Negative plasma Epstein-Barr virus DNA nasopharyngeal carcinoma in an endemic region and its influence on liquid biopsy screening programmes

John Malcolm Nicholls1, Victor Ho-Fun Lee2,3, Sik-Kwan Chan2, Ka-Chun Tsang2, Cheuk-Wai Choi2, Dora Lai-Wan Kwong2,3, Ka-On Lam2,3, Sum-Yin Chan2, Chi-Chung Tong2, Tsz-Him So2, To-Wai Leung2,3, Mai-Yee Luk2, Pek-Lan Khong4 and Anne Wing-Mui Lee2,3

BACKGROUND: Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC) in endemic regions may have undetectable plasma EBV DNA.

METHODS: We prospectively recruited 518 patients with non-metastatic NPC and measured their pre-treatment plasma EBV DNA. The stage distribution and prognosis between pre-treatment plasma EBV DNA-negative (0–20 copies/ml) and EBV DNA-positive (>20 copies/ml) patients following radical treatment were compared.

RESULTS: Seventy-eight patients (15.1%) were plasma EBV DNA-negative, and 62 in this subset (12.0%) had 0 copy/ml. Only 23/78 (29.5%) plasma EBV DNA-negative patients with advanced NPC (stage III-IVA) had strong EBV encoded RNA (EBER) positivity (score 3) in their tumours compared to 342/440 (77.7%) EBV DNA-positive patients of the same stages (p < 0.001). Though EBV DNA-negative patients had more early-stage disease (p < 0.001) and smaller volumes of the primary tumour and the positive neck nodes (p < 0.001), they had similar 5-year overall survival and cancer-specific survival to those EBV DNA-positive counterparts by stage. Similar results were also seen when plasma EBV DNA cut-off was set at 0 copy/ml.

CONCLUSIONS: Patients with low-volume NPC may not be identified by plasma/serum tumour markers and caution should be taken in its utility as a screening tool for NPC even in endemic regions.

CLINICAL TRIAL REGISTRATION: Clinicaltrials.gov Identifier: NCT02476669.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is endemic in southern China including Hong Kong. Over the past 20 years, plasma Epstein-Barr Virus (EBV) deoxyribonucleic acid (DNA) has been advocated for the diagnosis of NPC. The rationale for this methodology has been based on the concept that since EBV has been associated with NPC, the circulating viral or tumour associated DNA (ctDNA) may be detected in the blood as a measure of tumour presence or disease burden. However, in NPC there is a sensitivity between 53 and 96%, and this wide variation has been attributed to three main factors—the analytical method of detection, disease stage and the World Health Organization (WHO) histology of the disease. There has been an attempt to develop standardised protocols of analysing plasma EBV DNA, but meta-analyses of plasma EBV DNA in the diagnosis of NPC have not reported the proportion of plasma EBV DNA-positive patients for each WHO type, and only four studies looked at the clinical stage. Squamous cell carcinomas have typically been separated by WHO from non-keratinising carcinoma, as previous studies suggested that the former was usually negative for EBV by in-situ hybridisation (ISH), while the other subtypes were positive. In Hong Kong, however, tumours with squamous differentiation can be EBV positive, and this has also been shown in NPCs from Malaysia.

Hong Kong is one of the first regions in the world discovering the association of plasma EBV DNA with NPC, and devised the first quantitative assay for accurate and reliable measurement. Over the years, the lowest detection limit as a representation of improved sensitivity has decreased from 60 copies/ml to 20 copies/ml, and in a large NPC screening programme this low detection limit has been advocated as an initial screening tool. In this publication, we focused our attention on the incidence of plasma EBV DNA-negative (≤20 copies/ml) NPC patients, their clinico-pathological characteristics and survival outcomes, in an attempt to determine whether there are unique features concerning these patients which may account for the absence of EBV DNA in their plasma. Furthermore, since there have been recent publications on the value of liquid biopsy in screening...
programmes, we sought to determine whether the same concerns on the usefulness of these screening programmes could be applied to NPC.

