitis, rheumatoid arthritis, SLE and pregnancy were excluded from the study as in these systemic conditions cfDNA levels are affected. With the exception of the healthy volunteers and Sub Group I, all samples were drawn from patients at the time of surgery, but before any surgical incisions. Case history Performa with TNM staging was taken in oral cancer patients. Only histologically proven oral cancer and precancerous subjects were taken in the study and cancer patients who had received and neoadjuvant radiotherapy or chemotherapy before sample collection, were excluded from the study.

Sample collection

Five ml peripheral blood samples were preoperatively collected from patients and processed within an hour of collection. Blood samples was subjected to the isolation of cell-free nucleic acids in Micro Centrifuge EPPENDORF–5804 R by a 3 spin protocol (1500 rpm for 10 min, 3000 rpm for 5 min and 4500 rpm for 5 min) to prevent cross-contamination from cellular nucleic acids. Following centrifugation, the plasma samples were separated and stored at -80°C until further analysis.

Isolation and quantification of cell free DNA from plasma

DNA extraction was carried out from 200 μl aliquots of plasma by commercially available Blood Purification Kit (HiPurATM Blood Genomic DNA Miniprep Purification Spin Kit of HIMEDIA–MB504) followed by quantitative and qualitative assessment of extracted cfDNA by Picodrop UV–Spectrophotometer. Results of cfDNA quality was measured by calculating the ratio of absorbance at 260 nm and 280 nm and ratio between 1.6 and 1.8 indicates the presence pure DNA. Assessment of quantity of short and long fragmented DNA was done by using Polymerase chain reaction (PCR) with two different primer sets for the beta–actin gene, amplifying short (102 bp) and long (253 bp) products. Both short and long PCR fragments were amplified using the same forward primer (5'-GCACACACCTTCTACACGA-3') and two different reverse primers (reverse primer for short product (102 bp)
bp) was 5′-GTCATTTCTCGGTTGC-3′ and for long product (253 bp) was 5′-GAACCTGCAGAGTTCCA-3′.

Primers used in this study were procured from Integrated DNA Technologies (IDT, USA).

PCR reaction components and quantity of chemicals used in reaction mixture were as follows: 2.5μl 10x PCR buffer, 0.5 μl each deoxynucleotide triphosphate, 2 μl MgCl2, 0.3 μl TaqDNA Polymerase, total 3μl PCR primer (1μl each PCR primer) and 5 μg of extracted DNA. DNA-free H2O was used to bring the reaction volume to 25μl. All samples were incubated for 15 minutes at 95°C after which time the amplification was carried out for 50 cycles under the following conditions: 95°C for 15 s, 58°C for 20 s, 72°C for 40 s; followed by final extension at 72°C for 10 min in single cycle. Gel electrophoresis of the genomic DNA was carried out in 2% agarose gel for qualitative analysis of samples prepared. Each PCR was conducted as an experiment, with controls (distilled water instead of template DNA) to test the purity and viability of reagents. The analysis was performed for all the samples at least three times with each selected primers to check the reproducibility. After running the gel, it was placed on gel documentation system and visualized by 302 nm high intensity UV light. Image was captured and analyzed by using Quantity One Software. Molecular weight was calculated by using the same software. The DNA integrity index was measured by ratio of quantity of long fragmented DNA to short fragmented DNA and calculated as the concentration of the 253 bp fragments / the concentration of the 102 bp fragments in each assay.

Statistical Analysis

The One–way ANOVA test was used to compare the differences in the concentrations of cfDNA (total, short and long fragmented DNA) and the DNA integrity between the different study groups and the healthy control group. If results were found to be statistically significant by using One–way ANOVA test, to establish significant pair wise correlation amongst different groups Tukey HSD statistical analysis was done. Evaluation for correlation of all quantitative parameters cfDNA with various clinicopathologic parameters of oral cancer were performed using the same statistical analysis.

