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MB based RT-qPCR increase the clinical application of cfEBV DNA for NPC in non-endemic area of China

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To compare the performance of magnetic bead (MB) and the concentrated precipitation (CP) based RT-qPCR to qualify cell free EBV DNA (cfEBV DNA) for nasopharyngeal carcinoma (NPC) in non-endemic area of China. From January 2014 to June 2024, a retrospective analysis of 2 cohort studies on cfEBV DNA in NPC patients was conducted to assess the diagnostic value, positive detection rate and clinical application. cfEBV DNA detection with CP based RT-qPCR in cohort 1 and MB based RT-qPCR method in cohort 2. The MB based RT-qPCR for the quantitative measurement of cfEBV DNA load was higher than the CP based RT-qPCR in the same plasma samples from NPC patients ($P < 0.001$). CP based RT-qPCR measured cfEBV DNA in 1405 NPC and 244 healthy controls in cohort 1 with 40.8% sensitivity (AUC = 0.704, 95% CI: 0.676–0.731). In cohort 2(683 naive NPC and 303 controls), cfEBV DNA had a sensitivity of 75.84% (AUC = 0.879, 95% CI: 0.86–0.90). There were no significant differences in TNM stage among NPC between the two cohorts ($P > 0.05$). The MB method considerably increased the positive detection rate of cfEBV DNA in NPCs at stages III-IV, T2-T4, N1-N3, and M0 ($P < 0.001$). At the end of treatment, 97.51% of patients had no detectable EBV and just 2.49% had detectable cfEBV DNA. Those who received ≤ 2 or ≥ 3 cycles of NAC had a median $t_{1/2}$ clearance rate of 9.8 days and 12.6 days, respectively. MB based RT-qPCR increased the quantity of cfEBV DNA. MB based RT-qPCR demonstrated superior sensitivity and positive detection rates for cfEBV DNA. cfEBV DNA can be more positively noticed, with a higher diagnostic value and a broader variety of clinical applications among NPC in non-endemic areas.

Keywords RT-qPCR, MB, CP, cfEBV DNA, NPC

Human herpesvirus Epstein-Barr virus (EBV) infection is linked to a variety of human cancers, including nasopharyngeal carcinoma (NPC), gastric cancers, and various lymphomas[1]. EBV infection has also been linked to autoimmune illnesses such as systemic lupus erythematosus and multiple sclerosis[23]. Cell-free EBV DNA (cfEBV DNA) is derived from apoptotic NPC cells, not intact viral particle-containing DNA, but naked viral DNA fragments (cell-free) of 150 bp[4]. Numerous studies have confirmed its close association with NPC screening, tumor load, efficacy and recurrent metastasis[5]. A study with 2,154 participants found that combining

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cfEBV DNA with standard TNM staging outperformed current TNM staging systems[6]. Furthermore, in addition to plasma, EBV DNA could be detected both in nasal and nasopharyngeal swab[7]. Nasopharyngeal cfEBV DNA loads were much higher than nasal swabs ($P < 0.001$)[8].

EBV DNA can be detected in whole blood, lymphocytes, peripheral blood mononuclear cells (PBMCs), serum and plasma. The specimens used to test for various diseases vary significantly. In a study of 2146 samples of virous illnesses, including NPC, plasma cfEBV DNA showed greater sensitivity and specificity in identifying individuals with ongoing systemic EBV1 disease compared to PBMCs[9]. Currently, cfEBV DNA can be detected by RT-qPCR, digital PCR, and sequencing. According to Lo et al.[10] RT-qPCR(real-time quantitative polymerase chain reaction) was utilized to detect cfEBV DNA in NPC. Multiple experiments with non-uniform extraction volumes (130-3000ul), various specimen types (plasma/serum/whole blood), and different EBV genome target areas (BamH1-W/EBNA2/EBNA1 /BALF5 /EBER1/BXLF1/BALF5/LMP2A/POL-1) have validated with this approach[11]. Lee et al. recently published a review with sensitivities from 31% to 99% and specificities from 83% to 100%[11]. Because of its absolute quantitative, digital PCR is used to identify EBV DNA. NPC PBMC prognosis is evaluated by a digital PCR log value of 1.98 IU/mL for cfEBV DNA detected by digital PCR and 15% PD-L1 expression in tumor-infiltrating cells[12]. A CRISPR/Cas12a-based digital DNA assay, targeting cfEBV DNA repetitive sequences, improved early-stage NPC diagnosis accuracy. The AUC (receiver operating characteristic curve) was 0.9883 (95% CI: 0.9753–1.0000)[13]. In 769 IIB-IVB NPC cases with 6–8 weeks post-radiotherapy, targeted sequencing of cfEBV DNA improved local recurrence and distant metastatic prediction sensitivity to 88.5% and 97.1%, respectively[14].

Although digital PCR and sequencing technologies have advantages, but their high testing costs limit their clinical application, and RT-qPCR remains the most common cfEBV DNA testing technology in clinics. Numerous studies on cfEBV DNA and NPC in endemic areas have relied on RT-qPCR detection methods[1115]. Due to the absence of EBV DNA measurement standards, clinical application of cfEBV DNA is limited by the disparities in processes and criteria used in different laboratories. In non-endemic regions of NPC, the clinical use of RT-qPCR for cfEBV DNA detection is complicated by variables such as varied specimen types, varying lower detection limits, and a range of detection reagents. For example, in a multi-center study, the plasma cfEBV DNA detected with RT-qPCR was limited by lower sensitivity compared to endemic regions[16]. This raises the question of whether it is due to detecting technologies or differences in the EBV infection burden between geographical areas. To answer this question, we conducted this study to investigate if alternative nucleic acid extraction methods affected the diagnostic sensitivity of cfEBV DNA for NPC in this location, potentially broadening its clinical application.

Materials and methods

Study design and participants

From January 2014 to June 2024, naïve NPC patients in Sichuan cancer hospital, West China hospital and Chong Qing University-Three Gorges Hospital were retrospectively reviewed. This study comprised pathologically diagnosed NPC patients suffering from pre-treatment plasma EBV DNA detected via CP (concentrated precipitation) or MB (magnetic bead) based RT-PCR. Basic clinical information, treatment, and dynamic cfEBV DNA were obtained from medical record system. Prior to anticancer treatment, patients had thorough examination and laboratory tests. Patients diagnosed with two or more types of malignant cancer were excluded. The cfEBV DNA detected with CP or MB based RT-qPCR were searched, screening of age-sex matched healthy controls.

