Unveiling mitochondrial DNA copy number alterations: Insights into progression from cervical intraepithelial neoplasia to cervical cancer

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Abstract. Mitochondrial dysfunction has been increasingly implicated in carcinogenesis, with alterations in mitochondrial DNA (mtDNA) copy number reported across various cancer types. However, the role of mtDNA copy number changes in the progression from cervical intraepithelial neoplasia to invasive cervical cancer remains insufficiently characterized. The present study aimed to elucidate the association between mitochondrial DNA copy number (mtCN) variations and the progression of cervical intraepithelial neoplasia (CIN) to cervical cancer, and to evaluate the potential of mtCN as a biomarker for cervical cancer risk stratification. A cohort of 100 participants from the Gynecology and Obstetrics Clinic of Ankara Etlik City Hospital (Ankara, Türkiye) was enrolled. Cervical samples from the participants were categorized into four groups as follows: Normal (n=32), low-grade squamous intraepithelial lesion (CIN1; n=21), high-grade squamous intraepithelial lesion (CIN2/3; n=23) and cervical cancer (n=8). The remaining 16 samples were excluded from the analysis due to inadequate DNA yield or quality. Quantitative PCR was employed to quantify mtCN relative to nuclear DNA. Differences in mtCN according to disease category, smoking status and human papillomavirus (HPV) status were analyzed, and logistic regression modeling was performed to identify independent predictors of high-risk cervical disease (HSIL and invasive cancer). The study revealed a statistically significant stepwise increase in mtCN concomitant with increasing disease severity, reaching the highest level in cervical cancer. Notably, HPV-positive samples exhibited elevated mtCN levels compared with HPV-negative samples. In addition, smoking was associated with a significant increase in mtCN within cervical tissues. A triple model comprising mtCN fold change, smoking status and HPV status demonstrated superior predictive performance for distinguishing high-risk cervical disease, with a sensitivity of 79% and specificity of 92%. The findings indicate that mtCN alterations are associated with the progression of CIN to cervical cancer, particularly in cases who are HPV positive and smoke. To substantiate these findings and evaluate their clinical utility, larger longitudinal studies with standardized assessment protocols are imperative. However, the present study underscores the potential of mtCN as a biomarker for cervical cancer risk assessment and highlights the necessity for continued exploration into its role in tumorigenesis and diagnostic applications.

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Abbreviations: CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; mtDNA, mitochondrial DNA; mtCN, mitochondrial DNA copy number; nDNA, nuclear DNA; HPV, human papillomavirus; FFPE, formalin-fixed paraffin-embedded; qPCR, quantitative polymerase chain reaction; ΔCq, difference in quantification cycle; ND1, NADH dehydrogenase subunit 1; HBB, hemoglobin subunit β; ROS, reactive oxygen species; ROC, receiver operating characteristic

Key words: mtDNA copy number alteration, cervical intraepithelial neoplasia, cervical cancer, logistic regression model, HPV, smoking

Introduction

Cervical cancer is the fourth most prevalent malignancy diagnosed in women worldwide, following breast, colorectal and lung cancer (1). Numerous risk factors have been associated with cervical cancer, including human papillomavirus (HPV) infection, early onset of sexual activity, multiple sexual partners, the use of oral contraceptives and smoking. In this context, HPV vaccination has demonstrated safety and effectiveness as a primary prevention strategy for cervical intraepithelial neoplasia (CIN) and cervical cancer (2). CIN is a premalignant stage in the development of cervical cancer and provides an opportunity for early detection and intervention. Although persistent infection with HPV is considered the major cause of the disease, only a small proportion of

HPV-infected women develop cervical malignancy, which limits the utility of HPV testing due to its low positive predictive value for high-grade lesions (3,4). Other main screening techniques for cervical cancer include the Pap smear, visual inspection with acetic acid and Lugol's iodine and liquid-based cytology. Several countries, including Türkiye, United Kingdom, Australia, Canada and The Netherlands, have implemented cytology-based testing and HPV testing as part of their cancer screening programs (5). There is increasing interest in the identification of novel molecular markers to improve the identification of individuals at high risk of cervical cancer, with the aim of expanding the reach of screening programs in the community, improving accessibility and increasing their diagnostic accuracy.

In almost every type of human cell, there are hundreds to thousands of mitochondria, each containing its own genome, a feature that distinguishes mitochondria from other organelles. Human mitochondrial DNA (mtDNA) is a circular, double-stranded DNA of ~16,569 nucleotides that is involved in a variety of cellular functions (6). It contains a total of 37 genes encoding 13 proteins essential for the oxidative phosphorylation system, as well as 22 transfer RNAs and 2 ribosomal RNAs (7). Several characteristics distinguish mtDNA from nuclear DNA (nDNA). The most well-known of these is the maternal inheritance of mtDNA, which differs from the Mendelian pattern of inheritance. Another feature is that the mitochondrial genome is polyploid, meaning that each cell has multiple copies of mtDNA depending on the cell type (8). mtDNA is present in multiple copies per cell, with the copy number typically ranging from 1,000 to 10,000 and varying according to the type of cell (9). Notably, mtDNA is particularly vulnerable to damage and copy number alterations, due to exposure to reactive oxygen species (ROS) and other metabolites generated within mitochondria, the lack of protective histones, and inadequate repair and protection mechanisms (10). Alterations in mtDNA copy number (mtCN) can potentially result in impaired mitochondrial function and increased ROS production. Variations in mtCN have been observed in numerous pathological conditions, including neuropsychiatric disorders, autoimmune diseases, chronic inflammation and various types of cancer (11-13). In this context, alongside well-characterized alterations in nDNA, mtDNA has also been extensively studied in a range of cancer types (9).

Changes in mtCN, reflecting the balance between mitochondrial biogenesis and degradation, have emerged as potential indicators of cellular dysfunction and tumorigenesis. It has been suggested that mtCN increases during the early stages of cancer to compensate for metabolic defects in damaged mitochondria, However, in advanced stages, cumulative damage may result in mtDNA degradation and a subsequent reduction in mtCN (14,15). In light of this, previous studies have elucidated the involvement of mtCN alterations in the pathogenesis of various cancer types in different patient cohorts. mtCN changes reported in the literature vary according to cancer stage, the characteristics of the study cohort and additional patient-related factors (16). This variability indicates that, when mtDNA is considered as a biomarker in cancer, expectations of increased or decreased copy numbers in tumor samples cannot be generalized and may not be independent of diagnosis, stage and patient characteristics. Therefore, it is essential to quantitatively determine the alterations of mtDNA within the specific population for which a predictive model is being developed.