Results

Results of present study showed that Total cfDNA quantity was highest in Sub–group III (cancer patients) followed by Sub–group II (precancerous patients), Sub–group I (tobacco users without any oral lesion) and control group. Details of pair wise comparison of different quantitative analysis of cfDNA in different groups based on Tukey HSD statistical analysis along with p–value of dependent variable are given in Table 2. Statistically significant difference was found between the Control group and subgroup II and III (P value <0.001). DNA quantity was 7 folds higher in cancer group as compared

<table>
<thead>
<tr>
<th>Pair Wise Comparison of Different Groups</th>
<th>P-Value of Dependent Variable (TUKEY HSD Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>comparison of mean total cfDNA</td>
</tr>
<tr>
<td>Control group Vs subgroup I</td>
<td>0.997</td>
</tr>
<tr>
<td>Control group Vs subgroup II</td>
<td>0.048</td>
</tr>
<tr>
<td>Control group Vs subgroup III</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Subgroup I Vs subgroup II</td>
<td>0.076</td>
</tr>
<tr>
<td>Subgroup I Vs subgroup III</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Subgroup II Vs subgroup III</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*HSD=0.071 *HSD=0.814 *HSD=0.116 *HSD=0.925

Table 2. Pair Wise Comparison of Different Quantitative Analysis of cfDNA in Different Groups (TUKEY HSD Test)

*HSD; The mean difference is significant at the 0.05 level.
Quantification of cf DNA in oral cancer and precancer, Ami Desai, et. al.

Applying ‘One-way ANOVA’ test. Test results showed that there is no correlation of tumor size and histologic grading with any quantitative parameters of cfDNA. Statistical analysis confirmed that among all quantitative parameters, only concentration of Total cfDNA was significantly correlated with nodal metastasis and clinical staging. Total cfDNA quantity in cancer patients with nodal metastasis is significantly higher as compared to cancer patients without nodal metastasis. Tukey HSD statistical analysis proved that cancer patients with stage III and stage IV have significantly higher total cfDNA quantity as compared to cancer patients with stage I and stage II. But there was no significant difference in cfDNA quantity between patients with stage I and II and also between patients with stage III and IV.

Discussion

Nucleic acids that are no longer confined within cells but are dispersed in body fluids or in circulation are termed circulating cell free nucleic acids. The first discovery of circulating nucleic acids was reported by Mandel and Metais in 1948. They demonstrated that extracellular DNA and RNA could be detected from the blood of healthy as well as sick individuals (11). However, their pioneering work had not awakened much interest at that time. Research on circulating DNA resumed in the 1960s following the discovery of high levels of circulating DNA in patients with systemic lupus erythematosus (12). Further interest in this field was developed in the 1970’s by Leon and Shapiro following the demonstration that increased concentrations of DNA in the serum could be detected in patients with cancer (2). It is now well established that measurable quantities of nucleic acids circulate in healthy individuals as well as in patients with various disease pathologies.

Results depicted that mostly all cases of cancer group showed positive amplification for both short and long fragments while only 25% of cases of remaining three groups (subgroup I, II and control) showed positive amplification. Mean quantity of both short and long fragmented DNA and DNA integrity index was significantly higher in cancer group as compared to other groups, but no difference was found between cfDNA quantity of control group and tobacco chewers without lesions.

Table 3. Correlation of Clinical Parameters with Total, Short and Long Fragmented cfDNA Quantity and DNA Integrity in Cancer Group (One Way ANOVA Test)

