Altered mitochondrial DNA copy number in cervical exfoliated cells among high-risk HPV-positive and HPV-negative women

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Abstract. The majority of cervical cancer cases are due to human papillomavirus (HPV) infection. However, certain cases of cervical cancer are not caused by HPV. Recent studies have shown a link between altered mitochondrial DNA (mtDNA) copy number, an indicative measure of mitochondrial dysfunction, and cervical cancer in women who test positive for HPV. However, the role of the mtDNA copy number in HPV-negative cervical cancer has remained elusive. In the present study, the mtDNA copy number was determined using quantitative PCR as the ratio between mtDNA and nuclear DNA in 287 ThinPrep cervical samples, including 143 cases with cervical abnormalities and 144 control subjects with high-risk (hr)-HPV positive or HPV-negative status. In an overall analysis of cases categorized based on the cytology diagnosis into squamous cervical carcinoma/high-grade squamous intraepithelial lesions (SCC/HSIL), low-grade squamous intraepithelial lesions (LSIL) and normal controls, the mtDNA copy number was significantly higher in all cases compared to the controls and a higher mtDNA copy number was observed in SCC/HSIL compared to LSIL cases. In the stratification analyses based on hr-HPV positive and HPV-negative status, an increased mtDNA copy number was observed in the cases compared with the controls regardless of their HPV status ($P<0.05$). When cases with cervical abnormalities were categorized based on histological diagnosis into cervical intraepithelial neoplasia (CIN)2/CIN3 and CIN1, an overall analysis indicated an increased mtDNA copy number in CIN2/CIN3 compared to CIN1 ($P=0.01$). Stratification analyses of these cases based on HPV status revealed a higher mtDNA copy number in CIN2/CIN3 compared to CIN1 regardless of HPV infection ($P<0.05$). These results showed that an elevated mtDNA copy number in subjects with cervical abnormalities was not influenced by the HPV status and suggested the possibility of its role in the progression of cervical cancer. The increased mtDNA copy number may be an adaptive response mechanism to compensate for mtDNA oxidative stress and energy deficiency, possibly induced by HPV infection and other environmental exposures.

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Introduction

Cervical cancer is the fourth most common malignancy and the leading cause of cancer-associated death in women worldwide (1,2). In the Gulf Cooperation Council States, which include Bahrain, Kuwait, Oman, Saudi Arabia, Qatar and the United Arab Emirates, cervical cancer is the ninth most prevalent female malignancy and the number of cases in these countries is expected to increase in the coming years (3). Several risk factors have been linked to cervical cancer, such as human papillomavirus (HPV), early age of sexual intercourse, multiple sexual partners, oral contraceptive use and smoking (4-6). However, since the isolation of HPV 6 DNA from human genital warts in 1980 (7), HPV has been identified as the major causative agent for the development of cervical cancer. The majority of cases of cervical cancer are caused by persistent HPV infection, involving 15 genotypes of high-risk HPV (hr-HPV) (8). In these cases, HPV vaccination has been proven to be safe and effective as a primary preventative strategy for cervical intraepithelial neoplasia (CIN) and cervical cancer (9). A secondary preventative method includes regular screening and prompt treatment for precancerous lesions (9,10). The primary methods of screening for cervical cancer include a Pap smear, visual inspection with acetic acid and Lugol's iodine, liquid-based cytology and HPV testing (10). In addition to cytology-based tests, HPV testing is a pivotal method for cervical cancer screening, allowing clinicians to assess cells for infection with hr-HPV (10). The use of cytology-based testing and HPV testing together or simultaneously has assisted in guiding women who are at risk of developing cervical cancer but has also led to an increase in the number of referral cases to colposcopy clinics, in particular, cases with low-grade cytological scores and hr-HPV infection (11,12). Several countries have adopted

the use of cytology-based testing and HPV testing, including Australia, The Netherlands, United Kingdom, Canada and Turkey (11,12).

In addition to HPV-positive cervical cancer, studies have shown that ~5% of cervical cancer cases are not associated with HPV infection (13-16). Thus, the new World Health Organization Female Genital Tumors classification subdivided cervical squamous and adenocarcinomas into HPV-associated and HPV-independent tumors (17). HPV-negative cervical cancer represents a biologically different subset of tumors with a distinct pathogenic pathway and a poorer prognosis than HPV-positive cervical cancer (18). Furthermore, HPV-negative and HPV-positive cervical cancers show differences in molecular profiles and no specific therapeutic strategies have been developed based on HPV status (19).