METHODS
Study population
The study cohort comprising 518 consecutively and prospectively recruited patients with previously untreated non-metastatic NPC (i.e. stage I–IVA) enrolled in another study which investigated the role of plasma EBV DNA in proposing a new staging system (ClinicalTrials.gov NCT02476669). All biopsy-confirmed patients had complete staging investigations, including positron-emission tomography with integrated contrast-enhanced computed tomography (PET-CT) scan, magnetic resonance imaging (MRI), serum haematology, biochemistry and lactate dehydrogenase, serology for EBV immunoglobulin A (IgA) for viral capsid antigen (VCA), and plasma EBV DNA to investigate the prognostic role of plasma EBV DNA to investigate the prognostic role of plasma EBV DNA genome copies. Circulating EBV DNA concentrations were measured using a real-time quantitative polymerase chain reaction (PCR) system with ABI Prism® 7000 Sequence Detection System (Applied Biosystems, USA) that amplified a DNA segment in the BamHI-W fragment region of the EBV genome. All samples were repeated twice on the same day by the same assay for accurate quantification and the results showed that the discrepancy was less than 2% for all repeated samples. The results were expressed as EBV DNA genome copies per ml with accuracy to the nearest 0.1 copies/ml. All pre-treatment investigations were performed within 14 days of the pathological diagnosis of NPC. All patients, within 14 days of these investigations, then received radical intensity-modulated radiation therapy (IMRT) with or without concurrent chemotherapy and adjunct (induction or adjuvant) chemotherapy based on the stage of the disease according to the 7th edition of American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM staging system. The details of treatment and follow-up surveillance were previously described. The TNM stage of each patient’s disease was re-staged according to 8th edition of AJCC/UICC TNM staging system for subsequent analysis in this study.

As there has been no consensus on what determines the lowest limit of plasma EBV DNA detection, we selected and analysed patients who had 0–20 copies of EBV DNA per ml in the plasma as this has been regarded as the lowest limit of detection threshold in a recently reported screening study, with plasma EBV DNA ≤20 copies/ml (i.e. 0–20 copies/ml) designated as plasma EBV-negative. In this publication, plasma EBV DNA-negative NPC and EBV DNA-positive NPC were defined as those diagnosed in patients who had pre-treatment ≤20 copies/ml and > 20 copies/ml respectively.

RESULTS
From October 2010 to May 2016, 518 patients were prospectively recruited with their dispositions shown (Fig. 1, Table 1). Seventy-eight (15.1%) patients were classified as pre-treatment plasma EBV DNA-negative (i.e. ≤20 copies/ml). Sixty-two (79.5%) patients had 0 copy/ml and 16 (20.5%) had 1–20 copies/ml of plasma EBV DNA before treatment. Pre-treatment plasma EBV DNA correlated very well with GTV_P, GTV_N, GTV_T and serum lactate dehydrogenase (all p < 0.01) (Fig. S2). However, no correlation was identified between pre-treatment plasma EBV DNA and GTV_P (p = 0.936), GTV_N (p = 0.900) and GTV_T + N (p = 0.910) in plasma EBV DNA-negative patients. Plasma EBV DNA-negative patients tended to have an earlier stage of their NPC, as well as smaller volumes of the primary tumour in the nasopharynx and the positive neck nodes. The overall stage distribution of these 78 plasma EBV DNA-negative patients was: stage I in 18 (23.1%); stage II in 17 (21.8%); stage III in 34 (43.6%) and stage IV in 9 (11.5%). No plasma EBV DNA-negative patients had stage IVB metastatic disease at the time of initial diagnosis. The T- and N-classification of the plasma EBV DNA-negative patients were shown (Table S1).

After a median follow-up of 5.2 years (range 1.2–6.4 years), the 5-year PFS, OS and CSS of the whole population were 72.9%, 79.8% and 86.1%, respectively. Except for PFS (92.7% vs 70.0%, 95% CI = 83.3–97.2% vs 73.1–82.8%, p = 0.023), OS (91.9% vs
Non-metastatic NPC patients enrolled for baseline investigations before commencement of treatment (n = 554)

Had baseline investigations including hematology, biochemistry, plasma EBV DNA, MRI, 18F-FDG PET-CT scan and nasopharyngoscopy and biopsy (n = 521)

Received radical IMRT with or without adjunct chemotherapy (n = 521)

Excluded (n = 3)

Died of sepsis during IMRT (n = 2)

Committed suicide during IMRT (n = 1)

Underwent post-IMRT 8th week biopsy and plasma EBV DNA measurement (n = 518)

Had persistent NPC at 8th week after IMRT (n = 5)