As showed in Fig. 1, we designed two cohort studies: in multicenter cohort 1, cfEBV DNA was identified using nucleic acid isolated by CP (Fig. 1A-B). Cohort 2 consisted of NPC patients with similar TNM staging to cohort 1 in the same region. RT-qPCR detected cfEBV DNA with nucleic acid extracted by MB method (Fig. 1C-D). Cohort 1 includes patients from January 2014 to December 2021, whereas Cohort 2 covers January 2022 to June 2024. Health controls were enrolled in both cohorts, and the same detection method employed for NPC was applied to detect cfEBV DNA from health controls. We investigated the sensitivity of cfEBV DNA for NPC diagnosis, the positive detection rate, and the differences in clinical application values between the two cohorts.

This study was approved by the institutional Ethics Committee for Medical Research and New Medical Technology of Sichuan Cancer Hospital (SCCHEC-02-2019-10). This study followed the principles of the Declaration of Helsinki. All experimental procedure used in this study was carried out in compliance with the manufacturer's instructions.

Nucleic acid extraction

Peripheral venous blood samples were collected in EDTA tubes from NPC patients and healthy individuals. After centrifuging the blood sample at 3000 rpm for 5 min, the plasma was separated into a 2 ml EP tube and store it at -20℃ for nucleic acid extraction. cfEBV DNA nucleic acid was isolated with CP or/and MB in accordance with the manufacture's instructions for EBV viral nucleic acid extraction or amplification kit (Sansure Biotech Co. Ltd., Hunan, China).

CP based RT-qPCR

Nucleic acid extracted by CP method was performed in accordance with the manufacturer's instructions for EBV viral nucleic acid amplification kit (Sansure Biotech, Hunan,

China). As reported previously[16], 100ul plasma sample and 100ul concentration agent were blended and centrifuged at 12,000 r/min for 5 min. After discarding the supernatant liquid, 100uL of releasing agent was added and vortexed quickly. After 10 min of centrifugation at 12,000 r/min for 5 min, the isolated nucleic acid in the supernatant was ready for the amplification procedure.

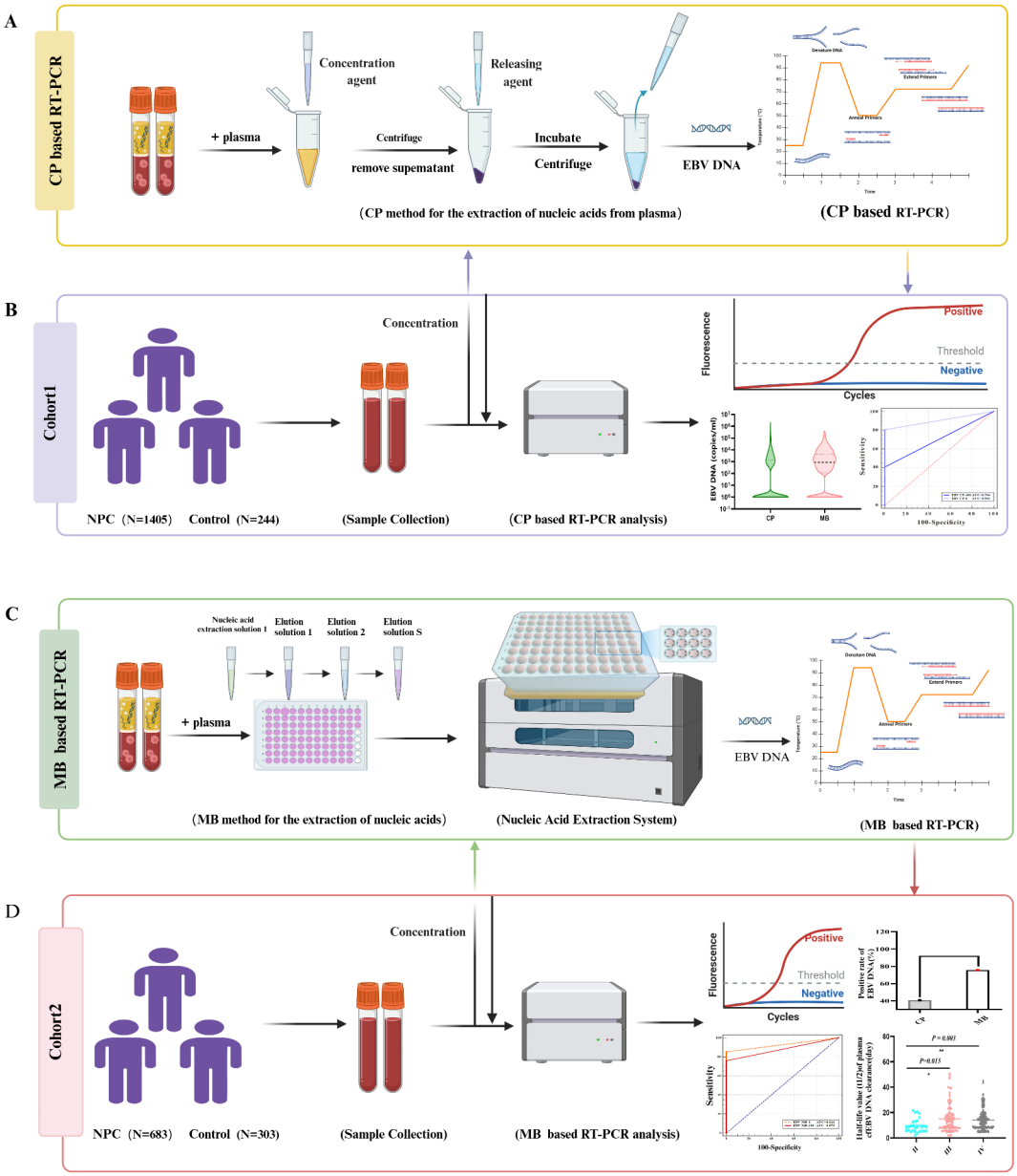

Fig. 1. Design of the study. **(A)** The procedure of CP method for the extraction of nucleic acids from plasma. cfEBV DNA was detected with RT-qPCR and nucleic acid extracted with CP (CP based RT-qPCR). **(B)** From January 2014 to December 2021, 1405 NPC and 244 healthy controls were included in Cohort 1. CP based RT-qPCR was used to detect cfEBV DNA in this Cohort, and the cfEBV DNA positivity rate and diagnostic value were assessed. **(C)** The procedure of MB method for the extraction of nucleic acids from plasma. cfEBV DNA identified with RT-qPCR and nucleic acid extracted with MB (MB based RT-qPCR). **(D)** Cohort 2 included 683 naive NPC patients and 303 healthy controls (January 2022 to June 2024), whose cfEBV DNA were detected with MB based RT-qPCR. cfEBV DNA positive rate, diagnostic value, and half-life value of plasma cfEBV DNA clearance were analyzed. CP: concentrated precipitation; MB: magnetic bead. The figure was created with BioRender.com.