Dynamic genetic alterations are the primary causative mechanisms in the initiation and progression of human cancer (20). In addition to nuclear changes, the role of mitochondrial DNA (mtDNA) has been widely investigated in various types of cancers, including cervical cancer (21-24). The mitochondria generate the majority of cellular energy through the oxidative phosphorylation (OXPHOS) system and are also involved in other cellular functions, such as the regulation of cell death and generation of reactive oxygen species (ROS). The human mtDNA is a 16.6 kb circular double-stranded DNA that contains 37 genes coding for proteins essential for cellular respiration and normal mitochondrial function (25). mtDNA is present in multiple copies per cell, ranging from 1,000-10,000, and the mtDNA copy number varies by cell type (26). Several factors make the mtDNA particularly susceptible to oxidative stress, including its proximity to the electron transport chain, lack of histone protection and reduced DNA repair capacity (27). Thus, mtDNA is a particularly susceptible target of ROS, and this may result in mutations or copy number alterations (27). Changes in mtDNA copy number can potentially lead to a decrease in mitochondrial function with increased ROS production (27,28). Variation in the mtDNA copy number has been reported in a wide range of pathological conditions, such as neurodegenerative diseases (29,30), autoimmune diseases (31) and different types of cancer (32,33). Certain previous studies have indicated an association between mtDNA copy number and the development of cervical cancer. Sun *et al* (34) reported an increase in mtDNA copy number in exfoliated cervical cells of women who tested positive for HPV and hypothesized an association between mtDNA copy number and cervical carcinogenesis. A study by Warowicka *et al* (23) showed an association between increased mtDNA copy number (and mtDNA mutations) and cervical cancer development. However, these studies focused only on HPV-positive cervical cancer cases, and thus far, no studies have investigated the mtDNA copy number in HPV-negative cervical cancer. Therefore, the aim of the present study was to examine the changes in mtDNA copy number among HPV-positive and HPV-negative cervical cancer cases. Knowledge regarding mtDNA copy number alterations in HPV-positive and HPV-negative cervical cancer may assist in understanding the role of mtDNA in the pathogenesis of the disease.

Patients and methods

Sample collection. A total of 287 ThinPrep cervical samples were collected from female patients who attended the Obstetrics and Gynecology outpatient clinic at the Maternity Hospital (Al-Sabah Health Area, Kuwait) and Mubarak Al Kabeer Hospital (Jabriya, Kuwait) between January 2022 and January 2023, and the samples were analyzed in the hospitals' Cytology Laboratory. Informed consent was obtained from all participants and the study was approved by the Health Science Center Ethics Committee at Kuwait University, Kuwait and the Ministry of Health the Standing Committee for Coordination of Health and Medical Research, Kuwait (no. VDR/EC/3746). Samples were prepared and results were reported using The Bethesda System 2014 guidelines (35). Samples with abnormal cytological results (n=143) included the following: Squamous cell carcinoma (SCC), high-grade squamous intraepithelial lesion (HSIL) and low-grade squamous intraepithelial lesion (LSIL). Samples with SCC and HSIL were categorized together as the SCC/HSIL group. Follow-up histological information for cases with cervical abnormalities was recorded when possible. Histological reports of SCC/HSIL included the following diagnosis: CIN grade 1 (CIN1), CIN2 and CIN3. Cases with LSIL with two sequentially abnormal cytology reports were also referred for histological analysis. The median age of the cases group was 39 years (age range, 19-79 years).

Control samples (n=144) were without any intraepithelial lesions or malignancy. The HPV status (HPV-positive and HPV-negative) for both cases and controls was determined by genotyping. The median age of the control group was 38.5 years (age range, 19-64 years).

DNA extraction. DNA was isolated from cervical cells using the MagNA Pure LC DNA Kit I (Roche Diagnostics GmbH), based on magnetic-bead technology, and it was performed using an automated MagNA Pure LC Instrument (Roche Diagnostics GmbH). In brief, the sample was mixed with a lysis/binding buffer for DNA lysis. Proteinase K was added to complete the digestion of protein. Magnetic Glass Particles (MGPs) were added for DNA binding to the surface and unbound substances were discarded by several washing steps. The DNA was eluted using a low salt buffer and MGPs. The DNA samples were checked for purity using a NanoDrop 1000 system (Thermo Fisher Scientific, Inc.) and the DNA concentration was determined using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Inc.).

