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The impact of plasma epstein–barr virus DNA and fibrinogen on nasopharyngeal carcinoma prognosis: an observational study

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Background: The impact of combining plasma fibrinogen levels with Epstein–Barr Virus DNA (EBV DNA) levels on the prognosis for patients with nasopharyngeal carcinoma (NPC) was evaluated.

Methods: In this observational study, 2563 patients with non-metastatic NPC were evaluated for the effects of circulating plasma fibrinogen and EBV DNA levels on disease-free survival (DFS), distant metastasis-free survival (DMFS), and overall survival (OS).

Results: Compared with the bottom biomarker tertiles, TNM stage-adjusted hazard ratios (HR, 95% confidence intervals (CIs)) for predicting DFS in fibrinogen tertiles 2 to 3 were 1.26 (1.00 to 1.60) and 1.81 (1.45 to 2.26), respectively; HR for EBV DNA tertiles 2 to 3 were 1.49 (1.12 to 1.98) and 4.24 (3.27 to 5.49), respectively. After additional adjustment for established risk factors, both biomarkers were still associated (P for trend <0.001) with reduced DFS (HR: 1.79, 95% CI, 1.43 to 2.25 for top fibrinogen tertiles; HR: 4.04, 95% CI: 3.10 to 5.27 for top EBV DNA tertiles compared with the bottom tertiles). For patients with advanced-stage disease, those with high fibrinogen levels ($\geqslant 3.34\,g\,l^{-1}$) presented with worse DFS, regardless of EBV DNA $\geqslant 4000$ or <4000 copies ml^{-1} subgroup. Similar findings were observed for DMFS and OS.

Conclusions: Circulating fibrinogen and EBV DNA significantly correlate with NPC patients survival. Combined fibrinogen and EBV DNA data lead to improved prognostic prediction in advanced-stage disease.

Nasopharyngeal carcinoma (NPC) is endemic in Southern China and Southeast Asia, where a peak incidence of 50 cases per 100 000 has been reported (Wee *et al*, 2010). Radiotherapy is the primary treatment modality, and concurrent chemoradiotherapy with or without adjuvant chemotherapy is the primary regimen for patients with locoregionally advanced NPC. However, patients with similar stages and histologic classifications have markedly different survival outcomes given the heterogeneity of protein expression profiles (Ludwig and Weinstein, 2005; Wei and Sham, 2005). Various studies have attempted to identify molecular

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biomarkers to predict NPC progression, and numerous promising biomarkers have been evaluated as potential prognosis predictors of NPC. Recently, pretreatment plasma Epstein–Barr Virus (EBV) DNA levels were clinically employed as a useful tool for NPC diagnosis, risk stratification, monitoring and prognosis (Lo *et al*, 1999; Chan *et al*, 2002; Leung *et al*, 2003a; Lin *et al*, 2004; Leung *et al*, 2006). EBV DNA levels are considered the most attractive potential biomarker that complements TNM classification in NPC (Ng *et al*, 2014). Given the biological heterogeneity of cancer, the present staging system, even in combination with plasma EBV DNA levels, remains inadequate for predicting NPC patient prognosis. Therefore, we hypothesise that additional biomarkers could complement EBV DNA levels. These biomarkers could be used in combination to improve the prognostic stratification of NPC patients.

Fibrinogen, a circulating glycoprotein that is a nonspecific acute-phase reactant and important clotting factor, might serve as a useful biomarker in this context. Fibrinogen converts to insoluble fibrin via thrombin, thereby significantly affecting inflammatory response, fibrinolysis, blood clotting, wound healing and neoplasia (Mosesson, 2005). Increased fibrinogen levels influence cancer cell growth, progression and metastasis. Hyperfibrinogenaemias are associated with various human malignancies, including oesophageal (Takeuchi *et al*, 2007), colorectal (Yamashita *et al*, 2009), ovarian (Polterauer *et al*, 2009a), cervical (Polterauer *et al*, 2009b), and pancreatic cancer (Guo *et al*, 2009). Nasopharyngeal carcinoma is associated with EBV infection and hence chronic inflammation. Nevertheless, little information is known regarding the clinical significance of fibrinogen and the potential complementary role of fibrinogen and EBV DNA levels in predicting NPC carcinogenesis and progression. Therefore, this large cohort study compared the efficacy of EBV DNA and fibrinogen alone and in combination for predicting NPC patient survival. In addition, this study provides information regarding personalised therapy.

MATERIALS AND METHODS

Two thousand seven hundred and sixty-seven patients with primary NPC were consecutively recruited from January 2007 to December 2011 at the Sun Yat-sen University Cancer Center, Guangzhou, China. Patients were excluded from this study if they met the following criteria: (1) previously received any anticancer therapy ($n=24$); (2) <18 years old ($n=12$); (3) pregnant or lactating ($n=8$); (4) unsuitable for chemotherapy as a result of a liver, kidney, lung or heart deficiency ($n=15$); (5) a history of previous or synchronous malignant tumours ($n=17$); (6) have primary NPC metastasis ($n=90$); or (7) lost during follow-up ($n=38$). In total, 2563 patients with non-metastatic primary NPC were eligible for analysis.