Considering that high-risk HPV oncoproteins E6 and E7 may induce DNA damage by affecting ROS production by different mechanisms (17), it can be hypothesized that alterations in mtCN associated with mtDNA damage in cervical cancer cells may contribute to cervical carcinogenesis. Supporting this hypothesis, Sun *et al* (18) demonstrated that the mtCN in cells from cervical smear samples from patients with cervical cancer was significantly higher than that in corresponding samples from cancer-free controls. Furthermore, Warowicka *et al* (19) reported an association between increased mtCN, as well as mtDNA mutations, with the pathogenesis of cervical cancer. A recent study has demonstrated a significant association between altered mtCN and cervical cancer, even in HPV-negative cases. Al-Awadhi *et al* (20) demonstrated elevated mtCN levels in both high-risk HPV-positive and HPV-negative cervical samples, suggesting that this increase may represent an adaptive response to mitochondrial oxidative stress and energy deficiency.

Despite accumulating evidence that high-risk HPV oncoproteins (E6/E7) can increase oxidative stress and perturb mitochondrial homeostasis, the direction and magnitude of mtCN changes appear to vary across settings. In cervical pathology, previous studies have primarily compared cancer or high-grade squamous intraepithelial lesions (HSILs) with samples from control subjects without evaluating mtCN alterations across the full spectrum of CIN. In addition, they have rarely included HPV status in the same analysis. Consequently, it remains unclear whether mtCN exhibits a stepwise pattern with increasing histological severity and whether such a pattern is influenced by HPV positivity. To address this gap, the present study quantified mtCN across normal, CIN1 [low-grade squamous intraepithelial lesion (LSIL)], CIN2/3 (HSIL) and invasive cervical cancer tissues. In addition, the combined contribution of mtCN, HPV status and smoking status was evaluated in relation to the classification of high-risk disease.

Materials and methods

Study group. A total of 100 participants who presented at the Gynecology and Obstetrics Clinic of Ankara Etlik City Hospital (Ankara, Türkiye) between July 2023 and September 2023 and provided cervical samples were included in the present study. All participants were female, and their median age was 53 years, with an age range of 45-65 years. Prior to intervention, written informed consent was obtained from each patient. The patients were selected based on histopathological diagnosis, and included 32 healthy individuals and 52 patients with cervical pathology. The latter comprised 21 cases of CIN1 (LSIL), 23 cases of CIN2/3 (HSIL) and 8 cases of cervical cancer. The remaining 16 samples were excluded due to insufficient DNA yield or suboptimal quality.

Personal and clinical data of the participants, including age, medical history, histopathological features and HPV genotype were collected from the digital medical archive. The samples were collected from patients aged 45-65 years, with

efforts made to minimize age-related variations in mtDNA by ensuring a comparable age distribution across groups. To standardize mtCN, individuals with a history of chronic exposure to environmental or occupational agents, narcotic substances or drugs, as well as those with chronic inflammation of the internal genital organs or adjacent tissues, were excluded from the study. Smoking status was recorded and evaluated as a variable in the analysis. The control group included both HPV-negative and HPV-positive cases; HPV16 and HPV18 were classified as high-risk. The present study was approved by the Ethics Committee of Ankara Etlik City Hospital (AEŞH-EK1-2023-015).

DNA extraction. Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) cervical tissue sections using the AmoyDx® FFPE DNA Kit (Amoy Diagnostics Co., Ltd.), following the manufacturer's protocol. The FFPE tissue blocks had been archived for ≤12 months. Serial 5-μm FFPE sections were cut for nucleic acid extraction. Briefly, deparaffinization was performed using the standard xylene/ethanol method and DNA was isolated via the silica column-based procedure of the kit. The purified DNA was eluted and stored at -20˚C until further analysis. DNA concentration and purity were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and samples with an optical density (OD)260/OD280 ratio between 1.8 and 2.0 were considered suitable for downstream sequencing.

HPV status assessment. The HPV status of the cervical samples was determined. This was performed by analysis of the extracted DNA using the cobas® HPV Test on the cobas® 4800 testing system according to the manufacturer's instructions (Roche Diagnostics for both). This qPCR-based assay detects 14 high-risk HPV genotypes, providing individual results for HPV16 and HPV18 and pooled detection for 12 additional types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). The specific primer sequences used in this commercial assay are proprietary to the manufacturer and not disclosed. The analytical sensitivity and specificity of the cobas® HPV Test for the detection of CIN2+ lesions have been reported in clinical validation studies to be 90-99 and ~87%, respectively (21). All analyses were performed in the institutional laboratory following standard quality control procedures.

Measurement of mtCN. The mtCN normalized to nDNA in cervical cells was determined using quantitative PCR (qPCR). The difference in quantification cycle (ΔCq) values of the NADH dehydrogenase subunit 1 (ND1) and hemoglobin subunit β (HBB) genes were used to evaluate the mtCN and nDNA copy number, respectively. Amplification was performed using QuantiNova SYBR Green PCR Master Mix (Qiagen, Inc.) and a commercial primer assay (RT^2 qPCR Primer Assay; Qiagen, Inc.) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The primer sequences are presented in Table SI. The reference gene, hemoglobin subunit beta (HBB), was selected as a single-copy nuclear locus with stable representation in cervical epithelial DNA and no known regulation by mitochondrial pathways, consistent with previous validations (11-13).

qPCR was performed for 40 cycles using a two-step protocol, comprising denaturation at 95˚C for 15 sec, followed by combined annealing and extension at 60˚C for 60 sec, in accordance with the manufacturer's recommendations for SYBR Green chemistry. The assay results were accepted when the following criteria were met: i) Cq values <35 for both the mtDNA target ND1 and the single-copy nuclear reference (HBB); ii) no amplification in no-template controls (Cq undetermined or >40); iii) melting curve analysis demonstrated a single, specific peak with a consistent melting temperature across replicates (±0.5˚C); and iv) technical replicates differed by ≤0.3 Cq.

The relative mtCN differences between the study groups and controls were calculated using the $2^{-\Delta\Delta Cq}$ method and expressed as fold change (22).

