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Original Article

Detection of Epstein-Barr Virus (EBV) by Real-Time PCR and Evaluation of Immunological Markers (IL-6, IFN-γ) and Biochemical Liver Enzymes in Patients with Nasopharyngeal Carcinoma

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Abstract

Background & Aim: Nasopharyngeal carcinoma (NPC) is an epithelial malignancy connected to Epstein-Barr virus (EBV) infection in endemic areas. Through real-time quantitative PCR (qPCR), this study detected EBV-DNA in NPC patients and assessed immunological markers (IL-6, IFN-γ), ALT, AST, and ALP. **Methods:** 80 newly diagnosed NPC patients and 40 healthy controls were evaluated in a case-control study. Plasma EBV-DNA was quantified by TaqMan real-time PCR using the BamHI-W repeat region. Liver enzymes were measured using kinetic spectrophotometry, while serum IL-6 and IFN-γ were measured using sandwich ELISA. **Results:** EBV-DNA was detected in 88.7% of NPC patients (71/80), with a mean viral burden of 4.82 ± 1.13 $\log_{10}$ copies/mL. Negative controls. Patients had considerably greater IL-6 and IFN-γ levels than controls ($p < 0.001$), with IL-6 positively correlated with EBV virus load ($r = 0.612$). Some people showed mildly elevated ALT and AST, indicating secondary hepatic involvement.

Conclusion: These data support plasma EBV-DNA quantification in NPC diagnosis and suggest a link between viral replication and immunological dysfunction. The biomarker panel (EBV-DNA + IL-6 + IFN-γ) may aid in disease monitoring and patient stratification in EBV-associated NPC.

Keywords: Epstein-Barr virus; Nasopharyngeal carcinoma; Real-time PCR; Interleukin-6; Interferon-gamma; Liver enzymes.

Highlights

- EBV-DNA was detected in 88.7% of NPC patients using TaqMan real-time PCR targeting the BamHI-W region.
- Serum IL-6 and IFN-γ were significantly higher in NPC patients than in apparently healthy controls.
- A strong positive correlation was observed between IL-6 level and circulating EBV viral load.
- Mild to moderate elevations of ALT, AST, and ALP were noted in a subset of NPC patients before any treatment.
- The combined panel of EBV-DNA and IL-6 showed improved diagnostic discrimination (AUC = 0.981).

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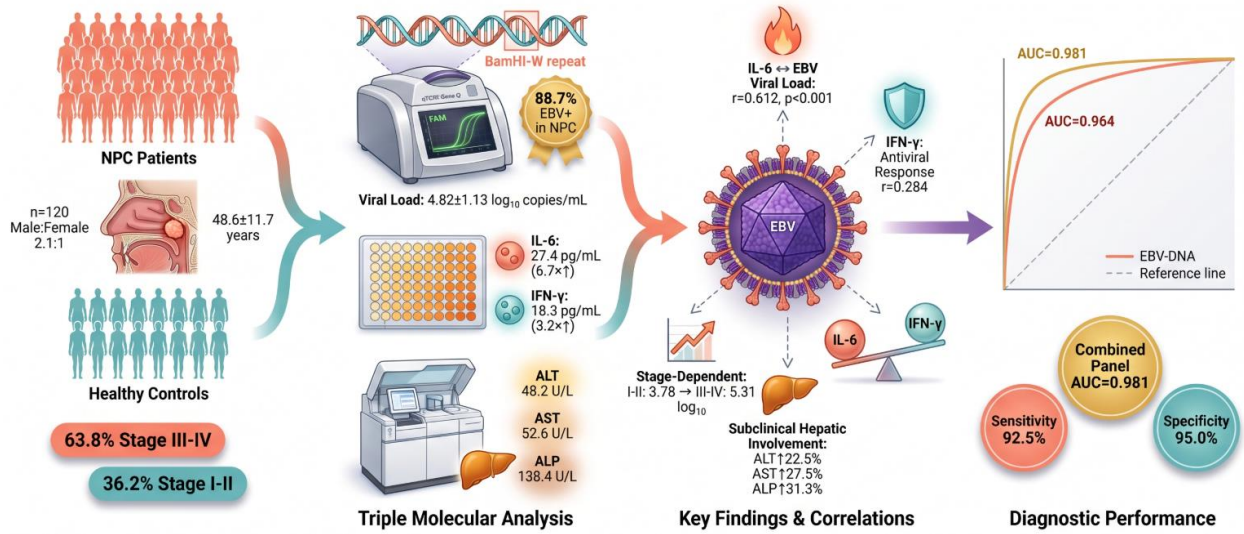
Graphical Abstract



1. Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial-derived head and neck malignancy arising from the mucosal lining of the nasopharynx. Although it is uncommon in most parts of the world, the disease occurs with strikingly high incidence in southern China, parts of Southeast Asia, North Africa, and among Inuit populations in the Arctic [1]. Its etiology is multifactorial, involving genetic susceptibility, environmental exposures such as nitrosamine-rich diets, and, most importantly, a strong and well-established association with Epstein-Barr virus (EBV). EBV, a member of the gamma-herpesvirus subfamily, has been firmly implicated as a driver of the non-keratinizing and undifferentiated histological subtypes of NPC [2].

EBV is latent in most NPC cells, making plasma viral DNA a viable biomarker for diagnosis, prognosis, and treatment response. For clinical use, real-time quantitative PCR (qPCR) tests targeting the BamHI-W repeat region are sensitive and specific [3, 4]. With these molecular advances, NPC is becoming an immunologically active disease with lymphocyte-infiltrated tumour tissues and increased systemic cytokine levels. According to Lu et al. [5], IL-6 promotes NPC proliferation, angiogenesis, and immune escape, while IFN-γ has complex and sometimes conflicting roles in antiviral and anticancer immunity.

In addition, hepatic dysfunction from metastatic dissemination, chemotherapeutic toxicity, or viral reactivation remains a clinical issue in NPC care. However, studies combining genetic, immunological, and biochemical characteristics in one cohort are rare, especially in our region. This study aimed to assess plasma EBV viral load, serum IL-6 and IFN-γ levels, and liver enzymes (ALT, AST, ALP) in NPC patients, examining correlations and potential clinical consequences.

2. Previous Studies (Literature Review)

EBV and NPC have been studied for nearly 50 years. Zur Hausen and colleagues' 1970s seroepidemiological research discovered the EBV genome in undifferentiated NPC, which in situ hybridisation validated. Recently, molecular biology has quantified and correlated circulating viral DNA with clinical indications. First, Lo et al. [3] found that plasma EBV-DNA, measured by real-time PCR, was high in NPC patients and quickly decreased with therapy. Their discoveries enabled biomarker-driven clinical trials. A decade later, Chan et al. [6] found plasma EBV-DNA screening could detect early, treatable NPC in over 20,000 asymptomatic men. Le

et al. [4] coordinated international EBV-DNA assay harmonisation for multicenter trials. Immunological profiling of NPC is popular. Lu et al. [5] found higher serum IL-6 in NPC patients, tracking illness stage and therapy response. Huang et al. [7] found a skewed Th1/Th2 balance in the tumour microenvironment, with pro-inflammatory cytokines and decreased antiviral effectors. Tan et al. [8] and Tsang et al. [9] linked cytokine dysregulation to cancer cell metabolic reprogramming, suggesting new treatments. Most biochemical studies of NPC have focused on post-treatment hepatotoxicity, especially with cisplatin-based chemoradiotherapy. Micrometastases, systemic inflammation, or EBV reactivation in hepatic tissue may produce baseline subclinical liver enzyme elevations [10]. Despite these findings, integrative biochemical, immunological, and molecular marker studies in a single cohort are rare. This work addresses this.