The routine staging work-up included clinical examination of the head and neck region, magnetic resonance imaging scan from the suprasellar cistern to the collarbone, fibreoptic nasopharyngoscopy, chest radiography, abdominal sonography and whole-body bone scan or whole-body FDG PET/CT. All patients were restaged according to the seventh American Joint Committee on Cancer (AJCC) TNM staging manual. In total, 1126 (43.9%) patients were treated with conventional two-dimensional (2D) or three-dimensional (3D) conformal radiotherapy radiotherapy, and 1437 (56.1%) patients were treated with intensity-modulated radiotherapy. In addition, 2183 (87.5%) patients with stage II–IV disease received platinum-based chemotherapy. Concurrent chemoradiotherapy with or without neoadjuvant or adjuvant chemotherapy was administered for advanced-stage disease (stages III and IV). A stratified multitherapeutic protocol was used. Radiation alone was administered for stage I disease, and radiation alone or with concurrent platinum-based chemotherapy was administered for stage II disease (Chen *et al*, 2011). Concurrent chemoradiotherapy with or without neoadjuvant or adjuvant chemotherapy was administered for advanced-stage disease (stages III and IV). Neoadjuvant or adjuvant chemotherapy consisting of cisplatin plus 5-fluorouracil or cisplatin plus taxane was administered every 3 weeks for two or three cycles (Chen *et al*, 2012). Concurrent cisplatin chemotherapy was administered on weeks 1, 4 and 7 of RT. All patients were treated according to the principles of treatment for NPC patients at the Sun Yat-sen University Cancer Center, Guangzhou, China.

Collection of data. Before treatment, baseline clinical data were collected regarding sex, age, hereditary NPC, smoking status and PS as assessed by the Eastern Cooperative Oncology Group (ECOG). Information regarding relevant concurrent diseases, such as cardiovascular disease, diabetes and chronic hepatitis, was collected as previous studies have indicated that these factors promote increased plasma fibrinogen levels (Mora *et al*, 2006; Sinning *et al*, 2006; Calvaruso *et al*, 2008; Lowe *et al*, 2013; Sapkota *et al*, 2013). These comorbidities and smoking status were defined as follows: chronic hepatitis B: HBsAg-positive >6 months and serum HBV-DNA $\geqslant 2000\,\mathrm{IU\,ml^{-1}}$ (10^4 $\mathrm{copies\,ml^{-1}}$) with or without increased alanine transaminase/aspartate transaminase levels; diabetes: fasting plasma glucose level $7.0\,\mathrm{mmol\,l^{-1}}$ and/or 2 h plasma glucose level $11.1\,\mathrm{mmol\,l^{-1}}$ after a 75 g glucose load or a previous diagnosis of diabetes by a healthcare professional; cardiovascular disease: coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, pulmonary embolism, hypertension (systolic blood pressure 140 mm Hg, diastolic blood pressure 90 mm Hg) or a previous diagnosis of any of these diseases made by a healthcare professional; smoking: patients were identified as current, former or never smokers. Patients who smoked or reported smoking cessation within 1 year at the time of the diagnosis were considered current smokers. Patients who had smoked less than 100 cigarettes during their lifetime were considered never smokers.

Plasma fibrinogen evaluation. A 3 ml fasting blood sample was collected from each patient before treatment and processed within 3 h of collection. The plasma was stored at $-80\,^{\circ}\mathrm{C}$ until use in assays. Fibrinogen values were measured by the Clauss method using an Automatic Analyser Sysmex CA7000 (Sysmex Corporation, Kobe, Japan) and reagents according to the SIEMENS AG guidelines (Munich, Germany) (Clauss, 1957; Cook and Ubben, 1990). The results were obtained using a standard curve prepared according to the manufacturer's instructions, and the interassay imprecision (coefficient of variation) was $<10\%$. Plasma D-dimer concentrations $<3.5\,\mathrm{g\,l^{-1}}$ were considered normal based on the manufacturer's instructions.

EBV DNA, VCA-IgA and EA-IgA measurement. As previously described (Lo *et al*, 1999; Shao *et al*, 2004; An *et al*, 2011), patient plasma EBV DNA concentrations were routinely measured by q-PCR before treatment. EBV-specific VCA/IgA antibodies and EBV-specific EA/IgA antibodies were assessed using a previously described immunoenzymatic assay (Liu *et al*, 2012b).

Clinical outcome assessment and patient follow-up. Our primary endpoint was disease-free survival (DFS), and our secondary endpoints were distant metastasis-free survival (DMFS) and overall survival (OS). Disease-free survival was calculated from the date of the first NPC diagnosis to the date of the first relapse at any site, death from any cause or the date of the last follow-up visit. DMFS was determined from the date of the first NPC diagnosis to the date of distant relapse or patient censoring at the date of the last follow-up. Overall survival was calculated from the date of the first NPC diagnosis to the date of death from any cause or patient

Table 1. Patient demographics and clinical characteristics

Number of patients (fibrinogen and EBV DNA tertiles, $n = 2563$)						
	Fibrinogen, g l^{-1}			EBV DNA, copies ml^{-1}		
Characteristic	**<2.67**	**2.67–3.34**	**⩾3.34**	**<326**	**326–11 333**	**⩾11 333**
Age, years						
Median	44	47	48	46	46	47
Mean	44.82	47.61	48.33	46.27	47.15	47.31
Sex						
Male	635	607	645	608	635	644
Female	226	256	194	246	220	210
Histology, WHO type						
III	820	830	812	810	824	828
II	39	28	26	39	30	24
I	2	5	1	5	1	2
ECOG						
0–1	856	861	837	853	851	850
2	5	2	2	1	4	4
Clinical stage						
I	40	23	6	59	8	2
II	158	100	46	210	61	33
III	474	482	446	439	542	421
IV	189	258	341	146	244	398
Tumour stage						
T1	103	62	27	111	50	31
T2	237	164	113	221	150	143
T3	390	435	420	398	448	399
T4	131	202	279	124	207	281
Node stage						
N0	167	167	105	292	97	50
N1	333	310	257	367	322	211
N2	298	309	370	168	384	425
N3	63	77	107	27	57	168
Treatment						
Radiotherapy	168	125	80	229	83	61
Chemotherapy and radiotherapy	693	738	759	625	772	793
Radiotherapy technique						
2DRT/3DCRT	414	355	357	355	367	404
IMRT	447	508	482	499	488	450
VCA-IgA						
<1 : 80	246	249	231	321	223	182
⩾1 : 80	615	614	608	533	632	672
EA-IgA						
<1 : 10	391	375	343	465	356	288
⩾1 : 10	470	488	496	389	499	566
LDH, U l^{-1}						
<170	489	428	340	494	439	324
⩾170	372	435	499	360	416	530

