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Plasma cfDNA VILL gene methylation as a diagnostic marker for nasopharyngeal carcinoma

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Abstract

Currently, the non-invasive diagnostic methods for nasopharyngeal carcinoma (NPC) continue to grapple with the challenge of low sensitivity. The hypermethylation of tumor suppressor genes is an established early event in NPC pathogenesis. Consequently, we conducted whole-genome methylation sequencing on plasma cell-free DNA (cfDNA) from six NPC cases and four healthy controls, integrating Illumina Human Methylation 450 K microarray data from the GEO database comprising six NPC cases and six samples of non-cancerous nasopharyngeal tissue (NP). As result, we screened only one CpG island associated with cell type-specific regulation within the candidate tumor suppressor gene VILL (Vilin Like), which exhibits specific methylation patterns in NPC. We validated our findings using 25 pairs of NPC and NP samples from GEO, alongside 9,736 pan-cancer tissues from TCGA and 656 healthy human leukocyte samples sourced from GEO through methylation microarray analysis. Based on this, we designed a methylation-specific qPCR (qMSP) system for the VILL gene, and then tested it on 192 primary NPC and 154 NC plasma samples. The new qMSP system when compared with EBV DNA qPCR revealed a sensitivity for primary NPC of 80.2% vs.81.3% (78.8% vs.54.5% for early-stage NPC), and a specificity of 100% vs. 93.5%. Notably, employing a combined methodology further enhanced sensitivity to 94.8%, including a sensitivity rate of 90.9% for early-stage NPC diagnosis. Therefore, VILL methylation assessment combined with EBV DNA detection presents a promising avenue for non-invasive diagnosis of NPC, particularly beneficial for early detection.

Keywords Nasopharyngeal carcinoma (NPC), Cell-free DNA (cfDNA), VILL, DNA methylation, Diagnosis

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Background

Nasopharyngeal carcinoma (NPC) is a prevalent malignant neoplasm that arises from the mucosal lining of the nasopharynx, with the superior aspect of the pharynx and the pharyngeal recesses being its most frequent sites of manifestation [1]. According to data from the GLOBOCAN project conducted by the International Agency for Research on Cancer, there were approximately 133,354 new cases of NPC globally in 2020, resulting in over 80,000 fatalities [2]. The incidence of NPC has regional characteristics, and it is mainly concentrated in East Asia, Southeast Asia and North Africa, especially in southern China, such as Guangdong, Guangxi, Hunan and Fujian. According to the latest statistics from the National Cancer Center of China, there was more than 50,000 new NPC patients and more than 20,000 deaths in China in 2022 [3].

Due to the subtle and nonspecific local symptoms associated with NPC and the inherent challenges in conducting routine nasopharyngeal examinations, over 70% of NPC patients are diagnosed at an advanced stage. The five-year survival rate for early-stage NPC exceeds 90%, accompanied by a high quality of life, whereas the five-year survival rate for advanced-stage NPC ranges from 50 to 70%, often resulting in a diminished quality of life [1].

Although plasma EBV-DNA has been used as an auxiliary diagnostic marker, its sensitivity for diagnosing early-stage NPC is only around 50% and false positives are common [4]. Therefore, there is an urgent need to develop other auxiliary diagnostic markers.

Methylation of tumor suppressor genes is recognized as an early event in cancer, especially NPC [5]. Plasma cfDNA-based methylation testing has been used for early diagnosis of a variety of cancers [6, 7]. The aim of this study is to screen NPC-specific methylation regions by whole genome methylation sequencing of plasma cfDNA for early diagnosis of NPC.

Materials and methods

Data sources

We collected blood samples from 211 patients with primary NPC and 174 healthy controls (NC) from General Hospital of Southern Theater Command in Guangzhou from 2019 to 2024. The exclusion criteria for NPC patients were: (a) combined with other malignant tumors; (b) history of chemotherapy in the last three months; (c) uncertain clinical stage. Healthy controls were eligible if they were older than 18 years of age and had no history of major illness (cancer or chronic disease). After applying these criteria and the following quality controls (Additional file 1: Figure S1), a total of 198 primary NPC samples and 158 NC samples were included in this study. Six cases of NPC and four cases of NC were used for whole genome methylation sequencing. A total of 192 NPC and 154 NC samples were used for validation (Table 1). All participants provided written informed consent. This study was approved by the Ethics Committee of the General Hospital of Southern Theater Command.

In addition, 9736 pan-cancer methylomics data were downloaded from TCGA database. The methylomic data GSE52068 (6 NPC vs. 6 NP) and GSE62336 (25 NPC vs. 25 NP) of two groups of primary NPC and nasopharyngeal non-cancer tissues (NP) were downloaded from the GEO database, and the methylomic data GSE40279 of leukocytes from 656 healthy people were downloaded. The above Methylation data were based on Illumina Human Methylation 450 K microarray.