Detection of HPV in cervical samples. HPV DNA in cervical samples was detected by reverse transcription-quantitative (q)PCR using two different sets of primers, MY09/MY11 (MY09 reverse, 5'-CGTCCMARRGGAWACTGATC-3' and MY11 forward, 5'-GCMCAGGGWCACAAYAATGG-3') and Gp5+/Gp6+ (Gp5+ forward, 5'-TTTGTTACTGTGGTAGAT ACTAC-3' and Gp6+ reverse, 5'-GAAAAATAAACTGTA AATCATATTC-3') (custom design; Thermo Fisher Scientific, Inc.), as described previously (36). Samples with positive PCR amplification results were subjected to a conventional PCR assay then Sanger-based sequencing analysis was carried out using MY09/MY11 and Gp5+/Gp6+ universal primers. The sample results were analysed using in-house Sequencing

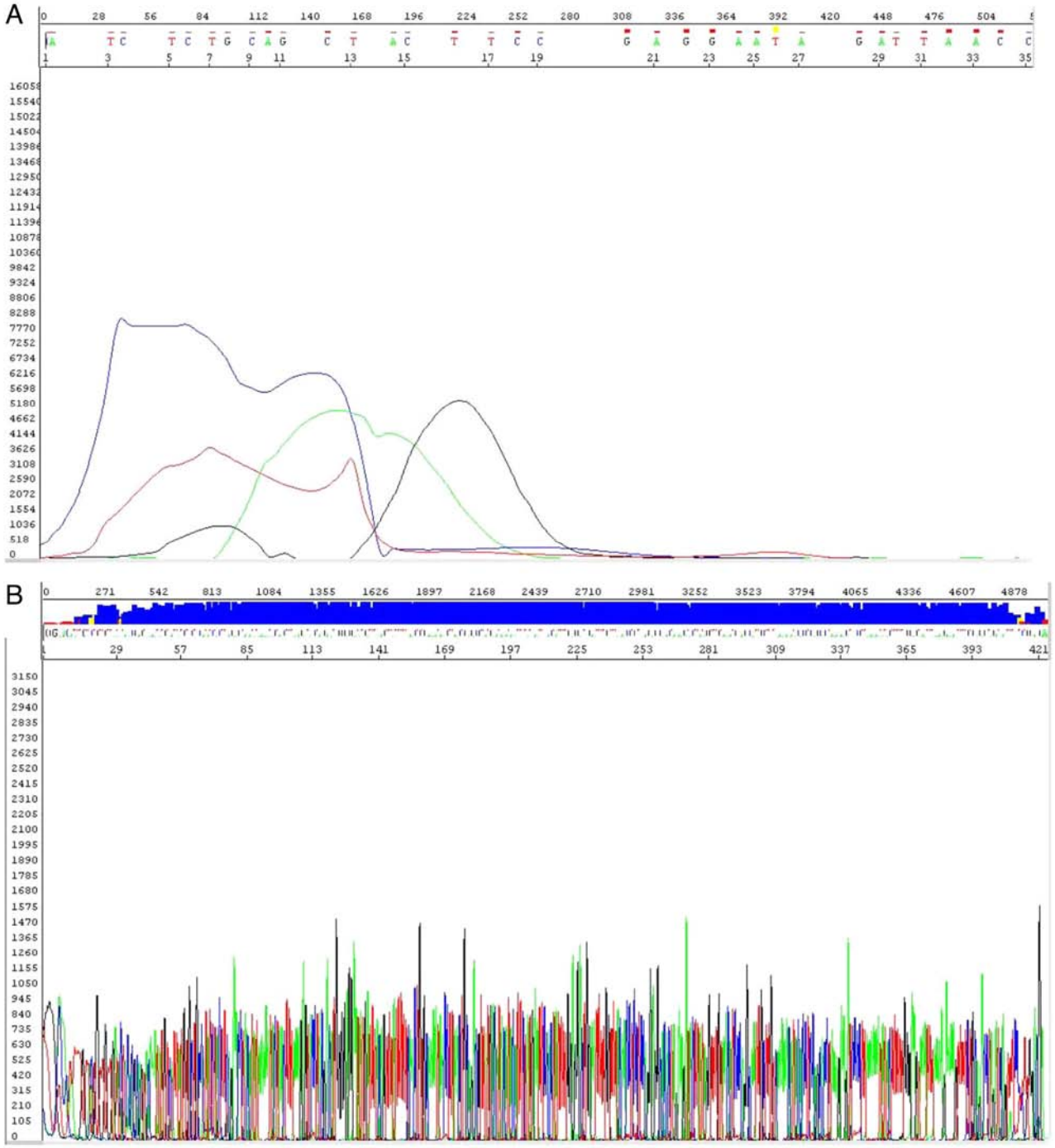

Figure 1. Sanger sequencing chromatograms of the L1 region of the HPV genome amplified by universal primers. (A) Negative result: Absence of specific sequences. (B) Positive result for HPV16 with the presence of high-quality sequences. The specificity of sequences was confirmed using BLAST, where similarity with HPV sequences was >98%. HPV, human papillomavirus.