MB based RT-qPCR

The following steps were taken to extract nucleic acid using MB (Nucleic acid extraction kits, Sansure Biotech, Hunan, China) with nucleic acid extraction system (Sansure Biotech, Hunan, China). 200ul of plasma, 50ul proteinase K and MB mixture, and 450ul of nucleic acid extraction solution were mixed at 75 °C for 5 min. Aspirate magnetically for 180s, then discard the waste solution. Add 450ul of cleaning solution 1, shake and mix at 75 °C for 5 min. Magnetically aspirate for 180s, discard the waste solution, and add 300ul of cleaning solution 2. Magnetically aspirate, remove the waste solution, and add 60ul of TE as elution solution.

RT-qPCR

cfEBV DNA extraction solution extracted by CP and MB were measured with the same RT-qPCR regent targeting the BamHI-W fragment region of the EBV genome (Sansure Biotech, Hunan, China). The forward and reverse amplification primers were 5-TGCAGCTTTGACGATGGA

GTAG-3 and 5- TCACTCCTGCCCTTCCTCAC-3. The fluorescent probe labeled with FAM was 5-TTT GCCTCCCTGGTTTCCACCTATG-3. PCR amplification was conducted in ABI7500 real-time PCR system (Thermo Fisher Scientific, ABI, America), and parameters were set as 50∘C for 2 min, initial denaturation at 94 °C for 5 min, 45 cycles of denaturation at 94 °C for 15 s and extension at 57 °C for 30 s. Calibrators for standard curves for cfEBV DNA were performed as previous described[16].

Interlaboratory comparisons

NPC patient plasma ($n=45$) was divided equally and tested by Sichuan Cancer Hospital and Sun Yat-Sen University Cancer Center with the same amplification kit (nucleic acid extracted by MB), respectively. The experiments evaluated the correlation and consistency in different cfEBV concentration, ranging from 0 to 10^7 copies/ml.

Digital PCR

For RT-qPCR results less than 400copies/ml, digital PCR was performed with the same DNA extraction solution using EBV Nucleic Acid Quantification Test Kit(Rainsure Scientific, China) by SG-2000 PCR Amplifying Apparatus (Rainsure Scientific, China). The final volume of the PCR mix for each test was 20ul, consisting of 10ul digital PCR buffer, 2ul primer probe mix, and 8ul cfEBV DNA extraction solution. After loading 75ul of droplet generation oil, 20ul PCR mixture was added into the sample well. The cycling conditions included preheating at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 60 s, and final heating at 98 °C for 10 min, followed by cooling at 20℃ for 2 min. After amplification, the cartridge was relocated in a droplet scanner (DScanner4-1000 Biochip Scanner, Rainsure Scientific, China). The data analysis was conducted with GeneCount Analysis System software version v1.63.0222 by RainSure Scientific in Suzhou, China.

Statistical analysis

Categorical variables were summarized by count and percentage, and statistical significance was determined using the Chi-square test. Continuous variables were compared using Mann-Whitney U-tests in two groups. The cutoff value, sensitivity, specificity, and AUC were determined using the receiver operating characteristic curve (ROC). AUC was utilized to evaluate the prediction performance of cfEBV DNA for NPC obtained through the two nucleic acid extraction techniques. The percentage of patients with cfEBV DNA clearance upon treatment (PEC) was estimated by calculating the number of patients with undetectable cfEBV DNA (cfEBV DNA = 0 copies/mL) by the total number of patients evaluated*100%[17]. The quantity of undetectable plasma cfEBV DNA was changed from 0 to 1 by applying logarithmic transformation. The half-life value ($t_{1/2}$) of plasma EBV DNA clearance was calculated with the equation of [$t_{1/2}$ = 0.693/k][18]. When plasma EBV DNA concentration was plotted against time[19], an exponential model predicted a slope of –k. Graph Prim 8.0.2 or SPSS (SPSS 27.0, Chicago, Illinois, USA) were used for all statistical analyses. Statistical significance was defined as a P-value of <0.05.

Results

Clinical characteristics

This study included 2088 NPC patients and 547 age- and gender-matched healthy controls from January 2014 to June 2024. Participants were separated into two cohorts. In Cohort 1, CP based RT-qPCR detected cfEBV DNA in 1405 NPC and 244 healthy controls(Fig. 1A-B). Cohort 2 comprised 683 naive NPC patients and 303 healthy controls, and MB based RT-qPCR detected cfEBV DNA (Fig. 1C-D). There were no significant differences in age or gender distribution among NPC patients and healthy controls ($P>0.05$). In cohorts 1 and 2, the NPC group had male-to-female ratios of 2.45:1 (998/407) and 2.52:1 (489/194), respectively. There were no differences in T, N, M, WHO pathological type, or clinical stage between the cohorts ($P>0.05$). When 400 or 100 copies/ml were used as the limitation, the NPC positive rates for the two groups differed significantly (40.78% versus 60.61% (400 copies/ml); 58.36% versus 75.85% (100 copies/ml)) (Table 1).

Nucleic acid extracted using the MB for quantitative measurement of cfEBV DNA concentration is higher than that extracted with CP

Plasma from NPC patients was extracted using CP and MB techniques ($N=60$). We then used the same amplification kit to detected cfEBV DNA concentration. The results divided into 3 groups based on CP's cfEBV DNA data. Group 1: cfEBV DNA < 100 copies/ml (Fig. 2A); Group 2: 100–400 copies/ml (Fig. 2B); and Group 3: ≥ 400 copies/ml (Fig. 2C). CP and MB had significantly different cfEBV DNA concentrations at various loads (Fig. 2A-C). The two nucleic acid extraction methods show a smaller difference in the high load of cfEBV DNA group (cfEBV DNA ≥ 400 copies/ml, $P=0.004$, Fig. 2C) and a greater difference in the low load group (cfEBV DNA < 400 copies/ml, $P<0.001$, Fig. 2A-B). Table S1 shows the average CV from the two extraction methods: 27.14%, 16.22%, and 9.07%.

Digital PCR was also used to evaluate MB based RT-PCR (Fig. 2D-E). The findings showed a significant association ($P<0.0001$, $r=0.90$). Sichuan Cancer Hospital and Sun Yat-Sen University Cancer Center compared MB-extracted nucleic acid for cfEBV DNA detection. Bland-Altman plots revealed strong agreement, with all occurrences falling inside the 95% LoA (Fig. 2F). Both interlaboratory tests showed similar cfEBV DNA detection results ($P<0.0001$, $r=0.98$, Fig. 2G).