Microarray data analysis

The microarray data were initially preprocessed and normalized using R. Subsequently, the ChAMP package

Table 1 Clinical information of NPC patients and healthy controls

	Whole genome methylation sequencing		Validation by qPCR	
	NC(n = 4)	NPC(n = 6)	NC(n = 154)	NPC(n = 192)
Gender				
Male	2	4	108	144
Female	2	2	46	48
Age(years)	48 ± 8.1	55.5 ± 8.9	38.5 ± 9.6	47.6 ± 10.4
T stage				
T1	N/A	2	N/A	22
T2	N/A	3	N/A	46
T3	N/A	1	N/A	90
T4	N/A	0	N/A	34
N stage				
N0	N/A	0	N/A	10
N1	N/A	4	N/A	64
N2	N/A	2	N/A	68
N3	N/A	0	N/A	50
M stage				
M0	N/A	6	N/A	181
M1	N/A	0	N/A	11
TNM stage				
I-II	N/A	4	N/A	33
III-IV	N/A	2	N/A	159
EBV DNA				
Positive	0	3	10	156
Negative	4	3	144	36

NPC, nasopharyngeal carcinoma; NC, healthy control

in R was employed to analyze differential methylation levels (beta value, i.e. ratio of the methylated probe intensity and the overall intensity) between cancerous and normal groups with unpaired t test. This analysis aimed to identify regions exhibiting methylation-specific differences, specifically regions with hypermethylation in cancer tissues and hypomethylation in normal tissues. Additionally, regions with hypomethylation in the leukocyte genome were identified to exclude background interference, thereby ensuring the specificity of VILL (Vilin Like) methylation differentials. After analyzing the methylation levels, the distribution of CpG sites was combined to obtain two regions of the VILL gene for which probe primers could be designed.

Whole genome methylation sequencing analysis

10 ml whole blood was collected from the subjects and centrifuged to obtain 4–5 ml plasma, which was then sent to Shenzhen Ace Gene Technology Co., LTD for whole genome methylation sequencing analysis according to the standard process. The standard process is as follows: (1) cfDNA extraction and quality control, (2) end repair, (3) methylation linker ligation, (4) bisulfite processing, (5) PCR amplification, (6) library purification, (7) next generation sequencing (Illumina HiSeq4000, sequencing depth 30X). The sequencing results were uploaded to the national genetic resources database GSA (HRA000621). Bismark (v0.23.0) software was used to perform methylation numerical analysis according to default parameters, and the reference genome was hg19. Unpaired t test was used to analyze differential methylation levels (beta value) between cancerous and normal groups with R.

Plasma cfDNA extraction and transformation

We collected 5–10 ml of whole blood from the subjects in cfDNA preservation tubes (Ardent BioMed, GSP2601-10). Blood was centrifuged at 3000 g and 4 °C for 10 min to extract plasma into centrifuge tubes, and further centrifuged at 16000 g and 4 °C for 10 min to extract the liquid supernatant into new centrifuge tubes. The samples were then stored at − 80 °C or directly subjected to cfDNA extraction.

50ul of cfDNA was extracted from 1000ul of plasma with the use of a magnetic bead-based cfDNA extraction kit (Megi, IVD5435-10) according to the manufacturer's instructions (Additional file 1: Figure S2). The quality of plasma cfDNA was assessed using the Agilent 4200 Bioanalyzer (Additional file 1: Figure S3). Samples exhibiting degradation or genomic DNA contamination were excluded from further analysis. A no template control (NTC) was included in each run to evaluate potential contamination during the testing process. The concentration of cfDNA was quantified using the Qubit 4.0 (Thermo Fisher Scientific, Shanghai, China), and only samples with cfDNA concentrations ranging from 2 to 100 ng/ml were retained for subsequent analysis.

The cfDNA extract was split into two fractions. One fraction was used for EBV detection; the other was further transformed with a bisulfite conversion kit (in 25ul volume) (Simplite Bio, JSM530-50) (Additional file 1: Figure S4). During the bisulfite conversion of cfDNA, both the NTC and a positive control were processed in parallel with the samples in each run.

Detection of VILL methylation

We detected VILL methylation in plasma cfDNA based on TaqMan probe qPCR, and designed primer probes (Additional file 2: Table S1). ACTB was used as an internal control. The 25ul reaction system was comprised of 12.5ul of qPCR premix (CW3332ZXQ), 2.5ul of primer and probe, 5ul of DNA template, and 5ul of ddH_2O. The positive control was ssDNA extracted and transformed from NPC cell line CNE2. The negative control was ssDNA extracted and transformed from healthy human leukocytes. The reaction conditions were as follows: predenaturation at 95 ℃ for 30 s; 60 cycles at 95 ℃ for 15 s, and fluorescence signal collection at 60 ℃ for 15 s. The qPCR instrument was a Yarui MA-6000. We defined a CT value of VILL less than 50 with a clear S-type amplification curve as being positive for methylation. All valid samples must meet the quality control criterion of $CT_{ACTB} \leq 36$. Positive controls, negative controls, and NTC were processed concurrently with the samples in each experimental run. Figure S5 (Additional file 1) presents representative amplification curves for VILL and ACTB in a randomly selected case, as well as in the positive control, negative control, and NTC.

