FULL PAPER

BJC

British Journal of Cancer (2015) 113, 99–106 | doi: 10.1038/bjc.2015.184

Keywords: mitochondrial DNA content; glioma; prognosis; immunophenotype; cytokine

High leukocyte mitochondrial DNA content contributes to poor prognosis in glioma patients through its immunosuppressive effect

Y Chen[1,4], J Zhang[2,4], X Huang[1], J Zhang[1], X Zhou[1], J Hu[1], G Li[3], S He[*,3] and J Xing[*,1]

[1]State Key Laboratory of Cancer Biology and Experimental Teaching Center of Basic Medicine, Fourth Military Medical University, 169 Changle West Road, Xi'an, Shaanxi 710032, China; [2]Department of Oncology, The First affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan 610500, China and [3]Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, 1 Xinsi Road, Xi'an, Shaanxi 710038, China

Background: Epidemiological studies have indicated significant associations of leukocyte mitochondrial DNA (mtDNA) copy number with risk of several malignancies, including glioma. However, whether mtDNA content can predict the clinical outcome of glioma patients has not been investigated.

Methods: The mtDNA content of peripheral blood leukocytes from 336 glioma patients was examined using a real-time PCR-based method. Kaplan–Meier curves and Cox proportional hazards regression model were used to examine the association of mtDNA content with overall survival (OS) and progression-free survival (PFS) of patients. To explore the potential mechanism, the immune phenotypes of peripheral blood mononuclear cells (PBMCs) and plasma concentrations of several cytokines from another 20 glioma patients were detected by flow cytometry and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: Patients with high mtDNA content showed both poorer OS and PFS than those with low mtDNA content. Multivariate Cox regression analysis demonstrated that mtDNA content was an independent prognostic factor for both OS and PFS. Stratified analyses showed that high mtDNA content was significantly associated with poor prognosis of patients with younger age, high-grade glioma or adjuvant radiochemotherapy. Immunological analysis indicated that patients with high mtDNA content had significantly lower frequency of natural killer cells in PBMCs and higher plasma concentrations of interleukin-2 and tumour necrosis factor-α, suggesting an immunosuppression-related mechanism involved in mtDNA-mediated prognosis.

Conclusions: Our study for the first time demonstrated that leukocyte mtDNA content could serve as an independent prognostic marker and an indicator of immune functions in glioma patients.

Glioma is the most common form of primary brain tumour, accounting for 7% of cancer-related death before the age of 70 years (Furnari *et al*, 2007; Gladson *et al*, 2010). Despite significant improvement in treatment for glioma patients, the median survival remains not optimistic, particularly for those with glioblastoma (Wen and Kesari, 2008). Patients with newly diagnosed glioblastoma exhibit a median survival of $\sim$1 year, with generally poor responses to all therapeutic modalities. Thus, the elucidation of glioma survival factors is imperative and could potentially give support to the treatment of glioma patients.

*Correspondence: Dr J Xing or Dr S He ; E-mail: xingjinliang@163.com or hshimin@fmmu.edu.cn
[4]These authors contributed equally to this work.

Received 22 December 2014; revised 13 April 2015; accepted 27 April 2015; published online 28 May 2015

Mitochondria are organelles generally found in all nucleated cells. Its major role is to generate cellular adenosine triphosphate (ATP) and reactive oxygen species (ROS) through oxidative respiration, to synthesise organic molecules and regulate the concentration of intracellular calcium and cellular apoptosis (Chan, 2006). Mitochondria have their own genome (mitochondrial DNA (mtDNA)), an ~16.6 kb circular double-stranded DNA that encodes 13 polypeptide subunits of the respiratory chain complexes, 2 rRNAs and a set of 22 tRNAs (Chen and Butow, 2005). Generally, each human cell contains several hundred to 1000 mitochondria, and each mitochondrion has 2 to 10 copies of mtDNA. Under physiological conditions, intracellular mtDNA content has a steady-state level in each specific tissue that is related to the energy demand of the host cells (Capps *et al*, 2003). Interindividual variations of mtDNA copy number in cells exist in the general population (Moraes, 2001).

