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Research Article

Somatic Mutations in Circulating Cell-Free D-Loop Region of Mitochondrial DNA: A Study from North-East India

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Abstract: Head and neck Squamous cell carcinoma (HNSCC) is highly prevalent in North east India. The widespread use of tobacco exposure is a known risk factor, making mitochondrial DNA (mtDNA) more susceptible to damage by oxidative stress in comparison to nuclear DNA. Mitochondrial dysfunction being a hallmark of cancer, the study aims to evaluate liquid biopsy involving circulating cell-free mitochondrial DNA (cfmtDNA) as an early diagnostic marker by reducing the dependability over tumor tissue biopsy specimen. A total of 50 HNSCC cases reported at Cancer Hospital, Guwahati Medical College from January 2018 to August 2018 were included in this study. Cell-free DNA was isolated using QIAamp Circulating Nucleic Acid Kit. PCR based amplification of mitochondrial D-loop, followed by direct sequencing. Our result indicated the presence of somatic mutations (73(A/G), 93(G/A), 146(T/C) and 207 (G/A)). Polymorphism was also observed in the sequences (263A>G, 275G>A, 318T>C, 16034T>C, 16257C>A and 16519T>C) upon comparison with reference sequence. Analysis of c-tract region showed the presence of an additional cytosine nucleotide at position 309.Identifying somatic mutations in cfmtDNA using liquid biopsy approach will certainly minimize the dependency of clinicians and molecular biologist over the availability of tumor tissue specimens. The identified somatic variations from our study will help in the implementation of preventive measure. Therefore, our study provides an early mtDNA diagnostic marker using liquid biopsy approach.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is one of the predominant causes of cancer related casualties worldwide [1]. The term head and neck carcinoma encompasses all malignancies arising in the nasal and oral cavities, pharynx, larynx and the paranasal sinuses. Majority of these (approximately 95%) epithelial cancers are squamous cell carcinoma [2]. Smoking and alcoholism are two well-known predisposing factors. Whereas, smokeless tobacco and betel nut are etiological agents responsible for it in the Asian population [3]. Overall, 2.5 lakh new patients are diagnosed every year with HNSCC, of whom about three-fourths are in an advanced stage [4]. In Northeast India, HNSCC is most familiar in the states of Assam, Manipur, Mizoram, Tripura and Nagaland with an incidence of 54.48%, that acquires 30–40% cancers at all sites and is the sixth most common cause of death in males and seventh in females [5]. The disease mortality rate is still high that is partly due to the late diagnosis and inefficient therapy [6]. In order to minimize the mortality, it is critical to identify novel HNSCC biomarkers with potential utility in early diagnosis to reduces the suffering and cost associated with the disease [7].

In the past decade, management of cancer patients based on the molecular features of tumor tissue (tissue biopsy) has given submissive growth in cancer diagnosis and monitoring [8]. Further, the limitations associated with tumor tissue, such as heterogeneity, tumor accessibility and in disease monitoring has limited the reliability of clinicians

[9,10]. Therefore, to overcome some of the challenges described above, cancer-derived circulating cell-free DNA (cfDNA) has become an appealing alternative against the conventional tumor tissue biopsy [11]. Cell-free DNA, fragments in human blood was first described in 1948 by Mandel and Métais. The origins and characteristics of cfDNA were later studied intermittently in subsequent decades [12].Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer. The mitochondrial genome is susceptible to ROS and other types of genotoxic damage due to lack of protective histones and its limited mtDNA repair capabilities [13]. Earlier, mitochondrial DNA mutation has been reported in oral cancer [14].

In this study, we have examined 50 cases of HNSCC with a habit of consuming smokeless tobacco and betel nut on cell-free mitochondrial DNA (cfmtDNA), D-loop region to establish an early diagnostic marker in HNSCC patients.