Table 1. (Continued)

Number of patients (fibrinogen and EBV DNA tertiles, $n = 2563$)						
	Fibrinogen, g l^{-1}			EBV DNA, copies ml^{-1}		
Characteristic	**<2.67**	**2.67–3.34**	**⩾3.34**	**<326**	**326–11 333**	**⩾11 333**
Body mass index, kg m^{-2}						
<23	504	449	448	431	470	500
⩾23	357	414	391	423	385	354
Smoking						
Never	558	550	467	566	535	474
Ever	40	50	46	45	41	50
Current	263	263	326	243	279	330
Chronic HBV infection						
Yes	69	60	54	58	62	63
No	792	803	785	796	793	791
Cardiovascular disease						
Yes	35	57	65	51	47	59
No	826	806	774	803	805	795
Diabetes mellitus						
Yes	15	21	21	22	18	17
No	846	842	818	832	837	837
Family history of NPC						
Yes	97	84	83	92	90	82
No	764	779	756	762	765	772
Median follow-up (months)	40	35	35	37	36	37
Outcome features						
PR	129	159	225	81	122	310
Non-PR	732	704	614	773	733	544
DM	80	104	160	40	82	222
Non-DM	781	759	679	814	773	632
LR	57	68	83	44	50	114
Non-LR	804	795	756	810	805	710
Deaths	45	57	113	27	40	148
Non-deaths	816	806	726	827	815	706

Abbreviations: DM = the number of patients presenting with distant metastasis at the last follow-up; EA = early antigen; ECOG = Eastern Cooperative Oncology Group; HBV = chronic hepatitis B virus; IMRT = intensity-modulated radiotherapy; LDH = serum lactate dehydrogenase levels; LR = the number of patients presenting with local or regional relapse at the last follow-up; Non-DM = the number of patients without distant metastasis at the last follow-up; Non-LR = the number of patients without local or regional relapse at the last follow-up; Non-PR = the number of patients who had not progressed at the last follow-up; NPC = nasopharyngeal carcinoma; PR = the number of patients who progressed at the last follow-up; VCA = viral capsid antigen; WHO, World Health Organization; 2DRT = two-dimensional radiotherapy; 3DCRT = three-dimensional conformal radiotherapy. Deaths = the number of deceased patients at the last follow-up; Non-deaths = the number of patients alive at the last follow-up.

censoring at the date of the last follow-up. Patients still alive on 31 December 2013 (end of follow-up) were censored at the date of the last contact. After the treatment was complete, the patients were evaluated at 3-month intervals for the first 3 years and every 6 months thereafter.

Statistical analysis. Spearman rank correlation coefficients (r_s) were calculated for continuous variables, and fibrinogen and EBV DNA values were divided into tertiles. The Kaplan–Meier method was used to estimate the cumulative survival plot in relation to the variables divided according to their tertiles. The survival among groups was compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) for EBV DNA and fibrinogen tertiles were estimated using Cox proportional hazards regression. We first adjusted for TNM stage and then further adjusted for age (years), sex, ECOG performance, pathological type, disease stage, treatment allocation, LDH, VCA-IgA, EA-IgA, smoking (never, former, current), cardiovascular disease (yes, no), diabetes mellitus (yes, no), familial history of NPC (yes, no) and body mass index. To assess potentially confounding variables or effect mediation by other biomarkers, the models assessing the association of fibrinogen with survival were further adjusted for EBV DNA and vice versa. We then evaluated the combined association of EBV DNA and fibrinogen with NPC patient survival by dividing participants into prespecified groups. We analysed the combined association according to high or low fibrinogen (above or below top tertile) and high or low EBV DNA (⩾4000 copies ml^{-1} or <4000 copies ml^{-1}; Chan *et al*, 2002; Leung *et al*, 2006). In addition, we repeated this analysis of the combined association of EBV DNA and fibrinogen using tertiles of the above two biomarkers. Finally, statistical tests for

interaction between fibrinogen and EBV DNA tertiles were performed using the TNM stage-adjusted Cox regression models. All reported probability values were two tailed, and $P<0.05$ was considered significant. Statistical analyses were performed with STATA version 8.2 and SPSS 17.0.

RESULTS

Patient characteristics and association with clinical variables. The characteristics of the 2563 NPC patients are listed in Table 1. The median follow-up time was 37 months (interquartile range (IQR): 28–46).The median fibrinogen level in patients with ($n=513$) and without ($n=2050$) relapse were 3.21 (IQR: 2.67–4.06) $g\,l^{-1}$ and 2.95 (IQR: 2.49–3.47) $g\,l^{-1}$, respectively ($P<0.001$). The median EBV DNA levels were 22 500 (IQR: 3280–130 000) copies ml^{-1} and 1940 (IQR: 0–14 225) copies ml^{-1} in patients with and without relapse, respectively ($P<0.001$). When examined as continuous variables, fibrinogen was positively correlated with EBV DNA ($r_s=0.222$, $P<0.001$). In addition, both fibrinogen and EBV DNA concentrations were significantly correlated with TNM staging ($r_s=0.231$, $P<0.001$; $r_s=0.369$, $P<0.001$; respectively). In total, 208 patients developed locoregional recurrences, 344 patients had distant metastases and 215 were deceased at the last follow-up.