Recent experimental and epidemiological studies have demonstrated that alterations of mtDNA content are involved in the development of cancer (Yu, 2011). It has been found that alterations of mtDNA content occur early in premalignant lesions (Lin *et al*, 2010; Cormio *et al*, 2012). Continuous changes of mtDNA content may last all through the procedure of carcinogenesis. When compared with their corresponding normal tissues, tumour tissues have the altered (increased or decreased) mtDNA content in many types of malignancies, including glioma (Wang *et al*, 2005; Yu *et al*, 2007; Mizumachi *et al*, 2008). Moreover, mtDNA content in cancer tissues are associated with the progression and chemosensitivity (Yu *et al*, 2007; Hsu *et al*, 2010; Lin *et al*, 2010; Cui *et al*, 2013; Guo *et al*, 2013) as well as the prognosis of patients (Yu *et al*, 2007; Cui *et al*, 2013; Cheau-Feng Lin *et al*, 2014). A series of epidemiological evidence suggest that mtDNA content variations in peripheral leukocytes can be used as a biomarker to predict the risks of cancers, such as renal, breast, lung, colorectal and liver cancers as well as non-Hodgkin's lymphoma (NHL) (Lan *et al*, 2008; Hosgood *et al*, 2010; Shen *et al*, 2010; Qu *et al*, 2011; Zhao *et al*, 2011). Our previous study has also demonstrated that higher peripheral leukocyte mtDNA content is associated with increased risk of glioma (Zhang *et al*, 2014). However, whether leukocyte mtDNA content can predict the prognosis of glioma patients has never been investigated.

In this study, we measured the leukocyte mtDNA content using a real-time PCR-based method and assessed its association with the overall survival (OS) and progression-free survival (PFS) of glioma patients after tumour resection. Furthermore, we explored the potential immunological mechanisms of glioma patients with different mtDNA content. To the best of our knowledge, this is the first study to assess the value of mtDNA content in predicting prognosis of glioma patients.

MATERIALS AND METHODS

Patients. A total of 336 glioma patients were enrolled at the Department of Neurosurgery, Tangdu Hospital in Xi'an, China, from January 2000 to April 2012. Patient eligibility criteria were as follows: (1) with histologically confirmed primary glioma, (2) received surgical resection, (3) availability of complete clinical and follow-up data, (4) no preoperative anticancer treatment, (5) no history of other synchronous malignancy and (6) alive at least 3 months after surgical operation. Moreover, 20 additional glioma patients were enrolled for the detection of peripheral blood mononuclear cell (PBMC) immunophenotypes and plasma cytokine concentrations at Tangdu Hospital from October to December in 2013. Before surgery, 5 ml venous blood sample was collected from each patient and subjected to genomic DNA extraction using the RelaxGene Blood DNA System (TIANGEN, Beijing, China) according to the manufacturer's instructions. The study was approved by the Ethic Committee of Fourth Military Medical University and informed consents were obtained from all participants. All experimental procedures were performed according to the Helsinki Declaration.

Patient data collection. Epidemiological information was collected using a standardised questionnaire through in-person interviewing by well-trained staffs. Clinical data were obtained by medical record review. Follow-up data were obtained through on-site interview, telephone calling or medical chart review. The latest follow-up date was March 2014. Overall survival time was defined as the interval from surgery to death or last follow-up. Progression-free survival time was defined as the interval from surgery to the date of the first magnetic resonance imaging (MRI)-confirmed tumour progression according to the RANO criteria (Brandsma and van den Bent, 2009).

Determination of mtDNA content by quantitative real-time PCR. Relative mtDNA content was measured by a quantitative real-time PCR-based method in the Mx3005P QPCR System (Agilent, Santa Clara, CA) as previously described, with the same primers that were used for the mitochondrial ND1 gene (ND1-F and ND1-R) and the single-copy nuclear gene human globulin (HGB-1and HGB-2) (Xing *et al*, 2008). In short, two pairs of primers were used in the two steps of relative quantification for mtDNA copy number. The primer sequences were as follows: ND1 forward (ND1-F), 5′-CCCTAAAACCCGCCACATCT-3′; ND1 reverse (ND1-R), 5′-GAGCGATGGTGAGAGCTAAGGT-3′; HGB forward (HGB-1), 5′-GTGCACCTGACTCCTGAGGAGA-3′; HGB reverse (HGB-2), 5′-CCTTGATACCAACCTGCCCAG-3′. In the first step, the ratio of mtDNA copy number to HGB copy number was calculated for each sample from standard curves. In the second step, the ratio for each sample was normalised to a calibrator DNA in order to standardise between different runs, and then defined as the measurement of relative mtDNA content. The calibrator DNA is a genomic DNA sample from a healthy control subject. It was measured in each run and used to standardise different real-time quantitative PCR runs. The value of this only scaling factor varied from 0.92 to 1.14 across different runs. The intra-assay or interassay variations were evaluated by assaying one sample in eight replicates or in three different runs, respectively.

The PCR reaction system (20 μl) consisted of 1 × SYBR green mastermix (TaKaRa, Dalian, China), 10 nM ND1-R (or HGB-1) primer, 10 nM ND1-F (or HGB-2) primer and 8 ng of genomic DNA. The thermal cycling conditions for both primer pairs were 95 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s with signal acquisition. The PCRs were always performed on separate 96-well plates, with the same samples in the same well positions. All samples were assayed in duplicate using the Mx3005P QPCR System (Agilent, Santa Clara, CA, USA). In each run, negative and positive controls, a calibrator DNA and a standard curve were included. For each standard curve, one reference DNA sample (the same DNA sample for all runs) was diluted with a three-fold increment per dilution to produce a 5-point standard curve between 0.593 and 48 ng DNA in each reaction. The R^2 for each standard curve was ≥0.99, with acceptable s.d. values set at 0.25 (for the Ct values). Otherwise, the test was repeated.