Had persistent NPC at 10th week after IMRT (n = 2)

Had persistent NPC at 12th week after IMRT (n = 1)

Had event and survival follow-up (n = 518)

Fig. 1 Study flowchart

78.1%, 95% CI = 81.1–97.1% vs 73.1–82.8%, p = 0.242) and CSS (96.2% vs 84.6%, 95% CI = 85.2–99.0% vs 80.0–88.3%, p = 0.293) of the plasma EBV DNA-negative patients were not statistically better than their EBV DNA-positive counterparts (Table S2). When compared by stage, their survival outcomes (PFS, OS and CSS) were also not different from the plasma EBV DNA-positive patients (Figs. 2–4).

Since there were 62 and 16 patients who had 0 copies/ml and 1–20 copies/ml of pre-treatment plasma EBV DNA, respectively, we also compared the stage distribution, tumour and nodal volumes and survival endpoints with those who had pre-treatment plasma EBV DNA >20 copy/ml (Tables S3–S6). Again, patients with pre-treatment plasma EBV DNA 0 copy/ml had more early-stage disease, smaller tumour and nodal volumes but similar survival outcomes (except PFS) as compared to those who had pre-treatment plasma EBV DNA >0 copy/ml (Figs. S3–S5 and Table S7).

Association of EBER with plasma EBV DNA and overall stage of disease

The association of EBER by ISH with plasma EBV DNA and NPC T-, N- and overall stage was shown (Tables S8–S13). Though statistical significances on the association between EBER scores and overall stage were identified in the whole study population and plasma EBV DNA-positive patients, they were not detected in plasma EBV DNA-negative patients. Intriguingly, only 23 out of 78 (29.5%) of advanced NPC (stage III-IVA) patients who were plasma EBV DNA-negative had diffuse strong EBER positivity (score 3) in their tumours, as compared to 342 of 440 (77.7%) plasma EBV DNA-positive patients of the same stages (p < 0.001). Plasma EBV DNA-negative patients were also associated with a lower EBER intensity in their tumours (p < 0.001) (Table S11). The results were also similar when 0 copy/ml was set as the cut-off: 41 of 62 (66.1%) had EBER score 3 in patients with plasma EBV DNA 0 copy/ml compared to 412 of 456 (90.4%) in patients with plasma EBV DNA > 0 copy/ml (p < 0.001) (Tables S11–S13).

DISCUSSION

In view of the close association between NPC and EBV, antibodies to EBV have been used for NPC screening and treatment monitoring, and to differentiate NPCs from other head and neck cancers.17,18 Immunohistochemistry and ISH studies conclusively demonstrated that the EBV genome was incorporated into the tumour cells,19–21 resulting in EBV being classified as an oncogenic virus.22

In the late 1990’s several studies showed that cancer-derived cells or DNA could be detected in the blood of cancer
Table 1. Patient characteristics at baseline based on 8th edition of AJCC/UICC staging classification stratified by pre-treatment plasma EBV DNA (0–20 copies/ml vs >20 copies/ml)