Analysis Software version 3.7 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The HPV genotypes were identified using the BLASTn Software version 2.13.0 (http://www.ncbi.nlm.nih.gov/blast/htlm) and the Los Almos Data National Laboratory Theoretical Biology and Biophysics HPV database (https://pave.niaid.nih.gov/).

A total of 17 different HPV genotypes were detected in the samples, including 11 hr and six low-risk (lr) genotypes. hr-HPV included the following genotypes: HPV16, HPV18, HPV31, HPV33, HPV35, HPV45, HPV53, HPV56, HPV58, HPV66 and HPV73. lr-HPV included the following genotypes: HPV6, HPV11, HPV83, HPV90, HPV102 and HPV106. Only hr-HPV cases were included in the further analysis. Representative Sanger sequencing chromatograms of an HPV-negative and HPV16-positive sample are provided in Fig. 1A and B, respectively.

Determination of mtDNA copy number. qPCR was used to determine the mtDNA copy number relative to nuclear DNA (nDNA) in cervical exfoliated cells. The mtDNA-encoded human NADH dehydrogenase subunit 2 (mt-ND2) was used as a target gene and the nuclear β2-macroglobulin (β2-M) was

Table I. Demographic and clinical characteristics of study groups.

Variables	Cases (n=143)	Control (n=144)	P-value
Age, years	38.2±10.4	36.9±10.3	0.16
HPV status			0.47[a]
Positive	49 (34)	44 (31)	
Negative	94 (66)	100 (69)	
Cytology diagnosis	143	n/a	
SCC/HSIL	28 (20)	n/a	
LSIL	115 (80)	n/a	
Histology diagnosis	56	n/a	
CIN2/3	18 (32)	n/a	
CIN1	38 (68)	n/a	

[a]χ^2 test. Values are expressed as the mean ± standard deviation or n (%). n/a, not applicable; SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

used as a reference gene. Amplification was performed using the following primers: mt-ND2 forward, 5'-CACAGAAGC TGCCATCAAGTA-3' and reverse, 5'-CCGGAGAGTATA TTGTTGAAGAG-3'; β2-M forward, 5'-CCAGCAGAGAAT GGAAAGTCAA-3' and reverse, 5'-TCTCTCTCCATTCTT CAGTAAGTCAACT-3'. The qPCR was set up according to the manufacturer's instruction and optimized for >95% amplification efficiency using the SYBR1 Green PCR MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc.) as described previously (37). Reactions were run in duplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a non-template control was included in each run. The following thermocycling program was used: 1 cycle of 50˚C for 2 min, 1 cycle of 95˚C for 10 min, and 30 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative mtDNA copy number was calculated from the quantification cycle (Cq) values of the target and reference genes using the $2^{-\Delta\Delta Cq}$ method (38).

Statistical analysis. Statistical analysis was performed using SPSS version 20.0 (IBM Corp.). The distribution of data was assessed using a Kolmogorov-Smirnov test. Continuous variables were compared between cases and controls using a Student's t-test. The χ^2 test was used to determine statistical differences between categorical variables. Multiple groups were compared by using one-way ANOVA followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference. Graphs were plotted using GraphPad Prism version 5.0 (GraphPad Software; Dotmatics). The data are presented as the mean ± standard deviation (SD) or n (%).

Results

Demographic and clinical characteristics of study subjects. The demographic and clinical characteristics of the study subjects, including 143 cases with cervical abnormalities and 144 controls, are presented in Table I. There was no statistically significant difference in the respective mean ± SD and median age of cases (38.2±10.4; 39 years) and controls (36.9±10.3; 38.5 years; P=0.16). The distribution of the HPV genotypes, which included hr-HPV positive and HPV-negative, was also assessed. In the group of cases with cervical abnormalities, 49 (34%) were hr-HPV positive and 94 (66%) were HPV-negative. In the control group, 44 (31%) were hr-HPV positive and 100 (69%) were HPV-negative. There were no significant differences between the cases and controls among hr-HPV positive subjects (P>0.05). In terms of cytological diagnosis, 28 (20%) of the cases had SCC/HSIL and 115 (80%) had LSIL. SCC/HSIL cases, as well as LSIL cases with two sequential abnormal cytological reports, were further analysed based on the histological diagnosis. Histology diagnoses were available for 56 cases, including 18 (32%) CIN2/3 and 38 (68%) CIN1. Among the 28 SCC/HSIL cases, 18 (64%) had CIN2/3 and 10 (36%) had CIN1, and among the 115 LSIL cases 28 (24%) had CIN1.

