in the present studies; the differences are not statistically significant.

The major advantage of this method is its simplicity because only two PCR reactions are used to genotype four RSPs. This procedure replaces the three pairs of primers used by Cambien et al. *(3)* to amplify and determine the same polymorphisms (codons 10 and 25 are also amplified in the same PCR reaction) and the design and temperature optimization of eight ASOs. It also reduces the processing time per sample because the ASO method not only needs PCR amplification but also needs 6 h of hybridization, without taking into account the time for autoradiography. Digestion with restriction enzymes and polyacrylamide electrophoresis can be performed in 5 h. The present restriction enzyme method also avoids the use of radioactivity (^{32}P-labeled oligonucleotides).

Recently, a codon 10 polymorphism has been characterized by allele-specific amplification *(5)*. Allele-specific amplification is based on two primers, each differing from the other in the terminal 3′ nucleotide, which are specific for each allelic variant. Taq polymerase will not extend if a mismatch is present at the 3′ end of the primer-template duplex. This method involves a limited effort because only one PCR reaction is necessary for each allele-specific primer. Direct visualization on agarose gels is the only postamplification processing. However, primer design and PCR conditions are critical because under some conditions, a single 3′-mismatched base permits amplification *(6)*. Purine-purine and pyrimidine-pyrimidine mismatches are more refractory to extension than purine-pyrimidine mismatches; in such cases, additional mismatches can be introduced to destabilize the primer-template duplex and to increase specificity. Magnesium, dNTPs, Taq polymerase or primer concentrations, and the precise temperature of annealing must be carefully controlled to maintain the specificity of the reaction *(7)*.

Another three polymorphisms were described by Cambien et al. *(3)* in the *TGFB1* gene. A C→A transversion was detected at position −988; this could be considered as a variant because it was present in only 2 of 1000 individuals. A "C" insertion was also reported at position +72 in the 5′-untranslated region. This polymorphism is in mutual association with a codon 25 polymorphism (as shown by the strong linkage disequilibrium coefficient of −1.0 reported); therefore, we decided to exclude it and to analyze codon 25 polymorphisms. The +72 polymorphism is more difficult to detect than the C→G transversion at codon 25, the latter being easily detected by the loss of an *Fse*I site. This allows us to use only one pair of primers to amplify codons 25 and 10 in one PCR reaction. Another variant was reported in codon 263. In this case, a C→T transition produces a Thr^{263}→Ile substitution, the Thr allele being much more frequent, with only 6.7% of the subjects analyzed being heterozygous for this polymorphism. To our knowledge, there is no available enzyme capable of recognizing this substitution; therefore, the use of allele-specific primers or the design of a modified primer introducing an artificial restriction site *(8)* could be considered.

The genotyping system described here is simple, reliable, and relatively inexpensive when a large sample set needs to be genotyped, and it could be performed in laboratories without facilities for radioactivity. In the future, clinical studies on the *TGFB1* polymorphisms could confirm suspicions of the crucial role of TGF-β_1 and genetic predisposition to several fibrotic diseases.

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Analysis of Cell-free Epstein-Barr Virus-associated RNA in the Plasma of Patients with Nasopharyngeal Carcinoma, *Kwok-Wai Lo,[1] Y.M. Dennis Lo,[2] Sing-Fai Leung,[3] Yuen-Shan Tsang,[1] Lisa Y.S. Chan,[2] Philip J. Johnson,[3] N. Magnus Hjelm,[2] Joseph C.K. Lee,[1] and Dolly P. Huang[1*]* (Departments of [1] Anatomical and Cellular Pathology, [2] Chemical Pathology, and [3] Clinical Oncology, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region; * author for correspondence: fax 852 2637 6274, e-mail waisinhuang@cuhk.edu.hk)

Chen et al. *(1)* and Nawroz et al. *(2)* have reported that tumor-derived DNA is detectable in the plasma and serum of cancer patients and have opened up a new molecular approach for the early detection of malignancy. It is not known, however, whether circulating tumor-derived RNA is also present in plasma, because of the

lability of RNA. To address this possibility, we used nasopharyngeal carcinoma (NPC) as a model system and attempted to detect Epstein-Barr virus (EBV)-latent gene transcripts in cell-free plasma samples from NPC patients.

NPC constitutes one of the commonest cancers in Hong Kong and Southern China *(3)*. Previous studies have indicated that EBV is consistently detected in all undifferentiated NPC cases and is present in all cancer cells *(3)*. Latent EBV infection is an early event in the development of this cancer *(4)*. These findings suggested that the EBV genome and latency products may serve as potential markers for the screening and diagnosis of this cancer. Among the EBV-latent genes, the small EBV-encoded RNAs (EBERs) are expressed in all NPC cases and are the most abundant latency-associated transcripts in NPC cells ($\sim 10^5$ to 10^6 copies per cell) and are widely used for the detection of EBV-associated human tumors, using in situ hybridization *(5)*. We hypothesize that EBER RNA may also be detectable in the plasma of NPC patients.

