Quantitative Plasma Hypermethylated DNA Markers of Undifferentiated Nasopharyngeal Carcinoma

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ABSTRACT

Purpose: Gene-specific methylation is common in primary undifferentiated nasopharyngeal carcinoma (NPC). DNA released from apoptotic or necrotic cell death including those aberrantly methylated promoter DNA of cancer cells is absorbed into the circulation as cell-free plasma DNA of the patient. This study aims at evaluation of the potential use of methylated gene promoter DNA as a serological tumor marker of primary and potentially salvageable local or nodal recurrent NPC.

Experimental Design: The quantity of plasma hypermethylated gene promoters of CDH1, DAPKI, p15, p16, RASSF1A, and MLH1 of 41 NPC patients before treatment and 43 normal individuals were studied using real-time quantitative PCR. The post-treatment plasma hypermethylated CDH1, DAPKI, and p16 were also measured in 13 NPC patients with locoregional recurrence and 17 patients in remission.

Results: Concentrations of cell-free circulating DNA were significantly higher in NPC patients than normal controls (28.79 ng/ml versus 16.57 ng/ml, respectively). There was no significant difference in plasma DNA concentration of EBV-positive and -negative normal individuals. Methylated DNA was detectable in plasma of NPC patients before treatment including 46% for CDH1, 42% for p16, 20% for DAPKI, 20% for p15, and 5% for RASSF1A. Hypermethylated MLH1 was not detected in plasma of all of the NPC patients and normal individuals. aberrantly hypermethylated promoter DNA of at least one of the five genes was detectable in 29 of 41 (71%) plasma of NPC patients before treatment. Hypermethylated promoter DNA of at least one of the three genes (CDH1, DAPKI, and p16) was detectable in post-treatment plasma of 5 of 13 (38%) recurrent NPC patients and none of the patients in remission.

Conclusions: Our results suggested that cell-free circulating methylated gene promoter DNA is a possibly useful serological marker in assisting in screening of primary and potentially salvageable local or regional recurrent NPC.

INTRODUCTION

Poorly differentiated or undifferentiated nasopharyngeal carcinoma (NPC) is a common head and neck cancer in southern China and is the fourth leading cancer in Hong Kong (1, 2). NPC in Chinese is characterized by the association with EBV. EBV infection is associated with promoter methylation of tumor suppressor genes in cancers. Hypermethylation is common in many cancers including NPC and gastric cancers. In gastric cancers, EBV harboring gastric cancers demonstrated a 3-fold higher frequency of tumor suppressor gene hypermethylation than the EBV-negative gastric cancers (6).

Cell-free circulating DNA is an emerging source of biomarkers for cancer screening and treatment response monitoring. Elevated plasma DNA concentration has been reported in patients with epithelial malignancies including head and neck carcinomas (7). In patients with non-small cell lung cancer, elevation of plasma DNA concentration after surgery may indicate the presence of minimal residual disease (8). In addition, cell-free circulating DNA in plasma/serum of cancer patients shares many similarities with the DNA in the primary tumors such as microsatellite instability (9), mutations (10), and CpG methylation (11). The presence of cancer-derived DNA in circulation may possibly be used to screen early cancer and monitor cancer treatment noninvasively.

In view of the large population at risk of developing NPC in the Asian region, a cost-beneficial screening program may be considered. Patients with NPC have high titer of antibody against various EBV viral proteins such as early antigen (EA) IgA, viral capsid antigen (VCA) IgA, nuclear antigen 1 (EBNA1) IgA, and transactivator ZEB virus replication activator protein (EB1 or Zta) IgG (12). None of them is a stand-alone adequate serological screening marker for NPC due to either low sensitivity or low specificity (12). Raised plasma level of EBV DNA has also been reported previously to be a potential serological marker of recurrent distant metastatic NPC (13). It is well known that local and regional recurrences but not distant metastasis are salvageable after primary treatment of NPC. Unfortunately, many patients had late presentation of the locoregional recurrence and were too advanced for successful salvage at the time of detection. There is currently no satisfactory clinical or laboratory method for early detection of locoregional recurrence. EBV antibodies are not effective in screening recur-
Table 1 Detection rate and range of hypermethylated genes in plasma of NPC\(^a\) and controls

