Abstract. Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related mortality worldwide. Mitochondrial dysfunction has been postulated to render cancer cells resistant to apoptosis based on the Warburg hypothesis. However, few studies have investigated the prognostic value of mitochondrial DNA (mtDNA) content and G10398A polymorphism in NSCLC patients. mtDNA copy number and G10398A polymorphism in 128 NSCLC tissue samples were assessed by real-time PCR (RT-PCR) and PCR-RFLP respectively, and their relationship to prognosis were analyzed by survival analysis and Cox proportional hazards model. For the whole group, the median follow-up time and overall survival time were 22.5 and 23.4 months, respectively. Patients with high mtDNA content had a marginally longer survival time than patients with low mtDNA content (P=0.053). Moreover, patients with high mtDNA content plus 10398G had a significantly longer overall survival time compared with those having low mtDNA content plus 10398A (47 vs. 27 months, P<0.05). In addition, multivariate analysis showed that stage and low mtDNA content plus 10398A were the two most independent prognostic factors. In conclusion, the present study suggests that in patients with NSCLC, low mtDNA content plus 10398A could be a marker of poor prognosis which is associated with resistance to anticancer treatment caused by low mtDNA content plus 10398A polymorphism resulting in mitochondrial dysfunction.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide and is the most frequently diagnosed cancer in men (1). The two main types of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 80% of lung cancer patients and has a 5-year survival rate of <20% despite improvements in diagnosis and treatment (2). Most NSCLC patients are diagnosed at an advanced stage, in a poor performance status, and are often unresectable (3). It is crucial for improving the outcome of NSCLC to develop better molecular biomarkers for the prediction of disease progression and therapeutic resistance.

Mitochondrial dysfunction has been postulated to render cancer cells resistant to apoptosis based on the Warburg hypothesis (4). The mean copy number of mitochondrial DNA (mtDNA) in lung carcinoma tissue samples was statistically lower than that in adjacent histologically normal lung tissue samples (P<0.001) (5). Low copy number and low oxidative damage of mtDNA in lung cancer tissues are associated with tumor progression after neoadjuvant chemotherapy (6). In recent years, somatic mtDNA mutations including point mutation, deletion and insertion as well as decreased mtDNA copy number have been usually found in primary human cancer, including NSCLC (7). Also, 8701 and 10398 that code for ATPase6 and NADH dehydrogenase 3 were reported to be mutational hotspots in the mitochondrial genome of lung cancer (8). African-American women carrying 10398A had higher risk of invasive breast cancer (9). It has been proposed that these somatic mtDNA alterations in cancer can contribute to the switch of energy supply from mitochondrial oxidative phosphorylation to aerobic glycolysis and cancer progression (10). However, few studies have examined the prognostic value of mtDNA content and G10398A polymorphism in NSCLC patients.
In the present study, the mtDNA copy number and 10398

genotype were assessed in tumor tissues of 128 NSCLC

patients, and their prognostic values were analyzed. Furthermore, A549 cells were cultured in EB to get \( \varphi^0 \) cells. The radiosensitivity of these two cell lines (\( \varphi^0 \) and \( \varphi^+ \)) were investigated. The results showed that mtDNA content and G10398A polymorphism may play a role in patient prognosis by affecting cancer cell growth. Our studies suggest that the mtDNA content and G10398A polymorphism may provide new biomarkers to predict outcome of NSCLC. Intervention of the mtDNA content and G10398A polymorphism may also represent a novel approach for cancer treatment.