| Characteristic | Total (n = 518) | Pre-treatment plasma EBV DNA | p     |
|---------------|----------------|----------------------------|-------|
|               | Patients, No. (%) | 0–20 copies/ml (n = 78) | >20 copies/ml (n = 440) |       |
| Median age in years (range) | 53 (16–90) | 54 (16–86) | 53 (16–90) | 0.983 |
| Male/female   | 385 (74.3)/133 (25.7) | 61 (78.2)/17 (21.8) | 324 (73.6)/116 (26.4) | 0.395 |
| Histology     | 508 (98.1) | 78 (100) | 430 (97.8) | 0.405 |
| Keratinising squamous cell carcinoma | 1 (0.2) | 0 (0) | 1 (0.2) |       |
| Non-keratinising differentiated carcinoma | 9 (1.7) | 0 (0) | 9 (2.0) |       |
| Non-keratinising undifferentiated carcinoma | 508 (98.1) | 78 (100) | 430 (97.8) |       |
| ECOG performance status | 80 (15.4) | 14 (17.9) | 66 (15) | 0.983 |
| T-classification | 147 (28.4) | 40 (51.3) | 107 (24.3) |       |
| N-classification | 60 (11.6) | 25 (32.1) | 35 (8.0) |       |
| Overall stage | 231 (44.6) | 35 (44.9) | 196 (44.5) |       |
| Laterality of primary tumour | 60 (11.6) | 25 (32.1) | 35 (8.0) |       |
| Involvement of retropharyngeal node | 231 (44.6) | 35 (44.9) | 196 (44.5) |       |
| Median pretreatment plasma EBV DNA in copies/millilitre | 588.5 (0–1143750) | 0 (0–19) | 895 (22–1143750) | <0.001 |
| Stage I       | 12 (0–315) | 0 (0–19) | 65 (24–315) | 0.004 |
| Stage II      | 321 (0–8850) | 0 (0–19) | 547 (22–8850) | <0.001 |
| Stage III     | 494 (0–175000) | 0 (0–17) | 705.5 (22–175000) | <0.001 |
| Stage IVA     | 2012.5 (0–1143750) | 0 (0–14) | 2203 (38–1143750) | 0.584 |
| Median pretreatment serum lactate dehydrogenase in international units/litre | 196 (109–688) | 180.5 (125–310) | 196 (109–688) | <0.001 |
| Stage I       | 179.5 (121–310) | 178.5 (132–310) | 190.5 (121–260) | 0.655 |
| Stage II      | 185.5 (140–275) | 174 (143–275) | 188 (140–256) | 0.235 |
| Stage III     | 197.5 (109–521) | 191 (137–306) | 198 (109–521) | 0.593 |
| Stage IVA     | 200 (125–688) | 182 (125–254) | 204 (130–688) | 0.174 |
| Median Gross tumour volume of the primary tumour (GTV_P) (cm³) | 8.7 (0–136) | 4.4 (0–66.9) | 10.1 (0–136) | 0.004 |
| Median Gross tumour volume of the positive neck nodes (GTV_N) (cm³) | 17.1 (0–191.3) | 9.45 (0.4–191.3) | 18.55 (0–168.2) | <0.001 |
| Median Gross tumour volume of the primary tumour and the positive neck nodes (GTV_P + N) (cm³) | 31.4 (0.9–229) | 16.25 (2.3–199.9) | 34.9 (0.9–229) | <0.001 |
| Radical IMRT only | 71 (13.7) | 26 (33.3) | 45 (10.2) | <0.001 |
| Concurrent chemoradiation | 91 (17.6) | 14 (17.9) | 77 (17.5) | 0.194 |
| Induction chemotherapy then concurrent chemoradiation | 165 (31.9) | 12 (15.4) | 153 (34.8) | 0.022 |
| Concurrent chemoradiation then adjuvant chemotherapy | 191 (36.9) | 27 (34.6) | 164 (37.3) | 0.199 |

AJCC American Joint Committee on Cancer, EBV DNA Epstein-Barr virus deoxyribonucleic acid, ECOG Eastern Cooperative Oncology Group, IMRT intensity-modulated radiation therapy, UICC Union for International Cancer Control
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...failed extraction, inhibition, patient NPC. Using a detection limit of 60 copies/ml, EBV DNA was combination of plasma EBV DNA and IgA VCA in detecting et al in the same year also investigated the accuracy of which was suggested to be a measure of tumour burden. Leung Plasma EBV DNA also appeared to be linearly related to T-stage was better at detecting stage I compared to stage IV disease.

showed that the sensitivity has ranged from 53–96%. The false positive rate was 8%. A recent review, however, compared to 3 of 43 normal individuals, giving a detection rate of 96%.2 The superiority of plasma EBV DNA over serum IgA VCA was

patients, and this was applied to NPC first by Mutirangura et al and later by Lo et al. Using the BamH1-W and EBNA1 targets to amplify, 55 of 57 patients had plasma EBV DNA detected compared to 3 of 43 normal individuals, giving a detection rate of 96%. The false positive rate was 8%. A recent review, however, showed that the sensitivity has ranged from 53–96%.2