<table>
<thead>
<tr>
<th></th>
<th>41 NPC patients</th>
<th>43 normal controls</th>
<th>Statistical analysis of incidence Fisher’s test</th>
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<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Mean (range) copy/ml</td>
<td>Incidence</td>
</tr>
<tr>
<td>CDH1</td>
<td>19 (46%)</td>
<td>297 (25–1056)</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>DAP-Kinase p15</td>
<td>8 (20%)</td>
<td>338 (53–833)</td>
<td>0</td>
</tr>
<tr>
<td>p16</td>
<td>8 (20%)</td>
<td>267 (26–1000)</td>
<td>0</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>17 (42%)</td>
<td>539 (43–2334)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>MLH1</td>
<td>2 (5%)</td>
<td>71 (53–89)</td>
<td>0</td>
</tr>
<tr>
<td>At least one gene positive</td>
<td>29 (71%)</td>
<td>–</td>
<td>4 (8%)</td>
</tr>
</tbody>
</table>

\(\text{a}\) NPC, nasopharyngeal carcinoma.  
\(\text{b}\) Copy number in 1-ml plasma sample of those positive patients or controls.

rent NPC. The sensitivity and specificity of plasma EBV DNA, however, was found to be unsatisfactory in screening primary and potentially salvageable local or regional recurrent NPC in more recent studies (12–15). A better screening tumor marker, which can replace or complement the currently used EBV antibodies or DNA, are desirable.

We have demonstrated previously that tumor suppressor genes are differentially methylated in undifferentiated NPC, and the genes with high methylation frequency could also be detected in the peripheral blood of NPC patients (16, 17). In this study, we first evaluated the proposed diagnostic value of quantitative measurement of plasma DNA concentration and hypermethylated DNA markers [CDH1, DAPK1, p15, p16, RASSF1A, and MLH1] in patients with undifferentiated NPC. Because the serum EBV IgA antibody or EBV DNA level are not satisfactory for screening local or regional recurrences, we also try to evaluate the potential use of plasma hypermethylated DNA markers in detection of locoregional recurrences after treatment of NPC.

MATERIALS AND METHODS

Patients. Plasma samples were collected from 41 NPC patients (20 male and 21 female; ages 30–66 years; mean, 46 years) before radiotherapy. All of the patients were confirmed to have primary undifferentiated NPC. Peripheral blood was also collected from 14 normal individuals with positive EBV serology (positive to at least one of the three antibodies including VCA, EA, or EBNA1) and 29 normal individuals with negative EBV IgA serology. These normal controls came to the clinic for screening of NPC and all were proven clinically negative by nasoendoscopy. All of the normal controls with high serum EBV antibody titer also had random nasopharyngeal biopsies of right, left, and midline of nasopharynx, and all had proven negative histology. Plasma samples were also collected from 13 recurrent NPC patients (10 local and 3 neck recurrence) and 17 individuals in remission for 5–11 years after treatment of NPC. The study was approved by the ethnic committee of the faculty. Written consent for donation of blood and tissue for medical research was obtained from patients before blood collection.

The procedure for plasma preparation has been reported previously (16). All of the plasma samples were stored at \(-70^\circ\text{C}\) until use. The clinicopathologic characteristics of the NPC patients are showed in Table 1. Circulating DNA was extracted from 400 \(\mu\)l plasma using the QIAamp Blood and Tissue kit (Qiagen, Hilden, Germany) according to the blood and body fluid protocol.

Real-Time Quantitative PCR Measurement of Cell-Free Circulating DNA Concentration. The principles and procedures for real-time quantification were described previously (18). Circulating DNA concentration was derived from the quantity of \(\beta\)-globin gene using a conversion factor of 1 copy = 3.3 pg. Absolute quantity was derived from standard curve generated by serial dilution of normal human genomic DNA (Promega). The concentration of plasma DNA was expressed as ng/ml. One-tenth of the purified DNA was subjected to the assay. Quantification was performed using ABI/Prism 7700 Sequence Detector System (Applied Biosystem, Perkin-Elmer). Forty cycles were performed for each assay. The primers, fluorogenic probe sequences, and reaction conditions were reported previously (19). All of the plasma samples examined in this study were positive for \(\beta\)-globin gene amplification.