Power analysis. An *a priori* sample size calculation was performed using G*Power software (version 3.1.9.7; Heinrich Heine University Düsseldorf; https://www.gpower.hhu.de) based on a two-tailed independent t-test with an α value of 0.05 and statistical power of 0.80. The analysis was designed to compare a high-risk group, comprising patients with HSIL or invasive cancer, with a low-risk group, comprising patients with LSIL or normal cervical cytology based on mtCN fold-change ($2^{-\Delta\Delta Cq}$) as the continuous outcome measure. A conservative effect size corresponding to a large Cohen's d of 0.80 was assumed to prevent the overestimation of results, based on previous studies reporting significant mitochondrial alterations, including elevated mtCN, in cervical cell exfoliates, and major mitochondrial genomic alterations in dysplastic and cancerous tissues (20,22,23). Under the assumption of an equal group allocation, the required sample size was 25 per group. A 20% attrition/mismeasurement buffer was established; therefore, the prespecified target sample size was increased to 30 per group (total, n=60). These assumptions were informed by published evidence on mitochondrial abnormalities in HPV-related cervical pathology (19,20).

Statistical analysis. Statistical analyses were performed using R (version 4.1.3; R Foundation for Statistical Computing; https://www.r-project.org/) via the RStudio interface (Posit Software, PBC; https://posit.co/). Descriptive statistics are presented as the mean ± standard deviation or median and interquartile range, as appropriate. One-way analysis of variance was applied to normally distributed numerical values, Associations between categorical variables were evaluated using Fisher's exact test.

In the model-building process, the normal and LSIL categories were merged to form a low-risk group, while the HSIL and invasive cancer categories were combined to form a high-risk group, yielding a binary outcome variable. Logistic regression with a logit function was used to predict the binomial target variable. As only one numerical predictor was available, univariate methods were used to identify potential outliers and extreme values prior to model fitting. Overall, seven models were constructed and used for prediction. The performance of all models was assessed using sensitivity, specificity, accuracy and receiver operating curve (ROC) analysis, and the area under the curve was determined. Optimal thresholds were determined using the Youden index. Statistical significance was defined as $P<0.05$, with an a set at 0.05 and β at 0.80 (24).

Table I. Comparison of demographic and clinical variables of the full cohort according to cervical disease group.

Variables	Total (n=100)	Normal (n=38)	LSIL (n=25)	HSIL (n=26)	Cancer (n=11)	P-value
Age, mean ± SD (min-max), years	53.45±5.74 (45-65)	53.9±5.84 (45-65)	54.5±5.30 (45-63)	51.7±5.58 (45-62)	53.5±6.55 (45-64)	0.316[a]
HPV, n (%)						0.057[b]
High-risk	55 (55.00)	15 (39.47)	15 (60.00)	16 (61.54)	9 (81.82)	
Low-risk	45 (45.00)	23 (60.53)	10 (40.00)	10 (38.46)	2 (18.18)	
Smoking status, n (%)						0.51[b]
Smoker	18 (18.00)	5 (13.16)	7 (28.00)	4 (15.38)	2 (18.18)	
Non-smoker	82 (82.00)	33 (86.84)	18 (72.00)	22 (84.62)	9 (81.82)	

[a]One-way analysis of variance; [b]Fisher's exact test. Two-sided α=0.05. High-risk HPV is HPV16 or HPV18, and low-risk HPV is any other type of HPV or HPV negativity. HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Results

Descriptive statistics. Descriptive characteristics of the full cohort of 100 patients according to disease status are presented in Table I, including age, HPV and smoking status. The mean age of all patients was 53.45±5.74 (range, 45-65) years. Patients positive for HPV16 or HPV18 were classified as the *high-risk oncogenic HPV group*, in accordance with established cervical cancer risk stratification. Patients positive for other HPV genotypes detected by the assay (including non-16/18 high-risk types) as well as HPV-negative individuals were analyzed together as a *non-HPV16/18 group* for comparative purposes. No statistically significant differences were observed between the groups with respect to age, HPV category or smoking status.

Comparison of mtCN results according to cervical disease category, HPV status and smoking status. Subsequent mtCN analyses were performed only in samples with adequate DNA yield and quality (n=84), after exclusion of 16 samples; Table I summarizes the baseline characteristics of the full enrolled cohort (n=100). In the first analysis, the groups were compared according to disease category. A statistically significant difference was identified among the groups in terms of mtCN (P<0.001). Post hoc analysis revealed significant differences between the cancer and control groups, the cancer and HSIL groups, the control and HSIL groups, the cancer and LSIL groups, and the HSIL and LSIL groups (P<0.001). However, no significant difference was detected between the control and LSIL groups. In addition, when HSIL and cancer cases were combined into a high-risk group and LSIL and control group patients were combined into a low-risk group, a statistically significant difference in mtCN was observed between these groups (P<0.001; Fig. 1).

In the second analysis, the participants were categorized according to their HPV status. Patients positive for HPV16 and/or HPV18 were classified as the high-risk HPV group, while patients positive for other HPV types or with no detectable HPV infection were classified as the low-risk HPV group. A statistically significant difference in mtDNA copy number was observed between these groups (P<0.05; Fig. 2).

In the third analysis, the mtCN was compared according to smoking status. A statistically significant difference was detected between non-smokers and smokers (P<0.05; Fig. 3).

Development of a model for prediction of high-risk and low-risk cervical lesions. A prediction model was developed to distinguish between high-risk and low-risk cervical disease groups. Prior to model development, outlier detection was performed using the local outlier factor method, and extreme values were excluded from the analysis. Logistic regression analysis was implemented for model development. Among the seven tested models, the triple model incorporating mtCN fold change, smoking status and HPV status showed the highest discriminative performance (AUC=0.93; Figs. 4 and 5). As the study restricted the ages of the participants to a narrow range of between 45 and 65 years, age was excluded from the modeling process.

Among the predictors included in the model, mtCN changes were identified as the strongest predictor of risk status. Smoking status and HPV status were evaluated as independent variables, demonstrated weaker associations and did not reach statistical significance (Table II). The triple model, including all three variables, demonstrated a superior overall

Table II. Triple logistic regression model results.

			95 CI%	
Variables	P-values	Odds ratio	Lower	Upper
Intercept	<0.001	1.146	1.038	1.471
mtCN fold change	0.003	3.551	3.013	4.548
HPV	0.083	1.426	1.062	5.108
Smoking	0.203	1.081	1.002	2.522

mtCN, mitochondrial DNA copy number; HPV, human papillomavirus.