EBV DNA testing

EBV DNA was detected in plasma cfDNA. We used the qPCR kit Z-OD-0023–02 from Liferiver Bio, Shanghai, which uses a TaqMan probe method for detection. The reaction conditions were as follows: predenaturation at 95 ℃ for 2 min; 45 cycles at 95 ℃ for 15 s, and fluorescence signal collection at 60 ℃ for 30 s. Analysis was performed using a Yarui MA-6000 qPCR instrument. According to the kit guidelines, samples were considered EBV-positive if they had a CT value below 38 and showed a clear S-type amplification curve in the FAM lane. Samples that did not meet these criteria were classified as EBV-negative. Positive controls and negative controls were processed concurrently with the samples in each experimental run. The test results of negative control product should be CT_{FAM} undetermined and $CT_{VIC} < 38$ with obvious S-type amplification curve. The test results

of positive control products should be $CT_{FAM} \leq 35$ with obvious S-type amplification curve. Otherwise, the experiment was deemed invalid. Representative amplification curves of EBV and internal control (EBV mimic) in four randomly selected case, positive control and negative control are shown in Figure S6 (Additional file 1).

Statistical analysis

Diagnostic accuracy of the VILL and EBV systems were assessed with the use of sensitivity, specificity, and accuracy rate with 95% confidence intervals (CI). ROC curve analysis was used to evaluate the performance of the VILL, EBV, and VILL + EBV detection systems. The comparison of ROC curves for either VILL or VILL + EBV versus EBV were performed with DeLong's test. Pearson's chi-square test or Fisher's exact test was used to evaluate the statistical difference in diagnostic accuracy between VILL methylation and EBV DNA methods in different groups and the correlation between the test results and demographic and clinical characteristics. Independent sample t-test was used to compare VILL methylation or EBV DNA status in different study groups (NPC and NC), both at the stage of marker selection and validation. Independent sample t-test was also used to compare age in different groups of VILL methylation levels (or EBV DNA status). All hypothesis tests were two-sided, and P values of less than 0.05 were considered to indicate statistical significance. All statistical analyses and data visualization were performed using R software, version 4.3.1 (The R Foundation; https://www.r-project.org/).

Result

The MHB5 region of the VILL gene is specifically methylated in NPC (tissue and plasma)

As shown in Fig. 1, first, we extracted cfDNA from the blood of 6 NPC patients and 4 NC for genome-wide methylation sequencing. Meanwhile, the methylation microarray data of 6 NPC and 6 NP tissues (GSE52068) were downloaded from the GEO database. A total of 4,429 differentially methylated CpG sites were identified by intersecting the differential sites from the two datasets. Further, 277 CpG sites (Additional file 2: Table S2) were selected to meet the following criteria: 1) In the two sets of data, the average methylation beta value of NPC—the average methylation beta value of

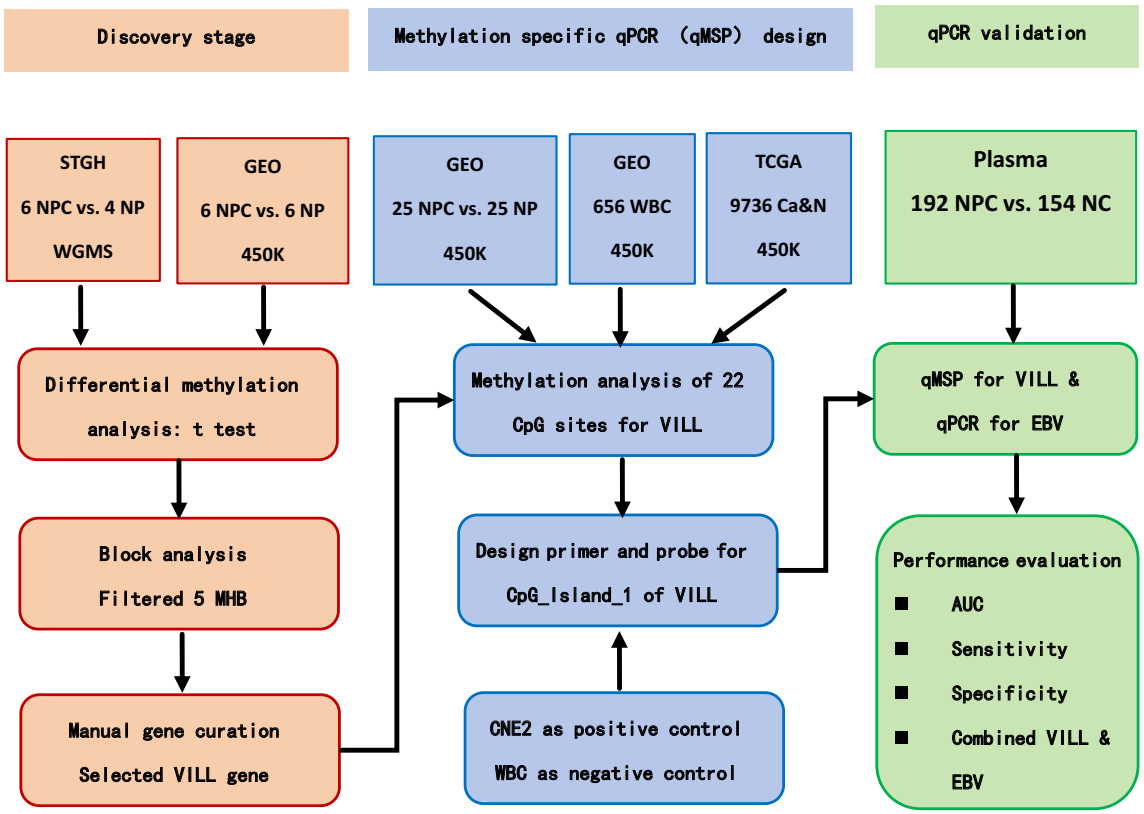

Fig. 1 Overall flow chart of the study. STGH, Southern Theater General Hospital; WGMS, whole genome methylation sequencing; 450 K, Illumina 450 K methylation microarray; MHB, methylation haplotype block; NPC, nasopharyngeal carcinoma; NP, nasopharyngeal tissue; NC, healthy control; WBC, white blood cells; CNE2, NPC cell line; qMSP, Methylation specific qPCR