Determination of lymphocyte immunophenotypes by flow cytometry. The PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ, USA). The cells were then fixed and stained with isotype control immunoglobulins or fluorescence-conjugated antibodies against the following immune markers: CD3, CD4, CD8, CD25, FOXP3, CD19 and CD56 (eBioscience, San Diego, CA). Finally,

subtype analysis of PBMCs was carried out on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Cytokine detection by enzyme-linked immunoassay (ELISA). Blood plasma from CRC patients was by centrifugation at 4000 r.p.m. under 4 °C. The concentrations of interleukin (IL)-2, IL-4, transforming growth factor (TGF)-β1, tumour necrosis factor (TNF)-α and interferon (IFN)-γ were examined by ELISA using commercial kits according to the manufacturer's instructions (eBioscience).

Statistical analysis. Because of the nonlinearity in the effects of mtDNA content on the progression of cancer, either higher or lower cutoff point would affect the statistical power. Therefore, mtDNA content should be converted into category variable by the optimal cutoff point. Finally, we found that patients in the second and third tertile had similar median OS (21 and 25 months, respectively) and PFS (14 and 15 months, respectively) time, whereas those in the first tertile showed much longer OS (39 months) and PFS (29 months) time. We therefore converted mtDNA content into category variable by the first tertile value. Mann–Whitney *U*-test was used to examine the frequencies of immune cell subsets and plasma cytokine concentrations between patients with different mtDNA content. Mann–Whitney *U*-test was also used to examine the difference in mtDNA between two subgroups and Kruskal–Wallis test was used to test difference in mtDNA among more than three groups. Kaplan–Meier survival curve was plotted and compared by log-rank test. Multivariate Cox proportional hazards regression model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for prognosis evaluation. All statistical analyses were performed using the SPSS Statistics 22.0 software (IBM, Armonk, NY, USA), and $P<0.05$ was considered to be statistically significant.

RESULTS

Host characteristics and mtDNA content distribution of glioma patients. The demographic and clinical characteristics of the study population are summarised in Table 1. Among the total 336 patients, 212 were diagnosed with low-grade glioma (LGG, WHO grade 1 or 2) including 142 astrocytomas, 3 oligodendrogliomas, 53 oligoastrocytomas and 14 ependymomas, whereas 125 were diagnosed with high-grade glioma (HGG, WHO grade 3 or 4), including 81 anaplastic astrocytomas, 13 anaplastic oligodendrogliomas, 2 anaplastic oligoastrocytomas, 4 anaplastic ependymomas and 23 glioblastomas. During the follow-up period (median, 25 months; range, 3–99 months), 201 patients died of glioma and 217 developed recurrence or progression. The median OS and PFS time of the total population was 26.8 and 22.3 months, respectively. We further analysed the prognosis of patients with different grade gliomas. Generally, the prognosis pattern in our study is very similar as previously summarised (Ricard *et al*, 2012). In grade II gliomas, ependymoma and oligoastrocytoma showed similar prognosis that was better than that of astrocytoma. In grade III gliomas, anaplastic oligoglioma showed better prognosis than anaplastic astrocytoma (detailed data not shown).

We measured mtDNA content using a real-time PCR-based method in all samples. The mean inter-assay coefficient variation (CV) of real-time PCR reaction was 7.8% (range, 4.4–10.1%), whereas intra-assay CV was 4.7% (range, 2.9–7.4%), indicating good assay reproducibility. We then explored the distributions of mtDNA by characteristics of glioma patients. As shown in Table 1, there was no significant difference in mtDNA between patients with different age, gender, lesion location, WHO grade or treatment regimen.

Prognostic analysis of mtDNA content in glioma patients. We first compared the OS and PFS between patients with different mtDNA content. As shown in Figure 1, patients with high mtDNA content had poorer OS and PFS than those with low mtDNA content (log-rank $P=0.023$ for OS and $P=0.027$ for PFS). Univariate and multivariate Cox regression analyses revealed that age, WHO grade, resection degree and adjuvant therapy regimen were all independent prognostic factors for both OS and PFS of patients (Table 2). Moreover, univariate Cox regression analysis showed that both risks of death and progression decreased in patients with high mtDNA content when compared with those with low mtDNA content. Multivariate Cox regression analysis further confirmed mtDNA content as an independent prognostic factor for both OS and PFS (HR 1.477, 95% CI 1.075–2.029,

Table 1. Associations between mtDNA content and clinical characteristics of glioma patients