MATERIALS AND METHODS

Sample collection and preparation

The study was conducted at the Life Sciences Division, Institute of Advanced Study in Science and Technology (IASST), Guwahati, India. Consent forms were collected from all patients included in the study. This study was approved (IEC (HS)/IASST/1082/2015-16/1) by the Institutional Review Board (IRB), Institute of Advanced Study in Science and Technology, Guwahati, India. All possible precautions were taken to avoid any cross contamination while collecting as well as processing of the samples.

DNA isolation

The study comprised of 50 HNSCC patients. The sample collection started from the time period 2015 to 2016. Blood samples were collected before any surgical interventions or therapeutic treatments. For clinical samples, 10ml peripheral blood was collected in cfDNA BCT® Streck tubes (Streck Inc., Omaha, NE, USA). Plasma was separated by centrifugation at 1000–2000g at 4°C for 10 mins and 10,000 –16,000g at 4°C for 10 mins. Plasma aliquots were processed immediately for isolation. The cfDNA were processed using QIAamp Circulating Nucleic Acid Kit (cat No. 55114) with QIAvac 24 Plus setup and peripheral blood sample were processed using DNeasy Blood and Tissue Kit (cat No. 69504).

PCR amplification

PCR amplification was done using Fermentas High Fidelity PCR Enzyme Mix (Cat No. K0192). The total reaction volume was 20 µl containing 2 µl of 10X PCR buffer with $MgCl^2$, 2 µl of 10 mM dNTPs, 0.2 µl (5 units/µl) of Taq DNA polymerase, 1 µl each of 20 pmol/ µl forward and reverse primer, 50–100 ng of genomic DNA and the volume make up to 20 µl by adding nuclease-free water. The amplified product was checked on 1.5% agarose gel with 1kb DNA ladder.

Primers for D-loop amplification

The primers used for D-loop amplification in this study were: forward primer F1-CACCATTAGCACCCAAAGCT and reverse primer R1- AGTGTATTGCTTTGAGGAGGT which amplify an initial 1122 bp PCR product. The PCR programme used for amplification was: initial denaturation step was done at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s; annealing at 55 °C for 45 s and elongation at 72 °C for 90 s. The amplified product was observed in 1.5% agarose gel.

DNA sequencing

The amplified PCR product was observed in 1.5% agarose gel. PCR product elution was done using Thermo Scientific Gene JET Gel Extraction and DNA Cleanup Micro Kit, Catalog Number- #K0831; #K0832. Sanger sequencing of d-loop was done using Genetic Analyzer 3500, Applied Bio systems (Molecular Medicine Lab, Department of Biotechnology, Assam University, Silchar, India). The resultant DNA sequences were compared with the published reference mtDNA sequence (NCBI Accession Number NC_012920 AC_000021). Any mtDNA sequences that differed between cfmtDNA sample and its matched blood or peripheral blood or germline mtDNA were scored as somatic mtDNA mutations specific to the tumor using nucleotide BLAST.

Software

The sequences were obtained in file format AB1 File (.ab1). Sequences visualization was done using BioEdit and SeqScanner. MEGA6.06 was used for sequence alignment.

RESULTS

Study of population

The study was conducted with 50 samples of HNSCC, to identify somatic DNA mutation against the germline DNA. The characteristics of the study population were summarized in Table 1. The patients include in this study were histologically confirmed to be cancerous.

Table 1: Clinical characteristic of HNSCC patients.

Characteristic	Values
Total Patients, n (%)	50 (100)
Age (Years) (Median)	54
Sex, N	
Male	31 (62)
Female	19 (38)
Diagnosis (%) (Head and Neck Cancer)	
Hypopharyngeal Cancer	4 (8)
Laryngeal Cancer	14 (28)
Lip and Oral Cavity Cancer	12 (24)
Nasopharyngeal Cancer	7 (14)
Oropharyngeal Cancer	6 (12)
Paranasal Sinus and Nasal Cavity Cancer	2 (4)
Salivary Gland Cancer	5 (10)
Tumor Grade (%)	
Well differentiated (low grade)	11 (22)
Moderately differentiated (intermediate grade)	14 (28)
Poorly differentiated (high grade)	16 (32)
Undifferentiated (high grade)	9 (18)

