Lymphocytic mitochondrial DNA deletions, biochemical folate status and hepatocellular carcinoma susceptibility in a case–control study

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Mitochondrial (mt) DNA deletions and low folate status, proposed characteristics of carcinogenesis, in relation to human hepatocellular carcinoma (HCC) susceptibility are not clearly understood. We hypothesised that low folate status may modify frequencies of mtDNA deletions in humans, both of which could predispose individuals to HCC development. Biochemical folate status of serum and lymphocytes, and frequencies of mtDNA deletions in lymphocytes were determined in ninety HCC cases and ninety cancer-free healthy controls, individually matched by age and sex. The data revealed that HCC patients had lower levels of serum folate (P = 0·0002), lymphocytic folate (P = 0·040) and accumulated higher frequency of lymphocytic mtDNA deletions (P < 0·0001) than the controls. In the total studied subjects, frequencies of lymphocytic mtDNA deletions were associated with hepatic B infection (P = 0·004) and HCC incidents (P = 0·001), and were correlated with serum folate (r = −0·314; P = 0·0001), lymphocyte folate (r = 0·163; P = 0·037) and α-fetal protein concentrations (r = 0·212; P = 0·005). After adjustment for age, sex, lifestyle and one-carbon metabolite factors, individuals with low blood folate (<11·5 nmol/l) or high mtDNA deletions (4 threshold cycle number (Ct) > 5·3) had increased risks for HCC (OR 7·7, 95% CI 1·9, 29·9, P = 0·003; OR 5·4; 95% CI 1·7, 16·8, P = 0·003, respectively). When combined with folate deficiency (serum folate < 14 nmol/l), the OR of HCC in individuals with high levels of lymphocytic mtDNA deletions was enhanced (OR 13·3; 95% CI 1·45, 122; P = 0·008). Further controlling for GOT and GPT levels, however, negated those effects on HCC risk. Taken together, the data suggest that biochemical folate status and liver injuries are important modulators to lymphocytic mtDNA deletions. The mt genetic instability that results from a high rate of mtDNA deletions and/or low folate status increased the risk for HCC, which is mediated by clinical hepatic lesions.

Biochemical folate status: Mitochondrial DNA deletions: Hepatocellular carcinoma: Liver injuries

Several studies have proposed that somatic mitochondrial (mt) DNA mutations of tissues may play an important role in tumorigenesis (1,2). The human mt genome comprises a 16·5 kb circular double-stranded DNA molecule encoding tRNA, rRNA and protein subunits for respiratory function. mtDNA is more vulnerable to oxidative damage than nuclear DNA due to the proximity of generated reactive oxygen species (ROS) and a low level of DNA repair (3). Mutations in mtDNA can accumulate to a greater extent than in nuclear DNA, being more than ten times as abundant as mutated p53 DNA (4). Among various types of mtDNA mutations, oxidative stress-associated large-scale deletions of mtDNA – a 4977 bp deletion in humans – are commonly found to accumulate in ageing tissues (5) and in tissues of patients with Kearns–Sayre syndrome and progressive external ophthalmoplegia (6). The mtDNA mutations including large deletions are also detected in bodily fluid (7) and tumours of several cancers (4,8–11). Although it is unclear whether mtDNA mutations underlie carcinogenesis in humans, the high sensitivity of mtDNA to oxidative stress and the higher rate of mtDNA mutations in pre-neoplastic lesions (10) suggest its usefulness as a marker for the early detection of genomic aberration.

Folate insufficiency is thought to influence DNA stability involved in cancer carcinogenesis (12,13). Folate deprivation results in DNA injuries such as increased uracil misincorporation (14,15), genomic DNA strand breaks (16) and global DNA hypomethylation (17). Several recent studies have demonstrated that folate status could also modulate mtDNA stability (18–20). Rats given folic acid supplementation have fewer mtDNA large deletions in hepatic tissue upon chemotherapeutic drug treatments (19) or in ageing liver tissues (18). Folate deficiency for 4 weeks increased the frequency of mtDNA large deletions in several tissues of young rats, and accumulation of such mtDNA deletions was inversely correlated with tissue folate levels (20). Low folate status or accumulation of the mtDNA

Abbreviations: Ct, threshold cycle number; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminases; HCC, hepatocellular carcinoma; mt, mitochondrial; ROS, reactive oxygen species.

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deletions beyond a certain threshold may result in mt dysfunction and ROS-generated vicious cycles leading to apoptotic signalling and cellular death \(^{(2,3,21)}\), all of which constitute plausible mechanisms in cancer development.