Variables	No. of cases	mtDNA content, median (range)	*P*-value
Age (years)			0.586
5–43	181	0.969 (0.100–2.927)	
44–81	155	0.980 (0.022–3.553)	
Gender			0.719
Female	147	0.969 (0.022–3.102)	
Male	189	0.979 (0.052–3.553)	
Location			0.213
Brain stem	9	0.882 (0.116–1.121)	
Cerebellum	12	0.909 (0.248–1.279)	
Diencephalon	25	1.190 (0.532–2.287)	
Telencephalon	260	0.957 (0.022–3.553)	
Ventricle	30	1.039 (0.122–2.867)	
WHO grade			0.835
I	18	0.962 (0.652–1.549)	
II	194	0.977 (0.022–3.102)	
III	100	0.929 (0.052–3.052)	
IV	24	1.059 (0.504–3.553)	
Surgery			0.422
GTR	276	0.967 (0.022–3.553)	
NTR	60	0.981 (0.116–2.361)	
Adjuvant therapy			0.116
None	30	0.916 (0.100–1.535)	
RT	165	1.008 (0.052–3.553)	
RCT	141	0.957 (0.022–2.959)	

Abbreviations: GTR = gross total resection; mtDNA = mitochondrial DNA; NTR = near total resection; RCT = radiochemotherapy; RT = radiotherapy; WHO = World Health Organisation.

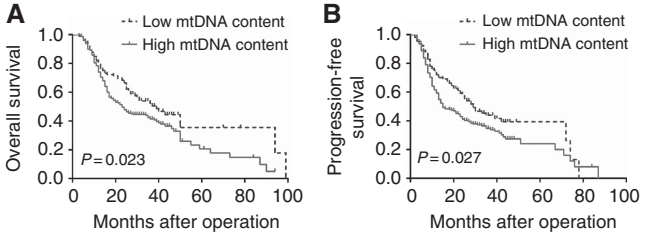

Figure 1. Kaplan–Meier curves of OS (**A**) and PFS (**B**) in glioma patients with different mtDNA content.

Table 2. Cox regression analysis of prognostic factors for glioma patients

	OS				PFS			
Variables	**Event/total**	**MST (months)**	**HR (95% CI)**	***P*-value**	**Event/total**	**MST (months)**	**HR (95% CI)**	***P*-value**
Univariate analysis								
mtDNA content								
Low	54/105	39.4	Ref		60/105	29.1	Ref	
High	147/231	22.9	1.43 (1.05–1.96)	0.025	157/231	15.2	1.39 (1.03–1.87)	0.030
Gender								
Female	80/147	34.0	Ref		87/147	25.2	Ref	
Male	121/189	24.5	1.23 (0.93–1.64)	0.148	130/189	18.8	1.27 (0.97–1.67)	0.082
Age (years)								
<44	89/170	40.3	Ref		103/170	29.1	Ref	
≥44	112/166	20.4	1.73 (1.30–2.29)	<0.001	114/166	14.3	1.49 (1.14–1.95)	0.004
Grade								
LGG	103/212	46.1	Ref		113/212	37.1	Ref	
HGG	98/124	13.2	2.72 (2.05–3.59)	<0.001	104/124	9.9	2.85 (2.17–3.74)	<0.001
Surgery								
GTR	161/276	30.4	Ref		175/276	23.8	Ref	
NTR	40/60	21.7	1.46 (1.03–2.07)	0.034	42/60	13.7	1.48 (1.05–2.07)	0.025
Adjuvant therapy								
RT	106/165	24.5	Ref		113/165	18.8	Ref	
RCT	78/141	34	0.75 (0.55–1.01)	0.060	86/141	25.2	0.76 (0.57–1.02)	0.064
Multivariate analysis								
mtDNA content								
Low	54/105	39.4	Ref		60/105	29.1	Ref	
High	147/231	22.9	1.48 (1.08–2.03)	0.016	157/231	15.2	1.53 (1.13–2.07)	0.006
Gender								
Female	80/147	34.0	Ref		87/147	25.2	Ref	
Male	121/189	24.5	1.09 (0.80–1.43)	0.661	130/189	18.8	1.13 (0.85–1.49)	0.401
Age (years)								
<44	89/170	40.3	Ref		103/170	29.1	Ref	
≥44	112/166	20.4	1.76 (1.32–2.34)	<0.001	114/166	14.3	1.54 (1.17–2.02)	0.002
Grade								
LGG	103/212	46.1	Ref		113/212	37.1	Ref	
HGG	98/124	13.2	2.74 (2.06–3.66)	<0.001	104/124	9.9	2.93 (2.21–3.89)	<0.001
Surgery								
GTR	161/276	30.4	Ref		175/276	23.8	Ref	
NTR	40/60	21.7	1.36 (0.95–1.94)	0.091	42/60	13.7	1.36 (0.96–1.92)	0.086
Adjuvant therapy								
RT	106/165	24.5	Ref		113/165	18.8	Ref	
RCT	78/141	34	0.74 (0.54–1.01)	0.058	86/141	25.2	0.73 (0.54–0.99)	0.042