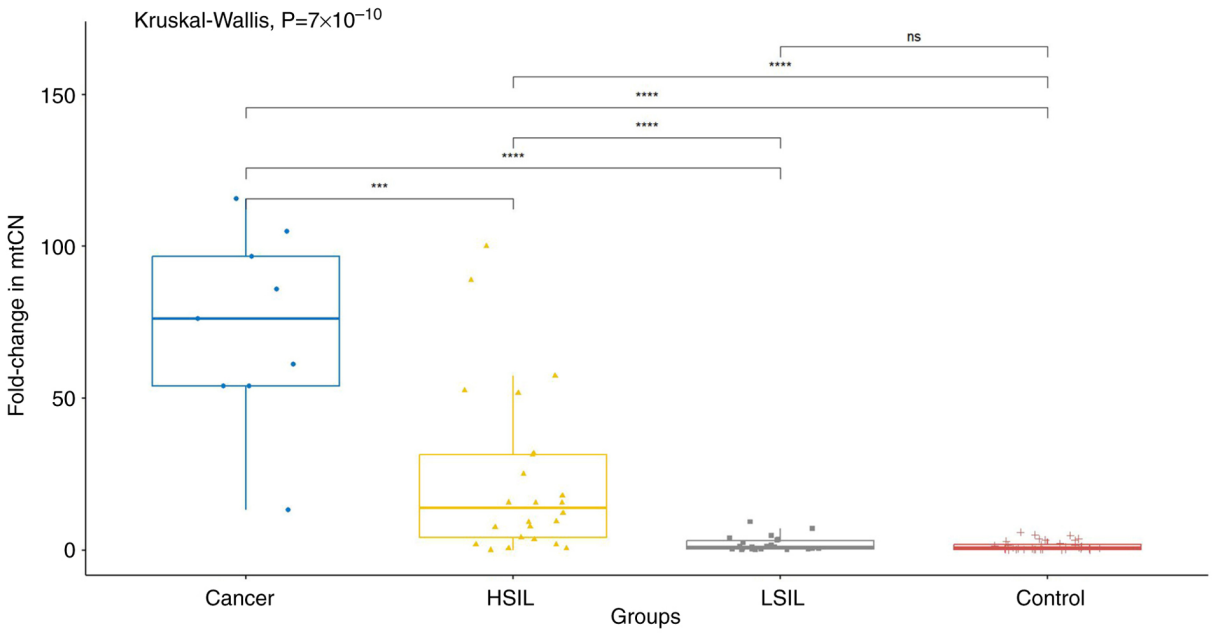

Figure 1. Fold change in mtCN level according to cervical disease category. ***P<0.001, ****P<0.0001. ns, not significant; mtCN, mitochondrial DNA copy number; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

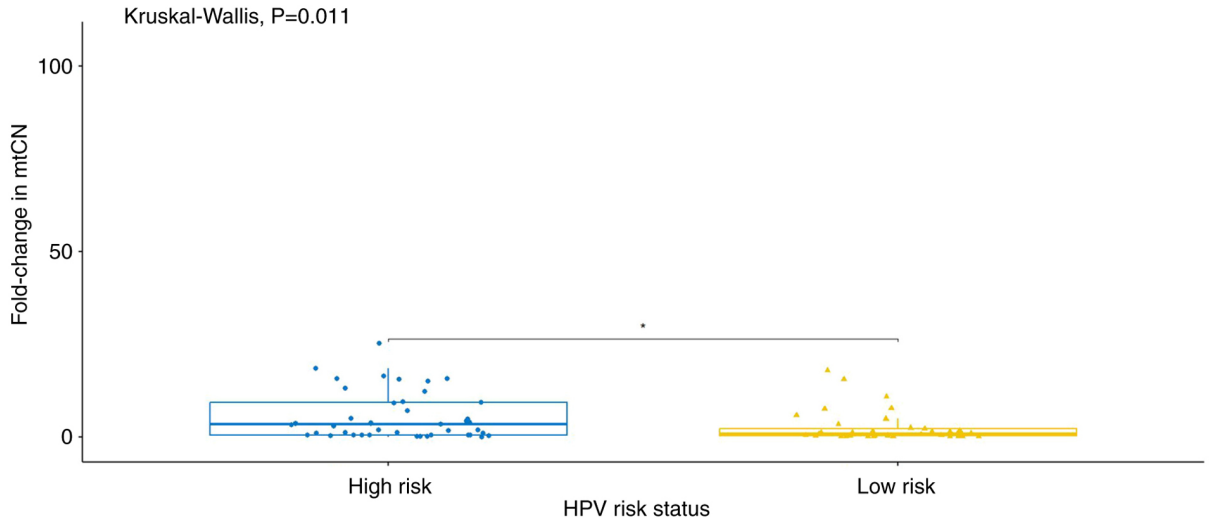

Figure 2. Fold change in mtCN level according to HPV risk status. *P<0.05. mtCN, mitochondrial DNA copy number; HPV, human papillomavirus.

performance. Consequently, this specific model was selected as the final predictive model. The optimal threshold value for successful differentiation using the triple model was determined, and a cutoff value of 0.17 was found to be optimum (Fig. 5). At this cutoff, the triple model exhibited a sensitivity of 79% and specificity of 92% (Table III).

Discussion

In the present study, the relationship between mtCN variations and the progression of CIN to cervical cancer was investigated. The findings provide important insights and are consistent with previous studies on mtDNA alterations in various cancer types. They also expand upon previous studies by providing a comprehensive understanding of the role of mtCN changes across the CIN spectrum. Notably, statistically significant increases in mtCN were observed with increasing disease severity, culminating in cervical cancer.

Previous studies have reported increased mtCN in cervical disease. In a cohort consisting exclusively of high-risk HPV-positive women, Sun *et al* (18) observed that mtCN levels in exfoliated cervical cells were higher in women with cervical cancer compared with cancer-free controls, supporting a potential role for mtCN alterations in cervical carcinogenesis. Similarly, based on tissue-based analyses, Warowicka *et al* (19) reported that the mtCN in HSIL and carcinoma is elevated compared with that in LSIL (19). In the cohort of the present study, mtCN increased in a stepwise manner with increasing histological severity, which was consistent with these previous observations.