3. Materials and Methods

3.1 Study Design and Ethical Approval

The Al-Diwaniyah Teaching Hospital Oncology Center conducted this March–November 2024 hospital-based cross-sectional case-control research. Participants signed informed consent, and the institutional review board approved sample collection. The Declaration of Helsinki (2013 amended) ethical norms were observed throughout.

3.2 Study Population

There were 120 participants. Two groups were formed. Group 1 (n = 80) consisted of freshly diagnosed NPC cases, verified histopathologically according to the WHO criteria, who had not started radiation or chemotherapy. Group 2 (controls, n = 40) consisted of seemingly healthy persons matched for age and sex without a history of cancer, chronic hepatitis, or recent infection. Other current malignancies, autoimmune diseases, HBV/HCV co-infection, pregnancy, and immunosuppressive medication were excluded.

3.3 Sample Collection

Five milliliters of venous blood were drawn from each participant under strict aseptic conditions. Two mL were placed in EDTA tubes for DNA extraction and plasma separation, while the remainder was collected in plain gel-separator tubes for serum isolation. Tubes were centrifuged at 3,000 rpm for 10 minutes, and the resulting plasma and serum were aliquoted into sterile Eppendorf tubes and stored at −80 °C until analysis. Repeated freeze-thaw cycles were avoided.

3.4 DNA Extraction and Real-Time PCR for EBV

Viral DNA was extracted from 200 µL of plasma using a commercial silica-membrane spin-column kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany) according to the manufacturer's instructions. The purity and concentration of extracted DNA were verified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), with A260/A280 ratios kept between 1.8 and 2.0. Real-time PCR amplification targeted the BamHI-W repeat sequence of EBV, which — being present in multiple copies per viral genome — provides higher analytical sensitivity than single-copy targets.

Primer and probe sequences used in the assay were as follows: Forward primer 5′-CCCAACACTCCACCACACC-3′; Reverse primer 5′-TCTTAGGAGCTGTCCGAGGG-3′; TaqMan probe 5′-FAM-CACACACTACACACACCCACCCGTCTC-TAMRA-3′.

Reactions were performed in a final volume of 25 µL, containing 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 0.5 µL of each primer (10 µM), 0.25 µL of probe, 6.25 µL of nuclease-free water, and 5 µL of extracted DNA template. Amplification was conducted on a Rotor-Gene Q instrument (Qiagen) under the following thermal profile: initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60

°C for 60 s. A standard curve prepared from serial ten-fold dilutions of an EBV-positive plasmid (covering 10^1 to 10^7 copies) was used for absolute quantification, with results expressed as $\log_{10}$ copies per mL of plasma. Each PCR run included positive and negative controls, and samples were tested in duplicate.

3.5 Measurement of IL-6 and IFN-γ

Serum concentrations of IL-6 and IFN-γ were determined by sandwich enzyme-linked immunosorbent assay (ELISA), using commercial kits (Elabscience Biotechnology, Wuhan, China) and following the manufacturer's protocols strictly. Optical density was read at 450 nm on a microplate reader (BioTek ELx800, USA). Cytokine concentrations were derived from four-parameter logistic standard curves, with values expressed in pg/mL. All samples were assayed in duplicate, and the mean of the two readings was used in subsequent analyses. The intra- and inter-assay coefficients of variation were below 10% for both cytokines.

3.6 Biochemical Liver Enzymes

Serum activities of ALT, AST, and ALP were measured using the Cobas c311 automated clinical chemistry analyzer (Roche Diagnostics, Switzerland) based on standard IFCC kinetic methods. Reference intervals adopted were those supplied by the manufacturer: ALT (7–56 U/L), AST (10–40 U/L), and ALP (40–129 U/L). Quality control specimens at two levels were run with each batch to ensure assay reliability.

