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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 hepatitic B infection ($P=0·004$) and HCC incidents ($P=0·001$), and were correlated with serum folate (r $-0·155$; $P=0·041$), lymphocyte folate (r $-0·314$; $P=0·0001$), levels of glutamate-oxaloacetate transaminase (GOT) (r 0·206; $P=0·006$), glutamate-pyruvate transaminase (GPT) (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 (Δ threshold cycle number (C_t) $>5·3$) 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: C_t, 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-neoplasmic 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 angiograph 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 hepatic 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*-cyclohexylamino]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* 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

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(H3532–3553) and probe (DYXL-5′-(3506)CCA-TCA-CC-CTC-TAC-ATC-ACC-GCCC-(3529)-3′-BHQ1). Forward primer (L12087–12109), reverse primer (H12140–12170) and probe (6FAM-5′-(12111)-CCG-ACA-TCA-TTA-CCG-GGT-TTTCCTCTTG(12138)-3′-BHQ1) were for the amplification of the *ND4* region. PCR amplification was carried out in a 50 μl reaction volume consisting of TaqMan Universal Master Mix, 200 nmol/l of deletion *ND4* primer, 100 nmol/l of each *ND1* primer, 100 nmol/l of each mtDNA deletion probe, and the *ND1* probe primer. The cycling conditions included an initial phase of 2 min at 50°C and 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 0·5 min at 72°C. The fluorescence spectra were monitored with a LightCycler Detection System with Sequence Detection Software (version 4; Roche Diagnostics, Mannheim, Germany). The cycle at which a statistically significant increase in normalised fluorescence was first detected was designated as the threshold cycle number (C_t). The C_t values were used to quantify the relative amount of *ND4* to *ND1* with the equation:

$$R = 2^{-\Delta Ct},$$

where $\Delta C_t = mtC_{tND4} - mtC_{tND1}$. A smaller value of ΔC_t indicates a higher relative ratio of *ND4*:*ND1*, which thus represents fewer mtDNA deletions.

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS/STAT version 6.12; SAS Institute, Inc., Cary, NC, USA). As lymphocytic mtDNA deletions of the studied subjects were stratified into low, moderate and high levels, the absolute frequencies of categorical variables such age, sex and genotypes were compared using the χ^2 test. Demographic and laboratory data of continuous variables were compared using one-way ANOVA followed by Duncan's multiple-range test or Student's *t* test between cases and controls. Dependence between the one-carbon metabolic markers and lymphocytic mtDNA deletions was evaluated using Pearson's correlation coefficient. Logistic regression models were used to examine the associations between folate status, mtDNA deletions and risk for HCC. The strength of a given parameter associated with HCC was measured by its OR and the corresponding 95 % CI and two-sided *P* value. Non-normally distributed dependent variables were first transformed using a logarithmic function. Differences were considered to be statistically significant at $P < 0·05$.

Results

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

Table 1 presents the baseline data, clinical markers, one-carbon metabolites and mtDNA mutations of the 180 study subjects. The controls (*n* 90) and HCC cases (*n* 90) exhibited similar distribution in age, sex, BMI and alcohol intake. A higher proportion of tobacco smokers were observed in the HCC patients than in the controls ($P < 0·0001$). As compared with the controls, HCC patients had significantly higher levels of GOT, GPT and α-fetal protein as compared with the controls ($P < 0·05$). For markers of one-carbon metabolites, lower levels of serum folate ($P = 0·0002$) and lymphocytic folate ($P = 0·040$), and elevated homocysteine concentrations ($P = 0·0002$) were detected in the HCC patients than in the controls. HCC patients accumulated an increased frequency of mtDNA deletions in lymphocytes than the controls ($P < 0·0001$).

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

(Mean values and standard deviations)

	Controls (*n* 90)		HCC cases (*n* 90)	
Variable	Mean	SD	Mean	SD
Age (years)	60	9·8	62·1	10·8
Sex (*n*)				
Male	66		65	
Female	24		25	
BMI (kg/m²)	23·9	0·5	24	0·3
Regular alcohol intake‡				
Subjects (*n*)	38		44	
Subjects (%)	42		49	
Smoking habit§				
Subjects (*n*)	21		48*	
Subjects (%)	23		53	
GOT (IU/l)	29·4	11·2	122·2*	116
GPT (IU/l)	33	19·8	96·4*	82·2
α-Fetal protein levels (μg/l)	3·4	1·5	2347·8*	1543
Serum folate (nmol/l)	26·1	15·6	16·8*	16·8
Serum homocysteine (μmol/l)	9·2	2·6	11·6*	5·5
Lymphocytic folate (ng/mg protein)	1·7	1·9	1·2*	1·6
mtDNA deletions (ΔC_t)‖	3·5	1·6	4·9*	2·2

HCC, hepatocellular carcinoma; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; C_t, threshold cycle number.
* Value was significantly different from that of the controls ($P < 0·05$).
† Statistical differences were determined by Student's *t* test for continuous variables and by the χ^2 test for categorical variables.
‡ Regular alcohol intake was defined as one or more drinks per week.
§ Smoking habit was defined as ever or never smoking 6 months before the diagnosis of HCC presence.
‖ For the formula for calculating relative levels of mtDNA deletions of lymphocytes, see the Materials and methods section.