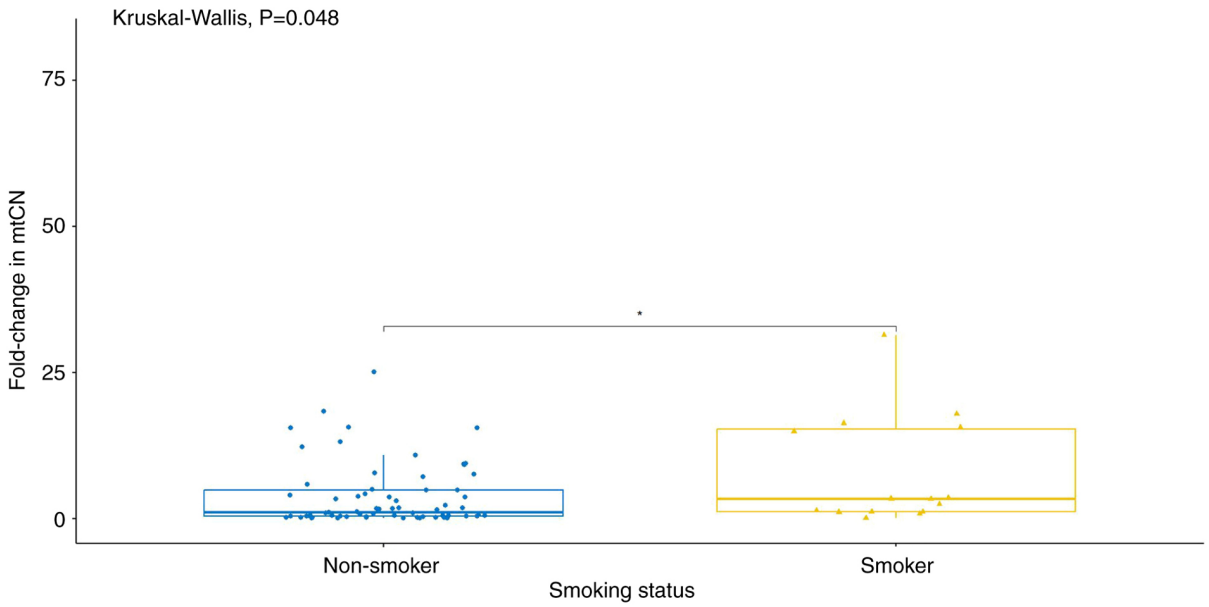

Figure 3. Fold change in mtCN level according to smoking status. *P<0.05. mtCN, mitochondrial DNA copy number.

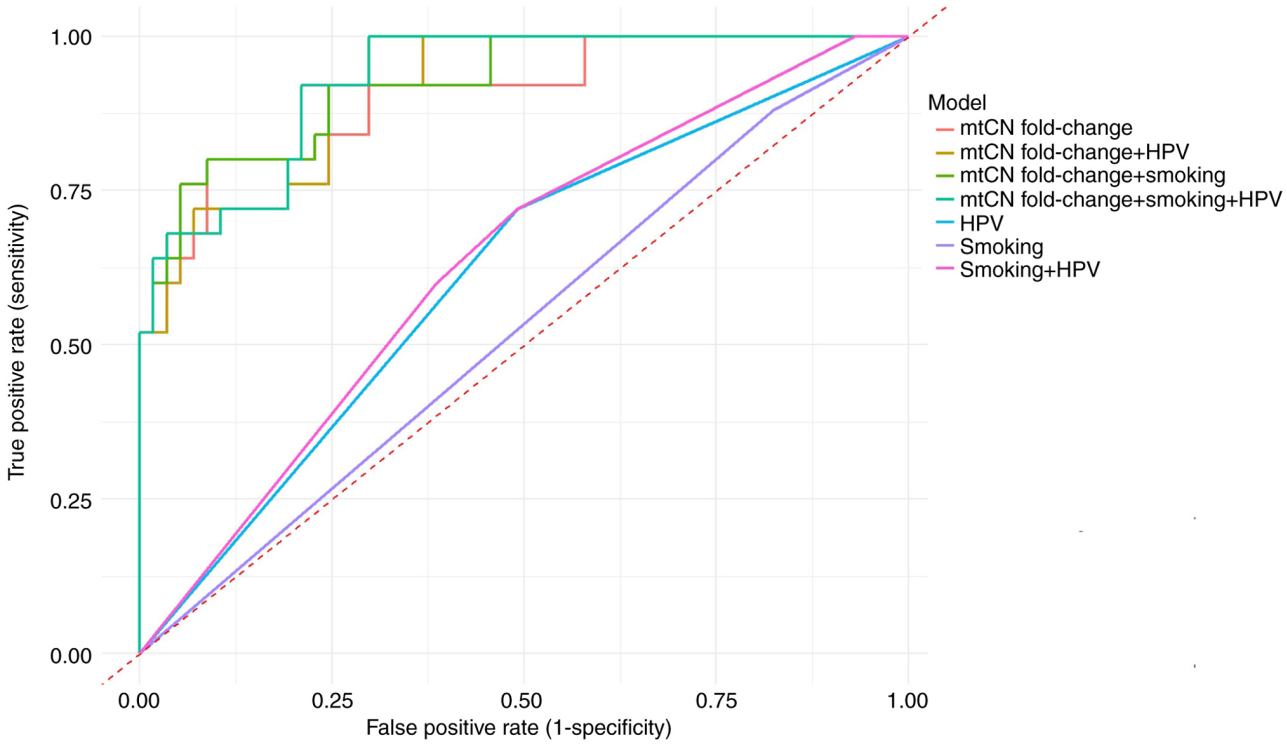

Figure 4. Comparison of receiver operating characteristics curves for all possible models. The triple model comprising cervical disease category, smoking and HPV status is the most successful for cervical cancer risk classification. HPV, human papillomavirus.

Al-Awadhi *et al* (20) reported elevated mtCN in cervical abnormalities irrespective of HPV status, suggesting an adaptive response to mitochondrial stress. The present study extends the results provided in the literature in the following three aspects: i) mtCN was evaluated across the full CIN spectrum in tissue, that is, in normal, CIN1, CIN2/3 and cancerous tissues, rather than by the comparison of only extreme disease categories; ii) HPV status was explicitly incorporated, demonstrating that HPV positivity amplifies the stepwise increase in mtCN; and iii) these findings were integrated into a joint predictive framework combining mtCN with HPV status and smoking, with defined operating characteristics (threshold, 0.17; sensitivity, 79%; specificity, 92%), thereby positioning mtCN within a clinically oriented risk-stratification context (Fig. 5).