PCR Amplification and elution of mitochondrial d-loop region

Mitochondrial non-coding region, d-loop was amplified using PCR amplification (Fig. 1). PCR products for mtDNA d-loop region on agarose gel showed a fragment of about 1122bp. After the visualization of amplified d-loop region on agarose gel. The identified bands were eluted for the removal of remaining, such as dNTPs, primers, Taq, and $MgCl^{2+}$ ion. As they could interfere with subsequent manipulations such as DNA sequencing.

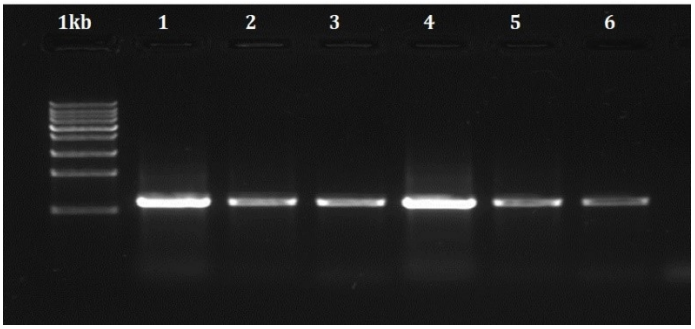

Fig. 1: Visualization of amplified D-loop region of mitochondria with 1.5% agarose gel. 1kb DNA ladder was used to determine the amplified PCR product of 1124bp.

Mutation detection in mitochondrial D-loop region

All the patients included in this study were of different tumor grade. Somatic variants with base substitution was identified in the D-loop region between 1-576 bp and 16024-16569bp. Out of the 50 patients, somatic variation was found in 12 positions. Among the identified positions the most commonly observed base substitution was in position 73(A/G), 93(G/A) and 146(T/C) (Table 2, Figure 2). The mtDNA sequences that differed between cfDNA and matched peripheral blood or germline DNA samples were regarded as somatic mutations.

Table 2: Mutation detection in D-loop region of mtDNA

Positions	Reference Sequence	Peripheral Blood DNA	Cell-Free DNA
73	A	A	G
93	A	A	G
146	T	T	C
207	G	G	A
16130	G	G	A
16154	G	G	A
16224	C	C	T
16279	C	C	T
16288	C	C	T
16328	C	C	C
16363	T	T	C
16520	T	T	C

Mitochondrial d-loop polymorphism

The d-loop sequence variation, was compared with reference sequence against peripheral blood and cfDNA of patients. The difference identified was reported as a polymorphism. In the present study, polymorphisms were identified at 25 positions (Table 3). The polymorphisms identified in the present study were in the nucleotides 263A>G, 275G>A, 16311T>C, 16034T>C, 16257C>A and 16519T>C.

Table 3: Polymorphism in d-loop region of mtDNA

Nucleotide position	Reference Sequence	Base Variation (cfDNA/Peripheral Blood DNA)
150	C	T
152	T	C
153	A	G
200	A	G
263	A	G
275	G	A
318	T	C
16034	T	C
16052	A	C
16076	T	C
16087	T	C
16093	T	C
16127	T	C
16159	A	G
16172	T	C
16257	C	A
16259	T	C
16261	C	T
16271	T	C
16304	T	C
16311	T	C
16362	T	C
16399	A	G
16519	T	C
16526	C	T

Mitochondrial C-tract region

The mitochondrial d-loop reference sequence was also aligned against the cfDNA (tumor DNA) and peripheral blood DNA to study the C-tract region, as it is found to be highly polymorphic. The number of cytosine bases in the long stretch of 7 bp tract varies from 6-13bp. The most frequent sequences for the D310 region are C7TC6, C8TC6 and C9TC6. In our study, we have observed cytosine stretch of is C7TC6, C8TC6 and C9TC6 in D310 region. The most common variations observed in our study was, the insertion of an additional cytosine nucleotide at position 309 (Fig. 3). The insertion of cytosine in C-tract region was observed when comparison was done between cfDNA (tumor source) and peripheral blood DNA within the same individuals. The variations in C-tract region was noted in different individual and reported as mutation.