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

Levels of lymphocytic mtDNA deletions of the total 180 studied subjects were stratified into low ($\Delta C_t \leq 2·53$), moderate ($2·53 < \Delta C_t < 5·32$) and high ($\Delta C_t \geq 5·32$) 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-fetal protein ($P = 0·03$) 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 the 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 del-

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Table 2. Potential factors associated with mitochondrial DNA (mtDNA) deletions in lymphocytes of the study subjects†
(Mean values and standard deviations)

	Frequencies of mtDNA deletions in lymphocytes (ΔC_t)					
	Low (*n* 44): $\Delta C_t \leq 2{\cdot}53$		Moderate (*n* 88): $2{\cdot}53 < \Delta C_t < 5{\cdot}32$		High (*n* 44): $\Delta C_t \geq 5{\cdot}32$	
Clinical variables	Mean	SD	Mean	SD	Mean	SD
Age (years)	63·5	10·1	59·7	10·5	61·2	10
Sex (*n*)						
Male	36		61		34	
Female	8		27		14	
BMI (kg/m^2)	23·0	6·9	24·1	2·7	23·8	3·7
Regular alcohol intake‡						
Subjects (*n*)	17		42		23	
Subjects (%)	38		47		47	
Smoking habit§						
Subjects (*n*)	13		35		21	
Subjects (%)	29		39		43	
Serum folate (nM)	21·9	13·0	23·1	19·9	17·7	13·4
Lymphocyte folate (ng/mg protein)	2·1	1·9	1·7	1·9	0·6*	0·9
Serum homocysteine levels (μM)	10·2	4·5	10·3	4·1	10·8	5·0
Hepatitis B surface antigen						
Subjects (*n*)	4		18		18	
Subjects (%)	9		20		37*	
Antibody to hepatitis C virus						
Subjects (*n*)	10		20		11	
Subjects (%)	22		22		22	
GOT (IU/l)	63·5	68·2	79·9	109	85·1*	87·8
GPT (IU/l)	63·4	62·1	63·7	69·6	67·6	69·8
Median α-fetal protein (μg/l)‖	3·7		4·7		16·1*	
Ascites						
Subjects (*n*)	4		14		12	
Subjects (%)	9		15		25	
Liver cirrhosis						
Subjects (*n*)	13		32		21	
Subjects (%)	30		36		44	
HCC						
Subjects (*n*)	15		43		32*	
Subjects (%)	34		49		67	

C_t, threshold cycle number; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; HCC, hepatocellular carcinoma.
* Value was significantly different from that of the low-mtDNA deletions group ($P < 0{\cdot}05$).
† Data of continuous variables were compared using one-way ANOVA (analysis of covariance) followed by Duncan's multiple-range test.
‡ Regular alcohol intake was defined as one or more drinks per week.
§ Smoking habit was defined as ever or never smoking 6 months before the diagnosis of HCC presence.
‖ Data of α-fetal protein levels were log-transformed.

etions in lymphocytes were inversely related to serum folate (r −0·155; P=0·041) and lymphocyte folate status (r −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, α-fetal protein levels and mtDNA deletions also existed in the control group and/or the HCC cases.

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

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

	Total subjects		Controls		Cases	
Variables	*R*	*P*	*r*	*P*	*r*	*P*
Serum folate level (nM)	−0·155	0·041	−0·024	0·820	−0·125	0·273
Serum homocysteine (μM)	0·123	0·103	−0·154	0·161	0·089	0·405
Lymphocyte folate (ng/mg protein)	−0·314	0·0001	−0·267	0·015	−0·315	0·003
GOT (IU/l)	0·206	0·006	−0·055	0·606	−0·068	0·525
GPT (IU/l)	0·163	0·037	0·010	0·926	−0·168	0·117
α-Fetal protein (μg/l)	0·212	0·005	0·086	0·434	0·210	0·049

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

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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, $P=0·03$; 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[11,33]. 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[21,34,35]. Given the fact that high rates of mtDNA deletions were associated with hepatitis B infections (Table 2), a pre-neoplasmic 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 *in vivo* and *in vitro* 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[35]. In mitochondria of folate-depleted livers, accumulated mtDNA deletions were associated with increased mt protein oxidative