**Materials and methods**

**Samples and clinical data.** A total of 128 NSCLC tissue samples were obtained from patients who underwent surgical resection (including lymph node biopsy operation) at Zhongnan Hospital of Wuhan University between July 2006 and July 2011. Clinical staging was assessed according to the American Joint Committee on Cancer (AJCC, seventh edition). None of the patients had received radiotherapy or chemotherapy prior to surgery. Formalin-fixed and paraffin-embedded surgical tissue samples were collected from the Department of Pathology. Pathological diagnosis was made according to the World Health Organization 2004 scheme. The study protocol was reviewed and approved by the Institutional Review Board of Zhongnan Hospital of Wuhan University. All participants (including 95 male and 33 female patients; age range, 20-80 years) provided written informed consent to participate in this study. Clinical information, including age, gender, pathological type, tumor node metastasis (TNM) stage, smoking status, EGFR mutation status and clinical follow-up data, was recorded prospectively (Table I). In addition to the scheduled follow-up examination within 6 months, patients were followed-up at 3-month intervals for up to 2 years, then every 6 months for 5 years, and annually thereafter. For the whole group, the median follow-up time and overall survival (OS) time were 22.5 and 23.4 months, respectively; 58 patients (45.3%) died during this period.

**Tissue genomic DNA acquisition.** Total DNA from formalin-fixed, paraffin-embedded tissues was extracted using the E.Z.N.A.® FFPE DNA kit (Omega), according to the manufacturer’s instructions. The extracted DNA was eluted in 100 µl TE buffer and stored at -20°C.

**Determination of mtDNA copy number.** mtDNA content was assessed by quantification of a unique fragment in human mitochondrial genome NC_012920 region relative to a single copy region of the nuclear gene \( \beta_2 \)M using a real-time PCR (RT-PCR) assay. NC_012920 forward, 5'-CTTCTGGGACAGCAGCTTTGAAAC-3' and reverse, 5'-GCTGGGTAGCTCTTTGTTTTT-3' (64 bp product); \( \beta_2 \)M forward, 5'-GCTGGGTAGCTCTTTGTTTTT-3' and reverse, 5'-GCTGGGTAGCTCTTTGTTTTT-3' (64 bp product); RT-PCR was carried out using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad) with a total volume of 25 µl reaction mixture containing 100 ng DNA template (2 µl), 12.5 µl QuantiTect SYBR-Green PCR Master mix (Takara), and 0.5 µl of each primer, 0.5 µl ROX Reference Dye II, 9 µl ddH₂O. The run protocol: initial ‘Hot Start’ activation step for 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C.

**Genotyping of mtDNA 10398.** DNA samples were PCR-amplified using primers located between 10284-10484 np, forward, 5'-CAAAACAATGACATCC-3' and reverse primer, 5'-ATGAGGAGCATTGTTGTA-3' (201 bp product), followed by digestion with Ddel at 37°C. On resolving the Ddel-digested products in a 2% agarose gel, the 10398A allele gave rise to bands of 128 and 73 bp, whereas the 10398G allele demonstrated bands of 90, 73 and 38 bp.

**Detection of EGFR mutations.** PCR-RFLP was performed for mutation analysis of EGFR exons 18, 19 and 21. The mutant allele of exon 18 was not digested by the restriction enzyme ApaI due to the base substitution of G to X at the second base of GGGGCC. Since the range of exon 19 deletions containing commonly deleted codons 746 to 751, differences in the sizes of the PCR products enabled us to distinguish mutant-type from wild-type. The restriction enzyme Msel was used to digest the TGGCCA sequence in the ampiclon of the wild-type allele of exon 21. By contrast, mutant type (L858R) was not digested due to the base substitution of T to G of TGGCCA. The digested-products were run in 2% agarose gel.