Characteristic+A2:G37	CP			MB		
	Control	NPC	*P*	Control	NPC	*P*
N	244	1405		303	683	
Median age in years (range)	49 (18-74)	50 (12-84)	0.981	49(28-81)	51 (12-81)	0.089
Gender			0.826			
Male	175 (71.72%)	998 (71.03%)		226(74.59%)	489(71.60%)	0.331
Female	69 (28.28%)	407 (28.97%)		77 (25.41%)	194(28.40%)	
Pathological type						
Undifferentiated non-keratinizing	/	1355(96.44 %)		/	664(97.22%)	0.426[a]
Differentiated non-keratinizing	/	11 (0.78 %)		/	4(0.59%)	
uncertain	/	39 (2.78 %)		/	15(2.20%)	
T category						0.959[a]
T1	/	98 (6.98%)		/	29 (4.25%)	
T2	/	367 (26.12%)		/	195 (28.55%)	
T3	/	483 (34.38%)		/	259 (37.92%)	
T4	/	457 (32.53 %)		/	200 (29.28%)	
Ncategory						0.272[a]
N0	/	41 (2.92%)		/	18 (2.64%)	
N1	/	199 (14.16 %)		/	146(21.38%)	
N2	/	783 (55.73%)		/	314(45.97 %)	
N3	/	382 (27.19%)		/	205(30.01%)	
M category						0.150[a]
M0	/	1294(92.10%)		/	641(93.85%)	
M1	/	111 (7.90%)		/	42 (6.15%)	
TNM stage						0.108[a]
I-II	/	86 (6.12%)		/	64 (9.37%)	
III	/	558 (39.72 %)		/	259 (37.92%)	
IV	/	761 (54.16%)		/	360 (52.71 %)	
EBV DNA (copies/ml)						
Limitation = 400[b]			<0.001			<0.0001
Negative	244 (100%)	832 (59.22%)		303(100%)	269 (39.39%)	
Positive	0	573 (40.78%)		0	414 (60.61%)	
Limitation = 100[c]			<0.0001			<0.0001
Negative	244 (100%)	585 (41.64%)		303(100%)	165 (24.15%)	
Positive	0	820 (58.36%)		0	518 (75.85%)	
Limitation = 0[d]			<0.001			<0.0001
Negative	242 (99.18%)	274 (19.50%)		300(99.01%)	100(14.64%)	
Positive	2 (0.82%)	1131 (80.50%)		3(0.99%)	583(85.36%)	

Table 1. Clinical characteristics [a]Statistical Comparison of NPC Staging Component Ratios in the MP and CP Groups [b]detected limitation for CP [c]detected limitation for MB [d]Combining with amplification curve, optimizing the baseline to 0 copies/mL

MB based RT-qPCR could increase cfEBV DNA positive rate among the pre-treatment NPC patients in the same region

The manufacturer of cfEBV DNA advised 400 copies/mL as the detectable limit for CP. Using 400copies/ml as the threshold, 40.78% and 60.61% of NPC patients had positive cfEBV DNA quantifications with CP and MB methods (Table 1). Positive rates increased to 58.36% and 75.85% based on the 100copies/ml threshold (MB). Optimizing the baseline to 0 copies/mL[16], the positive rates in CP and MB were 80.5% and 85.36%, showing a 5% difference. Healthy controls in each group had no cfEBV DNA at the 400 or 100 copies/ml threshold. When the cut-off value was 0 copy/mL, 1% of healthy controls in both groups had cfEBV DNA. Regardless of the threshold, the MB method had a higher cfEBV DNA positive rate than the CP method in pre-treatment NPC patients in the same area.

Two cohorts of naive NPC patients in the same region were tested for cfEBV DNA using CP or MP method. There was no difference in TNM staging, but the median pre-treatment cfEBV DNA (Fig. 3A) and positive rate of beyond 30% (Fig. 3B) differed significantly. NPC patients with CP and MB had median pre-treatment cfEBV DNA loads of 192.59 and 719 copies/mL, respectively ($P<0.001$, Fig. 3A). MB increased cfEBV DNA positive rates in several patient subgroups. Figure 3C-F showed that the MB extraction method significantly improved cfEBV DNA positive rate in T2-T4, N1-N3, M0, and III-IV stage NPC patients ($P<0.05$, >30%). Positive rates