NP > 0.2; 2) No signal was detected in NC in cfDNA data. We screened five methylation haplotype blocks (MHB, that is, at least three CpG sites within 100 bp of genomic DNA meet the above screening criteria [8], Additional file 2: Table S3) out of 277 CpG sites. Of these, only the MHB5 region (Additional file 2: Table S3) fell on the cell type-specific CpG island associated with the promoter of the candidate tumor suppressor gene VILL (Fig. 2). As shown in Fig. 2, the MHB5 region has a significant H3K27AC signal, binds to the transcription factor Txn, and the sequence is highly conserved. VILL localizes to chromosome 3p22 and its loss is an early event in NPC [1]. Therefore, MHB5 methylation in VILL may be an early diagnostic marker for NPC.

Primers and probes for methylation-specific qPCR (qMSP) were designed based on the MHB5 region of VILL

Since the length of MHB5 is only 60 bp, therefore, we had to design primers and probes upstream and downstream of MHB5. To this end, we comprehensively examined 22 CpG sites located in the VILL gene in the Illumina 450 K methylation microarray, including cg11431957 and cg04660410 fell on MHB5) in 25 pairs of NPC and paracancerous tissues (NP), 656 healthy human white blood cells (WBC) in GEO database, and 9736 pan-cancer tissues in TCGA database (Fig. 3).

As shown in Fig. 3A, two CpG islands are present for the VILL gene. In leukocytes from healthy individuals, these islands are significantly hypomethylated ($P < 0.0001$, Additional file 2: Table S4). MHB5 falls exactly on CpG island 1. As shown in Fig. 3B, two CpG islands were significantly hypomethylated in NP tissues ($P < 0.0001$, Additional file 2: Table S4); CpG island 1 was significantly hypermethylated in NPC compared to NP tissues ($P < 0.0001$, Additional file 2: Table S4). As shown in Fig. 3C, two CpG islands were significantly hypomethylated in pan-cancer tissues ($P < 0.0001$, Additional file 2: Table S4); however, CpG island 1 was found to be hypermethylated in some cancer tissues (e.g., colorectal cancer, bladder cancer and low-grade glioma) ($P < 0.0001$, Additional file 2: Table S4). Thus, we designed qMSP primers and TaqMan probes in the vicinity of CpG island 1, MHB5, using beacon designer (V8.14) software (Fig. 3D, Additional file 2: Table S1) with the ACTB gene as the internal control. Primers and probes were designed to contain as many CpG sites as possible [8].

Using our qMSP system, we found that VILL methylation was strongly positive in the genomic DNA of NPC cell line CNE2, but negative in the genomic DNA of healthy human WBC. Therefore, we used them as positive and negative controls.

VILL qMSP and EBV DNA qPCR were validated in clinical samples

We performed VILL qMSP detection in plasma cfDNA of 192 primary NPCs and 154 NCs, and compared it with plasma EBV DNA qPCR (Figs. 4 and 5, Additional file 1: Figure S7, Additional file 2: Tables S5 and S6). As shown in Table S5 and Fig. 4, the sensitivity of VILL and EBV for primary NPC was 80.2% vs. 81.2% ($P = 0.90$), the specificity was 100% vs. 93.5% ($P = 0.002$), and the accuracy was 89.0% vs. 86.7% ($P = 0.42$). For early stage NPC (clinical stage I and II), the sensitivity