Abbreviations: CI = confidence interval; GTR = gross total resection; HGG = high-grade glioma; HR = hazard ratio; LGG = low-grade glioma; MST = median survival time; mtDNA = mitochondrial DNA; NTR = near total resection; OS = overall survival; PFS = progression-free survival; RCT = radiochemotherapy; Ref = reference; RT = radiotherapy. Hazard ratio and 95% CI calculated in multivariate regression analyses were adjusted for age, gender, mtDNA content, World Health Organisation (WHO) grade, resection extent and adjuvant therapy.

$P = 0.016$ for OS; HR 1.528, 95% CI 1.130–2.066, $P = 0.006$ for PFS; Table 2). To exclude the confounding factors that might affect the prognostic value of mtDNA content, we further analysed the effects of mtDNA content on the OS and PFS of glioma patients stratified by clinical characteristics. As shown in Table 3, high mtDNA content was significantly associated with poor prognosis in patients with older age, HGG or concomitant radiochemotherapy, but not in those with younger age, LGG or radiotherapy only. Kaplan–Meier curve analysis further confirmed the results of Cox regression model (Figure 2 and Supplementary Figure S1).

Immunophenotypes of lymphocytes and concentration of plasma cytokines in glioma patients with different mtDNA content. To explore the underlying mechanisms that account for the different prognosis of glioma patients with different mtDNA content, we first examined the subtypes of lymphocytes in PBMCs from glioma patients by flow cytometry analysis. Our results showed that patients with high mtDNA content had significantly lower frequency of NK cells (9.8% *vs* 18.9%, $P = 0.037$, Figure 3G) than those with low mtDNA content. No significant difference on percentage of total, $CD4^+$ or $CD8^+$ T cells, B cells or Tregs was observed between the two patient subgroups (Figure 3).

Previous studies have demonstrated that mtDNA may affect the secretion of cytokines from immune cells, such as IL-2 and IL-4 (Kaminski *et al*, 2010). Interleukin-2 plays crucial roles in the survival, proliferation and activation of T and NK cells (Abbas *et al*, 2012). Interferon-γ and IL-4 are the key cytokines for cell differentiation in cell-mediated and humoral immune responses, respectively. Transforming growth factor-β1 is the main mediator of immunosuppressive effects conferred by Tregs. Tumour necrosis factor-α and IFN-γ may exert anticancer effects by directly killing target cells. We therefore examined the association between mtDNA content and plasma concentrations of these cytokines. As shown in Figure 3, high mtDNA content was significantly associated with higher concentration of IL-2 (50.3 *vs* 38.9 pg ml^{-1}, $P = 0.009$) and TNF-α (63.3 *vs* 41.6 pg ml^{-1}, $P = 0.023$). No significant concentration difference of IL-4, IFN-γ or TGF-β1 was noted between two patient subgroups.

Table 3. Stratified analysis of association between mtDNA content and glioma prognosis by Cox regression model

		OS				PFS			
Strata	**mtDNA content**	**Event/total**	**MST (months)**	**HR (95% CI)**	***P*-value**	**Event/total**	**MST (months)**	**HR (95% CI)**	***P*-value**
Age (years)									
<44	Low	20/52	94.2	Ref		27/52	39.2	Ref	
	High	69/118	35.2	2.49 (1.47–4.2)	0.001	76/118	22.3	1.92 (1.20–3.06)	0.006
≥44	Low	34/53	24.2	Ref		33/53	19.7	Ref	
	High	78/113	16.7	1.09 (0.72–1.65)	0.7	81/113	12.2	1.40 (0.92–2.13)	0.119
Grade									
LGG	Low	28/67	49.5	Ref		32/67	39.2	Ref	
	High	75/145	43.0	1.34 (0.86–2.09)	0.193	81/145	37.0	1.21 (0.80–1.83)	0.364
HGG	Low	26/38	23.4	Ref		28/38	14.0	Ref	
	High	72/86	12.0	1.80 (1.11–2.91)	0.017	76/86	8.6	2.12 (1.32–3.40)	0.002
Adjuvant therapy									
RT	Low	29/46	27.0	Ref		31/46	20.1	Ref	
	High	77/119	24.2	1.18 (0.75–1.84)	0.477	82/119	14.9	1.22 (0.79–1.87)	0.371
RCT	Low	21/49	94.2	Ref		25/49	32.6	Ref	
	High	57/92	20.9	2.10 (1.25–3.52)	0.005	61/92	15.0	1.96 (1.21–3.18)	0.006

Abbreviations: CI = confidence interval; HGG = high-grade glioma; HR = hazard ratio; LGG = low-grade glioma; MST = median survival time; mtDNA = mitochondrial DNA; OS = overall survival; PFS = progression-free survival; RCT = radiochemotherapy; Ref = reference; RT = radiotherapy. Hazard ratio and 95% CI were calculated by multivariate regression analyses with adjusting for age, gender, mtDNA content, World Health Organisation (WHO) grade, resection extent and adjuvant therapy where appropriate.

