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Mitochondrial DNA variation analysis in cervical cancer

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ABSTRACT

This study was undertaken to investigate the mitochondrial DNA (mtDNA) variation in non-malignant and malignant cervical tissue samples. We have identified 229 and 739 variations non-malignant and malignant tissues respectively distributed over 321 locations in the D-loop (50 in non-malignant and 166 in malignant; 216 variations), coding region (139 in non-malignant and 455 in malignant; 594 variations) tRNA and rRNA genes (39 in non-malignant and 119 in malignant; 158 variations). Besides, 77 novel and 34 various other disease associated variations were identified in non-malignant and malignant samples. A total of 236 tumor specific variations in 201 locations representing 30.1% in D-loop, 59.3% in coding regions and 10.6% in RNA genes were also identified. Our study shows that D loop (in 67 locations) is highly altered followed by ND5 (35 locations) region. Moreover, mtDNA alterations were significantly higher in malignant samples by two tailed Fisher's exact test ($P \leq 0.05$) with decreased mtDNA copy numbers. Bioinformatic analysis of 59 non-synonymous changes predicted several variations as damaging leading to decreased stability of the proteins. Taken together, mtDNA is highly altered in cervical cancer and functional studies are needed to be investigated to understand the consequence of these variations in cervical carcinogenesis and their potential application as biomarkers.

1. Introduction

Cervical cancer is the leading cause of mortality among female cancers accounting for approximately 9% (529,800) of the total new cancer cases and 8% (275,100) of mortality (www.globocan.iarc.fr). India's cervical cancer age-standardized incidence and mortality rates are 30.7 and 17.4 per 100,000 respectively which is highest in South Central Asia (Franco and Tota, 2010). HPV infection, multiple sexual partners, multiple childbirths and smoking are some of the risk factors associated with cervical cancer (Faridi et al., 2011). Despite the significance of mitochondria in tumorigenic process, information on mitochondrial DNA (mtDNA) variations is limited in cervical cancer.

With about 10^3–10^4 copies per cell, mtDNA is the target for high level of alterations in cancers (Mondal et al., 2013; Schon et al., 2012; Singh and Kulawiec, 2009). Despite the "aerobic glycolysis" hypothesis since 1920s, the interplay between nuclear and mitochondrial genes and their products is only begun to unravel. Several elegant studies have demonstrated the extent of alterations in mtDNA in cancers and how it contributes to carcinogenesis (Wallace, 2012). Outcome of many of these studies have led the investigators to suggest mitochondria as genetic harbor or have suggested that mtDNA mutations as being responsible for clonal development of tumors due to its ability to resist apoptosis by selecting for dominant mutations within the heteroplasmy. Consequently, discovery of mtDNA alterations is very attractive not only to understand the molecular mechanisms of carcinogenesis but also to use as diagnostic and prognostic markers (Lievre et al., 2005; Sharawat et al., 2010; Wallace, 2012).

High rate of mtDNA alterations could be due to several factors including absence of protective histones/non histone proteins, proximity to ROS generation site, rapid metabolic and replication rates and lack of an efficient DNA repair mechanisms (Sharma et al., 2005). Few pathways genes for base excision repair (BER), direct damage reversal, mismatch repair and recombination repair mechanisms have all been associated with mtDNA damage repair (Croteau et al., 1999; Larsen et al., 2005). It is also suggested that the mutations in mtDNA could alter the functions of respiratory chain proteins and there by contributing to increased mtDNA damage, mitochondrial genome instability and tumorigenesis (Chatterjee et al., 2006). Although few studies have reported the presence of mtDNA mutation in cervical cancer, its role in the pathogenesis is still unclear (Goia-Rusanu et al., 2011; Sharma et al., 2005; Wang et al., 2006; Zhao et al., 2010). Recently, occurrence of high incidence of somatic mutation in cervical dysplasia and cancer has been identified indicating that mtDNA alteration might be an early event in the pathogenesis of the disease in high risk HPV infected patients (Goia-Rusanu et al.,

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2011). However, information from entire mtDNA is not available for cervical cancer.

In the present study, we have (i) analyzed the entire mtDNA in non-malignant and malignant cervical tissues for sequence variations and (ii) performed detailed in silico analysis of impact of nucleotide substitution for its structure and function.

2. Materials and methods

2.1. Sample collection and DNA extraction

We collected 30 surgically resected cervical biopsy samples (10 unrelated non-malignant and 20 unrelated malignant), with informed written consent, from the Department of Obstetrics and Gynecology, Kasturba Medical College, Manipal, Karnataka, India. The non-malignant samples were cervicitis samples above 52 years of age and were free of cervical cancer. All the malignant samples were moderately differentiated squamous cell carcinoma types ranged from 45 to 68 years. Total DNA was isolated from the biopsy samples by treating the tissues with proteinase K (Sigma-Aldrich, USA) at 37 °C, followed by phenol–chloroform extraction and ethanol precipitation. DNA was dissolved in 1XTE buffer (pH8.0) and stored at −20 °C until used. The quality and quantity of the DNA was assessed on agarose gel and Nanodrop ND-1000 (Thermo scientific, USA).

2.2. HPV genotyping

HPV genotyping was performed as published previously by nested PCR using PGMY09/11 and GP5+/GP6+ primers (Evans et al., 2005; Gravitt et al., 2000). Positive samples were purified by Exo-SAP and sequenced using Big dye terminator kit in 3130 Genetic analyzer (ABI, USA). The HPV strains were identified by NCBI BLAST search.