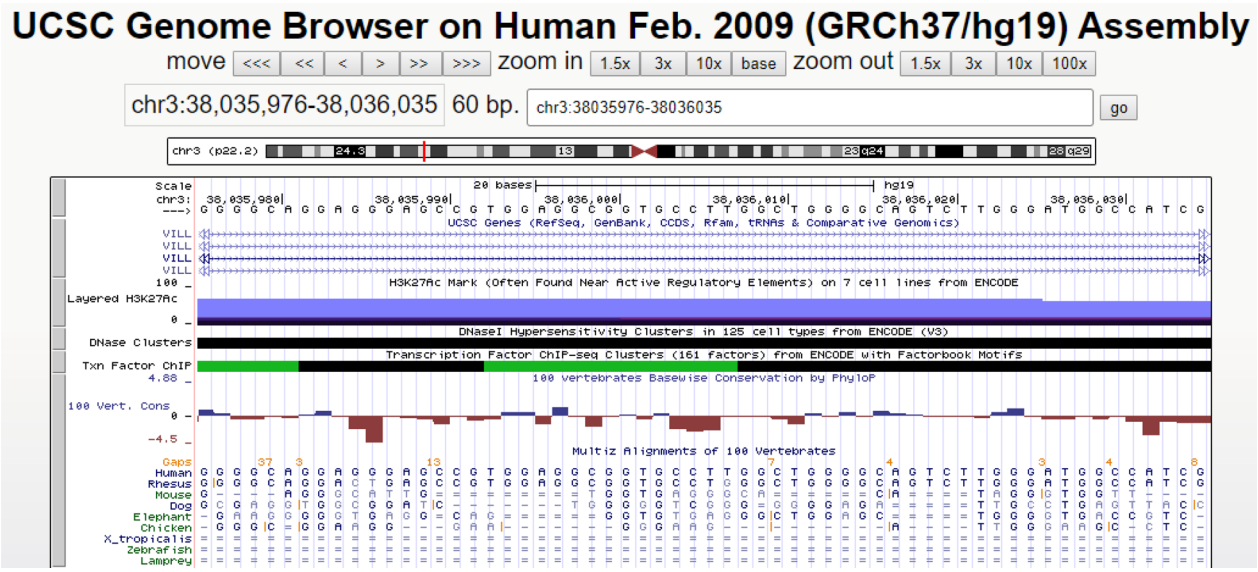

Fig. 2 UCSC Genome Browser mapping shows the genomic features of the MHB5 region

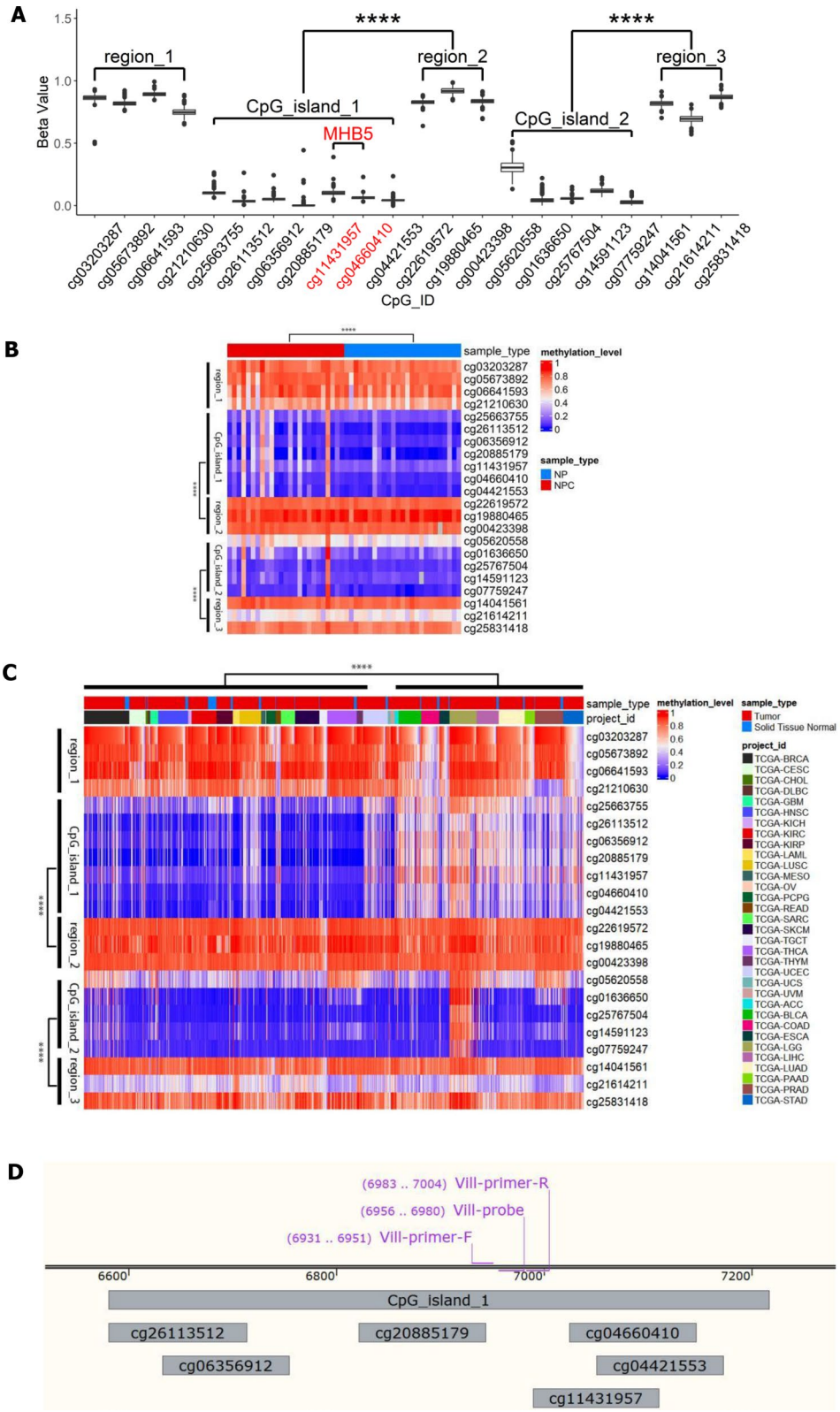

Fig. 3 Methylation profiles of the VILL gene in different tissues. A: White blood cells from 656 healthy individuals; B: 25 pairs of NPC and NP tissues; C: 9736 pan-cancerous tissues; D: Schematic design of primers and probes. T test was used to analyze statistical significance (****: $P < 0.0001$)

