Persistent Aberrations in Circulating DNA Integrity after Radiotherapy Are Associated with Poor Prognosis in Nasopharyngeal Carcinoma Patients

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Abstract

Purpose: Aberrations of circulating nucleic acid integrity have been observed in cancer patients. However, the clinical significance of such changes has not been completely elucidated. In this study, we investigated the plasma DNA integrity in nasopharyngeal carcinoma (NPC) patients and its association with patients' survival after radiotherapy.

Experimental Design: Plasma DNA integrity was analyzed for 105 NPC patients before and after curative-intent radiotherapy and for 40 healthy controls. The plasma DNA concentration of each sample was measured by two real-time PCRs targeting the *leptin* gene. The amplicon sizes of the two assays were 105 and 201 bp. The integrity index was calculated as the ratio of the two concentrations (201 bp/105 bp). More intact circulating DNA would give a higher integrity index.

Results: The plasma DNA integrity index of the NPC patients was significantly higher than that of the healthy controls (median, 0.356 versus 0.238; $P < 0.001$). After radiotherapy, a reduction in plasma DNA integrity index was observed in 70% NPC patients. Patients with persistent aberrations of plasma DNA integrity had significantly poorer survival probability than those with reduced DNA integrity after treatment ($P < 0.001$, Kaplan-Meier).

Conclusions: NPC is associated with disturbances in the integrity of circulating cell-free DNA. The persistence of DNA integrity aberrations after radiotherapy is associated with reduced probability of disease-free survival. Therefore, the measurement of plasma DNA integrity may serve as a useful marker for the detection and monitoring of malignant diseases.

Circulating nucleic acids have emerged as a new class of tumor markers (1). Tumor-associated genetic and epigenetic changes, including tumor-suppressor gene (2) and oncogene (3, 4) mutations, loss of heterozygozity (5), aberrant methylation of tumor suppressor genes (6, 7) and tumor-associated viral DNA (8, 9), have been detected in the plasma or serum of cancer patients. The detection of such changes in plasma or serum has been shown to be useful for the detection (10), monitoring (11), and prognostication of cancers (12). Recently, alterations in the integrity of circulating nucleic acids have also been observed in cancer patients (13, 14). The size of circulating DNA has been shown to be increased in patients suffering from gynecologic (13), colorectal (15), breast (16), and head and neck (17) cancers when compared with healthy subjects. Furthermore, the degree of alterations in circulating DNA integrity has been shown to reflect the extent of cancer involvement. For example, the integrity of serum DNA was shown to be significantly higher in breast cancer patients with lymph node metastasis or lymphovascular invasion than in those with less advanced disease (16). In this study, we further investigated if these tumor-associated aberrations in circulating DNA integrity are reversible after curative-intent therapy in nasopharyngeal carcinoma (NPC) patients and explored their potential clinical application in patient prognostication.

Patients and Methods

Patients. One hundred and five NPC patients under the care of the Department of Clinical Oncology at the Prince of Wales Hospital, Hong Kong were recruited. All patients were staged by a standard protocol including endoscopic examination of the nasopharynx and computed tomography of the head and neck; stage designation was according to the Unio Internationale Contra Cancrum and American Joint Committee on Cancer staging criteria (18, 19). Seventy-five patients had early-stage disease (stages I and II) and 30 patients had advanced stage (stages III and IV) disease. All patients were treated with radiotherapy, with or without chemotherapy, according to standard treatment protocols of the institution. Peripheral blood was taken before the commencement of treatment and at 6 wk after the completion of treatment. Forty healthy individuals were recruited as controls. The median time interval between blood sampling at presentation and after