**Cell line and treatment.** A human NSCLC cell line A549 was used in this study. \( \varphi^0 \) cells depleted of mtDNA were...
generated by incubating wild-type cells for 18 weeks in complete medium that was additionally supplemented with 100 ng/ml ethidium bromide (Sigma), 100 μg/ml pyruvate and 50 μg/ml uridine (11). Following selection, q0 cells were cultured in the medium specified above at 37°C in an incubator with 95% air and 5% CO2, with 110 mg/l sodium pyruvate and 50 μg/ml uridine. To verify mtDNA depletion in q0 cells, total cellular DNA from q+ and q0 cells was extracted and subjected to PCR amplification using two pairs of human mtDNA specific primers: i) COX-I F, 5’-ACACGAGCATATTTCACCTCGG-3’ and COX-I R, 5’-GGATTGTGGCGTAGTTTGGTC-3’, which gave a 337 bp product; ii) mtDNA-P1 F, 5’-AACATACCCCATGGCAACCT-3’ and mtDNA-P1 R, 5’-GGCAGGAGTAATCAGAGGTG-3’, which gave a 533 bp product. As a control, we measured the expression of β-actin F, 5’-TGGAAGGACTACCTCTGACA-3’ and R, 5’-TCAGGCTCAGGGATGACTGG-3’, which gave a 283 bp, which is coded by nuclear DNA.

Clonogenic survival. Cells were plated in triplicate into 60-mm tissue culture dishes at limiting dilutions. After irradiation (0, 1, 2, 4, 6, 8 and 10 Gy), the cells were incubated for 2 weeks to allow colony formation. The colonies were then fixed in 70% ethanol and stained with 1% crystal violet. A colony population should contain >50 cells. Plating efficiencies (PEs) for untreated control cultures were calculated using the following formula: PE = (number of colonies counted/number of cells seeded) x100. Surviving fractions (SFs) were calculated using the following formula: SF = (number of colonies counted)/(number of cells seeded xPE).

Cell cycle analysis. q+ and q0 cells in exponential growth were irradiated with 4 Gy and collected at 4, 8, 12, 16 and 24 h. Cell cycle phase distributions were measured by flow cytometry using propidium iodide (PI). Briefly, cells were collected and fixed in suspension in 70% ethanol on ice and then stored at 4°C. Cells were centrifuged at 500 g, washed with 1 ml PBS, centrifuged again, and resuspended in PBS containing 20 μg/ml PI and 10 μg/ml RNase A. After 30 min incubation in the dark at room temperature, PI-stained cells were analyzed for DNA content by flow cytometry, and the percentage of cells in G1, S and G2/M were calculated using MODFIT software (12).

Reactive oxygen species assay. q+ and q0 cells in exponential growth were plated in 96-well cell culture plates. Prior to 4 Gy radiation, DCFH-DA was added and then incubated for 20 min; cells were washed three times with serum-free cell culture in order to sufficiently remove DCFH-DA not up-taken by the cells. Modulus II Microplate was used to detect fluorescence immediately, and the value at this time was recorded as ft0, every 30 min afterwards, fluorescent values were denoted as ft30, ft60, ft90 and ft120. The fluorescent growth rate formula is: [(ft-f0)/ft0] x100% (13).

Statistical analyses. The relationships between mtDNA content, G10398A polymorphism and survival time were analyzed by the χ² test. Kaplan-Meier survival curves and the log-rank test were used to analyze OS. Multivariate analysis was performed using the Cox Proportional Hazards model.
XU et al: LOW mtDNA CONTENT PLUS 10398A COULD BE A MARKER OF POOR PROGNOSIS IN NSCLC