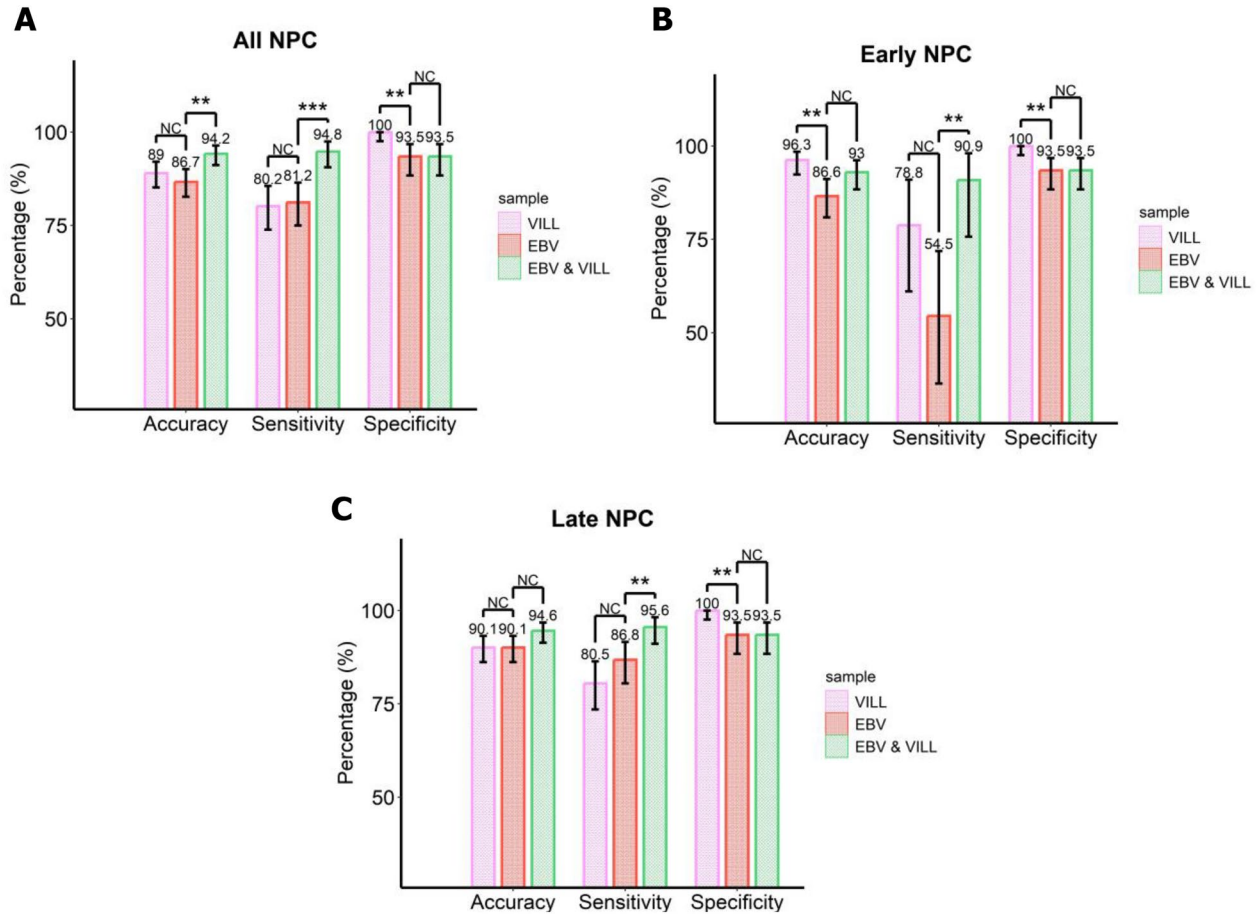

Fig. 4 Comparison of the diagnostic performance of VILL methylation and EBV DNA. A: All patients with nasopharyngeal carcinoma (NPC); B: Patients with early-stage nasopharyngeal carcinoma; C: Patients with advanced nasopharyngeal carcinoma. Chi-square test or fisher's exact test was used to analyze statistical significance (**: $P<0.01$; ***: $P<0.001$; NC: no significance)

for VILL versus EBV was 78.8% vs. 54.5% ($P=0.07$), and the accuracy was 96.3% vs. 86.6% ($P=0.0017$). For advanced NPC (clinical stage III and IV), the sensitivity for VILL versus EBV was 80.5% vs. 86.8% ($P=0.17$), and the accuracy was 90.1% vs. 90.1% ($P=1$). As shown in Table S6, VILL methylation was not correlated with age, gender and TNM stage of NPC. However, EBV DNA was significantly correlated with gender, T stage, N stage, and overall stage of NPC. As shown in Fig. 5, the AUC values of VILL vs. EBV in the diagnosis of NPC, early NPC and advanced NPC were 0.901 vs. 0.874 ($P=0.21$), 0.894 vs. 0.74 ($P=0.009$) and 0.903 vs. 0.901 ($P=0.96$), respectively. Therefore, compared with EBV DNA, VILL methylation shows better diagnostic performance for early stage NPC.