HRs and 95% CIs comparing fibrinogen and EBV DNA tertiles. Cumulative DFS, DMFS and OS probabilities for NPC patients indicate that EBV DNA tertiles are superior in survival prediction compared with fibrinogen tertiles (Figure 1), with $P<0.001$ from the log-rank significance tests across the tertile of either biomarker. As shown in Tables 2 and 3, both fibrinogen and EBV DNA are associated with DFS (TNM stage-adjusted HR: 1.81, 95% CI: 1.45 to 2.26 for top fibrinogen tertiles; TNM stage-adjusted HR: 4.79, 95% CI: 3.33 to 6.89 for top EBV DNA tertiles; both compared with the bottom tertiles). Linear associations were observed for fibrinogen tertiles 2 to 3 and EBV DNA tertiles 2 to 3. After adjusting for age and other risk factors (Tables 2 and 3), the upper tertiles of both biomarkers remained associated with DFS (P for trend <0.001).The HRs were 1.79 (95% CI: 1.43 to 2.25) for the upper fibrinogen tertiles and 4.04 (95% CI, 3.10 to 5.27) for the upper EBV DNA tertiles.

We further adjusted fibrinogen for EBV DNA in a Cox model. The HR comparing the top and bottom fibrinogen tertiles was slightly attenuated to 1.63 (95% CI: 1.30 to 2.04); however, the trend across tertiles remained significant (P for trend <0.001; Table 2). Similarly, in a Cox model that adjusted EBV DNA for fibrinogen, the HR comparing the top and bottom EBV DNA tertiles was also mildly attenuated to 3.91 (95% CI: 2.99 to 5.10), but the trend across tertiles remained significant (P for trend <0.001; Table 3). A similar finding was also observed for DMFS and OS regardless of HR adjusted for TNM stage or other risk factors.

Prognostic value of integrating plasma EBV DNA and fibrinogen levels. Combinatorial analyses of four prespecified groups of high and low EBV DNA or fibrinogen with DFS, DMFS and OS were performed (Figure 2). Reduced DFS, DMFS and OS were significantly associated with increased levels of both EBV DNA and fibrinogen. Increased DFS, DMFS and OS were significantly associated with low levels of both biomarkers (P log-rank <0.001). Of note, patients with high EBV DNA and low fibrinogen levels displayed increased event rates during follow-up compared with patients with low EBV DNA and high fibrinogen levels (Table 4). In subgroup analysis of advanced-stage disease (stages III and IV), the patients with high fibrinogen levels presented with worse DFS,

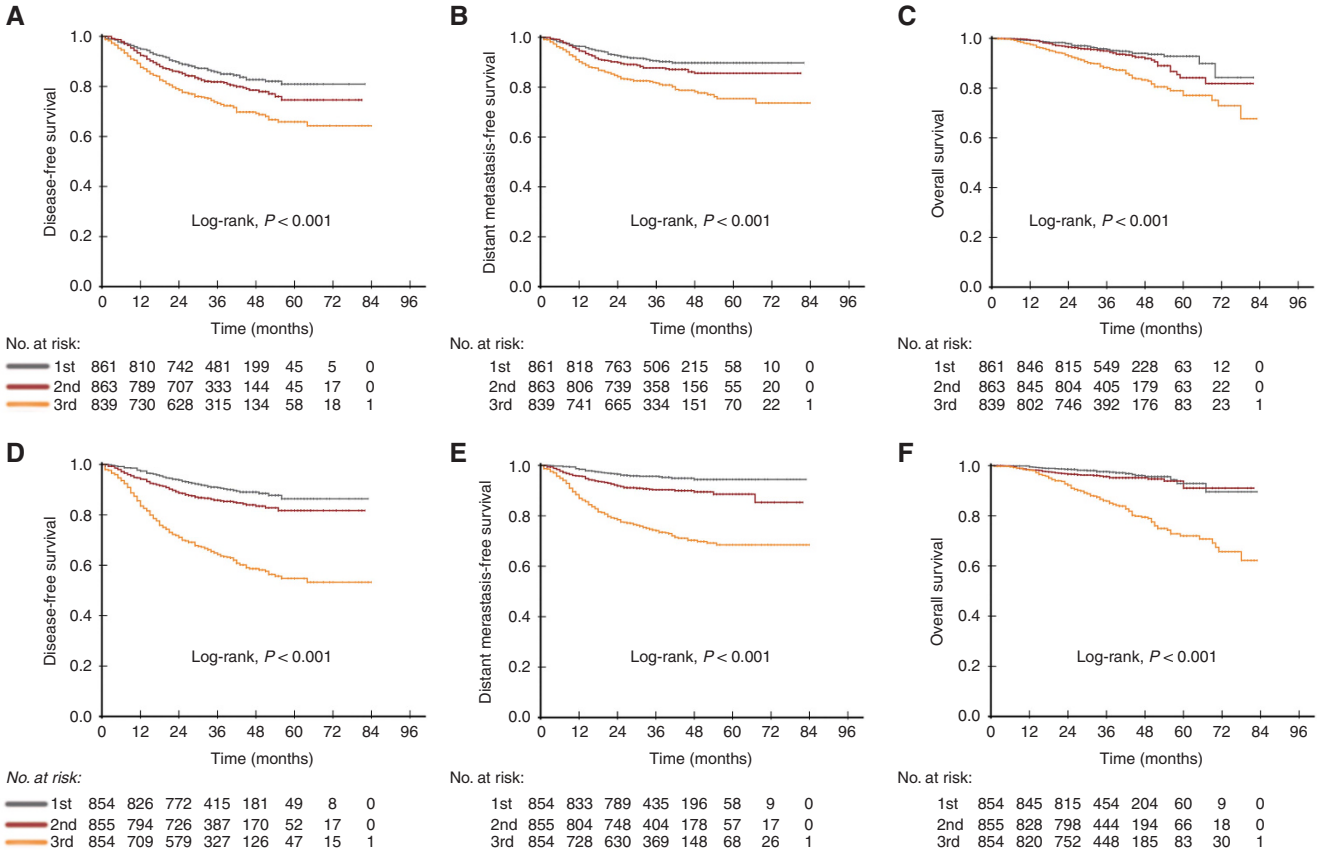

Figure 1. Upper fibrinogen (**A**–**C**) and EBV DNA (**D**–**F**) tertiles are associated with DFS, DMFS and OS. $P<0.001$ for both variables as determined by log-rank significance tests.