Hepatocellular carcinoma (HCC) is the third most frequent cause of death due to malignancies in men, and its incidence is increasing worldwide \(^{(22)}\). In rodent models, a methyl-deficient diet lacking in folate, choline and methionine promoted DNA instability and pre-neoplastic transformation of the liver, leading to spontaneous HCC \(^{(23–25)}\). A recent study in a prospective cohort showed an association of low blood folate with increased risks of liver damage and HCC \(^{(26)}\), suggesting a possible role of folate deficiency in the human liver carcinogenesis. However, the effects of folate status on mtDNA deletions, a characteristic of tumours, in relation to HCC risk are not clearly understood. To monitor for cancer-related mt genotoxicity and folate status in humans, tissue biopsy is not practical for clinical routine screening. Assays for mtDNA deletions and folate performed on samples of venous blood cells may serve as useful markers for the early detection of global genomic instability and cancer risk. We hypothesised that biochemical folate status may modify lymphocytic mtDNA deletions in humans, both of which could predict the susceptibility of individuals to hepatocarcinogenesis. The hypothesis was tested in a case–control study with ninety HCC patients and ninety cancer-free healthy controls, individually matched by age and sex. Potential mt genotoxic factors, serum folate levels, lymphocytic folate, homocysteine concentration and mtDNA deletions in lymphocytes were assayed. Their interaction and associations with HCC risk, simultaneously controlling for possible modifiers and/or confounders, were discussed.

Materials and methods

Study subjects

Between January 2005 and December 2006, the study subjects were recruited from Chi-Mei hospital, a medical centre to provide medical services to a defined population base in Tainan city and county in southern Taiwan. Eligibility criteria for cases from the Department of Internal Medicine included diagnosis with primary incident HCC. Patients were diagnosed with HCC by imaging examinations, including B-type ultrasonography, computed tomography, MRI and angiography by two physicians specialised in hepatology and oncology. For patients with a tumour size of 1–2 cm, the presence of HCC was histologically confirmed. All studied patients had primary HCC. Exclusion criteria for HCC patients were cardiac or renal diseases, overt diabetes, active intravenous drug abuse, and non-compliance. Of 120 eligible cases, those who declined to donate extra blood samples withdrew. Ninety HCC patients participated throughout the study. By matching age (±5 years) and sex to the cases, the controls were selected from enrolled lists of Health and Physical Examination Programmes provided by the Health Managing Centre of Chi-Mei Hospital. The controls were from a geographical background similar to the cases. In addition to the eligibility criteria for cases, ninety healthy controls free from HCC, viral infection, chronic liver disease and alcohol abuse were recruited into the study. The eligible controls and cases were interviewed by a trained professional for a complete medical and dietary history including HCC risk factors such as smoking and drinking habits. The study protocol was approved by the Joint Ethical Committee of Fu-Jen University and Chi-Mei Hospital. Informed consent was secured from all participants.

Blood biochemical determinations

Within 1 week following the diagnosis of HCC and before subsequent treatment, patients donated fasting blood samples. Blood of the controls was collected during their health examination. Peripheral blood samples were taken after a 12 h fasting period, chilled and transported to the laboratory. Plasma and serum samples were immediately separated upon arrival and were stored at −80°C until further analysis. Lymphocytes were purified from whole blood by standard Ficoll-Hypaque centrifugation, and were used for the folate assay and molecular genetic analysis. Folate and total homocysteine levels were measured in the serum samples with commercially available RIA kits (Becton Dickinson, Orangeburg, NY, USA) and by fluorescence polarisation immunoassay (Becton Dickinson) on an Abbott 130 AxSYM system (Becton Dickinson), respectively. Serum glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and α-fetal protein concentrations were measured by standard protocols (ITC Diagnostics, Taiwan). The detection of hepatitis B and C infection was established by seropositivity for hepatitis B surface antigen, or by antibody to the hepatitis C virus (anti-HCV). Serum hepatitis B surface antigen was tested using a RIA kit (Abbott Laboratories, North Chicago, IL, USA). Anti-HCV was detected by an enzyme immunoassay kit (Abbott Laboratories).

Lymphocytic folate assay

Samples for the lymphocytic folate analysis were prepared as described by Varela-Moreiras & Selhub \(^{(27)}\). Briefly, lymphocytes were added to an extraction solution containing 5 mmol 2-mercaptoethanol, 0.1 mol sodium ascorbate, 50 mmol HEPES and 50 mmol 2-[N-cyclohexy]lamin]ethanesulfonic acid per litre (pH 7.85). The mixture was heated in a boiling water-bath for 10 min and cooled on ice. The supernatant fraction extract was stored at −70°C in N\(_2\) for later analysis. After incubation of the thawed sample extracts with chicken pancreas conjugase (4:1, v/v) (DIFICO 6048505; Becton Dickinson Company, MD, USA) at 37°C for 6 h, a microbiological assay was performed using cryoprotected _Lactobacillus casei_ casei in ninety-six-well microtitre plates \(^{(28)}\). Absorbance was detected at 600 nm in an MRX model ELISA reader (Dynatech Laboratories, Billingshurst, West Sussex, UK).