mtDNA copy number in squamous intraepithelial lesions and controls. In the overall analysis, the mtDNA copy number was compared between cases with cervical abnormalities and controls. Cases were categorized based on the cytological diagnosis into SCC/HSIL and LSIL. As presented in Fig. 2, the mtDNA copy number was significantly higher in SCC/HSIL cases (788.8±102) and LSIL cases (399.4±37.5) compared with the controls (296.6±36.4; P<0.001 and P=0.02, respectively). Furthermore, cases with SCC/HSIL had a significantly higher mtDNA copy number compared with the cases with LSIL (P=0.0001).

mtDNA copy number in squamous intraepithelial lesions and controls based on HPV status. In subsequent analyses, SCC/HSIL and LSIL cases and controls were subdivided based on HPV status into hr-HPV-positive and HPV-negative. The mtDNA copy number was evaluated in these groups. In the hr-HPV-positive samples (Fig. 3A), the mtDNA copy number was significantly higher in SCC/HSIL cases (642.1±53.0) and LSIL cases (496.4±69.0) compared

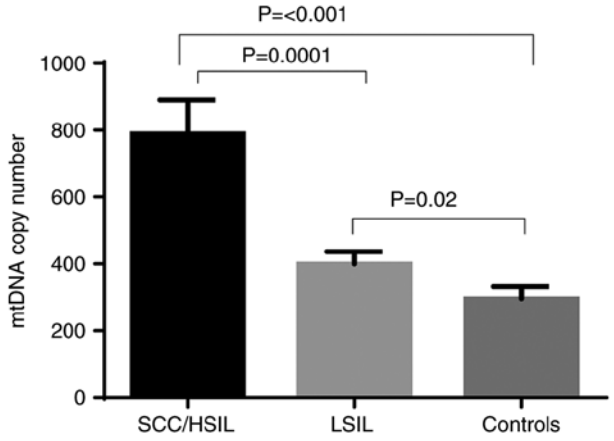

Figure 2. mtDNA copy number in cervical samples categorized based on cytology diagnosis as SCC/HSIL or LSIL compared to controls. HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; mtDNA, mitochondrial DNA.

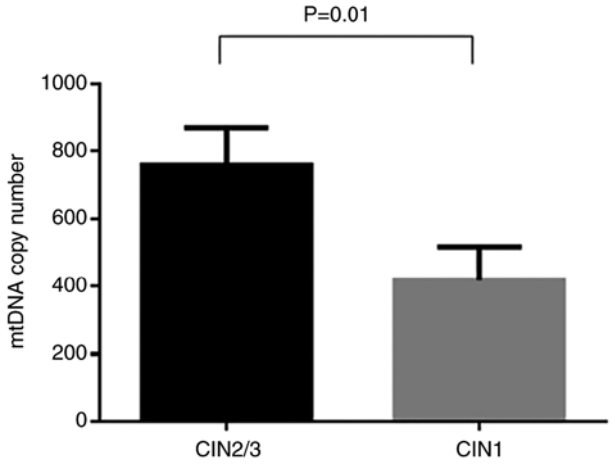

Figure 4. mtDNA copy number in cervical samples categorized based on histology diagnosis as CIN2/CIN3 or CIN1. CIN, cervical intraepithelial neoplasia; mtDNA, mitochondrial DNA.