Table II. Relationships between mtDNA content and clinical parameters (n=128).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low mtDNA content, n (%)</th>
<th>High mtDNA content, n (%)</th>
<th>$\chi^2$</th>
<th>P-value</th>
<th>OR (95% CI)</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>45 (47.4)</td>
<td>50 (52.6)</td>
<td>0.641</td>
<td>0.549</td>
<td>0.725 (0.329-1.595)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (57.6)</td>
<td>14 (42.4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td></td>
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</tr>
<tr>
<td>≤50</td>
<td>15 (62.5)</td>
<td>9 (37.5)</td>
<td>1.846</td>
<td>0.257</td>
<td>1.871 (0.752-4.655)</td>
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<tr>
<td>&gt;50</td>
<td>49 (47.1)</td>
<td>55 (52.9)</td>
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<td>Smoking status</td>
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<td>No</td>
<td>26 (54.2)</td>
<td>22 (45.8)</td>
<td>0.533</td>
<td>0.584</td>
<td>1.306 (0.637-2.677)</td>
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<tr>
<td>Yes</td>
<td>38 (47.5)</td>
<td>42 (52.5)</td>
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<td>Histology</td>
<td></td>
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<tr>
<td>AC</td>
<td>27 (46.6)</td>
<td>31 (53.4)</td>
<td>0.504</td>
<td>0.594</td>
<td>0.777 (0.387-1.560)</td>
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<tr>
<td>Others</td>
<td>37 (52.9)</td>
<td>33 (47.1)</td>
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<tr>
<td>EGFR mutation</td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>56 (87.5)</td>
<td>58 (90.6)</td>
<td>0.321</td>
<td>0.571</td>
<td>1.381 (0.450-4.234)</td>
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<tr>
<td>Yes</td>
<td>8 (12.5)</td>
<td>6 (9.4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Stage</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>I+II</td>
<td>18 (28.1)</td>
<td>32 (50.0)</td>
<td>6.433</td>
<td>0.018</td>
<td>0.391 (0.188-0.814)</td>
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<tr>
<td>III+IV</td>
<td>46 (71.9)</td>
<td>32 (50.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10398</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>34 (54.0)</td>
<td>29 (46.0)</td>
<td>0.781</td>
<td>0.480</td>
<td>1.368 (0.683-2.741)</td>
</tr>
<tr>
<td>G</td>
<td>30 (46.2)</td>
<td>35 (53.8)</td>
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<td></td>
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</tbody>
</table>

The $\chi^2$ tests were two-sided. mtDNA, mitochondrial DNA; AC, adenocarcinoma. OR, odds ratio; CI, confidence interval.

Figure 2. Survival analysis for mitochondrial DNA (mtDNA) content and G10398A polymorphism. (A) mtDNA content alone; (B) mtDNA content plus G10398A polymorphism. Overall survival time in NSCLC patients was significantly shorter in patients with low mtDNA content plus 10398A than in patients with high mtDNA content plus 10398G (median survival time 26.3 vs. 47.3 months; $\chi^2=6.010$; $P=0.0141$; Kaplan-Meier analysis, log-rank test).

Generation and verification of $q^0$ cell model. To verify the mtDNA depletion, total cellular DNA was extracted and subjected to PCR using two pairs of human mtDNA specific primers, as previously described. $q^+$ and $q^0$ cells contained equivalent amounts of GAPDH, indicating that nuclear DNA was similar between the cell lines. However, $q^+$ cells contained mtDNA, while $q^0$ cells contained no mtDNA (Fig. 3A). The growth defects experiment found growth inhibition in $q^+$ cells when the culture medium lacked sodium pyruvate and uridine. $q^0$ cells continued to proliferate at a slower speed in the complete medium containing sodium pyruvate and uridine (Fig. 3B).
Mitochondrial dysfunction results in radioresistance in human NSCLC cells. Colony formation assay was used to evaluate the radiosensitivity of A549 ρ₀ and ρ+ cells. A549 ρ₀ and ρ+ cells were treated with different doses of X-ray irradiation, and the cloning efficiency (PE) and survival fraction (SF) were calculated. The linear-quadratic model was used to fit the survival curves (Fig. 4). Surviving fraction of cells after irradiation in 2 Gy (SF₂) was 0.675±0.013 and 0.492±0.022 (P<0.01), suggesting that mtDNA deletion induced radioresistance of A549 cells.

mtDNA depletion suppresses radiation-induced G2 checkpoint activation in human NSCLC cells. Cell cycle analysis showed that both ρ₀ and ρ+ cells showed G2 arrest after 4 Gy radiation, but the G2 arrest in ρ₀ cells was inferior to ρ+ cells (39.55±0.050% vs. 48.08±2.92%, P<0.01). The A549 ρ+ cell G2 arrest peak appeared at 12 h after irradiation, while the A549 ρ₀ cell appeared at 16 h (Fig. 5); S and G1 phase changes had no significant difference.

mtDNA depletion suppresses radiation-induced reactive oxygen species (ROS) production in human NSCLC cells. The ROS levels of ρ₀ cells were significantly less than ρ+ cells. The rate of fluorescence increase (%) improved after the 4 Gy radiation exposure and peaked at 90 min both in ρ₀ and ρ+ cells (254.17±13.65% vs. 594.28±38.22%, P<0.01) (Fig. 6).