Real-time polymerase chain reaction analysis of mitochondrial DNA deletions

Lymphocytic DNA was extracted by a standard proteinase K digestion and the phenol–chloroform extraction procedure. According to the method of He _et al._ \(^{(29)}\), the mt _ND1_ gene with a rarely deleted region and the mt _ND4_ gene, which is commonly absent in the majority of patients with large deletions, was quantified by real-time PCR analysis. The PCR primers and fluorogenic probe for the _ND1_ region include the forward primer (L3485–3504), reverse primer...
Baseline data, one-carbon metabolites and mitochondrial DNA deletions in the study subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n = 90)</th>
<th>HCC cases (n = 90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean 60.9±8.6</td>
<td>Mean 61.1±9.8</td>
</tr>
<tr>
<td>Sex (Male)</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9±0.5</td>
<td>24.3±0.3</td>
</tr>
<tr>
<td>Regular alcohol intake‡</td>
<td>Subjects: 38</td>
<td>Subjects: 39</td>
</tr>
<tr>
<td></td>
<td>Subjects (%): 42</td>
<td>Subjects (%): 49</td>
</tr>
<tr>
<td>Smoking habit§</td>
<td>Subjects: 21</td>
<td>Subjects: 48*</td>
</tr>
<tr>
<td></td>
<td>Subjects (%): 23</td>
<td>Subjects (%): 53</td>
</tr>
<tr>
<td>GOT (IU/l)</td>
<td>33</td>
<td>112*</td>
</tr>
<tr>
<td>GPT (IU/l)</td>
<td>92</td>
<td>116*</td>
</tr>
<tr>
<td>α-Fetoprotein levels (μg/l)</td>
<td>3.4±1.5</td>
<td>2347±8*</td>
</tr>
<tr>
<td>Serum folate (nmol/l)</td>
<td>26.1±15.6</td>
<td>16.8*</td>
</tr>
<tr>
<td>Serum homocysteine (μmol/l)</td>
<td>9.2±2.6</td>
<td>11.6*</td>
</tr>
<tr>
<td>Lymphocytic folate (ng/mg protein)</td>
<td>1.7±1.9</td>
<td>1.2*</td>
</tr>
<tr>
<td>mtDNA deletions (ΔCt)</td>
<td>3.5±1.6</td>
<td>4.9*</td>
</tr>
</tbody>
</table>

HCC, hepatocellular carcinoma; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; Ct, threshold cycle number.

Table 1. Baseline data, one-carbon metabolites and mitochondrial DNA deletions in the study subjects

<table>
<thead>
<tr>
<th>Mean values and standard deviations</th>
</tr>
</thead>
</table>

Potential risk factors associated with lymphocyte mitochondrial DNA deletions in the studied 180 subjects

Levels of lymphocytic mtDNA deletions in the total 180 studied subjects were stratified into low (ΔCt < 2.53), moderate (2.53 ≤ ΔCt < 5.32), and high (ΔCt ≥ 5.32) categories, and the potential factors associated with the mtDNA deletions were examined (Table 2). The demographic and lifestyle factors such as age, sex, BMI, smoking and drinking habits did not correlate with frequencies of lymphocytic mtDNA deletions; neither were the clinical complications of ascites and liver cirrhosis. An increased frequency of lymphocytic mtDNA deletions was associated with hepatitis B infection (P = 0.004), levels of serum GOT (P = 0.02) and α-fetoprotein (P = 0.03) and HCC incidents (P = 0.001). A high frequency of lymphocytic mtDNA deletions was associated with low levels of lymphocytic folate (P = 0.02).

Table 3 demonstrates the dependence between one-carbon metabolic markers and lymphocytic mtDNA deletions, one-carbon metabolites and liver injury markers using Pearson’s correlation coefficient. In the total studied subjects, mtDNA deletions and hepatocellular carcinoma...
etions in lymphocytes were inversely related to serum folate \( r^2 = 0.155; P = 0.041 \) and lymphocyte folate status \( r^2 = 0.314; P = 0.0001 \), and positively correlated with GOT \( r = 0.206; P = 0.006 \) and GPT levels \( r = 0.163; P = 0.037 \). Correlation between lymphocyte folate, \( \alpha \)-fetal protein levels and mtDNA deletions also existed in the control group and/or the HCC cases.