Bisulfite-Modification and Real-Time Quantitative PCR Measurement of Hypermethylated DNA Concentration in Plasma. Bisulfite modification was carried out using CpGenome DNA Modification kit (Intergen, New York, NY). All of the purified DNA that remained after total circulating DNA analysis was subjected to bisulfite modification. The modified DNA was then eluted in 50 \(\mu\)l of water. Five \(\mu\)l of the eluted DNA was subjected to real-time quantitative PCR analysis.

Serial dilution of the universal methylated DNA (Intergen) was used to generate the four-pointed standard curve (range, 10,000–10 genomic equivalents). Again, 3.3 pg was used as a conversion factor for 1 copy of the target gene. The serial-diluted standard DNA was subjected to bisulfite modification together with the sample DNA at the same time. Multiple negative controls (water control and no template control) were included for each set of reaction. Reaction conditions for the real-time quantification were based on the MethyLight analysis (20). Forty cycles were performed for each assay. The primer and probe sequences for CDH1, DAPK1, MLH1, p15, p16, and RASSF1A have been reported previously (21–23).
RESULTS

Cell-Free Circulating DNA Concentration of NPC Patients versus Normal Controls. A total of 41 NPC patients and 43 normal individuals were studied. The mean plasma DNA concentration was higher in NPC patients than normal individuals (mean concentration, 28.79 ng/ml versus 16.57 ng/ml, respectively; Wilcoxon’s rank-sum test, P = 0.175). Among the 43 normal individuals examined, 14 were EBV serology positive, whereas 29 were EBV serology negative. No significant difference of plasma DNA concentration was observed between these two groups of normal controls (mean, 18.43 versus 15.67 ng/ml; P = 0.198, Wilcoxon’s rank-sum test).

Plasma DNA concentration of NPC patients before radiotherapy was not significantly correlated with sex (Wilcoxon’s rank-sum test, P = 0.193), stage (Fisher’s exact test, P = 0.196), and nodal metastasis status (χ² test, P = 0.869).

Hypermethylated Gene Promoter DNA Levels in Normal Controls and NPC Patients before Treatment. The plasma detection rate and concentration of hypermethylated gene promoter DNA in NPC patients are shown in Table 1. Five of the six genes were detectable in plasma of NPC patients. Overall, hypermethylation of at least one of the selected genes was found in 71% of the plasma of NPC patients. Low copy concentration (28–97 copy/ml) of hypermethylated CDH1 was found in 4 (9%) normal controls, 3 were serological positive for EBV IgA. One normal control who had positive EBV antibody titer and also positive plasma methylated CDH1 also had low copy concentration of hypermethylated p16 (60 copy/ml) detected in the plasma. Hypermethylated DAPK1, MLH1, p15, and RASSF1A were not detectable in plasma of all of the 43 normal individuals. The panel of five methylation markers including CDH1, DAPK1, RASSF1A, p15, and p16 had sensitivity of 71% (29 of 41), and specificity of 91% (39 of 43).

Hypermethylated gene levels in plasma of NPC patients were not correlated with sex, clinical tumor staging, and lymph node status as shown in Table 2.

Plasma Hypermethylated Genes in Patients with Locoregional Recurrence versus Remission. Hypermethylated CDH1 and DAPK1 were found in 2 of 13 (15%; 30–146 copy/ml) and 3 of 13 (23%; 22–58 copy/ml) recurrent patients, respectively, and were not detected in all patients of the control group in remission with 5–11 years of follow-up. Hypermethylated p16 was not detected in both the recurrent and control group. Hypermethylation of at least 1 gene was detected in 5 of 13 (39%) recurrent NPC patients. The sensitivity of the methylation marker was 39%, and the specificity was 100%.