Then, we combined EBV DNA with VILL methylation. As shown in Tables S5 and S7, the combination of the two detection methods further increased the sensitivity to 94.8% ($P=8.5e-05$). Among them, the sensitivity of early NPC was increased to 90.9% ($P=0.0024$). As shown in Fig. 5, the AUC values of the combination of the two in the diagnosis of NPC, early NPC and advanced NPC were 0.968 ($P=3.3e-10$), 0.948 ($P=2.7e-06$) and 0.972 ($P=5.4e-07$), respectively. Therefore, VILL methylation combined with EBV DNA for non-invasive diagnosis of NPC is significantly better than EBV DNA detection alone, especially for early diagnosis.

Discussion

NPC diagnostic studies based on plasma cfDNA methylation have been explored for a significant period of time, and many tumor suppressor genes with diagnostic potential have been found, such as DAPK1 [9], RIZ1 [10], RASSF1A [12], SLIT2 [13] and EBV DNA methylation [14]. Furthermore, people have also used gene combinations for NPC diagnostic studies, such as: five gene combinations (CDH1, DAPK1, p15, p16, RASSF1A and MLH1) [11], four gene combinations (RASSF1A, WIF1, DAPK1 and RARb2) [5], and two gene combinations (RERG and ZNF671) [15], All of

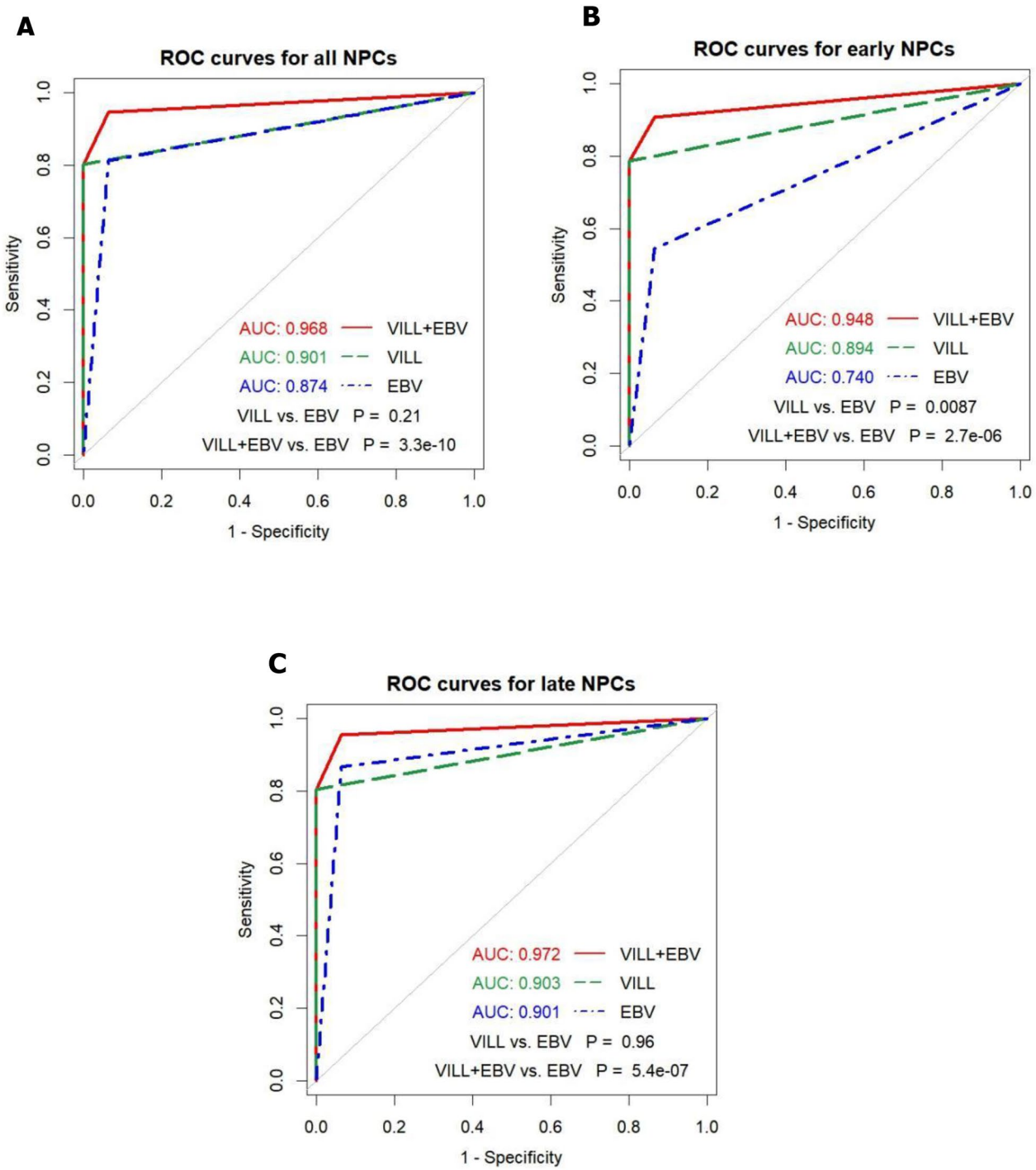

Fig. 5 Diagnostic performance of VILL methylation and EBV DNA was analyzed by ROC curve. A: All patients with nasopharyngeal carcinoma (NPC); B: Patients with early-stage nasopharyngeal carcinoma; C: Patients with advanced nasopharyngeal carcinoma. DeLong's test was used to analyze statistical significance

them also showed good diagnostic potential. However, these research results have not been further promoted in clinical practice, because they cannot effectively replace or supplement the existing detection methods based on plasma EBV DNA [16] or antibody [17].