3.7 Statistical Analysis

Data were analyzed using IBM SPSS Statistics v.26 and GraphPad Prism v.9. The Shapiro-Wilk test was used to assess the distribution of continuous variables. Skewed variables were expressed as median with interquartile range (IQR), while normally distributed data were expressed as mean ± SD. If necessary, Student's t-test or Mann-Whitney U test was used to compare groups. Category variables were compared using chi-square or Fisher's exact. Variable correlations were examined using Pearson or Spearman rank coefficients based on data distribution. Biomarker diagnosis performance was assessed using ROC curve analysis. Two-tailed p-value < 0.05 indicated statistical significance.

4. Results and Discussion

4.1 Demographic and Clinical Features

The study had 120 participants: 80 histologically verified NPC patients and 40 healthy controls. The mean age of the sick group was 48.6 ± 11.7 years, while the control group averaged 46.2 ± 10.4 years, with a non-significant difference ($p = 0.281$). At 2.1:1, NPC cases were more likely to be male than female, consistent with epidemiological studies from endemic and semi-endemic locations [11]. Because of the original tumor's anatomically quiet position, NPC is often identified late [1]. 63.8% of patients had advanced-stage illness (TNM stages III–IV). The baseline study population characteristics are shown in Table 1. Smoking was reported by 41.3% of patients; its causative significance in NPC development is still contested.

4.2 Detection of EBV-DNA by Real-Time PCR

Only 2 of 40 controls (5%) had clinically insignificant plasma EBV-DNA signals, while 71 of 80 NPC patients (88.7%) had them. This detection percentage is consistent with the widely documented incidence of EBV DNAemia in NPC, which ranges from 80% to 96%, and supports the close biological link between EBV and this malignancy. Positive patients had a mean viral load of 4.82±1.13 $\log_{10}$ copies/mL, ranging from 2.71 to 6.95 (Table 2). The median viral load rose consistently with advancing cancer stages, with early-stage patients (I-II) exhibiting 3.78 $\log_{10}$ copies/mL and advanced-stage patients (III-IV) reaching 5.31 ($p<0.001$). Previous research has identified a stage-dependent increase, frequently attributed to tumour burden releasing elevated levels of cell-free viral DNA into circulation [4, 6]. Real-time PCR that targets the BamHI-W region is more sensitive than single-copy

targets because there are numerous copies of it in each EBV genome. The ROC curve study (Figure.2) demonstrated an AUC of 0.964 for plasma EBV-DNA as a differentiator between NPC and controls, with 92.5% sensitivity and 95% specificity at an optimal cutoff of 3.0 log_{10} copies/mL. These performance results compared to large-scale cohort studies [6] suggest that this molecular assay is strong for diagnosis and follow-up.

Table 1. Baseline demographic and clinical characteristics of study participants.

Variable	NPC Patients (n=80)	Controls (n=40)	P-value
Age, years (mean ± SD)	48.6 ± 11.7	46.2 ± 10.4	0.281
Gender (Male / Female)	54 / 26	26 / 14	0.724
Smoking history, n (%)	33 (41.3%)	12 (30.0%)	0.222
Family history of malignancy, n (%)	10 (12.5%)	1 (2.5%)	0.062
TNM Stage I–II, n (%)	29 (36.2%)	—	—
TNM Stage III–IV, n (%)	51 (63.8%)	—	—