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)

	Cases		Controls		Model A†		Model B‡		Model C§	
Variables	*n*	%	*n*	%	OR	95 % CI	OR	95 % CI	OR	95 % CI
mtDNA deletion (ΔC_t)										
< 2·53	15	17	29	33	1·0		1·0		1·0	
2·53–5·32	43	48	45	52	2·0	0·8, 4·5	2·0	0·7, 5·4	3·1	0·5, 19·2
≥ 5·32	32	35	16	15	5·2	1·9, 13·7	5·4	1·7, 16·8	5·3	0·6, 45·2
P					0·0009*		0·003*		0·130	
Serum folate (nmol/l)										
> 25·9	12	13	33	37	1·0		1·0		1·0	
11·5–25·9	42	47	48	53	3·2	1·3, 7·5	2·3	0·8, 6·3	1·6	0·2, 12·5
< 11·5	36	40	9	10	12·2	4·0, 36·7	7·7	1·9, 29·9	3·8	0·3, 45·3
P					< 0·001*		0·003*		0·280	

C_t, 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 B_{12}, 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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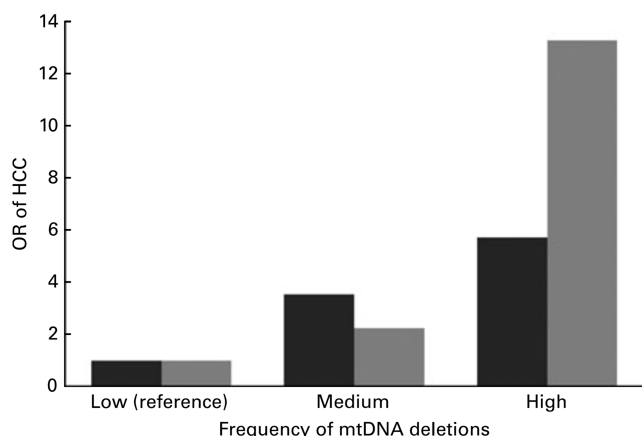

Fig. 1. Risk (OR) of hepatocellular carcinoma (HCC) by folate status and frequency of mitochondrial DNA (mtDNA) deletions. Folate status was classified into adequate (serum folate ≥ 14 nM; ■) and inadequate status (serum folate < 14 nM; ■). The numbers of cases and controls of the inadequate serum folate group with low (Δ threshold cycle number (C_t) < 2·53), moderate ($\Delta C_t = 2·53–5·32$) and high ($\Delta C_t \geq 5·32$) levels of mtDNA mutations were 11/4, 22/5 and 20/1, respectively. The numbers of cases and controls of the adequate serum folate group with low, moderate and high levels of mtDNA mutations were 4/25, 22/40 and 11/12, respectively.

injuries and reduced respiratory complex IV activities[40]. Hepatocytes with high levels of accumulated mtDNA deletions and/or low folate status lost mt respiratory function, leading to depolarisation of mt membrane potential and aberrant apoptotic signalling to promote premature cell death[40,41]. Increased mt genomic instability, elevated oxidative stress, and deregulation of apoptosis were contributable to pre-neoplastic transformation and HCC development[24]. The mechanisms proposed from the folate-depleted rodent model of HCC carcinogenesis may partially, if not all, explain why low serum folate and/or high lymphocytic mtDNA damage predict enhanced risk for HCC.

It is notable that adjustment for liver injury markers of GOT and GPT levels negated the association of folate and mtDNA deletions with HCC risk. The data suggest that the hepatic lesions are important confounders that modulate effects of low folate and mt genomic instability in HCC development. It has been known that HCC is usually preceded by chronic hepatic infection for numerous years. Individuals with viral hepatitis and impaired liver function reflected by high plasma levels of GOT and GPT may rapidly develop HCC[42]. Impaired liver function leads to altered folate-mediated methionine and homocysteine metabolism. Individuals with liver diseases commonly accompanied a low folate status, especially in patients with viral hepatitis[43]. Levels of hepatic injury markers were associated with serum folate levels[26,44]. Similarly, an increase in liver injuries from chronic hepatitis to cirrhosis impaired the mt respiratory activity and mtDNA replication in human liver[45]. We found that mtDNA deletions correlated with markers of liver dysfunction (GOT and GPT) levels of the studied subjects ($P < 0·05$). Collectively, the data suggest that low folate status and high mtDNA instability may be the secondary effect of liver lesions caused by the viral and hepatic pathogenic factors. In this case, folate status and lymphocytic mtDNA deletions may indicate an intermediate point of liver injury in relation to HCC development.

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

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