High-risk HPV oncoproteins establish a persistent pro-oxidant state that perturbs mitochondrial homeostasis. E6/E7-driven oxidative stress increases ROS production and DNA damage in infected epithelia; since mtDNA is in close

Table III. Performance metrics outcomes of all models.

Model variables	Accuracy	Sensitivity	Specificity	Positive predictive value
mtCN fold change[a]	88	91	80	91
HPV[b]	58	51	72	81
mtCN fold change + smoking[a]	88	92	80	91
mtCN fold change + HPV[a]	81	76	92	96
mtCN fold change + HPV + smoking[a]	83	79	92	95

[a]P<0.001, [b]P<0.05. Smoking alone and HPV + smoking models did not create a statistically significant model and are therefore excluded from the table. mtCN, mitochondrial DNA copy number; HPV, human papillomavirus.

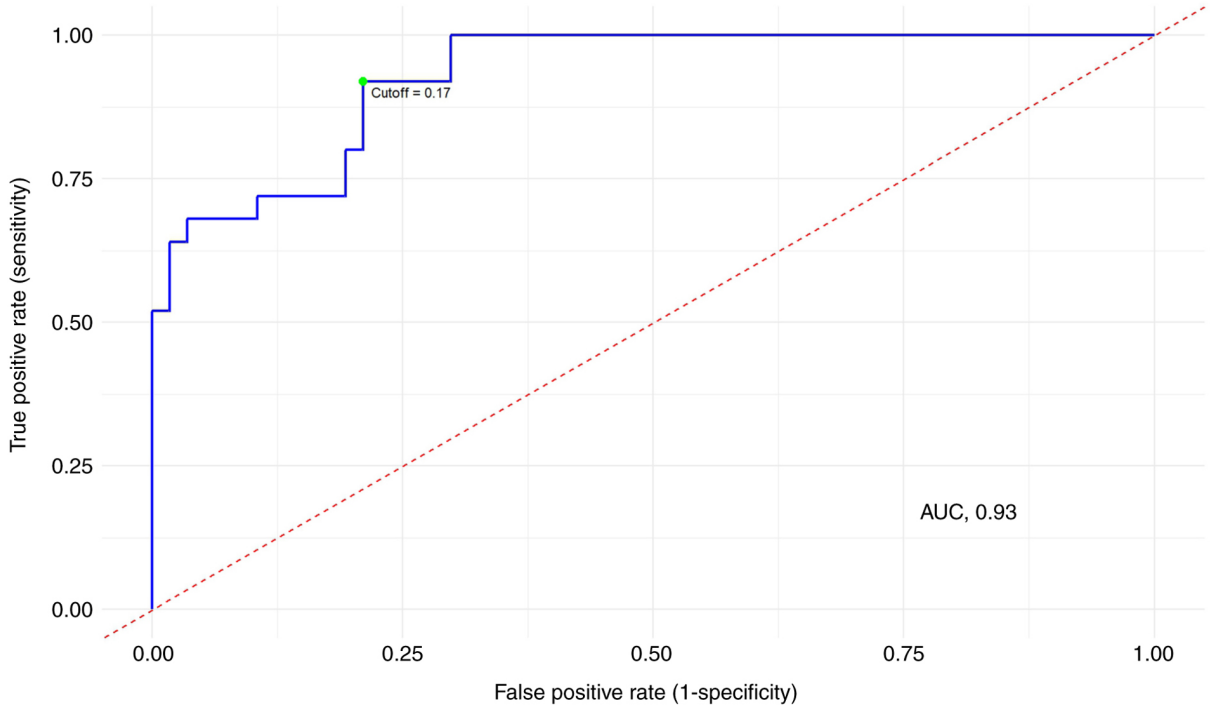

Figure 5. Receiver operating characteristic curve for the triple model, which combines cervical disease category, smoking and human papillomavirus status. AUC, area under the curve.

proximity to the respiratory chain, lacks protective histones and has limited repair capacity, it is particularly susceptible to damage. Such mitochondrial damage can activate mitochondrial biogenesis pathways, including the peroxisome proliferator-activated receptor g coactivator-1a (PGC-1α)/nuclear respiratory factor 1 (NRF-1)/mitochondrial transcription factor A (TFAM) axis, leading to an increase in mtCN as a compensatory response to maintain bioenergetic function. These mechanisms, described in previous reviews of HPV-related oxidative-stress and mitochondrial biology, provide a biologic rationale for the stepwise increase in mtCN observed with increasing cervical disease severity and for the higher mtCN detected in HPV-positive samples in the present cohort (18,25,26).

Previous studies have reported both increased and decreased mtCN across different tumor types (27). Elevated mtCN has been observed in breast, bladder, esophageal, head and neck squamous, kidney and liver cancers, with lung carcinoma as a notable exception, when compared with non-tumorous tissues (28). By contrast, reduced mtCN has been reported in kidney clear cell carcinoma, hepatocellular carcinoma and myeloproliferative tumors (29). The association between mtCN variations and the risk of cancer progression has also been documented. A meta-analysis by Hu *et al* (30) demonstrated a significant association between increased mtCN and the risk of lymphoma, melanoma and breast cancer, whereas an inverse relationship was observed for hepatic carcinoma. These divergent results suggest that mtCN may increase as a compensatory response to mitochondrial damage via increased mitochondrial biogenesis, whereas decreased mtCN may occur when mitochondrial damage becomes excessive and damaged cells are eliminated by mitophagy and increased cellular turnover. Which of these occurs depends on tissue context, tumor metabolic demands and disease stage. In cervical pathology, syntheses of recent evidence indicate a tendency toward mtCN elevation in high-grade lesions and cervical cancer, particularly in HPV-positive settings, consistent with compensatory

mitochondrial biogenesis under chronic ROS-induced stress. The findings of the present study align with this context-dependence and position cervical disease on the 'increased mtCN' side of this bidirectional framework (20,26).

Given the high prevalence of smoking in Türkiye, a comparison between cervical tissue samples from smokers and non-smokers was performed in the current study. Consistent with findings from large epidemiologic cohorts, smoking has been associated with a reduction in leukocyte mtCN (31,32), generally interpreted as systemic depletion associated with oxidative stress. However, in the present tissue-based analysis, upregulated mtCN was observed in the cervical samples of smokers. This apparent discrepancy supports a tissue-blood dissociation, whereby localized oxidative stress and increased bioenergetic demands in pre-neoplastic or tumor tissue may promote compensatory mitochondrial biogenesis via the PGC-1α/NRF-1/TFAM axis (33). This interpretation is consistent with previous literature on cervical cancer, which has reported the elevation of mtCN in high-grade cervical lesions or cervical cancer (20,26). It is important to note that this stratified, within-tissue observation, when combined with the CIN-graded analysis and the integrated predictive model combining mtCN, HPV status and smoking, constitutes a novel contribution of the present study. Together, these findings refine the interpretation of mtCN as a biomarker for risk stratification in HPV-related cervical disease.