Previous studies have screened differentially-methylated genes in tissues and then validated them

in plasma. Since the DNA present in tissues is genomic DNA, the DNA present in plasma is cfDNA (only about 167 bp in length and usually fragmented), which is formed by fragmentation through complex pathophysiological processes. As a result, hypermethylated genes in cfDNA is not as easily amplified as the same genes in genomic DNA found in tissues. Therefore, we performed genome-wide methylation sequencing directly on plasma cfDNA to screen for NPC-specific methylated cfDNA fragments, and then integrated and analyzed the results of tissue analysis for validation. Through this process, we screened the only candidate gene VILL. This gene has not been previously reported.

The VILL gene encodes a vilin-like protein that localizes to the early-event (−3p21-22) region of NPC [1]. By immunohistochemistry of 136 NPC and 67 NP tissues, we showed that VILL was highly expressed in 100% of normal nasopharyngeal mucosa, while it was down-regulated in 100% of NPC tissues. Furthermore, we demonstrated that VILL significantly inhibited the proliferation and stemness of NPC cells in vitro and in vivo, while silencing VILL significantly promoted the proliferation of immortalized NP69 cells. These studies suggest that VILL is an early key driver in NPC (unpublished data).

In general, we designed qPCR primers and probes to amplify the region covering the detection site. However, the research of Professor Zhang Kang's group in 2017 subverted our understanding [8]. This is the first study to show methylation linkage disequilibrium. That is, if one CpG site is functionally methylated, then its neighboring CpG sites will also be methylated. They identified 147,888 methylation-linked regions (MHBs) in tumor tissue, with an average length of 95 bp and each containing at least three CpG sites.

This study greatly liberated our design thinking of primers and probes. In the past, we designed primers and probes that had to cover the detection site. However, since qPCR has very strict requirements for the sequence of primers and probes, we often failed to design sufficiently-specific primers and probes even though the sequence of the target gene locus was known. Now, we simply ensure that our detection region is on the same CpG island as the target site and that it is not more than 100 bp away from the target site. This greatly improves the chances of designing sufficiently-specific primers and probes.

The sensitivity of our current test is only 80%, which is far less than the 100% expected with immunohistochemistry. In particular, our sensitivity for detecting early-stage NPC was comparable to that for late-stage NPC, indicating that the decreased sensitivity was not related to the cfDNA load released by tumor cells in the plasma. Because we transformed cfDNA with bisulfite at high temperature for an extended period of time, it has been shown that this process causes cfDNA to break down and become undetectable [18]. Therefore, in the future, new DNA transformation technologies or bisulfite-free methods may be used to reduce cfDNA degradation and thereby improve detection rates [15, 18]. Alternatively, we can extract genomic DNA from exfoliated nasopharyngeal cells via NP swab samples for testing. Recent studies have shown that this approach has a high application prospect [19].

This study is subject to several limitations. Firstly, the limited number of early stage NPC cases in the validated cohort may lead to an overestimation of the method's performance in early detection. Secondly, the collection of all NPC patient samples exclusively from the General Hospital of Southern Theater Command in Guangzhou may constrain the external validation and generalizability of our findings. Lastly, the extended assay times and the intricate chemical reaction required for bisulfite conversion of cfDNA pose challenges to the implementation of this approach in routine clinical screening.

In conclusion, this study shows for the first time that the VILL gene has a high sensitivity (80%) and specificity (100%) for the detection of NPC. The sensitivity can be further improved by combining VILL with EBV DNA status detection. In particular, the sensitivity of early NPC detection with combined testing can be improved from 54.5 to 90.9% when compared to EBV DNA alone. Therefore, we envision the application scenario of VILL combined with EBV DNA detection for auxiliary diagnosis in clinical suspected NPC patients with abnormal findings by MRI [20].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01847-7.

Additional file1

Additional file2

Acknowledgements
Not applicable.

Author contributions
Dr X-Y. F. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr X-Y. F. is the sole first author. Concept and design:Z-X. H. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: X-Y. F. and Y. Y. Critical review of the manuscript for important intellectual content: All authors. Statistical analysis:X-Y. F. Administrative, technical, or material support:D-B. L., Y-B. Z., F. Y. and Z-X. H. Supervision: Zhong-Xi Huang, Fei Ye and Yi-Bo Zhou.

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Availability of data and material
All data in this report are available by request from the corresponding or first author.

Declarations

Ethics approval and consent to participate
This study was reviewed and approved by the Scientific Research Ethics Committee of the General Hospital of Southern Theater Command of the People's Liberation Army under the ethics number NZLLKZ2024120.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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