**Table 4** shows the associations of lymphocytic mtDNA deletions and blood folate levels with HCC risk. After adjustment by demographic, lifestyle and one-carbon metabolite

**Table 3.** Univariate analysis for relationships between lymphocytic mitochondrial DNA deletions, one-carbon metabolic factors and liver injury markers in the case–control data

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total subjects</th>
<th>Controls</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R )</td>
<td>( P )</td>
<td>( r )</td>
</tr>
<tr>
<td>Serum folate level (nm)</td>
<td>0.155</td>
<td>0.041</td>
<td>0.024</td>
</tr>
<tr>
<td>Serum homocysteine (( \mu )M)</td>
<td>0.123</td>
<td>0.103</td>
<td>0.154</td>
</tr>
<tr>
<td>Lymphocyte folate (ng/mg protein)</td>
<td>0.314</td>
<td>0.0001</td>
<td>0.267</td>
</tr>
<tr>
<td>GOT (IU/l)</td>
<td>0.206</td>
<td>0.006</td>
<td>0.055</td>
</tr>
<tr>
<td>GPT (IU/l)</td>
<td>0.163</td>
<td>0.037</td>
<td>0.010</td>
</tr>
<tr>
<td>( \alpha )-Fetal protein (( \mu )g/l)</td>
<td>0.212</td>
<td>0.005</td>
<td>0.086</td>
</tr>
</tbody>
</table>

GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase.

Lymphocytic mitochondrial DNA deletion and folate levels in relation to risk for hepatocellular carcinoma

Table 4 shows the associations of lymphocytic mtDNA deletions and blood folate levels with HCC risk. After adjustment by demographic, lifestyle and one-carbon metabolite
factors, a positive mt genotoxicity–HCC association was observed in the highest tertile of lymphocytic mtDNA deletions (adjusted OR 5·4; 95 % CI 1·71, 16·8; P = 0·003; model B). A positive low-folate–HCC association was present in the lowest tertile of serum folate levels (adjusted OR 7·7, 95 % CI 1·98, 29·9; P = 0·003; model B). Both positive and significant associations were negated after further adjustment by liver injury markers (GOT and GPT levels) (model C). Subjects in the highest tertile of lymphocytic folate levels had a reduced risk for HCC, yet with no statistical significance (data not shown).

Modification of mitochondrial genotoxicity–hepatocellular carcinoma relationships by serum folate

We examined the mt DNA deletions–HCC risk associations stratified by serum folate status. Folate status was classified into adequate (serum folate ≥ 14 nmol/l or 6 ng/ml) or inadequate (serum folate < 14 nmol/l or 6 ng/ml), an indication of folate deficiency (30). Discernible modifying effects by folate factors on mt genotype–HCC risk were noted (Fig. 1). The significant association of lymphocytic mt deletions with increased HCC risk was observed among those with sufficient (OR 5·72; 95 % CI 1·5, 21·7; P = 0·008). When combined with folate deficiency (serum folate < 14 nmol/l), OR of HCC in individuals with high levels of lymphocytic mtDNA deletions was enhanced (OR 13·3; 95 % CI 1·45, 122; P = 0·008). However, the modifying effects of folate status on mt genotoxicity–HCC risk were negated after adjustment for liver injury factors.

Discussion

Our data show that the genetic instability that results in a high rate of mtDNA deletions increases susceptibility to HCC carcinogenesis. Individuals with high frequencies of mtDNA deletions in lymphocytes had increased risks for HCC (adjusted OR 7·7, 95 % CI 1·98, 29·9; P = 0·003).

As lymphocytic chromosomal damage may reflect similar lesions of target cells (31), lymphocytic mtDNA deletions were significantly correlated with hepatic mtDNA damage in a rodent model (Y.-F. Chou and R.-F. S. Huang, unpublished results). Indeed, it has been reported that mtDNA deletions were detected in cirrhotic livers (32), pre-neoplastic lesions of HCC and cancerous liver tissues of HCC patients (31,33). Accumulation of mtDNA deletions beyond a certain threshold may involve the altered synthesis of mt proteins, respiratory chain abnormalities, electron leakage from the mt respiratory chain, ROS overproduction, and releases apoptotic death signalling (33,34,35). Given the fact that high rates of mtDNA deletions were associated with hepatitis B infections (Table 2), a pre-neoplastic condition with increased oxidative stress (36), the increased mtDNA mutations in HCC patients may also be associated with increased hepatic oxidative stress. The resulting vicious cycles of increased mtDNA damage, respiratory dysfunctions, ROS overproduction and mitochondria-to-nucleus stress signalling in virus-infected livers may contribute to the early stages of hepatocarcinogenesis and tumour progression (1).