The superiority of plasma EBV DNA over serum IgA VCA was later shown in 2004 by Shao et al, who also demonstrated that plasma EBV DNA correlated with the TNM stage,27 though IgA VCA was better at detecting stage I compared to stage IV disease. Plasma EBV DNA also appeared to be linearly related to T-stage which was suggested to be a measure of tumour burden. Leung et al in the same year also investigated the accuracy of combination of plasma EBV DNA and IgA VCA in detecting NPC.11 Using a detection limit of 60 copies/ml, EBV DNA was detected in 132/139 patients, with an increased sensitivity seen in later stages (87/89) than early stages (45/50). The wide range of sensitivity previously reported may be due to many factors such as extraction technique, amplification technique and whether the BamH1-W region (which has more repeats for detection, but random in the number of repeats), or single-copy genes (e.g. EBNA1) are used as targets.28 Other potential factors that may contribute to assay include failed extraction, inhibition, patient age and the ambient temperature when blood is taken from patients.29 A previous report has shown low copy number of EBV in 3–7% of the general population when the BamH1-W target was used, and 2.03% when the EBNA1 target was used (1–512 copies/ml), so at these low numbers there is an overlap between normal subjects and patients with stage I NPC.31

In the screening programme performed by Chan et al., 9% of normal individuals had detectable plasma EBV DNA in the winter season and 5% in the summer, in which 20 copies/ml was the lowest limit of detection.30 On the other hand, when 0 copies/ml was used as a cut-off, plasma EBV DNA identified 17/24 NPC patients in a non-endemic (but high-risk population) area.32

The challenge of a screening tool for cancer is the balance between sensitivity and specificity. Recent publications have addressed the community acceptance of a false positive rate in cancer screening programs,33,34 where it has been recognised that there is a negative psychological outcome in patients with false positive screening in breast and lung cancer.35,36 Yip and colleagues commented that the lowest sensitivity of plasma EBV DNA for NPC is in the stage I patients—the major targets of screening programmes, but this review has been hampered by the very small number of patients included with this stage (ranging from 1 to 14 patients in each respective study) and the wide range...
of median EBV DNA levels present in these patients (14–2500 copies/ml). In a recent review of the usefulness of liquid biopsy for the detection of tumours, it was suggested that one of the factors affecting the effectiveness of a cancer screening test was the weight, or volume of the tumour. In patients with a tumour weight of 10 g and sampling 10 ml of blood, the cancer screening test appears effective, but in patients with tumour weight less than that, the effectiveness was called into question. Since Table 1 shows that the primary tumour GTV of our EBV negative cohort was 4.4 g compared to 10 g for the EBV positive patients, it is not surprising that the no EBV can be detected in the plasma. Further data has demonstrated that when the fraction of tumour DNA drops below 0.01%, then the use of 10 ml of blood (4 ml plasma) will not contain a single cancer genome.

Since detection of plasma EBV may be problematic in patients with low volume early-stage disease, one would expect a better correlation in high tumour volume patients and Figure 52 indeed shows a good correlation. Our ISH results show that one possible hypothesis to explain this low or absent detectable plasma EBV DNA is because of tumour heterogeneity in EBV copy number. The studies from Chan and Lo et al. have assumed that each tumour cell contains 50 copies EBV/cell. However, the original publication, found a great range of EBV genome equivalents (2–137) in NPC patients. In addition to variable but multiple copies of the genome in each cell, there are also variable but multiple copies of the promoter Wp within each genome. The standard B95.8 cell line contains 11 reiterations of Bam-W, Raji cell line 7 copies and clinical isolates a mean of 6, with a range of 5–11. We found in many tumours that had no detectable plasma EBV DNA there was a degree of variable EBER signal in the tumour cells—in some cases the signal was located within the nucleolus, in others the whole nucleus seemed to have positive signal, and some cells had no signal at all.

Our study demonstrated that 15.1% of histologically confirmed NPC patients were plasma EBV DNA-negative and that in these plasma EBV DNA-negative patients, 99.8% were either non-keratinising differentiated carcinoma or undifferentiated carcinoma. In response to a review article indicating 17.2–29.3% of NPC patients from endemic countries had undetectable plasma EBV DNA >20 copies/ml vs >20 copies/ml). Inconsistent sample analysis is
not likely as the samples in the current study were analysed by the same methodology used by Chan et al. in their screening programme. Technical error is also unlikely as all our cases were handled by the same laboratory with the same EBV target. All samples were analysed by two other laboratories. All cases had a positive detection when the same sample was handled by the same laboratory with the same EBV target. All cases had a positive detection when the same sample was handled by the same laboratory with the same EBV target. All cases had a positive detection when the same sample was handled by the same laboratory with the same EBV target. All cases had a positive detection when the same sample was handled by the same laboratory with the same EBV target. All cases had a positive detection when the same sample was handled by the same laboratory with the same EBV target.