DISCUSSION

In this study, we first demonstrated that mean plasma DNA concentration in NPC patients was significantly higher than normal controls. This result is concordant with those reported in other cancers (23–27). Because the additional plasma DNA of cancer patients mostly come from the tumor (28), it indicates the potential of using these tumor-derived plasma DNA as tumor markers for screening of early NPC or detection of minimal residual/recurrent cancer after treatment. Moreover, plasma DNA concentration of the normal controls was unaffected by EBV IgA serology level. Raised level of plasma tumor-derived DNA was found independent of sex, age, stage, and nodal status. It indicates the possibility of identifying potential bio-

<table>
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<th>Table 2</th>
<th>Associations of circulating DNA and clinicopathological parameters of NPC† patients</th>
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<tr>
<td></td>
<td>Mean DNA concentration (ng/ml)</td>
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<tr>
<td></td>
<td>CDH1</td>
</tr>
<tr>
<td>Sex</td>
<td>No</td>
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<tr>
<td>Male</td>
<td>30</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
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<td>Staging</td>
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<td>2</td>
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<td>II</td>
<td>5</td>
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<td>III</td>
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<td></td>
<td></td>
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<tr>
<td>Lymph node status</td>
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<tr>
<td>Positive</td>
<td>27</td>
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<tr>
<td>Negative</td>
<td>14</td>
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* NPC, nasopharyngeal carcinoma.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Serologic EBV IgA and methylation marker</th>
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<tbody>
<tr>
<td></td>
<td>EBV IgA+</td>
</tr>
<tr>
<td></td>
<td>Methylation+</td>
</tr>
<tr>
<td>NPC†</td>
<td>25 (89%)</td>
</tr>
<tr>
<td>Normal</td>
<td>3 (11%)</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
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* NPC, nasopharyngeal carcinoma.
markers from tumor-derived plasma DNA for screening of early
or recurrent NPC of both sexes and of any age group.

Promoter methylation of many tumor suppressor genes is
found frequently in cancer cells but is normally absent in normal
cells (29). This level of specificity is one of the desirable
requirements of tumor biomarkers for cancer screening. Meth-
ylated promoter DNA is one of the promising cancer biomarkers
of tumor-derived DNA present in plasma of patients. We have
shown previously in a preliminary study that methylated
DAPKI promoter DNA can be found in plasma of NPC patients
(16). We have reported previously a panel of differentially
methylated tumor-related genes in primary tumors of NPC (17).
Six genes with high frequency of methylation in NPC were
selected in this study. The frequencies of methylation in primary
NPC of these genes were DAPKI 74%, RASSF1A 65%, CDH1
50%, MLH1 40%, p15 35%, and p16 30% (17). In this study, we
have additionally studied quantitatively the plasma level of
these six methylated genes including CDH1, DAPKI, p15, p16,
RASSF1A, and MLH1. The results show that five of the six
genes including CDH1, DAPKI, p15, p16, and RASSF1A can be
found with high specificity in plasma of NPC patients but rarely
in both EBV IgA serological-positive and serological-negative
normal controls.

Serological EBV antibody screening of NPC has been
practiced for many years. However, it has the problem of either
low specificity or low sensitivity. In one of the recent reports by
Chan et al. (12), the sensitivities were found to be 93% for
VCA, 84% for EBNA1, 74% for Zta, and 73% for EA. The
EBNA1, Zta, and EA antibodies are not sensitive enough to be
used alone for screening purpose. The combination of EBNA1
and Zta antibodies has improved the sensitivity to 92%, which
is comparable with VCA IgA (30), and the combination of VCA
and EA has sensitivity of 93% (12). The conventional VCA IgA
is still the most sensitive serological EBV antibody compared
with the newer antibodies and their combinations. VCA IgA,
however, suffers from low specificity of only 60% (12). In our
previous study of serological surveillance of asymptomatic NPC
in southern Chinese, the positive NPC prediction rate of raised
EBV VCA IgA in the population was only ~5% (31). Low
positive NPC prediction rates of plasma EBV IgA VCA and EA
were also found in other studies (32). The recommended man-
agement of people with raised plasma EBV IgA is to have a
head and neck examination including nasoendoscopy and three
random biopsies from right, left, and middle nasopharyngeal
areas to depict microscopic NPC (31). If VCA IgA is to be used
in Hong Kong for surveillance of asymptomatic NPC in the
population, of the 7 million population being screened, a pro-
jected 2.8 million (with reference to 40% false positive as
published by Chan et al.; Ref. 12) people will be expected to
have raised plasma EBV VCA IgA level. The annual NPC
incidence in Hong Kong was, however, only ~1500 (1). The
projected correct positive prediction rate of EBV VCA in the
whole population is in fact only 0.0005% (1500/2.8 million),
which is 44% different from the selected samples as published
by Chan et al. (12). The low specificity and positive prediction
rate of serological EBV VCA IgA will mean the requirement of
a huge number of normal people for the need of unnecessary
invasive nasoendoscopy and nasopharyngeal biopsies. Labora-
tory screening tests are also used clinically to exclude the
presence of a disease or diagnosis. If a serological marker is to
be used to assist a clinician to exclude the diagnosis of NPC in
individuals with suspicious clinical features or in high risk of
having asymptomatic NPC, a 92–93% sensitivity (or 7–8% false-negative detection rate) of EBV VCA or combination of
EBNA1/Zta are unsatisfactory to exclude confidently the diag-
osis of NPC in clinical management of the patient. Both the
patient and the clinician would certainly still worry about pos-
sibly missing the diagnosis of NPC with a negative serological
EBV antibody result. The patient would not be satisfied with a
conclusion of being unlikely to have NPC, but with 7–8% chance of missing the NPC.