Table 2. DFS, DMFS and OS HRs according to fibrinogen tertiles

	Fibrinogen tertile, g l^{-1}, $n = 2563$			
	1	**2**	**3**	
	<2.67 (Bottom)	**2.67–3.34**	**⩾3.34**	***P*-value(trend)**
DFS				
TNM stage-adjusted[a]	1.00	1.26 (1.00–1.60)	1.81 (1.45–2.26)	<0.001
Plus risk factors[b]	1.00	1.30 (1.03–1.65)	1.79 (1.43–2.25)	<0.001
Plus EBV DNA[c]	1.00	1.25 (0.98–1.58)	1.63 (1.30–2.04)	<0.001
DMFS				
TNM stage-adjusted[a]	1.00	1.27 (0.95–1.70)	1.93 (1.47–2.53)	<0.001
Plus risk factors[b]	1.00	1.31 (0.97–1.76)	1.89 (1.43–2.50)	<0.001
Plus EBV DNA[c]	1.00	1.24 (0.92–1.67)	1.68 (1.27–2.23)	<0.001
OS				
TNM stage-adjusted[a]	1.00	1.26 (0.85–1.86)	2.31 (1.63–3.29)	<0.001
Plus risk factors[b]	1.00	1.21 (0.81–1.80)	2.08 (1.45–2.98)	<0.001
Plus EBV DNA[c]	1.00	1.16 (0.78–1.73)	1.85 (1.29–2.65)	<0.001

Abbreviations: DFS = disease-free survival; DMFS = distant metastasis-free survival; EBV DNA = Epstein–Barr Virus DNA; HR = hazard ratio; OS = overall survival; TNM stage = clinical stage for NPC based on the seventh American Joint Committee on Cancer (AJCC) TNM staging manual. The values represent hazard ratios (95% confidence interval).
[a]Obtained from Cox proportional hazard regression models adjusted for TNM stage (IV *vs* III *vs* II *vs* I).
[b]Obtained from Cox proportional hazard regression models adjusted for age (⩾46 years *vs* <46 years), sex (male *vs* female), WHO pathological type (undifferentiated non-keratinising *vs* differentiated non-keratinising *vs* keratinising squamous cell), ECOG performance status (2 *vs* 0–1), chemoradiotherapy (yes *vs* no), radiation technique (intensity-modulated radiotherapy *vs* 3D-CRT/2D-CRT), lactate dehydrogenase (⩾170 U l^{-1} *vs* <170 U l^{-1}), viral capsid antigen (⩾1 : 80 *vs*<1 : 80), early antigen (⩾1 : 10 *vs*<1 : 10), body mass index (⩾23 kg m^{-2} *vs* <23 kg m^{-2}), smoking status (yes *vs* no), concurrent cardiovascular disease (yes *vs* no), diabetes (yes *vs* no), chronic hepatitis disease (yes *vs* no) and family history of nasopharyngeal carcinoma (yes *vs* no). The lowest tertile of each biomarker served as the reference category for the hazard ratios. *P*-values were obtained from models, which were used to assess linear trends.
[c]Adjusted for all the above variables and EBV DNA.

Table 3. DFS, DMFS and OS HRs according to EBV DNA tertiles

	EBV DNA tertile, copies ml^{-1}, $n = 2563$			
	1	**2**	**3**	
	<326	**326–11 333**	**⩾11 333**	***P* (trend)**
DFS				
TNM stage-adjusted[a]	1.00	1.49 (1.12–1.98)	4.24 (3.27–5.49)	<0.001
Plus risk factors[b]	1.00	1.46 (1.10–1.95)	4.04 (3.10–5.27)	<0.001
Plus Fibrinogen[c]	1.00	1.45 (1.09–1.94)	3.91 (2.99–5.10)	<0.001
DMFS				
TNM stage-adjusted[a]	1.00	1.94 (1.32–2.85)	5.51 (3.88–7.84)	<0.001
Plus risk factors[b]	1.00	1.95 (1.32–2.87)	5.34 (3.72–7.66)	<0.001
Plus Fibrinogen[c]	1.00	1.92 (1.31–2.83)	5.12 (3.57–7.35)	<0.001
OS				
TNM stage-adjusted[a]	1.00	1.29 (0.78–2.11)	4.38 (2.86–6.70)	<0.001
Plus risk factors[b]	1.00	1.28 (0.78–2.11)	3.98 (2.57–6.16)	<0.001
Plus Fibrinogen[c]	1.00	1.25 (0.76–2.06)	3.76 (2.42–5.82)	<0.001

Abbreviations: DFS = disease-free survival; DMFS = distant metastasis-free survival; EBV DNA = Epstein–Barr Virus DNA; HR, hazard ratio, OS = overall survival; TNM stage = clinical stage for NPC based on the seventh American Joint Committee on Cancer (AJCC) TNM staging manual. The values represent hazard ratios (95% confidence interval).
[a]Obtained from Cox proportional hazard regression models adjusted for TNM stage (IV *vs* III *vs* II *vs* I).
[b]Obtained from Cox proportional hazard regression models adjusted for age (⩾46 years *vs* <46 years), sex (male *vs* female), WHO pathological type (undifferentiated non-keratinising *vs* differentiated non-keratinising *vs* keratinising squamous cell), ECOG performance status (2 *vs* 0–1), chemoradiotherapy (yes *vs* no), radiation technique (intensity-modulated radiotherapy *vs* 3D-CRT/2D-CRT), lactate dehydrogenase (⩾170 U l^{-1} *vs* <170 U l^{-1}), viral capsid antigen (⩾1 : 80 *vs*<1 : 80); early antigen (⩾1 : 10 *vs*<1 : 10), body mass index (⩾23 kg m^{-2} *vs* <23 kg m^{-2}), smoking status (yes *vs* no), concurrent cardiovascular disease (yes *vs* no), diabetes (yes *vs* no), chronic hepatitis disease (yes *vs* no) and family history of NPC (yes *vs* no). The lowest tertile of each biomarker served as the reference category for the hazard ratios. *P*-values were obtained from models used to assess linear trends.
[c]Adjusted for all the above variables and fibrinogen.