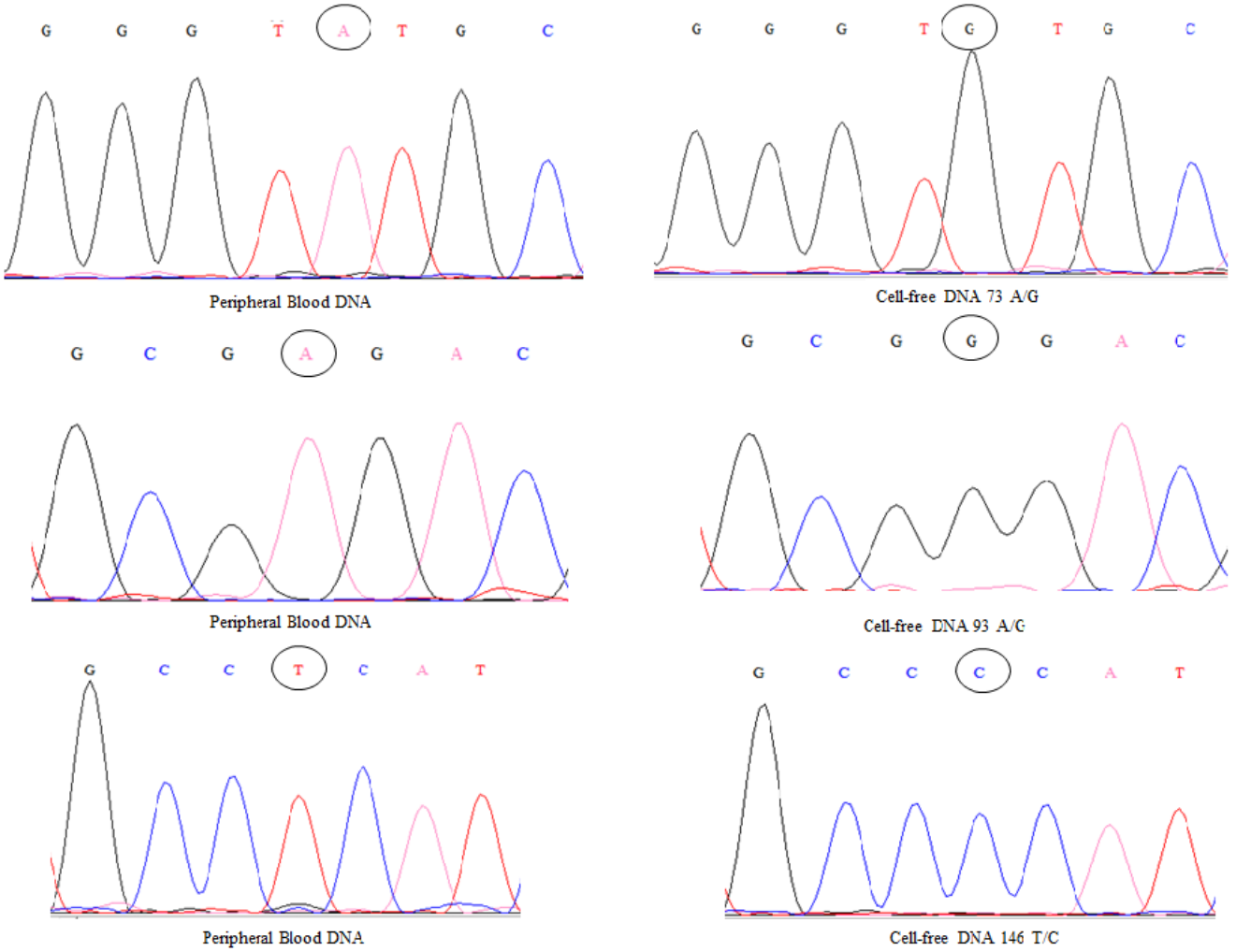

Fig. 2: Sequence analyses of the mitochondrial D-loop region. The displayed electropherogram shows the identified somatic mutation identified in cfmtDNA at positions 73(A/G), 93(G/A) and 146(T/C).