The plasma EBV DNA is another possible biomarker of
NPC. The sensitivity and specificity of EBV DNA are not
duplicative in different laboratories by different investigators,
and it was, however, found to have low sensitivity or high
false-positive rates in recent reports (12, 14, 15). The sensitivity
of EBV DNA was found to be 56% by Chan et al. (12) and is
much more inferior than EBV antibodies. EBV DNA is also
present in most EBV-associated benign or malignant diseases
apart from nasopharyngeal carcinoma, including infectious
mononucleosis, EBV-associated hematological malignancies,
chronic active EBV infection, EBV-associated hemophagocytic
syndrome, and transplantation-related lymphoproliferative dis-
eease, and is also detectable in a small percentage of the normal
population (33–35).

In the absence of a reliable EBV antibody or DNA biomar-
ker with both high sensitivity and high specificity, a second
biomarker or combination of several markers has to be consid-
ered to allow better selection of patients for additional expensive
and invasive nasoendoscopy and biopsy procedures or to ex-
clude the presence of primary NPC. The plasma DAPKI, p15,
and RASSF1A-methylated DNA had high specificity and were
found mostly in NPC patients but not in normal controls. Low
copies of methylated CDH1 and p16 were also found in a small
percentage of normal individuals. Methylated promoter also
suffers from the problem of low sensitivity and can also be
found in other cancers with a similar methylation pattern. The
present study, however, shows that the combination of quanti-
tative measurement of plasma-methylated DNA and serological
EBV antibody together is superior than either alone.

A recommended algorithm of clinical management after
serological screening of both EBV antibody and methylation
markers would be: (a) high-risk group for nasoendoscopy and
nasopharyngeal biopsy, to have nasoendoscopy and nasopa-
ryngeal biopsies for all patients with positive methylation irre-
spective of EBV antibody-positive or -negative status, as these
patients would have high chance of having NPC; (b) low-risk
group for reassurance without nasoendoscopy, individuals hav-
ing both EBV antibody and methylation marker negative can be
confidently reassured of exclusion of NPC, and nasoendoscopy
is not necessary, as the chance of missing the diagnosis of NPC
is almost zero; and (c) moderate risk group for nasoendoscopy
alone without routine nasopharyngeal biopsy, individuals hav-
ing discordant results of positive EBV antibody and negative
methylation marker should be investigated with nasoendoscopy,
random nasopharyngeal biopsy is not necessary unless suspected
area in the nasopharynx is visualized during nasoendoscopy, the
blood tests and nasoendoscopy should be repeated regularly.
until both EBV antibody titer and methylation marker become negative, and nasopharyngeal biopsy should be taken only in case the methylation marker becomes positive on follow-up. Other EBV-related diseases or other cancers associated with a similar pattern of aberrant methylation should also be considered in the differential diagnosis and interpretation of abnormal blood test results by the clinician. In comparison with the serological EBV antibody or EBV DNA alone, the use of a combination of serological EBV antibody and the panel of methylation markers has markedly increased the overall diagnostic accuracy in both detection and exclusion of NPC (Table 3). It can assist the clinician in correctly selecting patients for the invasive nasoendoscopy and multiple biopsies according to the risk. With the correct interpretation of two test results and appropriate management algorithm, all of the high-risk patients can be selected for invasive nasopharyngeal biopsy, and the moderate risk individuals are also selected for follow-up reassessment screening program. The patient and clinician can also be confidently reassured of the exclusion of NPC with a concordant finding of negative antibody and methylation.