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Correlation with Quantitative Parameters of cfDNA</th>
<th>F-Value</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Size</td>
<td>Total cfDNA quantity</td>
<td>1.163</td>
<td>0.351</td>
</tr>
<tr>
<td></td>
<td>Short fragmented DNA quantity</td>
<td>0.528</td>
<td>0.669</td>
</tr>
<tr>
<td></td>
<td>Long fragmented DNA quantity</td>
<td>0.265</td>
<td>0.850</td>
</tr>
<tr>
<td></td>
<td>DNA integrity</td>
<td>0.261</td>
<td>0.853</td>
</tr>
<tr>
<td>Nodal Metastasis</td>
<td>Total cfDNA quantity</td>
<td>10.721</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Short fragmented DNA quantity</td>
<td>0.843</td>
<td>0.446</td>
</tr>
<tr>
<td></td>
<td>Long fragmented DNA quantity</td>
<td>0.909</td>
<td>0.420</td>
</tr>
<tr>
<td></td>
<td>DNA integrity</td>
<td>1.421</td>
<td>0.266</td>
</tr>
<tr>
<td>Clinical Staging</td>
<td>Total cfDNA quantity</td>
<td>5.745</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Short fragmented DNA quantity</td>
<td>1.091</td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>Long fragmented DNA quantity</td>
<td>0.502</td>
<td>0.686</td>
</tr>
<tr>
<td></td>
<td>DNA integrity</td>
<td>0.991</td>
<td>0.419</td>
</tr>
<tr>
<td>Histologic Grading</td>
<td>Total cfDNA quantity</td>
<td>0.803</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>Short fragmented DNA quantity</td>
<td>0.546</td>
<td>0.588</td>
</tr>
<tr>
<td></td>
<td>Long fragmented DNA quantity</td>
<td>0.350</td>
<td>0.709</td>
</tr>
<tr>
<td></td>
<td>DNA integrity</td>
<td>1.764</td>
<td>0.198</td>
</tr>
</tbody>
</table>

Table 3 demonstrates the correlation of various clinicopathologic parameters with total, short and long cfDNA quantity and cfDNA integrity in cancer group by applying ‘One-way ANOVA’ test. Test results showed that there is no correlation of tumor size and histologic grading with any quantitative parameters of cfDNA. Statistical analysis confirmed that among all quantitative parameters, only concentration of Total cfDNA was significantly correlated with nodal metastasis and clinical staging. Total cfDNA quantity in cancer patients with nodal metastasis is significantly higher as compared to cancer patients without nodal metastasis. Tukey HSD statistical analysis proved that cancer patients with stage III and stage IV have significantly higher total cfDNA quantity as compared to cancer patients with stage I and stage II. But there was no significant difference in cfDNA quantity between patients with stage I and II and also between patients with stage III and IV.

Discussion

Nucleic acids that are no longer confined within cells but are dispersed in body fluids or in circulation are termed circulating cell free nucleic acids. The first discovery of circulating nucleic acids was reported by Mandel and Metais in 1948. They demonstrated that extracellular DNA and RNA could be detected from the blood of healthy as well as sick individuals (11). However, their pioneering work had not awakened much interest at that time. Research on circulating DNA resumed in the 1960s following the discovery of high levels of circulating DNA in patients with systemic lupus erythematosus (12). Further interest in this field was developed in the 1970’s by Leon and Shapiro following the demonstration that increased concentrations of DNA in the serum could be detected in patients with cancer (2). It is now well established that measurable quantities of nucleic acids circulate in healthy individuals as well as in patients with various disease pathologies.

Even though the origin of the free circulating DNA has been researched for the last 30 years, the exact mechanism of its emergence remains unknown. Various possibilities for origin and sources of cfDNA have been postulated and supported by theories and observations. Most commonly accepted theory is the release of DNA as a consequence of cell death in the tumour microenvironment (3). Necrotic and apoptotic cells are usually phagocytosed by macrophages or other scavenger cells that engulf cells and release digested or fragmented DNA into the tissue environment and circulation (2, 3). Another theory postulates that release of intact cells in the bloodstream and their subsequent lysis in blood might be the source of cfDNA in circulation (3). Intact cells have been retrieved from the bloodstream of patients with cancers. In various studies of cancer patients, a good concordance has been observed between patterns of DNA hypermethylation in circulating tumor cells and in cfDNA. It is important that these cells
might have acquired the ability to enter the blood stream but are not metastatic cells and do not extravasate and colonize other organ sites (3). Anker et al. (1975) have suggested that cells may actively release DNA in the form of a nucleoprotein complex into the blood (13). Jarh et al (2001) have hypothesized that a large majority of cfDNA comes from dying healthy cells that surround the tumor rather than from neoplastic cells (3).