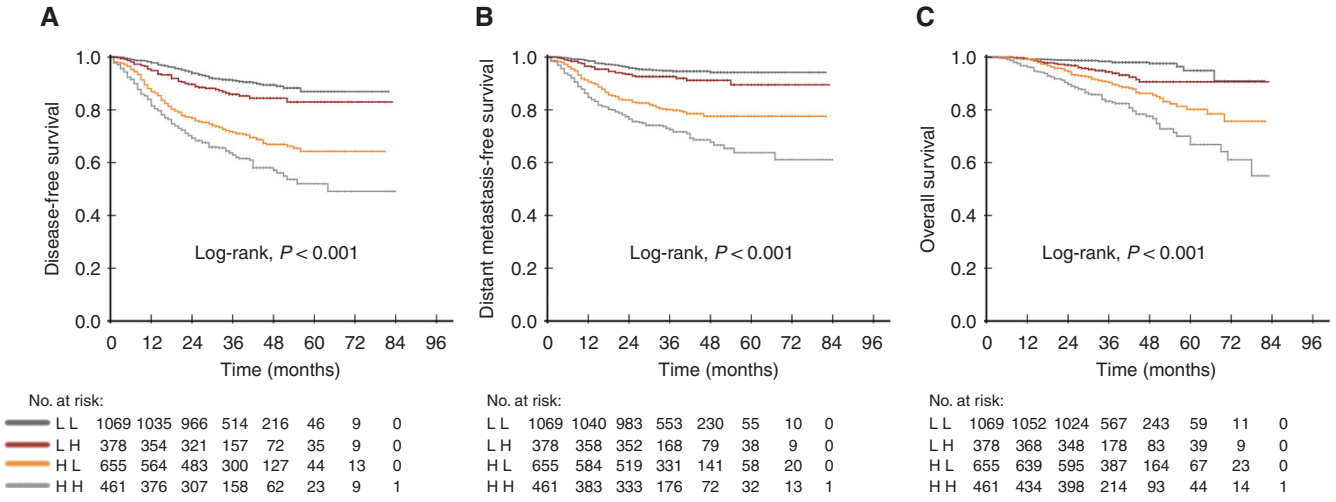

Figure 2. Kaplan–Meier curves of DFS, DMFS and OS according to the combination of pretreatment EBV DNA and fibrinogen levels in NPC patients. DFS (**A**), DMFS (**B**) and OS (**C**) values for 2563 patients. LL, <4000 copies ml^{-1} EBV DNA and <3.34 g l^{-1} fibrinogen; LH, <4000 copies ml^{-1} EBV DNA and $\geqslant 3.34$ g l^{-1} fibrinogen; HL, $\geqslant 4000$ copies ml^{-1} EBV DNA and <3.34 g l^{-1} fibrinogen; HH, $\geqslant 4000$ copies ml^{-1} EBV DNA and $\geqslant 3.34$ g l^{-1} fibrinogen.

DMFS and OS regardless of EBV DNA $\geqslant 4000$ or <4000 copies ml^{-1} subgroups. However, a significant difference for predicting distant metastasis in the low DNA group was not achieved. For patients with early stage disease (stages I and II), in the low DNA subgroup, high fibrinogen levels were associated with reduced DFS, DMFS and OS. Statistical significance was achieved for DMFS, but not for DFS and OS. In the high DNA subgroup, patients with high hs-CRP levels maintained reduced DFS, DMFS, and OS. However, statistical significance was only achieved for OS, but not for DFS and DMFS (Table 4).

We then divided the participants into nine categories according to EBV DNA and fibrinogen tertiles. Using patients with the lowest EBV DNA and fibrinogen levels (EBV DNA <326 copies ml^{-1} and fibrinogen <2.67 g l^{-1}) as the reference group, the TNM stage-adjusted HRs associated with DFS, DMFS and OS in patients with EBV DNA $\geqslant 11\,133$ copies ml^{-1} and fibrinogen $\geqslant 3.34$ g l^{-1} were 8.51(95% CI, 5.34–13.56), 14.86 (7.13 to 30.95) and 12.77 (5.08 to 32.12), respectively (Figure 3). When examined separately using the above cut-offs of high *vs* low fibrinogen and EBV DNA levels, the TNM stage-adjusted HRs associated with DFS, DMFS and OS were 1.60 (95% CI: 1.34 to 1.91), 1.69 (95% CI: 1.36 to 2.10) and 2.05 (95% CI: 1.56 to 2.69), respectively, for fibrinogen and 3.35 (95% CI: 2.78 to 4.03), 3.64 (95% CI: 2.89 to 4.59) and 3.77 (95% CI: 2.80 to 5.08), respectively, for EBV DNA (data not shown). Patients with either upper values for EBV DNA but lower or intermediate values for fibrinogen or upper values for fibrinogen but lower or intermediate values for EBV DNA displayed poorer survival compared with patients with lower values for both biomarkers. Therefore, similar results were obtained when we employed tertile cut-offs to define increased EBV DNA levels compared with previously reported cut-offs. Multiplicative interactions between fibrinogen tertiles and EBV DNA categories with regard to relapse, distant metastasis and death were not observed; the *P*-values for these interactions were 0.554, 0.760 and 0.067, respectively.