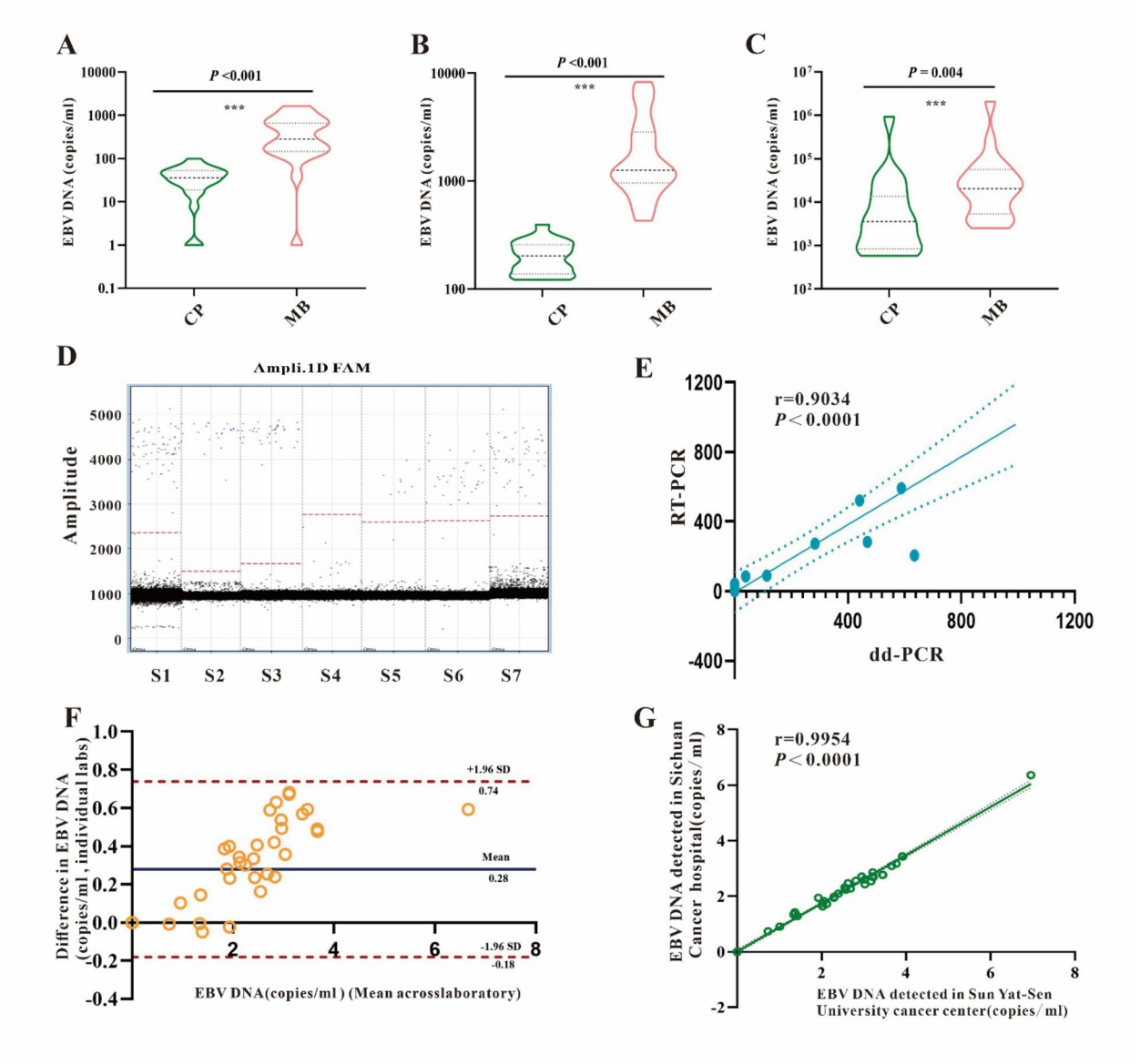

Fig. 2. Comparing cfEBV DNA quantitation with nucleic acid extracted by MB and CP. **(A-C)** Nucleic acid extracted from the same plasma by CP and MB method, respectively ($N = 60$). The same amplification kit measured cfEBV DNA. Different cfEBV DNA loads were compared. Group 1: cfEBV DNA < 100 copies/ml **(A)**; Group 2: 100 copies/mL ≤ cfEBV DNA < 400 copies/ml **(B)**; Group 3: cfEBV DNA ≥ 400 copies/ml **(C)**. **(D)** Digital PCR was used to replicate the outcomes of MB based RT-qPCR. Representative digital PCR amplification results of cfEBV DNA in samples (S1–S7) are displayed. **(E)** The relative significance of the two techniques was investigated ($P < 0.0001$, $r = 0.9034$). **(F)** Bland-Altman plots displayed the comparison between Sichuan Cancer Hospital and Sun Yat-Sen University Cancer Center. **(G)** The correlation analysis of two libraries.

rose by 17.82% and 27.64% in M1, I, and II stages. There was also a 10% increase in the T1 and N0 categories, but this has yet to achieve statistical significance.

MB based RT-qPCR improves the diagnostic value of cfEBV DNA for NPC in non-endemic areas.

To analyze the diagnostic value of pre-treatment cfEBV DNA detected by CP or MB, the ROC curve was utilized to evaluate sensitivity and specificity. In cohort1, cfEBV DNA detected with CP had a sensitivity and specificity were 40.8% and 100%, respectively (Fig. 4A, EBV CP-400). The AUC was 0.704 (95% CI: 0.676–0.731). In cohort2, the pre-treatment cfEBV DNA was detected with MB RT-qPCR, which increased sensitivity to 75.84% and AUC to 0.879 (95% CI: 0.86–0.90) (Fig. 4B, EBV MB-100). As shown in Fig. 4A, the AUC of EBV CP-400 was much lower than that of EBV CP-0. Figure 4B suggested that the AUC of EBV MB-100 was close to that of