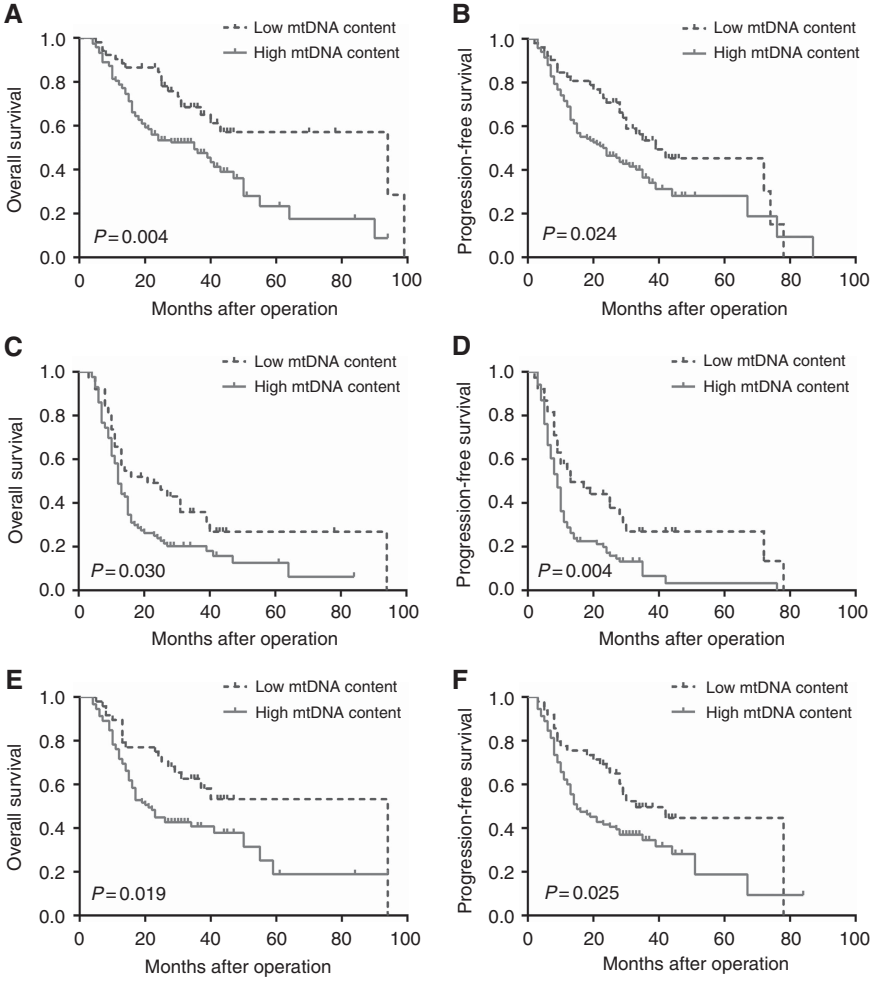

Figure 2. Kaplan–Meier curves stratified by age, WHO grade or adjuvant therapy. (**A**, **C** and **E**) Overall survival (OS) of younger patients, patients with HGG and receiving radiochemotherapy, respectively. (**B**, **D** and **F**) Progression-free survival (PFS) of younger patients, patients with HGG and receiving radiochemotherapy, respectively.