DISCUSSION

In this large-scale observational study of 2563 patients with non-metastatic primary NPC, we found that increased EBV DNA and fibrinogen levels alone and in combination are associated with reduced DFS, DMFS and OS. Despite the positive correlation between EBV DNA and fibrinogen, increased levels of these biomarkers together were associated with reduced survival. The predictive value of EBV DNA was superior to that of fibrinogen, and the combinatorial effect was greater than the individual effects of either biomarker alone, without evidence of multiplicative interactions. Patients with $\geqslant 11\,133$ copies ml^{-1} of EBV DNA and $\geqslant 3.34$ g l^{-1} of fibrinogen display a greater than eightfold increased risk of disease progression compared with patients with <326 copies ml^{-1} of EBV DNA and <2.67 g l^{-1} of fibrinogen.

Previous studies (Lo *et al*, 1999; Chan *et al*, 2002; Lin *et al*, 2004; Leung *et al*, 2006; An *et al*, 2011) have examined the association between EBV DNA and NPC prognosis. In addition, the prognostic value of fibrinogen has also been demonstrated in other cancers (Takeuchi *et al*, 2007; Yamashita *et al*, 2009; Polterauer *et al*, 2009a; Polterauer *et al*, 2009b). However, the clinical value of fibrinogen alone or in combination with plasma EBV DNA has not been assessed in NPC. Interestingly, our findings demonstrate that EBV DNA and fibrinogen display an additive effect even upon adjustment for established risk factors. These results suggest a complementary role for these biomarkers in risk prediction that is not provided by standard risk factors or either biomarker alone.

Recent advancements in NPC patient classification and NPC molecular alterations, including microRNA signatures and the NPC-SVM classifier (Wang *et al*, 2011a; Liu *et al*, 2012a), have been made. However, these developments required expensive and complicated procedures, and rapid clinical implementation was difficult to achieve. To date, routine prognostic risk assessment of NPC patients still relies on traditional clinico-pathological prognostic variables and EB virus-associated blood tests. Plasma fibrinogen levels are established, routinely measured blood-based parameters that are reproducibly detected without additional laborious efforts. Thus, fibrinogen is an attractive biomarker that is potentially useful for improving the prognostic stratification of NPC patients. Although EBV DNA combined with fibrinogen did not enhance prognostic prediction compared with EBV DNA alone in patients with early stage disease, the combination improved prognostic stratification in patients with advanced NPC (Table 4).These results suggest that these biomarkers may be selectively useful in advanced stage NPC. Although other established roles of plasma/serum EBV DNA were

Table 4. Log-rank test on DFS, DMFS and OS for TNM stages split by EBV DNA and fibrinogen combination

		DFS			DMFS			OS		
Patients	No. of patients	Events (No.)	DFS (%) at 3 years	*P*-value	Events (No.)	DMFS (%) at 3 years	*P*-value	Events (No.)	OS (%) at 3 years	*P*-value
All patients										
Low DNA										
Low Fbg	1069	96	89 (97.0–90.1)		54	94 (92.0–96.0)		22	98 (96.0–100.0)	
High Fbg	378	51	85 (81.1–88.9)	0.007	30	91 (87.1–94.9)	0.031	23	91 (87.1–94.9)	<0.001
High DNA										
Low Fbg	655	192	67 (63.1–70.9)		130	78 (74.1–81.9)		80	86 (82.1–89.9)	
High Fbg	461	174	58 (52.1–63.9)	0.001	130	68 (82.1–73.9)	<0.001	90	78 (74.1–81.9)	<0.001
I + II										
Low DNA										
Low Fbg	277	15	94 (92.0–96.0)		5	98 (96.0–99.9)		4	99 (97.0–100.0)	
High Fbg	44	5	85 (71.3–98.7)	0.132	1	95 (85.0–100)	0.041	2	93 (83–100.0)	0.192
High DNA										
Low Fbg	44	16	59 (41.4–76.6)		12	73 (59.3–86.7)		2	98 (94.1–100.0)	
High Fbg	8	5	30 (0.0–67.2)	0.084	3	44 (0.00–93.0)	0.431	4	63 (29.7–96.3)	0.001
III + IV										
Low DNA										
Low Fbg	792	81	87 (85.0–89.0)		49	93 (91.0–95.0)		18	97 (95.0–99.0)	
High Fbg	334	34	85 (81.1–88.9)	0.054	29	90 (86.1–93.9)	0.108	21	91 (87.1–94.9)	0.001
High DNA										
Low Fbg	611	176	68 (64.1–71.9)		118	78 (74.1–81.9)		78	85 (81.1–88.9)	
High Fbg	453	169	58 (52.1–63.9)	0.001	127	69 (63.1–74.9)	<0.001	86	78 (74.1–81.9)	0.001

Abbreviations: DFS = disease-free survival; DMFS = distant metastasis-free survival, EBV DNA = Epstein-Barr Virus DNA; HR = hazard ratio; OS = overall survival; TNM stage = clinical stage for NPC based on the seventh American Joint Committee on Cancer (AJCC) TNM staging manual. Low DNA defined as <4000 copies ml^{-1} EBV DNA; high DNA defined as $\geqslant 4000$ copies ml^{-1} EBV DNA; low Fbg defined as <3.34 g l^{-1} fibrinogen; high Fbg defined as $\geqslant 3.34$ g l^{-1} fibrinogen; *P*-values compared for overall log-rank trend test. Events (No.) = the total number of events that occurred at the last follow-up. Values in parentheses indicate 95% confidence interval ranges.

confirmed in monitoring relapse (Leung *et al*, 2003b) and response to treatment (Chan *et al*, 2004), in patient follow-up (Wang *et al*, 2011b) (Hsu *et al*, 2013) and in population screening (Chan *et al*, 2013), the value of fibrinogen alone or combined with EBV DNA in these fields will require further exploration.