The potential of mtCN as a biomarker for cancer prognosis is well-documented. Distinct mtCN levels have been shown to differentiate control populations from patients with solid tumors and other diseases (9,23). Despite this, the routine clinical assessment of mtCN is not possible due to the lack of a standardized method (34). Although the qPCR technique remains the gold standard for mtCN quantification, variations in its application among different laboratories contribute to inconsistent results. In particular, differences in reference genes, which often vary based on the sample type and disease context, complicate standardization (28,34). An additional technical limitation is that the nuclear genome can replicate a significant portion of the mtDNA genome, creating nuclear mtDNA segments that may interfere with mtCN measurement (35,36). This genetic overlap necessitates the careful selection of mtDNA target regions to avoid the co-amplification of these pseudogenes and ensure accurate mtCN quantification (36,37). In the present study, mtCN was demonstrated to be a significant biomarker for the progression of CIN to cervical cancer. The observed mtCN alterations in cervical tissues, notably the increased mtCN in HPV-positive cases and among smokers, further highlight its potential utility in clinical practice. However, the lack of standardized methods for mtCN assessment underscores the requirement for the development of consistent and reproducible techniques for clinical implementation. The present study contributes to the growing body of evidence supporting the importance of mtCN as a biomarker and emphasizes that standardized protocols are necessary to enhance its utility in cancer prognosis and screening.

The interaction between mtCN and HPV positivity was evaluated using the Wald test within logistic regression models, which is similar to the analytical approach previously described by Sun *et al* (18). Logistic regression models have also been employed in other studies investigating mtCN in various cancer types, for example, in a study on head and neck squamous cell carcinoma in which mtCN modeling in leukocytes was performed (38). In the present study, ROC curve analysis was used to compare all possible model combinations. Among these, the triple model incorporating mtCN fold-change, smoking status and HPV status demonstrated the strongest classification performance. Notably, mtCN fold-change alone was the strongest contributor to the model. The ROC curve analysis for the triple model indicates a discrimination threshold of 0.17, corresponding to a sensitivity and specificity of 79 and 92%, respectively.

Several limitations of the present study must be acknowledged. Firstly, the sample size was relatively small, which may limit the generalizability of the findings. Therefore, larger studies are required to validate these results and provide more robust evidence. Second, the cross-sectional design of the study with single-time-point sampling precludes the determination of whether mtCN alterations are a cause or a consequence of CIN progression. Prospective longitudinal cohort studies with serial cervical tissue sampling are required to clarify the temporal and causal relationship between mtCN dynamics and cervical disease development. Furthermore, although qPCR is considered the gold standard for mtCN quantification, the lack of standardized protocols across laboratories may introduce variability and limit comparability. The development of standardized methods for mtCN assessment will be essential to ensure consistency and reproducibility in clinical settings. In addition, the cancer group included only 8 patients; consequently, the power analysis was conducted by combining the HSIL and cancer groups into a single high-risk category and comparing this with a low-risk category combining the LSIL and normal groups. While this approach ensured sufficient statistical power to detect overall differences in mtCN, it may obscure variations specific to invasive cancer. Therefore, the results should be interpreted with caution, as this grouping limits the ability to draw distinct conclusions with regard to the cancer subgroup alone. Finally, the study did not account for all potential confounding factors, such as environmental exposures and genetic susceptibility, that may influence mtCN levels. Future studies should consider these variables to provide a more comprehensive understanding of the factors affecting mtCN in cervical cancer.

In conclusion, the present study provides compelling evidence that alterations in mtCN are significantly associated with the progression of CIN to cervical cancer. The findings highlight the potential of mtCN as a biomarker for cervical cancer risk assessment, particularly in HPV-positive cases. However, larger longitudinal studies with standardized protocols are essential to validate these results and facilitate their clinical translation. Overall, the insights gained from the study contribute to the growing body of evidence supporting the importance of mtCN in cancer biology and underscore that it is necessary to continue to investigate its role in tumorigenesis and its potential use as a diagnostic tool.

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Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