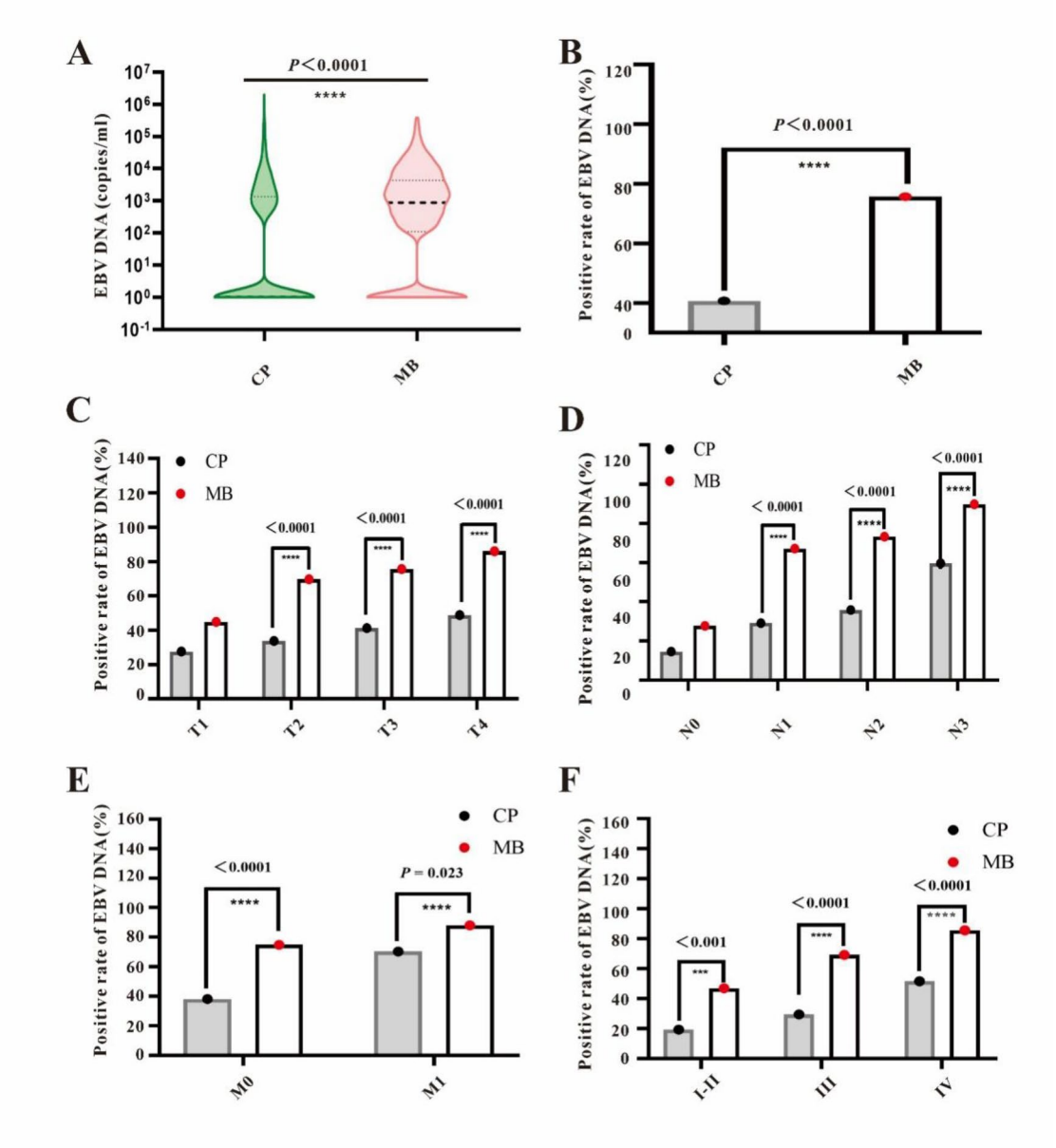

Fig. 3. MB basedRT-qPCRThe positive rate of cfEBV DNA detected with CP or MB based RT-qPCR in the same location. **(A-B)** Pre-treatment cfEBV DNA loads and positive rates in NPC patients from two cohorts. Cohorts 1**(A)** and 2 **(B)** were detected with CP based and MB based RT-qPCR, respectively. **(C-F)** Comparing the positive rate of EBV DNA (%) among T category, N category, M category, and TNM stage subgroups. The thresholds for CP and MB were 400 and 100 copies/ml, respectively.

EBV MB-0. These results revealed that MP improved the sensitivity of the diagnosis value of cfEBV DNA for NPC in this area.

MB-based RT-qPCR raised the pre-treatment positivity of cfEBV DNA and allowed for dynamic monitoring during therapy.

In cohort 2, 401 patients were positive for cfEBV DNA prior to therapy. After treatment, 391 (97.5%) patients had no detectable cfEBV DNA, whereas 10 patients (2.5%) remained positive. For patients who received ≤ 2 cycles of NAC ($N = 125$), the PEC ranged from 11.2%(Post-NAC1) to 100%(RT-END)(Fig. 5A). The PEC ranged from 8.5% (Post-NAC1) to 95.95% (RT-END) in patients with ≥ 3 NAC cycles ($N = 247$) (Fig. 5B).Plasma cfEBV DNA clearance half-life ($t_{1/2}$) was calculated in 391 NPC patients with undetectable cfEBV DNA after therapy. The median half-life value ($t_{1/2}$) of plasma cfEBV DNA clearance was 12.04 days. Plasma cfEBV DNA clearance half-life ($t_{1/2}$) differed significantly among patients with different N-stages (Fig. 6B) and clinical stages (Fig. 6D). The plasma cfEBV DNA clearance half-life ($t_{1/2}$) did not differ between the various M groups (Fig. 6C), although there was a difference between groups T1-2 and T3 (Fig. 6A). In addition, cf EBV DNA was considerably lower

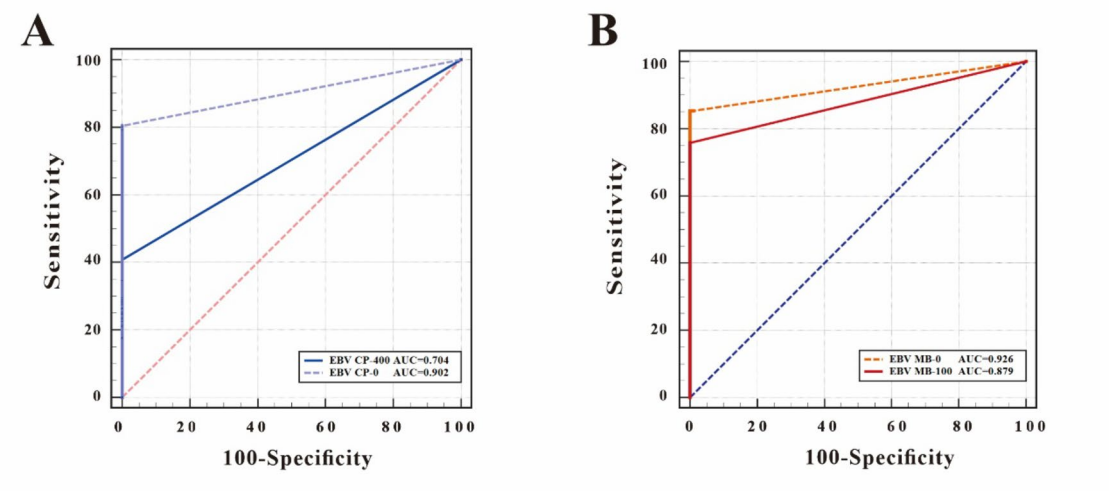

Fig. 4. ROC curve analysis based on the detected limitation of CP based RT-qPCR **(A)** and MB based RT-qPCR **(B).** EBV CP-0 or EBV MB-0: 0 copies/mL regarded as detected limitation; EBV CP-400: 400 copies/mL regarded as detected limitation. EBV MB-100: 100 copies/mL regarded as detected limitation.

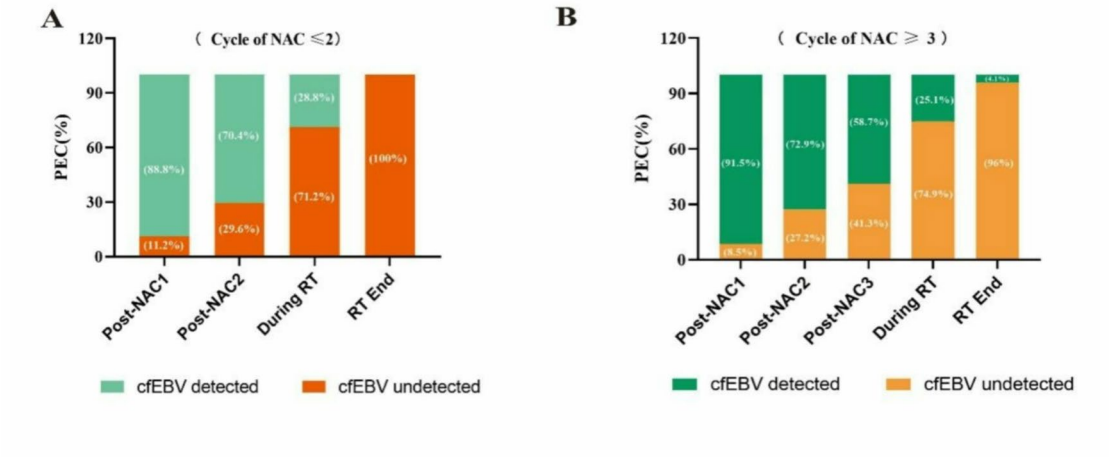

Fig. 5. The dynamic study of PEC among individuals who tested positive for cfEBV DNA in cohort 2. **(A)** The PEC of NPC patients at different time points who underwent neoadjuvant therapy for ≤ 2 cycles ($N = 371$). **(B)** PEC of NPC patients at different time points who received more than 2 cycles of neoadjuvant treatment. PEC: The proportion of patients with cfEBV DNA clearance upon treatment.

in EBER-negative patients than positive patients (Fig. 6E). Patients with KI67 < 40% showed decreased EBV DNA burdens (Fig. 6F).