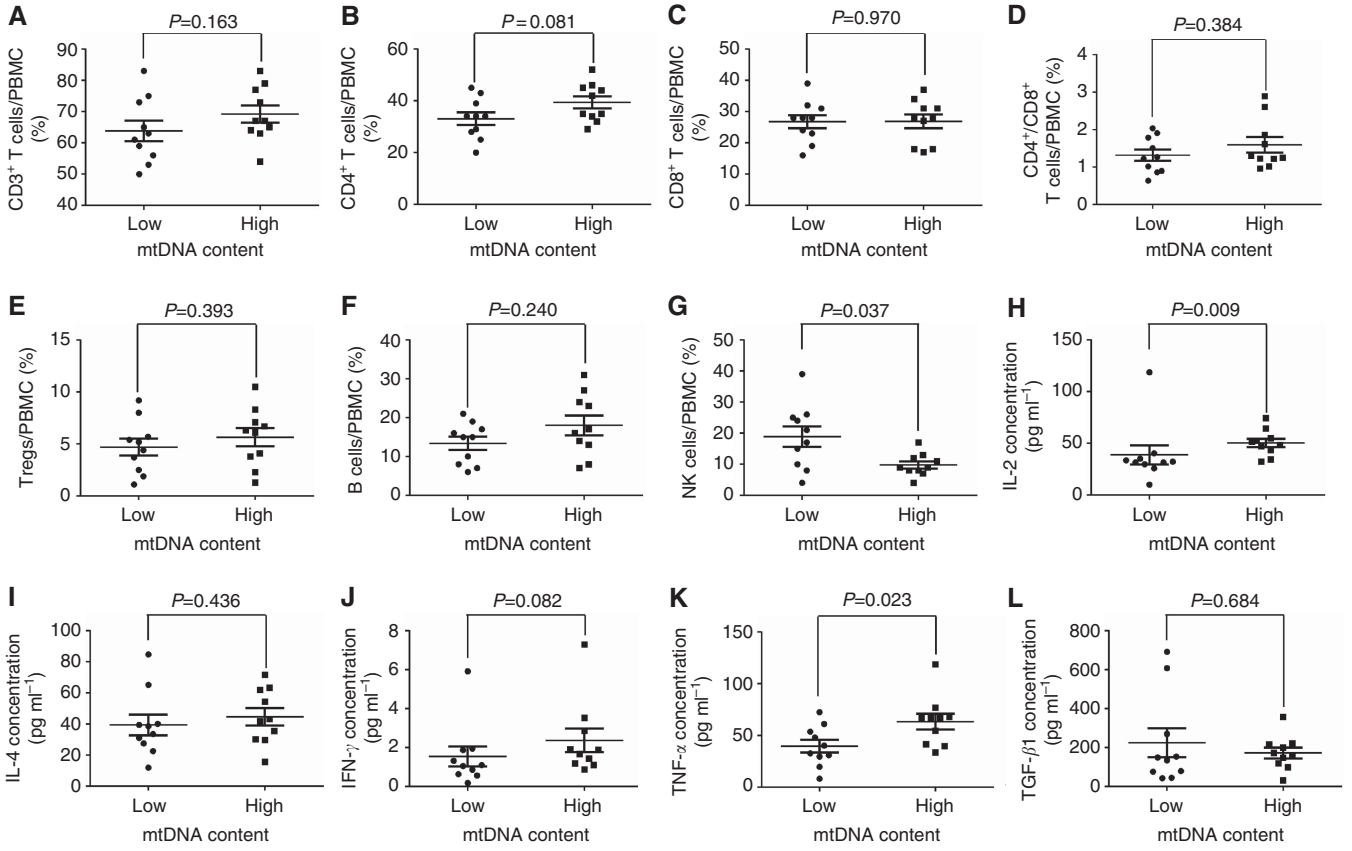

Figure 3. Immunophenotypes of glioma patients with different mtDNA content. (**A–G**) Frequencies of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$, Treg, B and NK cells in PBMCs, respectively. (**H–L**) Plasma concentrations of IL-2, IL-4, IFN-γ, TGF-β1 and TFN-α, respectively.

DISCUSSION

In this study, we explored the prognostic value of leukocyte mtDNA content in 336 glioma patients. We found that patients with high mtDNA content showed significantly poorer OS and RFS than those with low mtDNA. We also evaluated the association of mtDNA content with immune functions of glioma patients and found that high mtDNA content was associated with lower NK cell frequency and higher IL-2 and TNF-α concentrations. These data suggest that high mtDNA content may contribute to the progression of glioma possibly through the abnormal alteration of immune functions.

Alteration of mtDNA content plays an important role in the development of several malignancies (Yu, 2011). High mtDNA content may increase the intracellular bioenergenesis and anabolism, leading to a proliferative advantage and enhanced chemoresistance in cancer cells (Cavalli *et al*, 1997; Hsu *et al*, 2010). Progressive increase of mtDNA content has been observed during the malignant transformation from normal epithelia to endometrial, oesophageal or head and neck cancers (Kim *et al*, 2004; Lin *et al*, 2010; Cormio *et al*, 2012). Furthermore, high mtDNA content in cancer tissues has been shown to be associated with progression of oesophageal and laryngeal squamous cell carcinoma (Lin *et al*, 2010; Guo *et al*, 2013). Dickinson *et al* (2013) have demonstrated that higher mtDNA content facilitates the proliferation of glioblastoma cells. Moreover, Cheau-Feng Lin *et al* (2014) have shown that high mtDNA content predicts poor prognosis in patients with head and neck cancer. In line with these findings, our study demonstrated that higher leukocyte mtDNA content was associated with poor prognosis of glioma patients. All these results collectively suggest the critical role of mtDNA content increase in the initiation and progression of glioma.