In this study, we used reverse transcription-PCR (RT-PCR) and oligonucleotide hybridization to analyze the presence of *EBER-1* RNA in cell-free plasma samples of 26 NPC patients and 29 healthy subjects. The project was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong, and informed consent was obtained from all participants. Total RNA was extracted from 250 μL of fresh plasma sample from each individual, using a Trizol LS kit (Life Technologies) and following the manufacturer's recommendations. The RNA was then dissolved in 5 μL of RNase-free water, and 2 μL of plasma RNA was subjected to 40 cycles of RT-PCR amplification, using an EZ r*Tth* RNA PCR kit (PE Applied Biosystems). The *Tth* DNA polymerase had both reverse transcriptase and DNA polymerase activities *(6)* and thus was able to perform both reactions in a single tube. For each sample, duplicate aliquots were subjected to RT-PCR analysis. Primers specific to the *EBER-1* gene were used as described previously (sense, 5′-AAAACATGCGGACCACCAGC-3′; antisense, 5′-AGGACCTACGCTGCCCTAGA-3′) *(7)*. The PCR products were analyzed using 3% agarose gel electrophoresis, and their identities were confirmed by Southern blotting and hybridization using an *EBER-1*-specific internal probe (5′-ACGGTGTCTGTGGTTGTCTT-3′) *(7)*. A 167-bp RT-PCR product was detected in the plasma samples containing *EBER-1* RNA (Fig. 1).

Among the 26 NPC patients, *EBER-1* RNA was detected in 23 of 26 (88.5%) plasma samples. Control reactions without reverse transcriptase were negative, confirming that the products were amplified from RNA. In the three negative cases, transcripts of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), were detectable, demonstrating the integrity of RNA in these samples. Among the healthy control subjects, 6 of 29 (20.7%) plasma samples showed detectable *EBER-1* RNA. The difference in the proportion of cases in which *EBER-1* RNA was detectable in NPC and non-NPC cases was statistically significant (χ^2 test, $P < 0.001$). The detection of *EBER-1* RNA in the plasma of the healthy individuals is probably attributable to the presence of latent EBV-infected B lymphocytes in healthy carriers of the virus *(3)*.

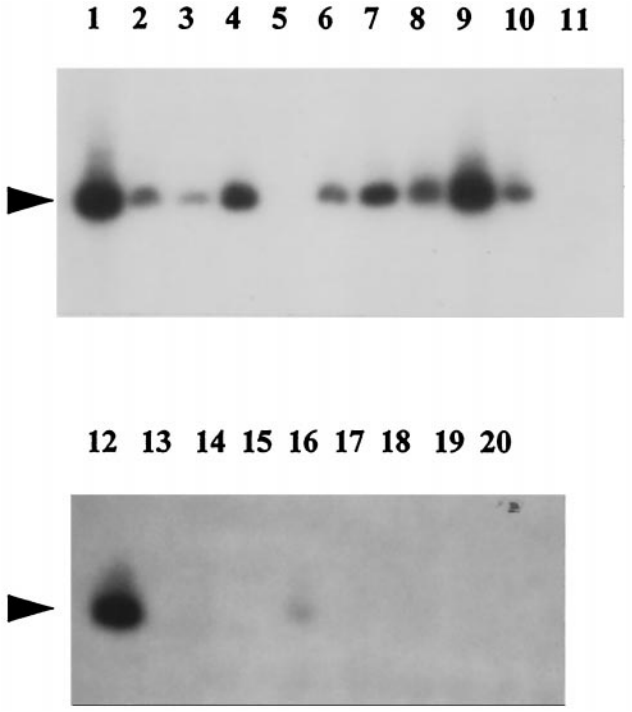

Fig. 1. Detection of *EBER-1* RNA in the plasma of NPC patients (*lanes 2–10*) and healthy controls (*lanes 13–19*) by RT-PCR.

Lanes 1 and *12*, an EBV-transformed lymphoblastoid cell line (CB14022) as a positive control; *lanes 11* and *20*, reagent control without RNA. The 167-bp *EBER-1* RT-PCR product (*arrows*) is indicated in the positive control lanes (*lanes 1* and *12*), plasma samples from NPC patients (*lanes 2–4* and *6–10*), and a non-NPC control (*lane 16*).

This study shows for the first time that cell-free tumor-related RNA can be detected in the plasma of patients with NPC. The sensitivity and specificity of the plasma RNA assay for NPC were 88.5% and 79.3%, respectively. This novel approach may be useful for early detection of NPC and other EBV-associated malignancies. The specificity of the detection method could potentially be increased by testing other NPC-related genes.