Discussion

EBV is linked to various human cancers, such as malignant lymphoma and NPC, making precision diagnostics in EBV particularly interesting[20]. To detect EBV, EBER, antibodies, and cfEBV DNA can be employed quantitatively or semiquantitatively[21]. The most common biomarker for quantifying EBV-related illness was cfEBV DNA[22]. Dynamic plasma cfEBV DNA analysis can adjust treatment strategies and predict outcomes for EBV-related illnesses[1723]. Due to the expensive cost of digital PCR and NGS, RT-qPCR became common in most countries. However, laboratory inconsistency and a lack of standardized cfEBV DNA detection methods make it challenging to utilize in clinical practice. Using CP based RT-qPCR, a multi-center study reported that only 40.8% of NPC patients in non-endemic southwest China had detectable cfEBV DNA[16]. Plasma cfEBV DNA for NPC needs better detection methods to increase its clinical utility and reduce its detection limitations.

Research indicates that cfEBV DNA is helpful for NPC diagnosis and prognosis[2425]. In this study, cfEBV DNA were measured with CP and MB extracted nucleic acid from the same samples. The results indicated that the cfEBV DNA loads were higher detected with MB based RT-qPCR (Fig. 2A-C). Inter-laboratory comparisons and digital PCR confirm the accuracy of the test results. In cohort 2 MB based RT-qPCR increased median pre-

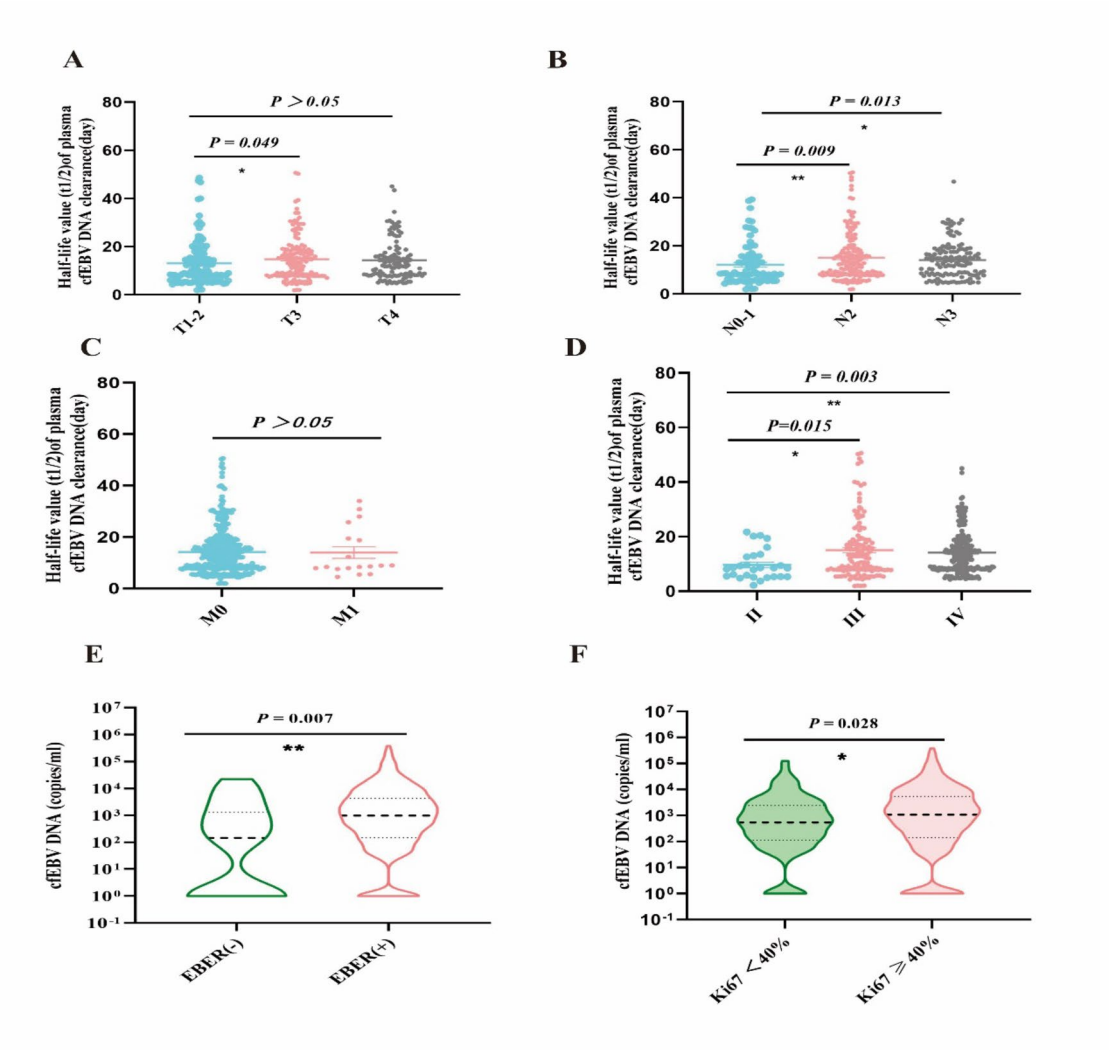

Fig. 6. Subgroup analysis of plasma cfEBV DNA clearance half-life ($t_{1/2}$) among NPC patients with undetectable DNA after therapy(cohort 2, $n = 391$). The plasma cfEBV DNA clearance half-life value ($t_{1/2}$) was analyzed in the T category (**A**), N category (**B**), M category (**C**), and clinic stage subgroups (**D**). cfEBV DNA loads were compared in different EBER status (**E**) and Ki67 subgroup (**F**).

treatment cfEBV DNA load (719 copies/ml versus 192.59 copies/ml) and pre-treatment positive detection rate (75.85% versus 40.78%, Fig. 3B). Since nucleic acid extraction methods vary, 59.22% of cohort 1 patients (CP RT-qPCR) were treated without markers for prognosis and efficacy. Higher positive detection rates for clinical stages I-II, M0, and N1 NPC are critical for early screening and efficacy monitoring. These results indicated that MB could increase the positive detection rate and the load of cfEBV DNA in NPC. Similarly, Zheng et al.[26] assessed plasma EBV DNA with three nucleic acid extraction methods (membrane spin column, boiling and automated magnetic bead). The magnetic bead showed a higher positive detection rate, lower limit of detection, and mean load of EBV DNA in plasma from NPC than the membrane spin column and boiling methods. Zhou et al.[27]. compared the CP to the spin column extraction for peripheral blood EBV DNA detection. The CP had a higher positive detection rate than spin column extraction method (900.0% versus 62.6%, respectively). This is the first study that compares the CP and MB extraction methods for detecting cfEBV DNA. It also confirms the actual statistics on the positive rate in NPC with no difference in TNM staging in the same location. Both suggest that the MB extraction approach is superior than the CP method, which could improve NPC screening in this region and broaden the clinical utility of cfEBV DNA.