The way clinical blood samples are handled before reaching the laboratory has significant impact on cfDNA yields. Therefore, plasma was separated within 10 minutes of blood sample collection as delay in separating the plasma could change the amount of recovered DNA. In the present study, plasma was preferred over serum for DNA extraction as findings have shown that the cfDNA levels in serum fluctuated more than those in the plasma and the plasma was more suitable source of DNA for biomarker screening (14). PCR was carried out to assess amplification and quantity of obtained DNA by two different primer sets (amplifying short (102 bp) and long (253 bp) products) for the beta-actin gene, which is the most common housekeeping gene found in almost all human cells. As there are 20 or more DNA segments in the human genome that contain beta-actin sequences, it has been widely used in different studies for quantification of cfDNA in cancer patients and controls (10, 15, 16, 17, 18).

**Comparison of the total cfDNA quantity and quality in different study groups:**

Significantly higher levels of cfDNA quantity have found in patients with lung cancer, breast cancer, prostate cancer, ovarian cancer, esophageal cancer, colorectal cancer, renal cancer etc than normal subjects (4, 5, 15, 19). Various studies have reported increased cfDNA levels, up to 20 folds higher in the cancer patients when compared with healthy controls (4, 5, 15, 19). In present study levels of cfDNA in precancerous patients were intermediate to cancer group and control group/subgroup I (tobacco chewers without lesions). These findings propose that quantitative analysis of total cfDNA may have application as a screening marker in tobacco chewers without lesions for early detection of precancer and in patients with precancerous lesions for early detection of oral cancer.

Results showed that short and long fragmented cfDNA concentration and the mean DNA integrity index tended to be higher in the oral cancer patients than other three groups. These findings are similar to the results reported by Kim J et al. (2006) (22), Tomita H et al. (2007) (10), Chan KC et al. (2008) (23), Hauser S et al. (2012) (17) and Zaher ER et al. (2012) (18); who have found that DNA integrity is significantly higher in patients with malignancy as compared to normal patients or patients with benign diseases. However, we found no significant difference in these three quantitative assays among normal controls, tobacco chewers without lesion and precancer group.

The long fragmented DNA concentration showed the most significant difference among all assays in distinguishing between cancer patients and other groups. Studies have suggested that the DNA retrieved from the blood of normal subjects and of patients with benign diseases or sepsis show a typical short apoptotic DNA ladder, indicating that apoptotic cell death is the main mechanism contributing to cfDNA in physiological conditions as well as in a non-cancer pathological conditions, while in malignancy necrotic cell death frequently occurs in tumor tissue, resulting in various lengths of longer DNA fragments (3). Thus, these differences in cfDNA nature are responsible for diverse results of DNA integrity index in different pathological conditions. Various studies have indicated that the rise in long DNA fragments is responsible for increased measurement of DNA integrity in cancer patients which is in accordance with the findings of the present study (12, 23, 24, 25).

We found no more than 5% samples exhibiting presence of pure DNA, whereas 86% samples showed protein contamination which is due to presence of various plasma proteins in plasma/blood samples. Similar levels of cfDNA degradation by proteins have been reported in other studies (3, 15).

Correlation of quantitative cfDNA parameters with clinicopathologic parameters of oral cancer:

As regards release of cfDNA and its association to tumor size, we found no correlation, as has been the case in studies by Hauser S et al (2012) (17) and Zaher ER et al. (2012) (18). But, this finding contradicts several reports in the literature which showed increase in levels of cfDNA with increase in tumor size (21, 26). Cell necrosis is suggested as a possible mechanism for release of cfDNA, therefore we can presume that it is the amount of tumor necrosis which may be responsible for the higher levels of cfDNA and not the size of tumor (2, 3).