The mechanism by which these biomarkers combination improves the prognostic stratification of NPC patients is unclear. In addition to its role as an inflammatory biomarker, fibrinogen is the predominant coagulation factor in blood plasma and has an important role in platelet aggregation and fibrin formation (Hackam and Anand, 2003; Kerlin *et al*, 2004). Increased platelet counts promote distant metastases in NPC patients (Gao *et al*, 2013). Increased plasma fibrinogen levels may result from the production of tumour-associated cytokines or endothelial cells via the host *vs* tumour response, the endogenous production of fibrinogen by tumour cells or tumour growth-induced hypercoagulation and hypoxia (Wenger *et al*, 1995; Palumbo *et al*, 2000; Wang *et al*, 2005; Sahni *et al*, 2008). Tumour growth increases hypoxia and induces tumour cell apoptosis. Previous studies demonstrated that EBV DNA molecules are released into the circulation by apoptosis and represent the tumour load (Mutirangura *et al*, 1998; Chan *et al*, 2003). This finding partially explains why EBV DNA levels correlate with fibrinogen levels. In addition, our findings potentially suggest that fibrinogen and EBV DNA may represent different signalling pathways or biological behaviours that contribute to NPC progression.

The major drawback of our study is the single measurement of both EBV DNA and fibrinogen. A single measurement potentially underestimates the magnitude of the association between the biomarkers and survival. The second limitation is that the data were obtained exclusively from one centre. Although our cancer centre treats a large number of NPC patients, these results need to be validated in other data sets. The third limitation is that the median follow-up time was 37 months. Patients remain closely followed, and we will report 5-year follow-up results as available.

The study's strengths include reliable fibrinogen and EBV DNA measurements with high accuracy in a core laboratory and well-characterised risk factor profiles of patient that allowed for the control of potential confounding variables. In addition, the large sample size allowed both biomarkers to be examined individually and in combination for incident events and subgroup analysis.

In conclusion, our results suggest that plasma fibrinogen may serve as a new tool for assessing prognosis of NPC patients. In addition, the baseline assessment of both plasma EBV DNA and serum fibrinogen levels significantly improves DFS, DMFS and OS predictions as well as prognostic stratification of NPC patients according to TNM staging.

If the results from the forthcoming large collaborative studies confirm our results, this simple noninvasive approach may prove beneficial in prognostic stratification of NPC patients in clinical

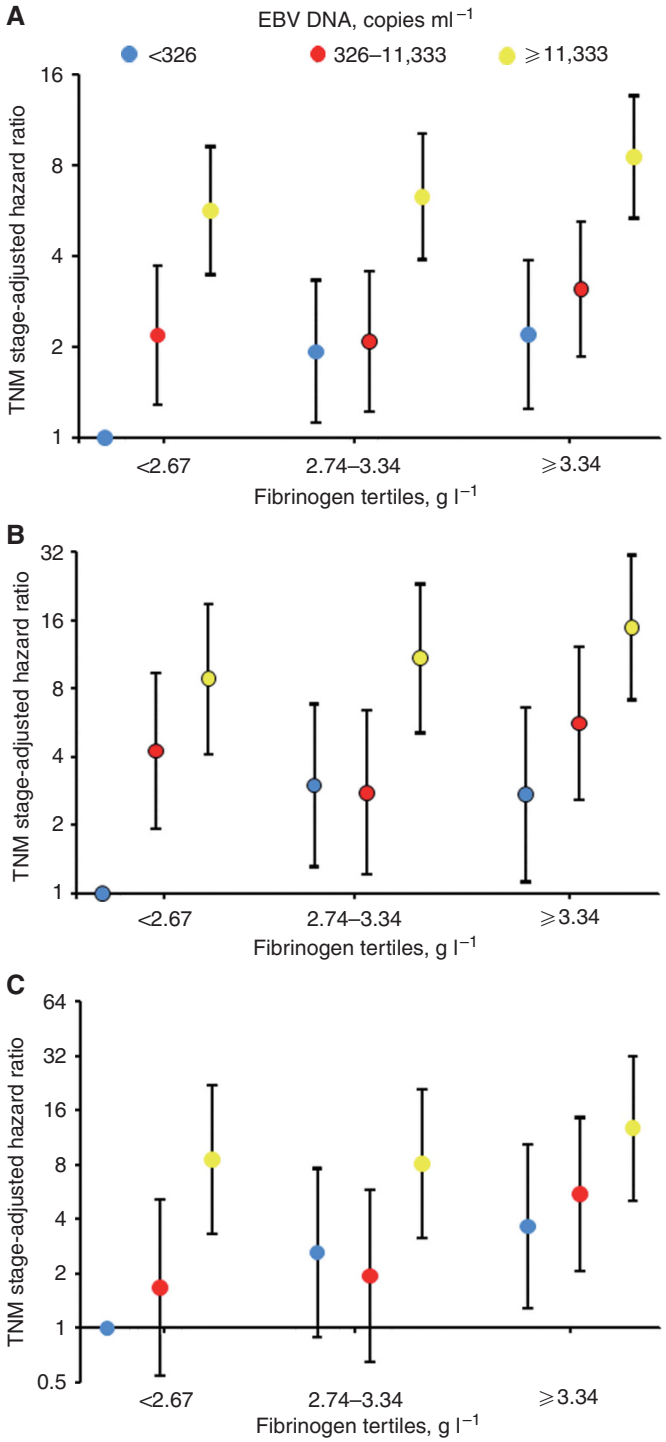

Figure 3. TNM stage-adjusted HRs predicting recurrence (A), distant metastasis (B) and death (C) are presented for EBV DNA and fibrinogen on the *y* axis. The fibrinogen tertile limits were $<2.67\,g\,l^{-1}$, 2.67 to $3.34\,g\,l^{-1}$ and $\geqslant 3.34\,g\,l^{-1}$. The EBV DNA tertile limits were <326 copies ml^{-1}, 326 to 11 333 copies ml^{-1} and $\geqslant 11\,333$ copies ml^{-1}.

trials. The approach could be used to guide individual treatment, ultimately improving NPC outcome. However, randomised biomarker trials are required to determine whether this molecular staging strategy can improve NPC management compared with the conventional staging approach before implementation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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