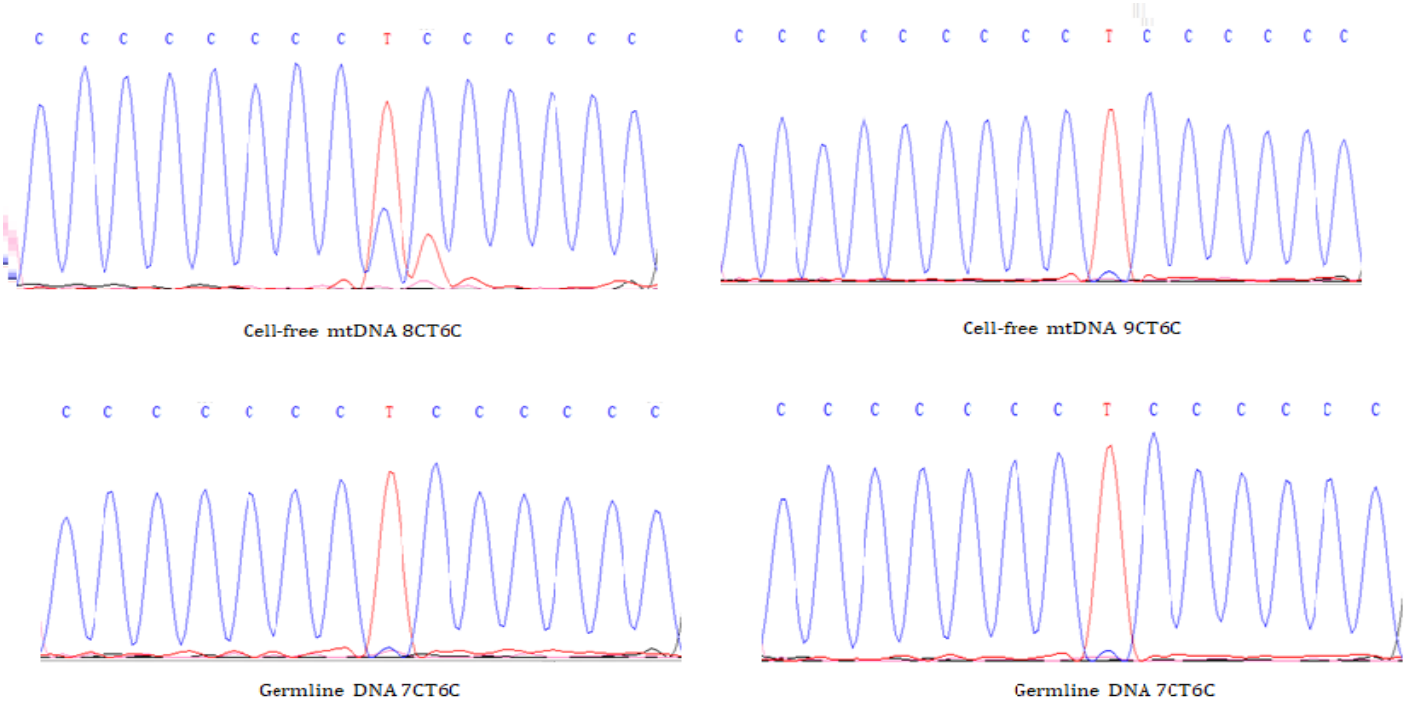

Fig. 3: Sequence analysis of the mitochondrial c-tract region. The displayed electropherogram shows the presence of an additional cytosine at position 309 in cfmtDNA, along with sequence variations of C7TC6, C8TC6 and C9TC6 in D310 region.