Results of the correlation of nodal metastasis with all quantitative parameters of cfDNA demonstrated that not all but only total cfDNA quantity was significantly correlated with nodal metastasis. It was 2 folds elevated in cancer patients with nodal metastasis as compared to patients without nodal metastasis. This finding is similar to the results of Jung K et al. (2004) (6), Gal S et al. (2004) (26), Bastian PJ et al. (2007) (27) and Schwarzenbach H et al. (2009) (28), who found positive correlation between nodal involvement and total cfDNA level. Some studies have showed increase in total cfDNA levels and DNA integrity in cancer patients with distant metastasis (2, 6, 27, 29).
Quantification of cf DNA in oral cancer and precancer, Ami Desai, et. al.

two patients with distant metastasis were present in our study; therefore, statistical analysis was not applicable to assess correlation of distant metastasis with cfDNA quantitative parameters.

We have found that cfDNA levels were significantly elevated from stage II to stage III. Results of correlation of overall clinical staging with total, short and long cfDNA quantity and cfDNA integrity in cancer patients depicts that positive correlation of clinical staging with cfDNA quantity is only due to inclusion of nodal involvement in staging parameter, while other parameters in stages have no significance. Positive correlation of staging with total DNA level is contradictory to findings in few studies of cfDNA \(^6, 17, 18, 20, 23\). But several studies have showed that there is a positive correlation between clinical staging and total DNA level as is the case here \(^7, 27\).

No association was found between histologic grading of oral carcinoma and quantitative data of cfDNA, which is in accordance with the earlier studies \(^3, 5, 6, 16, 26\). Correlation of histologic type or grading with cfDNA is still not fully understood and further cfDNA studies with individual histologic parameters such as amount of tumor necrosis, dysplasia, mitosis, pattern of invasion, local bone invasion, vascular and lymphatic invasion and extracapsular invasion in lymph nodes may better define origin as well as mechanism of release of cfDNA and its association with disease progression \(^3, 27\).

**Conclusion**

To the best of our knowledge, there have been no studies demonstrating correlation of different quantitative parameters of cfDNA and DNA integrity with oral cancer and precancer. Our findings suggest that quantitative analysis of total cfDNA may be applied as a screening marker in tobacco chewers without lesions for early detection of precancer and in patients with precancerous lesions for early detection of oral cancer. Additionally, plasma levels of short and long fragmented cfDNA and DNA integrity index can also be applied for early detection of oral cancer. Increase in total cfDNA levels is a risk factor of nodal metastasis in oral cancer patients. Therefore, quantitative analysis of plasma cfDNA and DNA integrity analysis can serve as a marker for noninvasive early detection and prognostication of oral cancer and precancer.

Although our results are preliminary, they emphasize a need towards further investigation of the potential usefulness of cfDNA in prediction of distant metastasis, recurrence, survival rate and in monitoring the effectiveness of treatment by further large–scaled follow–up studies. Future studies should consider the inclusion of assessment of cancer related genetics and epigenetic alterations in cfDNA to increase both sensitivity and specificity for oral cancer and precancer detection and assessment. Standardization of the protocols of sample collection, storage, DNA extraction and quantification method are also essential for better assessment of cfDNA.

In addition, further work is needed to evaluate the possible origin of circulating DNA and its precise correlation with each clinicopathologic parameters. Establishment of proper range values of cfDNA in different cancers, precancers and in various systemic conditions other than cancer with proper standardization of technique protocols can lead to better clinical and therapeutical application of cfDNA as an easy and effective screening and monitoring tool.

**Abbreviations**

OSCC=Oral squamous cell carcinoma; cfDNA= cell–free DNA; PCR= Polymerase chain reaction

**Authors’ Contributions**

Dr. Ami D, Dr. Abin M, Dr. Shreenivas K, Dr. Manisha T and Dr. Samar K conceived and carried out experiments and analysed the data. Dr. Vidhi M and Dr. Vijay S collected the blood sample and clinical details. Histopathological examination of tumor sections and assessment of histological grading were accomplished by Dr. Ami and Dr. Megha J. Dr. Ami and Dr. Shreenivas drafted the manuscript. All authors approved the submitted manuscript.

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