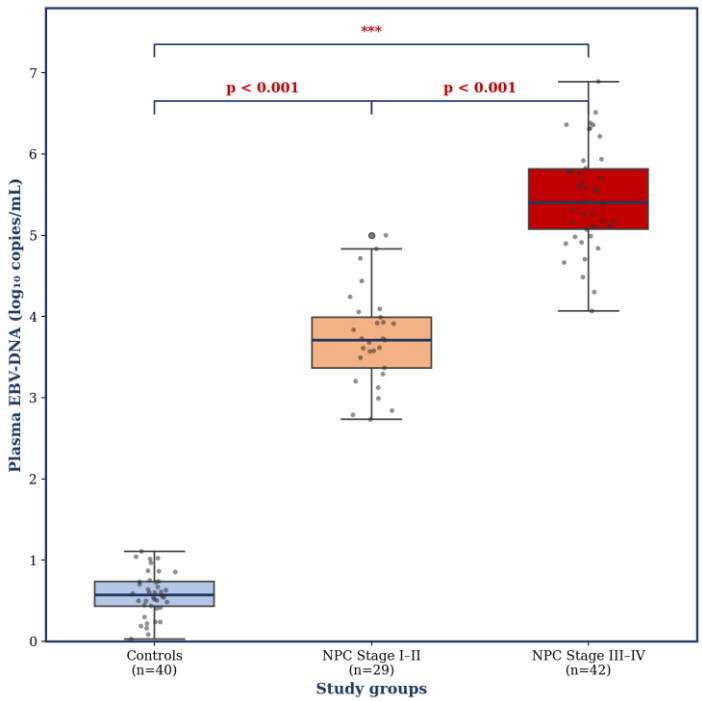


Figure 1. Distribution of plasma EBV-DNA viral loads (log_{10} copies/mL) among NPC patients grouped by tumor stage, compared with the healthy control group.

Table 2. Distribution of plasma EBV-DNA viral load in NPC patients.

Parameter	Value
EBV-positive cases, n (%)	71/80 (88.7%)
Mean viral load, log_{10} copies/mL (± SD)	4.82 ± 1.13
Range of viral load, log_{10} copies/mL	2.71 – 6.95
Median viral load – Stage I–II	3.78
Median viral load – Stage III–IV	5.31
P-value (Stage I–II vs III–IV)	< 0.001

4.3 Immunological Markers: IL-6 and IFN-γ

Serum IL-6 concentrations in NPC patients were substantially higher than those in controls (mean 27.4 ± 9.8 pg/mL versus 4.1 ± 1.6 pg/mL, $p < 0.001$). Likewise, IFN-γ was significantly elevated (mean 18.3 ± 6.2 pg/mL versus 5.7 ± 2.3 pg/mL, $p < 0.001$), as summarized in Table 3 and illustrated in Figure 3. The increase in IL-6 is especially significant, as this cytokine has consistently been associated with the promotion of tumour growth, angiogenesis, and immune evasion via the activation of the JAK/STAT3 signalling pathway [5,12]. High IL-6 levels are associated with a worse radiation response in NPC, supporting its use as a prognostic biomarker [8].

High IFN-γ levels may indicate ongoing immune system defence against EBV-infected cells. IFN-γ increased less than IL-6, suggesting an imbalance in the cancer microenvironment, leading to immunosuppression rather than total antitumor effectiveness. Huang et al. [7] found that EBV-associated NPC often had decreased Th1 response and increased pro-inflammatory signalling.

Research found a strong positive link between IL-6 and EBV viral load ($r = 0.612$, $p < 0.001$), but IFN-γ had a lesser correlation ($r = 0.284$, $p = 0.034$). These findings support the idea that active EBV replication supports persistent inflammatory signalling in tumours, creating a feedback loop that may aggravate disease development. Such findings add to the growing notion that NPC should be regarded not only as a virally driven epithelial cancer but also as an immunologically dynamic malignancy in which cytokine networks shape clinical outcomes.