DISCUSSION

Mitochondria DNA mutation is one of the most common genetic alterations in cancer [15]. These alterations were reported to be frequently activated via certain nucleotide changes in the control region, also known as the D-loop region [16]. It has also been identified as a hot spot region for somatic mtDNA mutations in various types of human cancer, including breast [17], colorectal [18] and lung cancer [19]. In addition the habitual practice of consuming tobacco and betel nut in north east, India (Assam) [20] , is also considered as one of the major etiological risk factors for the development of HNSCC by increasing the level of reactive oxygen species (ROS) [21]. These ROS initiates DNA and RNA damage with carcinogenic effects in the mouth of tobacco consumers, with concomitant increased risk of 50-folds and as mtDNA lacks the protective histone layer, it's more vulnerable towards oxidative stress induced genetic variability [22]. These variability in the D-loop region has also been suggested to affect the function of the respiration chain that is responsible for high ROS levels and could contribute to cancer initiation and mutagenesis [23].

Identification of these genetic variability in the control region, till now was dependent on the availability of tumor tissue specimen [24]. Although the method of identifying somatic mutation from tumor tissue specimen has given subversive growth in identification of somatic mutation. But it is also associated with certain limitations such as availability tumor specimen during the course of treatment and heteroplasmy condition of the tumor tissue specimens (mixed population of cancerous and non-cancerous cells) [11]. To overcome these limitations in identification of Somatic mtDNA mutations, cfDNA from the blood (plasma) of individuals with cancer; may therefore serve as potential markers [25].

In our study, somatic cfmtDNA mutations within the mitochondrial d-loop region of HNSCC cases were found in almost all the patients include in this study. In the hypervariable region (HV1 16024 – 16383 and HV2 57-372), somatic mutations were harbored in the HV1 region in position 16130, 16154, 16224, 16279, 16288, 16328 and 16363. Among the identified positions, only three positions were found to be common in most of the patients (73 A-G, 93 A-G and 146 T-C) (Table 2). In addition, the d-loop sequences were also aligned along with the reference sequence, in order to determine the polymorphic sites. There were altogether 25 polymorphic positions identified (Table 3).

It is also said that in the D-loop region, a poly-C stretch (poly-C tract) termed the D310 region is more susceptible to oxidative damage and electrophilic attack compared with other regions of mtDNA [26]. Therefore we screened the poly-C stretch region to identify any mutations present in this region. Our result indicated the presence of three type of sequences in the D310 region that is C7TC6, C8TC6 and C9TC6. Further, the mutation found in our study, showed the presence of an additional cytosine nucleotide at position 309 (Fig. 3) (C7TC6 - C8TC6- C9TC6). Among different cases of HNSCC, this kind of change was observed in all the selected group of patients (low grade to high grade tumor). This indicates that the patter C7TC6 - C8TC6- C9TC6 was initiated at the onset of HNSCC and continued its prevalence till the high stage tumor grade. Therefore this change in the c-tract region can serve as a prognosis marker in early diagnosis of HNSCC.

CONCLUSION

In conclusion, our study highlights the negative effects of tobacco and betel nut consumption on mtDNA. In addition it also shows the promising potential of liquid biopsy as cfmtDNA in identification of early somatic mutations in HNSCC patients. Therefore, detecting mutations in cfmtDNA of HNSCC patients may serve as a potential marker for screening, diagnosis, prognosis and monitoring of treatment response in early detection of disease progression. This study will also minimize the reliability of biologist over tumor tissue specimens for identifying biomarkers in different disease pathogenesis.

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AUTHORS CONTRIBUTION

MK, RM and SKG conceived and designed the study, MKS contributed to the sample collection and histopathology, MK and RM contributed in writing the manuscript. YC, SKG, MK, MKS and RM contributed in proofreading of the manuscript. All authors have read and approved the final version of this manuscript.

CONFLICT OF INTEREST: None

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