Several studies have reported the opposite results, indicating that decreased mtDNA content is associated with progression and worse prognosis of patients with HCC (Yamada *et al*, 2006), CRC (Cui *et al*, 2013), breast cancer (Yu *et al*, 2007), prostate cancer (Koochekpour *et al*, 2013) and early-stage laryngeal cancer (Dang *et al*, 2014). These contradictory findings suggest that the homeostasis of mitochondrial content plays important roles in tumourigenesis. When this homeostasis is broken down by various oncogenic factors, either increased or decreased mtDNA content might damage mitochondrial functions and thus promote tumour development and progression. Previous studies have reported both increased and decreased mtDNA content in different cancer types (Yu, 2011). Therefore, it is easy to understand that mtDNA content may affect cancer progression and prognosis of patients in a disease-specific manner. However, the underlying mechanisms that account for mtDNA content variations and their functional roles in different malignancies remain largely unknown. Moreover, differences in histology of samples, ethnic population and experimental procedures might also contribute to these discrepancies. Further investigations are needed to elucidate the mechanisms underlying different effects of mtDNA content on cancer progression.

The underlying mechanisms that account for prognostic effects of leukocyte mtDNA content in glioma remain unknown. One explanation is that mtDNA content may alter the immune functions of glioma patients, especially the functions of NK cells that play important roles in the first-line defense against tumours (Vivier *et al*, 2008). Recent studies have demonstrated that NK cells

may be a promising strategy for the treatment of glioma (Ogbomo *et al*, 2011). Our immunoassay indicated that glioma patients with high mtDNA content had low frequency of NK cells that may impair the immune defense against tumour cells and lead to poor prognosis.

Cytokines play pivotal roles in controlling the survival, expansion, activation and target cell killing of NK cells (Vivier *et al*, 2008). Immunological studies have shown that the combination of IL-2 and TNF-α promotes the apoptosis of NK cells (Jewett and Bonavida, 2000; Jewett *et al*, 2006). In this study, we found that glioma patients with high mtDNA content also had higher IL-2 and TNF-α levels that may contribute to NK cell reduction in these patients. Previous studies have demonstrated that mitochondria-derived ROS play an important role in the cytokine secretion of immune cells, such as IL-2 and TNF-α (Yang *et al*, 2013; Yu *et al*, 2014). Clearance of ROS by antioxidants has been shown to inhibit the secretion of IL-2 and TNF-α (Tatla *et al*, 1999; Li *et al*, 2014). In mammalian cells, mitochondria are the major source of ROS (Murphy, 2009), and intracellular ROS level is generally proportional to intracellular mtDNA content (Lee *et al*, 2000). Therefore, it is plausible that glioma patients with high mtDNA content have higher plasma concentration of IL-2 and TNF-α in our study. Collectively, these data suggest that high leukocyte mtDNA content might promote the tumour progression partially via ROS-induced NK cell inhibition.

In the stratified analyses, we found that high mtDNA content was significantly associated with poor prognosis in patients with younger age, HGG or adjuvant radiochemotherapy, but not in those with older age, LGG or adjuvant radiotherapy, indicating the modulating effects of host characteristics through unknown mechanisms. One possible explanation may be the immunodeficiency in patients with these characteristics: HGG patients have severer immunosuppression than LGG patients (Gomez and Kruse, 2006); the combination of radiotherapy and chemotherapy can cause long-lasting immunosuppression in glioma patients (Fadul *et al*, 2011); younger patients in our population received more adjuvant radiochemotherapy than older ones (81 *vs* 60 cases). All these factors may synergise the immunological effects of mtDNA content, leading to worse prognosis in patients. However, the detailed mechanisms need to be further investigated.

There are several limitations in our study. First, there were not enough patients receiving surgical treatment alone ($n = 30$), and this may limit the statistical power of our stratified analysis. Thus, our findings from stratified analysis need to be further validated. Second, we only performed association analyses between mtDNA content and immunological parameters of glioma patients. The underlying mechanisms through which mtDNA content affects the immune functions need further investigation. Third, all glioma patients were given standard-of-care treatment as suggested by the guidelines of European Society for Medical Oncology and China Health Ministry. However, because of the evolution of these guidelines in the study course and the differences in the status of patients, the details of adjuvant radiotherapy and chemotherapy such as dose, course and period greatly different among patients. Therefore, we could only classify the adjuvant therapy into radiotherapy and radiochemotherapy without further subdivision. The heterogeneity in adjuvant therapy thus could not be effectively adjusted in our multivariate analysis.

In summary, our study for the first time demonstrates that high mtDNA content is associated with poor OS and PFS in glioma patients partially through the inhibition of NK cells. Once confirmed, leukocyte mtDNA content may serve as a useful biomarker to improve the prognosis prediction for glioma patients. Further studies are needed to explore the mechanisms underlying the effects of mtDNA content on the clinical outcomes and immune functions in glioma patients.

ACKNOWLEDGEMENTS

This work was supported by Program for New Century Excellent Talents in University, National Natural Science Foundation (81171966) and National Key Technologies R&D Program (2011ZX09307-001-04) of China.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)