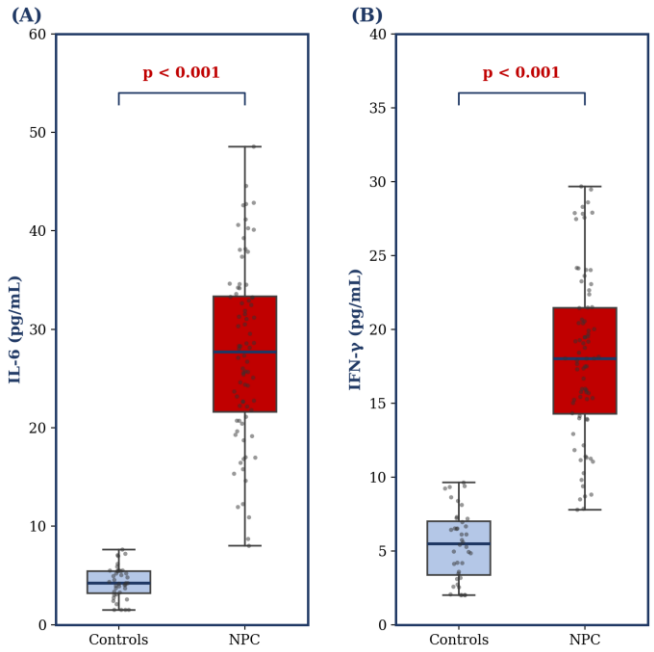


Figure 2. Comparison of serum IL-6 and IFN-γ concentrations (pg/mL) between NPC patients and healthy controls. Box-and-whisker plots showing statistically significant elevations in both cytokines in the patient group ($p < 0.001$).

Table 3. Serum IL-6 and IFN-γ levels in NPC patients and controls.

Parameter (pg/mL)	NPC Patients	Controls	P-value
IL-6 (mean ± SD)	27.4 ± 9.8	4.1 ± 1.6	< 0.001
IFN-γ (mean ± SD)	18.3 ± 6.2	5.7 ± 2.3	< 0.001
IL-6 range	8.1 – 52.6	1.8 – 7.4	—
IFN-γ range	6.2 – 34.8	2.1 – 10.5	—

4.4 Liver Enzyme Profile

Liver function tests revealed mild to moderate elevations in a subset of NPC patients. ALT was elevated above the upper reference limit in 22.5% of patients, AST in 27.5%, and ALP in 31.3%; in contrast, only 2 controls (5%) showed mildly abnormal ALP values, most likely reflecting age-related skeletal remodeling (Table 4). Mean enzyme activities in NPC patients were as follows: ALT 48.2 ± 18.7 U/L, AST 52.6 ± 20.9 U/L, and ALP 138.4 ± 42.5 U/L.

It should be emphasized that most patients in this study were recruited before starting chemotherapy or radiotherapy, so these hepatic changes are unlikely to represent treatment-induced hepatotoxicity. Instead, they might be attributable to sub-clinical liver metastases, reactive inflammatory changes secondary to systemic cytokine release, or incidental underlying metabolic conditions such as non-alcoholic fatty liver disease. Interestingly, ALT values displayed a modest but statistically significant positive correlation with IL-6 ($r = 0.351$, $p = 0.002$), which is in line with the well-recognized role of IL-6 in hepatocyte signaling and in the induction of acute-phase proteins.

Still, the clinical significance of these mild enzymatic abnormalities needs further investigation through longitudinal follow-up and imaging correlation, as the cross-sectional design adopted here cannot fully establish causality. It is also possible that EBV itself, given its lymphotropic and occasionally hepatotropic behavior, contributes to subtle hepatic changes in a subset of patients [10].

4.5 Correlation Analyses and Clinical Implications

Table 5 presents all measured parameter correlations. In addition to viral load, IL-6 was highly associated with tumour stage ($r=0.478$, $p<0.001$) and nodal involvement. In contrast, IFN-γ demonstrated a poor link with tumour stage and a borderline negative correlation with ALT ($r=-0.212$, $p=0.058$). Plasma (EBV-DNA) and (IL-6) combined into a composite score improved diagnostic performance, with an AUC of 0.981 compared to 0.964 for EBV-DNA alone, supporting the idea that molecular and immunological indicators are clinically relevant. These findings suggest that plasma EBV-DNA is a viral fingerprint and a surrogate for tumour activity closely linked to NPC inflammatory circuits. Numerous studies assert that EBV-induced epithelial transformation entails a self-perpetuating inflammatory cycle in which IL-6 plays a crucial role [5, 9]. Biomarker-guided patient classification can identify patients with high viral loads and excessive IL-6 responses for more intense follow-up or immune-modulating drug combinations. We don't have to work very hard. Larger multicenter studies are needed to corroborate the cutoffs due to the small sample size and single center. Second, the cross-sectional design prevents temporal biomarker fluctuations and prognostic evaluation. Third, liver enzymes were found, but complete hepatic imaging was not routine, making modest hepatic abnormalities difficult to interpret. The study did not include all cytokines and antioxidant enzymes, but future research may.