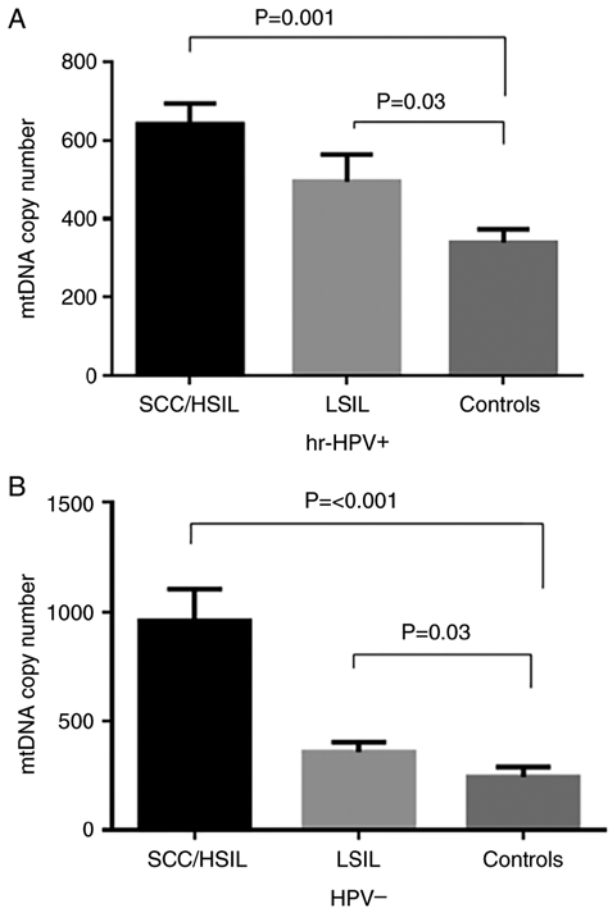

Figure 3. mtDNA copy number in squamous intraepithelial lesions with or without HPV infections. (A) Samples with HPV+, (B) Samples with HPV-. hr-HPV, high-risk human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; mtDNA, mitochondrial DNA.

with the controls (339.0±34.0; P<0.001 and P=0.03, respectively). Similar results were observed in the HPV-negative samples. A significant increase in mtDNA copy number was observed in SCC/HSIL cases (958.0±145.5) and LSIL cases (358.7±44.0) compared with the controls (244.7±46.0; P<0.001; Fig. 3B).

mtDNA copy number in squamous intraepithelial lesions of different histological stages. The mtDNA copy number in cases with squamous intraepithelial lesions based on their histological diagnosis (CIN1, CIN2 and CIN3) was determined. In the overall analysis (Fig. 4), the mtDNA copy number was significantly higher in CIN2/CIN3 cases (759.6±110.0) compared with the CIN1 cases (417.8±99.0; P=0.01).

mtDNA copy number in squamous intraepithelial lesions of different histological stages based on HPV status. Subsequent stratification analyses were performed by dividing CIN2/CIN3 and CIN1 cases based on HPV status into hr-HPV-positive and HPV-negative. Subsequently, the mtDNA copy number was compared between the groups. In hr-HPV-positive samples (Fig. 5A), there was a significant increase in the mtDNA copy number in cases with CIN2/CIN3 (722.0±127.0) compared to CIN1 (465.0±65.0; P=0.04). In HPV-negative samples (Fig. 5B), there was also a significant increase in the mtDNA copy number in cases with CIN2/CIN3 (693.0±123.0) compared to cases with CIN1 (418.0±73.0; P=0.02).

Discussion

Mitochondria, the critical organelles involved in various cellular functions, have their own multicopy genome (mtDNA). The regulation of the mtDNA copy number is significant for *maintaining the cellular energy* needs; thus, the mtDNA copy number is considered as an indicator of mitochondrial activity and function (39). Alterations in the mtDNA copy number have been reported in several human diseases such as Parkinson's disease, rheumatoid arthritis, multiple sclerosis and cancer (29-31,40). In particular, the role of mitochondria in tumor promotion and development has been widely investigated, and an altered mtDNA copy number has been shown to impact numerous cellular pathways associated with cancer. Alteration of mtDNA copy number may cause elevation of mtDNA oxidative stress and disruption of mtDNA gene

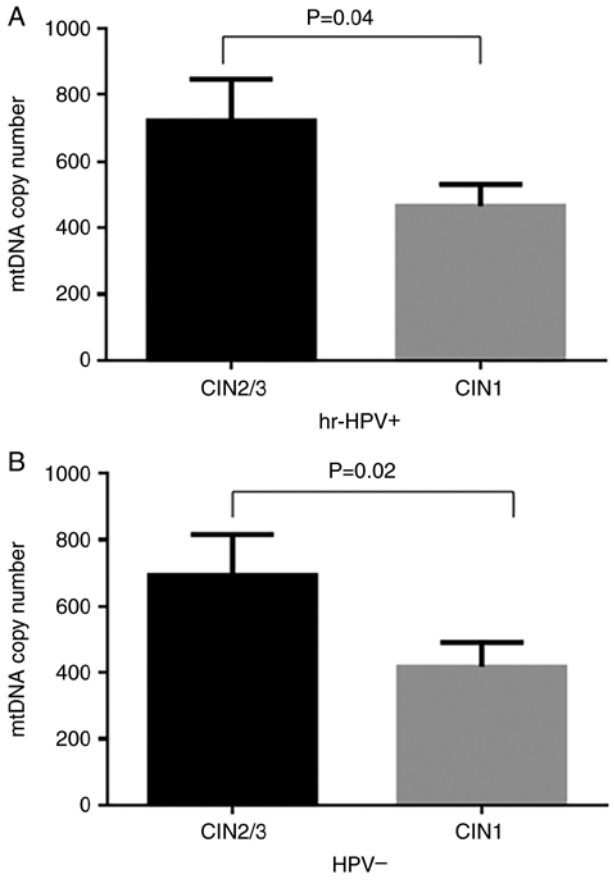

Figure 5. mtDNA copy number in cervical intraepithelial neoplasia with and without HPV infection. (A) Samples with hr-HPV+; (B) samples with HPV-. hr-HPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia; mtDNA, mitochondrial DNA.