Although many hypermethylated genes are found frequently in primary tumor (29), the sensitivity of individual gene in plasma is relatively low. In this study, the sensitivity of any single gene ranged from 14% to 29%. The sensitivity of the test was markedly improved when multiple genes were used as a panel of markers. With the use of a panel of five genes including DAPK1, CDH1, RASSF1A, p15, and p16, the sensitivity could be increased to 71%. With the expanding list of candidate genes found to be methylated in undifferentiated NPC, it may be possible to increase the sensitivity of the test with a smaller number of better-selected genes.

In an in vitro setting, the viral protein, LMP1, encoded by the EBV genome, could repress CDH1 expression by inducing de novo promoter methylation (35). It has been shown that serological positive EBV IgA can be found in NPC patients many years before clinical development of tumors of Chinese in Taiwan (36). Aberrant chromosomal abnormalities have also been found in nasopharyngeal tissue of normal individuals of high-risk southern Chinese susceptible to nasopharyngeal carcinoma (37, 38). Low concentration of CDH1 and p16 were found in a small percentage of mostly serological IgA-positive normal controls. It is unknown whether the presence of low concentration of aberrantly methylated gene promoter DNA indicates the higher risk of subsequent development of nasopharyngeal carcinoma or another malignancy of these clinically healthy individuals. We do not have long-term follow-up data of these apparently healthy normal controls with serological EBV IgA and methylation-positive results yet. These normal controls are currently closely monitored every 6 months in our NPC screening clinic with repeat blood test and nasoendoscopy according to our recommended management algorithm. A prospective follow-up study of larger scale is, however, required to answer the question.

Nasopharyngeal carcinoma is a radiosensitive cancer. Primary radiotherapy with or without concomitant chemotherapy has a high curative rate. Of the unfortunate patients who fail primary treatment, successful salvage with surgery or radiotherapy is still possible with a high curative rate for those local or regional recurrences provided that they could be detected in early stage. We have been able to salvage successfully locoregional recurrence with nasopharyngectomy, radioactive gold implantation, and radical neck dissection (38–42). There is, however, difficulty in clinical detection of residual or recurrent locoregional NPC in their early stage. Serological EBV IgA level is not reliable for detection of recurrent cancer (43). Plasma EBV DNA is suggested to be a serological marker of mostly distant metastasis by Lo et al. (44). The presence of distant metastasis, however, has no curative treatment. The sensitivity of EBV DNA was found to be only 38% for rT1 and 50% for rT2 local recurrence after radiotherapy by the same group of investigators recently (14). Low sensitivity (28%) for locoregional NPC recurrence was also reported by Hsiao et al. (15), and the increase of PCR cycle can increase the sensitivity to 89% but was also found to increase the false-positive rate to 37%. EBV DNA is, therefore, not a satisfactory marker for locoregional recurrence. Our results suggest that hypermethylated gene-specific promoter DNA in circulation might be potentially used as serological tumor marker for early detection of locoregional recurrence. Additional research in the refinement of methylation marker genes is necessary to increase the sensitivity. A prospective evaluation in a larger sample size and serial documentation of methylation markers are recommended to validate the diagnostic accuracy.

In conclusion, our results demonstrated that NPC patients have a higher cell-free circulating DNA level when compared with normal individuals. Hypermethylated genes could be detected in the plasma of NPC patients suggesting that hypermethylated gene might be used as a serological tumor marker in screening of primary NPC and to supplement the deficit of conventional plasma EBV antibody method. Hypermethylated DNA markers may also be used in detecting potentially salvageable local and regional recurrences. Additional refinement of the panel of markers and longitudinal studies are warranted to fully validate the diagnostic value of hypermethylated markers in patients with undifferentiated NPC.

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