In clinical practice, EBV-related NPC could be detected by analyzing cfEBV DNA or non-coding RNAs (EBER et al.)[2829]. In diffuse large B-cell lymphoma[30], EBV DNA in whole blood has good concordance with EBER. Two large-scale NPC screening studies indicated that a lower cutoff value of 0 or 20 copies/ml effectively identified early-stage NPC patients using cfEBV DNA[3132]. In this study, MB based RT-qPCR (AUC = 0.879 95% CI : 0.86–0.90) of cfEBV DNA demonstrated higher sensitivity and specificity for NPC screening. In early-stage NPC patients with low cfEBV DNA viral load (CP < 400 copies/ml), MB based RT-qPCR improved diagnostic value. EBER primary hybridization is commonly used by pathologists to diagnose NPC[33]. There are 683 NPC for

cfEBV DNA investigation, and 553 patients are accessible for EBER analysis. EBER was positive in 528 (95.48%) patients, whereas cfEBV DNA was positive in 584 (85.51%); the two analyses were 87.88% consistent in cohort 2. In addition, EBER negative patients had significantly lower EBV DNA loads than EBER positive patients (Fig. 6E).

cfEBV DNA is a crucial biomarker for therapy options, outcomes, and surveillance in NPC patients[24,34,35]. Changing cfEBV DNA cutoff values before treatment may impact survival, particularly in endemic regions. Few non-endemic Chinese research have related cfEBV DNA cutoff values to NPC prognosis. Based on CP RT-qPCR, the best pre-treatment cutoff value for survival in non-endemic locations was 262.7 copies/ml[25]. Lin et al. and Leung et al. identified 1500 and 4000 copies/ml as "best" cutoff for predicting overall survival[36,37]. Rapid cfEBV DNA clearance > 15 days was linked to worse DFMS, PFS, and OS in treatment-undergoing patients[38]. The presence of plasma cfEBV DNA during radiation therapy indicated a bad prognosis[39]. In this study, through a comparison of the two extraction methods, MB improved positive detection rate by 35%, providing patients a reference during treatment. This enables cohort 2 NPC patients to receive dynamic efficacy and prognosis monitoring.

Dynamic studies of plasma cfEBV DNA clearance showed a median half-life ($t_{1/2}$) of 12.04 days in cohort 2. The $t_{1/2}$ of plasma cfEBV DNA varied significantly between individuals with different N-stages (Fig. 6B) and clinical stages (Fig. 6D). These findings revealed that plasma cfEBV DNA $t_{1/2}$ was related with clinical phases, providing prognostic surveillance. Plasma cfEBV DNA varied by ki67 subgroup (Ki67 < 40% versus Ki67 ≥ 40%), suggesting a relationship to tumor progression. Changing dynamics of whole-course ctDNA have been linked to various survival outcomes in non-metastatic NPC[17]. In cohort2, cfEBV DNA clearance upon treatment (PEC) was identified. The PEC at different treatment times were lower than reported in the study of Jiawei Lv et al.[17]. The primary reason is that the investigators we included in the analysis did not strictly enforce routine cfEBV DNA testing, more patients were tested for conversion during or after radiation therapy. The PEC decreased and cfEBV DNA clearance extended.

These findings imply that employing the MB technique to extract nucleic acids can raise the cfEBV DNA load in NPC, increase the positive rate, and allow for dynamic monitoring of cfEBV DNA changes during therapy. This broadens the clinical use of cfEBV DNA for NPC efficacy and prognosis. Many previous endemic studies utilized nucleic acid extraction through spin column (Qiagen). But this extraction approach is time-consuming and expensive. The MB extraction process is simple and inexpensive, which can significantly improve labor efficiency. Currently, 200ul plasma is used in MB, but the filter column requires 400-800ul for extraction. More research centers in endemic and non-endemic NPC regions are encouraged to conduct related studies to optimal MB protocal to standardized cfEBV DNA detection.

The current study includes some limitations. First, this was retrospective study in nature, and potential bias could not be avoided. For example, the time intervals for cfEBV DNA monitoring during therapy were not uniform. This delay EBV clearance half-life and decrease the PEC in this study. In addition, samples were collected and stored in batches for testing after plasma collection. Some inaccuracies may arise as a result of sample storage. Because cohort 2 was only tracked for 2 years, this study lacks investigation between cfEBV DNA (MB-based RT-qPCR) and NPC prognosis. Few studies have focused on cfEBV DNA of NPC in non- endemic, therefore further research is needed to confirm this study's findings and make them useful in places with poor NPC awareness.

In conclusion, this work was able to convincingly show that MB based RT-qPCR was superior sensitivity and positive detection rates for cfEBV DNA. Comparing the 2 cohorts of NPC, the T, N, M, and clinical stage subgroups, cfEBV DNA-positive detection rates were higher in MB based RT-qPCR group. This indicates that cfEBV DNA can be more positively noticed, with a higher diagnostic value and a broader variety of clinical applications among NPC in non-endemic areas.

Data availability

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Author contributions

He Qiao: methodology, statistical analysis, manuscript writing of original draft. Tuo Yi and Yi Zhou: data collection, acquisition, and curation. Yan Yue and Dan Zhao: data collection, acquisition, and curation. Liu Xin: data curation. Wang Qiuju: methodology and validation. Luo Hao: methodology and validation. Zhang Zhengyao: data curation. Fanping Meng: resources. Binwu Ying and Dongsheng Wang: supervision, resources. Mu Yang: conceptualization, supervision, resources, writing – review & editing. Yecai Huang: supervision, conceptualization and design this study, funding acquisition, writing – review & editing.

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Declarations

Ethics approval and consent to participate

This respective study was approved by the institutional Ethics Committee for Medical Research and New Medical Technology of Sichuan Cancer Hospital (SCCHEC-02-2019-10). All patients included in this study

signed informed consent to the treatment protocol statements.

Competing interests
The authors declare no competing interests.

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