Mutirangura et al. *(8)* have evaluated the presence of cell-free EBV DNA in the serum samples of NPC patients. They reported that only 13 of 42 (31%) patients were positive for EBV DNA in their sera. Using real-time quantitative PCR, we have detected cell-free EBV DNA in the plasma of 96% of NPC patients *(9)*. Real-time quantitative PCR can potentially be applied to the detection of EBV-associated RNA in the plasma of NPC patients. This approach may potentially enhance the clinical usefulness of EBV-associated RNA detection for NPC diagnosis because of improved discrimination between NPC subjects and EBV RNA-positive subjects without NPC. This development is possible because the latter group of subjects generally has a smaller amount of plasma EBV-associated RNA (Fig. 1, lane 16, which shows a relatively weak RT-PCR signal) than those with NPC (Fig. 1, lanes 2–4 and 6–10). A further advantage of real-time PCR for

NPC diagnosis is that no post-PCR manipulation is necessary, which greatly increases throughput and reduces the risk of carryover contamination.

Our data highlight the concept that the detection of tumor-associated RNA in plasma may be a promising new direction for cancer detection. Recent advances in the expression genetics of cancer have successfully identified large panels of differentially expressed genes in human malignancies *(10)* and could potentially provide numerous new markers for plasma RNA-based molecular analysis.

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The Apolipoprotein E Content of HDL in Cerebrospinal Fluid Is Higher in Children than in Adults, *Takashi Miida,[1*] Fusako Yamazaki,[2] Miho Sakurai,[2] Reiko Wada,[2] Toru Yamadera,[3] Keiko Asami,[4] Mari Hoshiyama,[5] Akira Tanaka,[6] Koichi Inano,[1] and Masahiko Okada[1]* ([1] Department of Laboratory Medicine, Niigata University School of Medicine, Asahimachi 1-757, Niigata City, Niigata 951-8510, Japan; [2] Central Clinical Laboratory and [3] Division of Pharmacy, Niigata University Medical Hospital, Niigata 951-8520, Japan; [4] Department of Pediatrics, Niigata Cancer Center Hospital, Niigata 951-8566, Japan; [5] Department of Internal Medicine, Kashiwazaki Central Hospital, Kashiwazaki 945-0055, Japan; [6] Third Internal Medicine, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; * author for correspondence: fax 81-223-0996, e-mail miida@med.niigata-u.ac.jp)

The blood-brain barrier keeps the protein concentrations in cerebrospinal fluid (CSF) much lower than in serum *(1–4)*. However, the CSF apolipoprotein E (apoE) concentration is approximately one-tenth to one-twentieth of the serum apoE concentration *(5–13)*. Mainly glia cells secrete apoE in the central nervous system *(14–16)*. CSF apoE is carried exclusively on HDL, which is the major lipoprotein in the CSF *(17, 18)*. The CSF apoE concentration varies in neurological disorders such as central nervous system inflammatory diseases *(5, 19)* and Alzheimer disease *(7–13)*. However, the clinical significance of the CSF apoE concentration is still unclear.

Recent studies have suggested that the apoE content of CSF HDL is more important than the CSF apoE concentration *(20)*. HDL enriched with apoE promotes nerve growth factor-induced neurite outgrowth *(20)*. Because the number of synapses increases markedly in childhood *(21)*, the apoE content of CSF HDL might be higher in children than in adults. To address this question, we measured the apoE and phospholipid (PL) concentrations in CSF simultaneously.

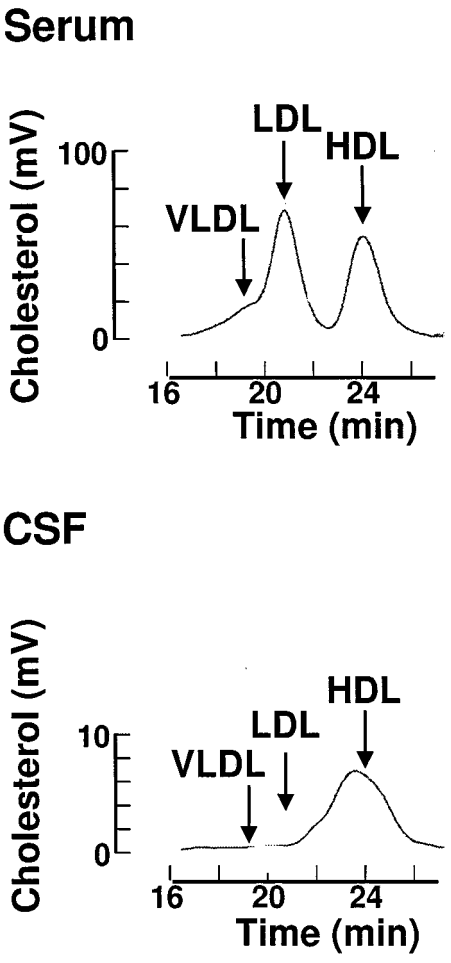

Fig. 1. HPLC elution profiles of CSF and serum.

Lipoproteins were detected by cholesterol monitoring. HDL was the main lipoprotein in the CSF, and the CSF HDL was larger than the serum HDL. Elution times for serum lipoproteins are indicated by *arrows*.