IO and HBE contributed to conceptualization, formal analysis, investigation and drafting the original manuscript. MTA performed statistical analysis, data curation (systematic data retrieval, clinical record verification, database management), designed scientific figures and assisted with graphical data presentation. OO contributed to clinical methodology, patient recruitment, clinical evaluation and tissue acquisition. FSD contributed to formal analysis and methodology. ECD contributed to analysis and interpretation of data and manuscript review and editing. AYT contributed to patient recruitment, clinical evaluation and tissue acquisition. HLK contributed to conceptualization, and manuscript review and editing. IO and HBE confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Ankara Etlik City Hospital (approval No: AEŞH-EK1-2023-015; date, 22.03.2023). All participants provided written informed consent prior to inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Lei J, Ploner A, Elfström KM, Wang J, Roth A, Fang F, Sundström K, Dillner J and Sparén P: HPV vaccination and the risk of invasive cervical cancer. N Engl J Med 383: 1340-1348, 2020.
3. Coquillard G, Palao B and Patterson BK: Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. Gynecol Oncol 120: 89-93, 2011.
4. Woodman CB, Collins SI and Young LS: The natural history of cervical HPV infection: unresolved issues. Nat Rev Cancer 7: 11-22, 2007.
5. Maver P and Poljak M: Primary HPV-based cervical cancer screening in Europe: implementation status, challenges, and future plans. Clin Microbiol Infect 26: 579-583, 2020.
6. Papa S and Skulachev V: Reactive oxygen species, mitochondria, apoptosis and aging. Mol Cell Biochem 197: 305-319, 1997.
7. Gustafsson CM, Falkenberg M and Larsson NG: Maintenance and expression of mammalian mitochondrial DNA. Annu Rev Biochem 85: 133-160, 2016.
8. Johns DR: Seminars in medicine of the Beth Israel Hospital, Boston. Mitochondrial DNA and disease. Mitochondrial DNA and disease. N Engl J Med 333: 638-644, 1995.
9. Wallace DC: Mitochondria and cancer. Nat Rev Cancer 12: 685-698, 2012.
10. Yakes FM and Van Houten B: Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci USA 94: 514-519, 1997.
11. Erdem HB, Ceylan AC, Sahin I, Sever-Erdem Z, Citli S and Tatar A: Mitochondrial DNA copy number alterations in familial mediterranean fever patients. Bratisl Lek Listy 119: 425-428, 2018.
12. Öğütlü H, Esin İS, Erdem HB, Tatar A and Dursun OB: Mitochondrial DNA copy number is associated with attention deficit hyperactivity disorder. Psychiatr Danub 32: 168-175, 2020.
13. Öğütlü H, Esin IS, Erdem HB, Tatar A and Dursun OB: Mitochondrial DNA copy number may be associated with attention deficit/hyperactivity disorder severity in treatment: A one-year follow-up study. Int J Psychiatry Clin Pract 25: 37-42, 2021.
14. Lee HC and Wei YH: Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol 37: 822-834, 2005.
15. Shokolenko I, Venediktova N, Bochkareva A, Wilson GL and Alexeyev MF: Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Res 37: 2539-2548, 2009.
16. Abd Radzak SM, Mohd Khair SZN, Ahmad F, Patar A, Idris Z and Yusoff AA: Insights regarding mitochondrial DNA copy number alterations in human cancer. Int J Mol Med 50: 104, 2022.
17. Cruz-Gregorio A, Manzo-Merino J, Gonzaléz-García MC, Pedraza-Chaverri J, Medina-Campos ON, Valverde M, Rojas E, Rodríguez-Sastre MA, García-Cuellar CM and Lizano M: Human papillomavirus types 16 and 18 early-expressed proteins differentially modulate the cellular redox state and DNA damage. Int J Biol Sci 14: 21-35, 2018.
18. Sun W, Qin X, Zhou J, Xu M, Lyu Z, Li X, Zhang K, Dai M, Li N and Hang D: Mitochondrial DNA copy number in cervical exfoliated cells and risk of cervical cancer among HPV-positive women. BMC Women's Health 20: 139, 2020.
19. Warowicka A, Kwasniewska A and Gozdzicka-Jozefiak A: Alterations in mtDNA: A qualitative and quantitative study associated with cervical cancer development. Gynecol Oncol 129: 193-198, 2013.
20. Al-Awadhi R, Alroomy M, Al-Waheeb S and Alwehaidah MS: Altered mitochondrial DNA copy number in cervical exfoliated cells among high-risk HPV-positive and HPV-negative women. Exp Ther Med 26: 521, 2023.
21. Fleider LA, de Los Ángeles Tinnirello M, Gómez Cherey F, García MG, Cardinal LH, García Kamermann F and Tatti SA: High sensitivity and specificity rates of cobas® HPV test as a primary screening test for cervical intraepithelial lesions in a real-world setting. PLoS One 18: e0279728, 2023.

22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung SCJ, Sukumar S and Sidransky D: Tumor-specific changes in mtDNA content in human cancer. Int J Cancer 116: 920-924, 2005.
24. Schwertman NC, Owens MA and Adnan R: A simple more general boxplot method for identifying outliers. Computational statistics & data analysis 47: 165-174, 2004.
25. Letafati A, Taghiabadi Z, Zafarian N, Tajdini R, Mondeali M, Aboofazeli A, Chichiarelli S, Saso L and Jazayeri SM: Emerging paradigms: unmasking the role of oxidative stress in HPV-induced carcinogenesis. Infect Agent Cancer 19: 30, 2024.
26. Pereira IOA, Silva NNT, Lima AA and da Silva GN: Qualitative and quantitative changes in mitochondrial DNA associated with cervical cancer: A comprehensive review. Environ Mol Mutagen 65: 143-152, 2024.
27. Lin CS, Wang LS, Tsai CM and Wei YH: Low copy number and low oxidative damage of mitochondrial DNA are associated with tumor progression in lung cancer tissues after neoadjuvant chemotherapy. Interact Cardiovasc Thorac Surg 7: 954-958, 2008.
28. Moraes CT: What regulates mitochondrial DNA copy number in animal cells? Trends Genet 17: 199-205, 2001.
29. Tang Y, Schon EA, Wilichowski E, Vazquez-Memije ME, Davidson E and King MP: Rearrangements of human mitochondrial DNA (mtDNA): New insights into the regulation of mtDNA copy number and gene expression. Mol Biol Cell 11: 1471-1485, 2000.
30. Hu L, Yao X and Shen Y: Altered mitochondrial DNA copy number contributes to human cancer risk: Evidence from an updated meta-analysis. Sci Rep 6: 35859, 2016.
31. Çakir M: Evaluation of smoking and associated factors in Turkey. Iran J Public Health 52: 766-772, 2023.
32. Wu S, Li X, Meng S, Fung T, Chan AT, Liang G, Giovannucci E, De Vivo I, Lee JH and Nan H: Fruit and vegetable consumption, cigarette smoke, and leukocyte mitochondrial DNA copy number. Am J Clin Nutr 109: 424-432, 2019.
33. Abu Shelbayeh O, Arroum T, Morris S and Busch KB: PGC-1α is a master regulator of mitochondrial lifecycle and ROS stress response. Antioxidants (Basel) 12: 1075, 2023.
34. Longchamps RJ, Castellani CA, Yang SY, Newcomb CE, Sumpter JA, Lane J, Grove ML, Guallar E, Pankratz N, Taylor KD, *et al*: Evaluation of mitochondrial DNA copy number estimation techniques. PLoS One 15: e0228166, 2020.
35. Hazkani-Covo E, Zeller RM and Martin W: Molecular poltergeists: Mitochondrial DNA Copies (numts) in sequenced nuclear genomes. PLoS Genet 6: e1000834, 2010.
36. Li B, Kalinowski P, Kim B, Pauls AD and Poburko D: Emerging methods for and novel insights gained by absolute quantification of mitochondrial DNA copy number and its clinical applications. Pharmacol Ther 232: 107995, 2022.
37. Filograna R, Mennuni M, Alsina D and Larsson NG: Mitochondrial DNA copy number in human disease: The more the better? FEBS Lett 595: 976-1002, 2021.
38. Wang L, Lv H, Ji P, Zhu X, Yuan H, Jin G, Dai J, Hu Z, Su Y and Ma H: Mitochondrial DNA copy number is associated with risk of head and neck squamous cell carcinoma in Chinese population. Cancer Med 7: 2776-2782, 2018.