Table III. Multivariate analysis of overall survival.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wald</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>6.235</td>
<td>0.013</td>
<td>2.186</td>
<td>1.152-4.147</td>
</tr>
<tr>
<td>mtDNA plus AG</td>
<td>18.515</td>
<td>0.000</td>
<td>0.392</td>
<td>0.256-0.601</td>
</tr>
</tbody>
</table>

Cox regression analysis, forward: Wald; P=0.05, entry; P=0.10, removal. OR, odds ratio; CI, confidence interval.

**Figure 3.** Generation and verification of ρ₀ Cells. (A) Mitochondrial DNA (mtDNA) and β-actin gene were amplified by PCR. The PCR products were visualized on 2% agarose gel by EtBr staining, showing the absence of mitochondrial genome in ρ₀ cells. (B) ρ₀ cells exhibited profoundly delayed growth kinetics compared to their parental cells.

**Figure 4.** The cell survival curve of A549 ρ₀ and ρ+ cells. Cells were irradiated with 0, 1, 2, 4, 6, 8, and 10 Gy, and then plated for colony formation for 2 weeks.

**Figure 5.** The change of G2 phase in A549 ρ₀ and ρ+ cells after 4 Gy irradiation. After radiation, ρ₀ cells showed delayed G2 arrest.

**Figure 6.** The change of reactive oxygen species (ROS) level after irradiation in A549 ρ₀ and ρ+ cells after 4 Gy irradiation. The ROS level of ρ₀ cells was significantly less than ρ+ cells. **P<0.01.**
Mitochondria are important and semi-autonomous organelles in eukaryotic cells. Each mitochondrion contains 2-10 copies of its genome. The copy number of mtDNA in each cell varies with cell type. In addition to plenty of somatic mutations in mtDNA, the increase or reduction of mtDNA copy number has been increasingly reported in a variety of primary human cancers, underscoring that accumulation of mtDNA alterations may be a pivotal factor in cancer pathogenesis and progression (14).

Previous studies indicated the potential involvement of both mutations and alteration of mtDNA content in the tumorigenesis of several malignancies (15). For instance, mtDNA content in patient tissues has been found to be increased in head and neck as well as in ovary cancer (8), but decreased in hepatocellular carcinoma (HCC) (16), renal cell carcinoma (RCC) (17) and advanced gastric cancer (18). These results highly suggested that the role of mtDNA in human cancer was cancer site-specific. A case-control study including 260 RCC patients and 281 matched control subjects showed that low mtDNA content was associated with a significantly increased risk of RCC (OR, 1.53; 95% CI, 1.07-2.19) (19).

Furthermore, mtDNA content was also found to be related to patient prognosis. After an anthracycline-based regimen, the disease-free survival time of breast cancer patients with higher mtDNA content was significantly shorter than that of patients with lower mtDNA content (P=0.03) (20). Patients with HCC harboring lower mtDNA quantity reportedly tended to have poorer prognosis and shorter 5-year overall survival (OS) rates in comparison with the cases with higher mtDNA quantity (13). Similarly, the decline in mtDNA levels was more prevalently identified in the ulcerated and in filtrating type (Borrmann's type III) and diffusely thick type (Borrmann's type IV) of gastric carcinoma, both of which are more likely to have adverse post-operative outcome (21).