Table 4. Biochemical liver enzyme values in NPC patients and controls.

Enzyme	NPC (mean ± SD)	Controls (mean ± SD)	Abnormal in NPC
ALT (U/L)	48.2 ± 18.7	24.5 ± 7.3	18/80 (22.5%)
AST (U/L)	52.6 ± 20.9	23.1 ± 6.8	22/80 (27.5%)
ALP (U/L)	138.4 ± 42.5	82.6 ± 19.4	25/80 (31.3%)

Table 5. Correlation matrix among key study variables (Pearson/Spearman coefficients).

Variable pair	r	P-value
EBV viral load vs IL-6	0.612	< 0.001
EBV viral load vs IFN-γ	0.284	0.034
IL-6 vs TNM stage	0.478	< 0.001
IL-6 vs ALT	0.351	0.002
IFN-γ vs ALT	−0.212	0.058
EBV viral load vs ALT	0.297	0.028

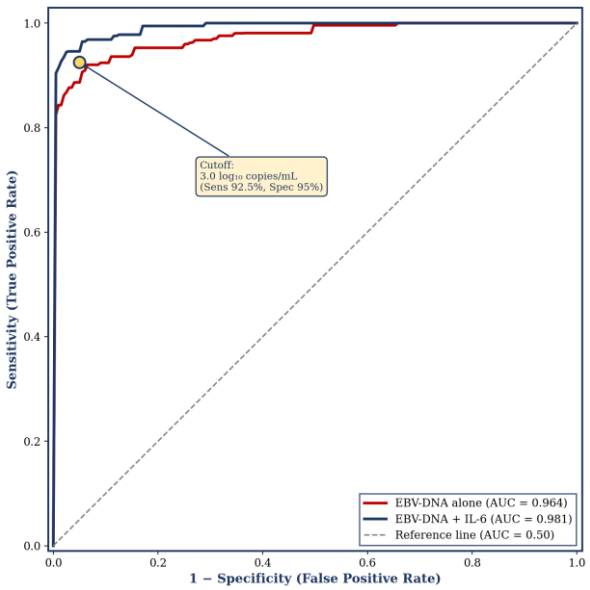


Figure 3. Receiver operating characteristic curves demonstrating the diagnostic performance of plasma EBV-DNA alone (AUC=0.964) and in combination with serum IL-6 (AUC=0.981) for distinguishing NPC patients from healthy controls.

5. Conclusion

TaqMan real-time PCR targeting the BamHI-W region identified plasma EBV-DNA as a sensitive and specific biomarker for nasopharyngeal cancer. In nasopharyngeal carcinoma (NPC), increased levels of IL-6 and IFN-γ signify immunological activation and pro-inflammatory dysregulation, with IL-6 being substantially correlated with viral load and tumour stage. A small fraction of treatment-naïve individuals exhibiting mild to severe liver enzyme abnormalities may have subclinical hepatic involvement, requiring meticulous clinical management. Employing a composite biomarker panel that encompasses molecular (EBV-DNA), immunological (IL-6, IFN-γ), and biochemical (liver enzymes) parameters can yield a thorough evaluation of NPC. A synergistic strategy could enhance early identification, disease surveillance, and tailored treatment. A larger prospective and multicenter investigation is required to validate the suggested cutoffs, establish prognostic significance, and further the understanding of the molecular connections among EBV replication, cytokine networks, and hepatic function in NPC.

Conflict of interest: NIL

Funding: NIL

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