2.3. Polymerase chain reaction and DNA sequencing

We sequenced complete mtDNA using previously defined 24 pairs of primers to generate 24 overlapping fragments (Rieder et al., 1998). Each PCR reaction (25 μl) contained 50 ng of genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10pmol each of sense and anti-sense primers and 1 U of Taq DNA polymerase (Finnzymes) in VERITI 96 well thermal cycler (Applied Biosystems, Foster City, CA). Purified PCR products were sequenced as described above. Electropherogram were read manually to identify the alterations present at low level. The sequences were aligned and compared with the revised Cambridge Reference Sequence (rCRS) using ClustalX standalone software. The observed variations were further analyzed by using Mitomap (http://www.mitomap.org), mtDB (www.mtdb.igp.uu.se) and HmtDB (www.hmtdb.uniba.it/). Variations are considered as novel if it is not reported previously in mtDB and/or hmtDB databases.

2.4. mtDNA copy number analysis

The mtDNA copy number was calculated using 378 bp fragment of the mitochondrially encoded cytochrome b (MT*CYB*) gene (sense primer: TGAAACTTCGGCTCACTCCT; anti sense primer: AATGTATGGG ATGGCGGATA) and 304 bp fragment of the nuclear encoded hemoglobin, beta (*HBB*) gene (sense primer: ACGTGGATGAAGTTGGTGGT; anti sense primer: GAGCCAGGCCATCACTAAAG) using SYBR green QPCR assay in ABI 7500 Fast (Applied Biosystems, USA) real time PCR instrument according to manufacturer's instruction. The values for the threshold for cycle number (Ct) for MT*CYB* and *HBB* were determined. mtDNA copy number/cell was calculated by using the formula $2 \times 2^{-\Delta Ct}$ [wherein ΔCt represents the Ct (MT*CYB*) − Ct (*HBB*)]. All the samples were run in triplicate. For each PCR run separate standard curves were generated using serially diluted plasmid DNA (pFM11) containing PCR products for both *HBB* and MT*CYB* gene fragments (pFM11 plasmid was generous gift from Dr. Phillip Nagley) (Miller et al., 2003).

2.5. Bioinformatic analysis

We have used Circos tool to plot the mtDNA variation across the mitochondrial genome (Krzywinski et al., 2009). The potential impact of non-synonymous substitutions was analyzed by PolyPhen and Pmut (http://www.ics.uci.edu/~baldig/mutation.html) and MuStab tool (Teng et al., 2010). The Protein Homology/analogY Recognition Engine V 2.0 and Project HOPE was used for building the three dimensional structure of the wild type and mutant proteins and to identify the impact of nucleotide substitution on protein structure (Kelley and Sternberg, 2009; Venselaar et al., 2010). The three dimensional structure analysis and visualization was performed by Chimera tool (Pettersen et al., 2004). Fisher's exact test was used for testing the significance differences in mtDNA variations between non-malignant and malignant samples. A p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Analysis of the entire mtDNA in cervical cancer patients

The analysis of the complete mtDNA genome in 20 cervical cancer and 10 non-malignant samples revealed 968 variation (229 and 739 in non-malignant and malignant samples respectively) which are distributed over 321 locations in the D-loop (216 variations), coding region (594 variations) and RNA (158 variations) (Fig. 1, Table 1, Tables S1–S3). We have identified 129 disease associated variations in 34 locations (Table 2). Furthermore, 77 positions showed novel variations which have not been reported in mtDB and/or HmtDB databases. The majority of the variations being single base substitution and few were one to two base deletions and insertions respectively. Furthermore, 240 tumor specific variations were also identified which are distributed in 201 positions (Tables 1–2 and Tables S1–S3). D loop was found to be highly altered (81 locations) followed by ND5 (35 locations), COI (26 locations), CytB (24 locations), ND2 (22 locations), ATP6 (20 locations),16s rRNA (18 locations), ND1 (15 locations), ND4 (15 locations), tRNA (12 locations), COIII (11 locations), 12s rRNA (10 locations), COII (7 locations), ND3 (7 locations),ND6 (7 locations), ND4L (6 locations) and ATP8 (3 locations) respectively (Fig. 2A and B).

3.2. D-loop and mtDNA variations

In D-loop, we have identified 216 alterations (50 in non-malignant and 166 in malignant samples) distributed over 81 locations (32 and 67 locations in non-malignant and malignant samples respectively). Further, 29 novel and 4 diseases associated variation were also identified (Table S1). Of the 216 variations, 179 were of single base substitutions (SBS), 30 were insertions (23 single base pair and 5 two base pair insertions respectively) and 7 were 2 bp deletion. D-loop variations at the nucleotide positions T16172T (5 out of 20; 25%) was found only in tumor samples. Additionally, the D310 mononucleotide repeat and polycytidine stretches (16183–16193) alteration were also observed in the samples analyzed. Moreover, addition or disruption of nucleotide which leads to new stretches of nucleotides of similar kinds (homopolymeric tracts) was also common in cervical cancer samples. In addition, significantly higher number of positions were found to be altered in malignant samples (67 out of 81) as opposed to non-malignant (32 out of 81) ones by two tailed Fisher's Exact test ($P \leq 0.0001$). The results are summarized in Fig. 2A and 2B.

3.3. Ribosomal RNAs and transfer RNA variation in mtDNA

In total, we have identified 27 rRNA location as altered in 30 samples sequenced which included 10 and 17 in 12s rRNA and 16s rRNA

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