expression. As a result, the overall mtDNA functions may be affected, including the OXPHOS system, ROS production, signal transduction, cell apoptosis and cell growth. Therefore, disturbances in the OXPHOS system may result in a reduction of intracellular ATP, which triggers glycolysis to compensate for the total ATP. Thus, the aberrant mtDNA copy number may reduce the rate of mitochondrial biogenesis and normal cellular function that eventually trigger tumorigenesis (41).

Previous studies have reported an increase or decrease in mtDNA copy number in different types of cancer (32,33,41), suggesting that the effects of alterations in mtDNA copy number may be cancer-specific, dependent on the energy needs of the specific cancer tissue. Biologically, a decrease in mtDNA copy number may result in a reduction in OXPHOS capacity, which triggers a compensatory increase in glycolysis, resulting in the disruption of cellular functions. A decrease in mtDNA copy number also promotes resistance to apoptosis of cancer cells and increases their sensitivity to chemotherapeutic drugs (42). Conversely, a high mtDNA copy number has been proposed as a marker for oxidative stress and impairment of aerobic pathways involved in the molecular mechanisms of carcinogenesis (27). In previous studies, an increase in mtDNA copy number was shown to be associated with the risk (31) and development (23) of cervical cancer. However, these studies focused only on cervical cancer cases who tested positive for HPV, the most common causative agent of cervical carcinogenesis (7,8). To date, no studies have explored the role of mtDNA copy number in HPV-negative cervical cancer, which occurs in ~5% of cases (13-16), to the best of our knowledge. No HPV-negative cervical cancer cases were included in the present study and all four squamous cervical carcinoma cases were HPV-infected, this could be explained by the small sample size of abnormal cases in this study, which could be avoided in future studies. The inclusion of only HPV-positive carcinoma cases is in line with previous mtDNA copy number studies (22,23,34). In the present study, the changes in mtDNA copy number in HPV-positive and HPV-negative cases with cervical abnormalities and controls were assessed.

In the overall analysis, the mtDNA copy number in cases with cervical abnormalities categorized based on cytological diagnosis into SCC/HSIL or LSIL, was compared with that in the controls. It was found that the mtDNA copy number was significantly higher in cases with SCC/HSIL and LSIL compared to the controls. Furthermore, cases with SCC/HSIL displayed a significantly increased mtDNA copy number compared with cases with LSIL. Stratification of SCC/HSIL and LSIL cases based on HPV status into hr-HPV+ and HPV-indicated that the mtDNA copy number was significantly higher in cases with SCC/HSIL compared to LSIL cases with and without HPV infection. These results suggest that the increase in mtDNA copy number was not influenced by HPV status in patients with different types of cervical abnormalities. In addition, the mtDNA copy number was compared among abnormal cervical cases with different stages. When these cases were stratified based on histological diagnosis into CIN2/CIN3 and CIN1, the mtDNA copy number was significantly higher in cases with CIN2/CIN3 compared to CIN1. Stratification of CIN2/CIN3 and CIN1 cases based on HPV status showed that the mtDNA copy number was significantly elevated in CIN2/CIN3 cases compared to CIN1 cases with and without HPV infection. These results suggested that a higher mtDNA copy number may be associated with the progression of cervical cancer, regardless of the HPV status.

These results of a higher mtDNA copy number in cases with cervical abnormalities are in agreement with those in previous studies, which reported an elevated mtDNA copy number in women with cervical cancer who tested positive for hr-HPV (34) and in SCC/HSIL compared to LSIL (23), whereas a low mtDNA copy number was reported in 20 cervical cancer tissues compared to 10 cervicitis samples (22). The small sample size in the study by Kabekkodu *et al* (22) may explain the discrepancy between their findings and the findings of other studies, including the present study.

The results of the present study extend on the previous above-mentioned findings of an elevated mtDNA copy number in HPV-positive cervical cancer cases (23,34), indicating that the mtDNA copy number in cervical abnormalities without HPV infection was also higher.