We demonstrated that EBER was poorly expressed in patients with low pre-treatment plasma EBV DNA (regardless of the cut-off set at 0 or 20 copies/ml). It was also poorly associated with T-, N- and overall stage of their disease even when it is advanced stage. In addition, EBER expression levels were strongly associated with pre-treatment plasma EBV DNA. Yet about half of these patients presented with advanced stage III to IVA disease at diagnosis. It can be inferred that EBV genome may be poorly incorporated in the tumour cells in this subgroup of patients leading to impaired expression of the EBER and plasma EBV DNA production. Since EBER has been demonstrated to influence the expression of RIG-I, inflammatory mediators and thus tumour progression, the link between EBER and tumour stage may be a reflection of the EBER induced stromal response to tumour growth leading to cell death and viral DNA release. The use of only a few slides from each patient’s FFPE tumour samples for subsequent ISH for EBER which may not give an overall picture of the extent of EBV genome incorporation into every tumour cell is one study limitation, though we have already selected the most representative slides containing the greatest number of tumour cells to ensure consistent EBER scoring. Though plasma EBV DNA has been proposed as a population screening tool for NPC by Chan et al., 62/518 (11%) of patients had 0 copy, and 78/518 (15%) of patients had between 0 and 20 copies/ml in our study. It appears that if this was used solely for screening in the general population, it would miss more than 130 patients with NPC each year in Hong Kong with a 7.5 million population. In other words, we can miss 60.0%, 23.0%, 14.5% of patients with NPC each year in Hong Kong with a 7.5 million population.

![Fig. 4 Cancer-specific survival of NPC patients in the study population stratified by pre-treatment plasma EBV DNA (0–20 copies/ml vs >20 copies/ml). a Stage I. b Stage II. c Stage III. d Stage IVA](image-url)
EBV DNA-negative patients by stage was not better than those who were EBV DNA-positive, as shown in our study.

CONCLUSIONS

In conclusion, it was not uncommon for previously untreated NPC patients in endemic regions to have plasma EBV DNA below or close to the lowest limit of detection,⁴⁹,⁵⁰ and while plasma EBV DNA has been shown to be a reliable predictive and prognostic factor, our findings reinforced the concerns of previous authors in adopting its use for the primary diagnosis or screening of NPC in a general population.⁵¹,⁵² The use of EBV as a "liquid biopsy" tool thus has many of the problems associated with other cancer screening tools in terms of both sensitivity and specificity for early cancer detection and additional investigations are warranted if NPC has to be safely ruled out.

ACKNOWLEDGEMENTS

We gratefully thank all the study participants and the staff of Department of Clinical Oncology and Department of Pathology of Queen Mary Hospital of The University of Hong Kong for their contribution to this study.

AUTHOR CONTRIBUTIONS

J.M.N.: conceptualisation, investigation, data analysis, manuscript preparation and editing. V.H.F.L.: conceptualisation, investigation, data analysis, manuscript preparation and editing. S.K.: investigation, data analysis, manuscript preparation and editing. D.L.K.: conceptualisation, investigation, data analysis, manuscript preparation and editing. A.W.M.L.: investigation, data analysis, manuscript preparation and editing. T.W.L.: investigation, data analysis, manuscript preparation and editing. C.C.T.: investigation, data analysis, manuscript preparation and editing. P.L.K.: investigation, data analysis, manuscript preparation and editing. T.H.S.: data analysis, manuscript preparation and editing. J.M.N.: conceptualisation, investigation, data analysis, manuscript preparation and editing. S.K.C.: investigation, data analysis, manuscript preparation and editing. V.H.F.L.: conceptualisation, investigation, data analysis, manuscript preparation and editing.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41416-019-0575-6.

Competing interests: The authors declare no competing interests.

Ethics approval and consent to participate: The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HK HKW IRB) (IRB/REC No. UW 12-153). The study was performed in accordance with the Declaration of Helsinki. Individual written informed consent was obtained from all participating patients at enrolment.

Funding: This work was supported by Croucher Foundation and SK Yee Medical Foundation (210212, 211259 and 213208). The funder has no role in study design and assessment, data collection, statistical analysis and interpretation, manuscript drafting, final manuscript approval and publication of this study.

Consent to publish: We have provided our consent to publish.

Data availability: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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