One of the major findings in the study was that low folate status, a well-known genotoxic factor to induce chromosomal aberration (2–7), had significant impact on mt genomic instability in humans. For the first time, our data showed that mtDNA deletions in lymphocytes of the studied subjects (n 180) were inversely related to serum folate (r = −0·155; P = 0·041) and lymphocyte folate status (r = −0·314; P = 0·0001). This finding is consistent with results of previous studies in rodent models showing that changes in mtDNA deletions in various tissues of rats including liver and lymphocytes were folate dependent (18–20). Folate is known to possess antioxidant capability (37,38). Reduced serum folate levels elevated hepatic oxidative stress as a result of impaired antioxidant defence, elicited ROS generation and lipid peroxidation (39), an important modulator to accumulated mtDNA deletions (33). In mitochondria of folate-depleted livers, accumulated mtDNA deletions were associated with increased mt protein oxidative

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### Table 4. Risk of hepatocellular carcinoma (HCC) by frequency of mitochondrial DNA (mtDNA) deletions in lymphocytes and serum folate status

(Odds ratios and 95 % confidence intervals)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases</th>
<th>Controls</th>
<th>Model A†</th>
<th>Model B‡</th>
<th>Model C§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>OR 95 % CI</td>
<td>OR 95 % CI</td>
<td>OR 95 % CI</td>
</tr>
<tr>
<td>mtDNA deletion (ΔCt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2·53</td>
<td>15 17</td>
<td>29 33</td>
<td>1·0</td>
<td>1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>2·53–5·32</td>
<td>43 48</td>
<td>45 52</td>
<td>2·0</td>
<td>0·8, 4·5</td>
<td>2·0</td>
</tr>
<tr>
<td>≥ 5·32</td>
<td>32 35</td>
<td>16 15</td>
<td>5·2</td>
<td>1·9, 13·7</td>
<td>5·4</td>
</tr>
<tr>
<td>P</td>
<td>0·0009*</td>
<td>0·003*</td>
<td>0·130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum folate (nmol/l)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 25·9</td>
<td>12 13</td>
<td>33 37</td>
<td>1·0</td>
<td>1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>11·5–25·9</td>
<td>42 47</td>
<td>48 53</td>
<td>3·2</td>
<td>1·3, 7·5</td>
<td>2·3</td>
</tr>
<tr>
<td>&lt; 11·5</td>
<td>36 40</td>
<td>9 10</td>
<td>12·2</td>
<td>4·0, 36·7</td>
<td>7·7</td>
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<tr>
<td>P</td>
<td>0·001*</td>
<td>0·003*</td>
<td>0·280</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ct, threshold cycle number.

* Statistically significant between the first and third tertiles (P < 0·05).
† Model A: adjusted for age, sex, BMI, alcohol intake and smoking habit.
‡ Model B: adjusted for all parameters in model A with addition of lymphocytic folate, serum B12, homocysteine levels, serum folate or mtDNA deletions.
§ Model C: adjusted for all parameters in model B with the addition of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase levels.

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The present study had a number of limitations. The most important one is the relatively small sample size, which reduces the statistical power for subgroup analysis. The unavailability of tumour specimens for the analysis of mtDNA deletions restricts the provision of direct evidence to elucidate relationships between mt genomic instability and HCC carcinogenesis. Since blood samples of HCC patients were collected after cancer diagnosis, nutritional and mt genomic parameters might have been changed by the disease condition and/or undeclared medical use such as Chinese herbs before the study period. Finally, the inherent limitations associated with retrospective study designs do not depict the causal effect of promoting HCC by mtDNA mutations and folate insufficiency.

Despite these limitations, our data provide several clinical implications. This case–control study helps validate the use of lymphocytic mtDNA deletions as the biomarker of genomic instability to monitor liver injuries and HCC progression. Our findings provide new insights on the effects of low folate in mt genomic instability as risk factors of hepatocarcinogenesis. The development of HCC, a high-mortality cancer with complex aetiology, may involve both genetic and environmental factors. Further prospectively designed studies with large sample sizes of subjects with precancerous lesions are warranted to confirm the interrelationship between folate status and mt genotoxic biomarkers in relation to HCC carcinogenesis.

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M.-Y. W., C.-S. K. and C.-Y. L. contributed equally to the present study.

None of the authors has any conflict of interest to declare.

References