For NSCLC, Wang et al (17) found that the mean copy number of mtDNA in lung carcinoma tissue samples was statistically lower than that in adjacent histologically normal lung tissue samples. Lin et al (7) reported that decreased relative value of mtDNA copy number was linked to the advancement of tumor progression. Similarly, our results revealed that patients with low mtDNA content had a marginally shorter survival time than those with high mtDNA content. The reason may be the small sample size of our study.

mtDNA content as well as mtDNA mutations have been reported to be potentially involved in cancer; 8701 and 10398 that code for ATPase6 and NADH dehydrogenase 3 were reported to be mutational hotspots in the mitochondrial genome of lung cancer. The mtDNA G10398A polymorphism alters the structure of Complex I in the mitochondrial electron transport chain, an important site of free radical production. This polymorphism is associated with several neurodegenerative disorders. American women with the 10398A allele had a significantly increased risk of invasive breast cancer (22). In addition, mtDNA G10398A variant in African-American women with breast cancer provides resistance to apoptosis and promotes metastasis in mice (23). However, the relationship between G10398A polymorphism and NSCLC prognosis has not been reported. In this study, in NSCLC patients with high mtDNA content plus 10398G, OS increased by 79.8% and death risk decreased compared to patients with low mtDNA content plus 10398A (median survival time 47.3 vs. 26.3 months; $\chi^2=6.010; P=0.0141$; OR, 0.392, 95% CI, 0.256-0.601).

To explore the possible mechanism of mtDNA influence on the prognosis of NSCLC, $\rho^0$ cell model was established by long-term exposure to low concentration of ethidium bromide. Our results showed that the A549 $\rho^0$ cells showed more resistance to radiation than $\rho^+$ cells. Upon irradiation, $\rho^0$ cells showed delayed G2 arrest and decreased ability to recover from the G2 checkpoint compared to $\rho^+$ cells. Moreover, loss of mtDNA inhibited cell growth and reduced the level of ROS (24). Recently, a growing body of functional experiments suggested that mtDNA content variations have enough capability to affect several aspects of malignant cell behaviors, such as anticancer drug sensitivity, cell growth, apoptosis as well as their invasive and metastatic potentials (14). Disruption of mtDNA integrity has been demonstrated to significantly influence cancer cell proliferation both in vitro and in vivo. For instance, mtDNA-depleted leukemia MOLT-4 $\rho^0$ cells grew markedly slower than their respective parental cells, probably due to reduced ROS generation (25). Similarly, researchers have shown that mtDNA loss considerably decreased proliferative rate and inhibited anchorage-independent growth and in vivo tumorigenicity of T47D breast cancer cells (26).

mtDNA copy number alterations have been revealed to facilitate cancer cells in acquiring resistance to a number of antitumor chemotherapeutic agents and radiation. SK-Hepl hepatoma cells lacking mtDNA exhibited markedly reduced apoptotic death when exposed to doxorubicin and two other oxidative stressors, menadione and paraquat (27). mtDNA depletion of human pancreatic tumor cells (MiaPaCa-2) suppressed radiation-induced G2 checkpoint activation, which was accompanied by increases in both cyclin B1 and CDK1, resulted in radioresistance (28). However, Kawamura (29) reported that $\rho^0$ cells were more resistant to irradiation than $\rho^+$ cells. The p53 status of the cell lines used were different, Kulawiec et al (30) reported that p53 could regulate mtDNA copy number and mitocheckpoint pathway, which may explain the different results.

In conclusion, our results highlight the complex relationships between mtDNA copy number and G10398A mutation in the prognosis of patients with NSCLC. There was no relationship between mtDNA content and gender, age, smoking status, histology, EGFR mutation status or G10398A polymorphism. Thus, other interfering prognostic factors in this paper have been eliminated. Low mtDNA content plus 10398A could be a marker of poor prognosis in NSCLC. mtDNA copy number decrease and 10398A mutation may lead to mitochondrial dysfunction, thereby influencing biological behaviors and the sensitivity to anticancer treatment so as to result in the poor prognosis. The pitfall of this study is its small sample size. Therefore, further research is required to verify the prognostic value of mtDNA copy number and G10398A polymorphism.

Acknowledgements

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References