HPV consists of a circular, double-stranded DNA of ~8 Kb pairs in size and encodes six early genes (E1, E2, E4, E5, E6 and E7) responsible for DNA maintenance, replication and transcription, as well as two late genes (L1 and L2) that constitute the viral capsid (4). Following HPV infection, the early genes E1, E2, E4, E5, E6 and E7 are expressed and

the viral DNA replicates from free DNA in the basal cells at the cervix and integrates into the host genome. As the infection progresses, upregulation of the E6 and E7 oncogenes occurs (43), and these two viral oncoproteins are necessary for malignant conversion (44). Studies have indicated that oncoproteins E6 and E7 of hr-HPV may induce a chronic oxidative stress response that increases the susceptibility to DNA damage (45,46). A previous report by Warowicka *et al* (23) showed higher mtDNA copy number and increased ROS generation during cervical cancer development.

Therefore, HPV infection may contribute to an increase in mtDNA copy number in cervical cancer. Conversely, ROS and free radicals contribute to changes in the mtDNA copy number and mtDNA integrity in human cells, and the increased mtDNA copy number is considered a biomarker of oxidative stress (27). Therefore, the observed increase in mtDNA copy number in the absence of HPV infection may suggest that oxidative stress associated with environmental exposure to pollutants, tobacco, smoke or radiation also has an influence on the elevated mtDNA copy number. In particular, the increased mtDNA copy number has been suggested as an adaptive response mechanism to compensate for oxidatively damaged mtDNA and energy deficiency. The mtDNA is preferentially clonally amplified by making more mitochondria and mtDNA to meet the energy demand of cells (27,28). These observations suggest that an increase in mtDNA copy number in cases with cervical abnormalities with and without HPV infection may be a consequence of impaired mitochondrial function.

A long-term follow-up study revealed that ~40% of CIN2 lesions progress to cervical cancer (47). In addition, women diagnosed with HSIL and CIN2 may have either an early progression that requires surgical intervention or a productive hr-HPV infection that may regress without treatment (48). Therefore, early detection is highly beneficial to prevent the progression of CIN2 lesions to cervical cancer. The current protocol followed by the Ministry of Health in Kuwait is a referral of women primarily diagnosed with cytologically abnormal lesions and positive hr-HPV infection for colposcopy. Applying this protocol to the present study, 49 women (34%) with hr-HPV positive results and abnormal cytology will be referred to colposcopy, including cases with LSIL. Nevertheless, the positive predictive value of HPV testing is <50% for high-grade lesions (49). Furthermore, there is no suitable screening test for HPV-negative cervical cancer and the identification of these cases is essential for the proper management of patients. The mtDNA copy number has been proposed as a potential biomarker in several diseases, including different types of cancers, such as breast cancer, colorectal cancer, cervical cancer and head and neck cancer (33,50,51).

Despite the small sample size in the present study, the results of increased mtDNA copy number in cases with cervical abnormalities may suggest a potential utility of the mtDNA copy number as a biomarker, particularly for HPV-negative cases. Further validation of these results in a larger sample size may highlight a novel avenue for the clinical utility of mtDNA copy number as a biomarker for the early detection of cervical lesions that are at a high risk of progression to cervical cancer.

In conclusion, the current study investigated, for the first time, changes in mtDNA copy number in cases of cervical abnormalities and controls with both hr-HPV-positive and HPV-negative status. The results of the present study revealed that the mtDNA copy number was higher in SCC/HSIL and LSIL cases compared to controls, as well as in SCC/HSIL cases compared to LSIL cases, and the increase in mtDNA copy number was not influenced by the HPV status in different types of cervical abnormalities. These results also showed an increase in mtDNA copy number in cases with CIN2/CIN3 compared to CIN1 for both hr-HPV positive and HPV-negative, suggesting that a higher mtDNA copy number may be involved in the progression of cervical cancer regardless of HPV status. The mtDNA copy number thus deserves further investigation for its role in cervical cancer and as a disease biomarker in a larger cohort.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to ethical restriction rules but are available from the corresponding author on reasonable request.

Authors' contributions

RAA, MSA and SAW conceived the study. MSA and MA developed the methodology. MSA and RAA performed data analysis. MSA, SAW and RAA confirmed the authenticity of all the raw data. RAA and SAW provided resources. RAA, MSA and MR curated data. MSA and RAA wrote the manuscript. RAA, SAW and MSA revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Health Science Center Ethics Committee at Kuwait University (Jabriya, Kuwait) and the Ministry of Health the Standing Committee for Coordination of Health and Medical Research (Safat, Kuwait) and registered under no. VDR/EC/3746. Written informed consent was obtained from all subjects involved in the study and all patients gave permission for the use of the remaining cytology material for research purposes.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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