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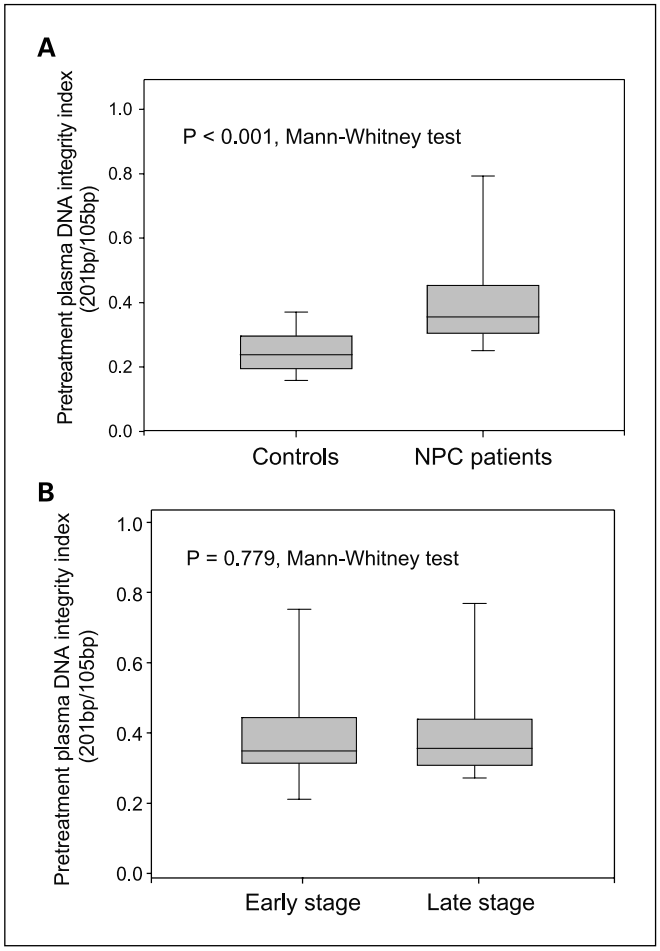

Fig. 1. Comparison of the plasma DNA integrity index (201 bp/105 bp) between NPC patients and healthy controls (*A*) and between early-stage and late-stage NPC patients (*B*).

radiotherapy was 3.6 mo. The median ages of the NPC patients and control subjects were 47 and 42 y, respectively. Seventy-six (72%) NPC patients and 27 (68%) control subjects were male. Informed consents were obtained from all patients and controls. The study was approved by the Clinical Research Ethics Committee of the institution.

Processing of blood samples. Blood samples were collected into EDTA-containing tubes. DNA was extracted from 800-μL freshly prepared plasma sample after centrifugation with the QIAamp mini kit (Qiagen) and eluted with 50 μL of H_2O (20, 21).

Real-time quantitative PCR. For the determination of the integrity of circulating DNA fragments, two real-time PCR assays targeting the *leptin* gene with amplicon sizes of 105 and 201 bp were used. The two PCR assays shared the same forward primer, 5′-CAGTCTCCTC-CAAACAGAAAGTCA-3′, and a common TaqMan minor groove binding (MGB) probe, FAM-CGGTTTGGACTTC-MGB. FAM and MGB represent 5-methylfluorescein and the proprietary MGB quencher (Applied Biosystems), respectively. The reverse primers for the 105 bp and 201 bp assays were 5′-ATCTGTTGGTAGACTGC-CAGTGTCT-3′ and 5′-CAGCTCTTAGAGAAGGCCAGCA-3′, respectively. PCR reactions were set up in a reaction volume of 50 μL. Each reaction contained 5 μL of 10× buffer A; 4 mmol/L $MgCl_2$; 200 μmol/L each of dATP, dCTP, and dGTP; 400 μmol/L dUTP; 1 μmol/L of each of the forward and reverse primers; 500 nmol/L of the TaqMan probe; 2 units of AmpliTaq Gold polymerase; 0.5 unit of AmpErase uracil *N*-glycosylase; and 5% DMSO (22). Five microliters of plasma DNA were used as template.

A common thermal profile was used for the two PCR assays. Following initial incubation at 50°C for 2 min and 95°C for 10 min, 50 cycles of thermal cycling at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min were carried out. A standard curve composed of serial 10-fold dilutions of buffy coat DNA from 10^5 to 10^{-1} copies was run for each assay. Each sample was analyzed in duplicate and the mean value was used for further analysis. Multiple negative water blanks were included in every analysis.

An integrity index was calculated for each plasma DNA sample as the ratio of concentration determined by the 201 bp assay divided by the concentration determined by the 105 bp assay. If all circulating DNA molecules are of high molecular weight and span the whole *leptin* gene, the concentrations determined by the two assays would be identical, thus giving an integrity index of 1. However, if the circulating DNA molecules are fragmented to <201 bp, the concentration determined using the 105 bp assay would be higher than that determined by the 201 bp assay, thus giving an integrity index of <1. Therefore, the integrity of circulating DNA could be reflected by the integrity index. The more intact the plasma DNA, the higher the integrity index would be.

The detection and quantification of plasma EBV DNA was done using a real-time PCR assay targeting the W fragment of the EBV genome as previously described (23).

Statistical analysis. Statistical tests were done using the SigmaStat 3.0 (SPSS, Inc.) and MedCalc (MedCalc) softwares.

Results

Increased plasma DNA integrity in NPC patients. The median plasma DNA integrity indices for the NPC patients before treatment and the control subjects were 0.356 (interquartile range, 0.304-0.453) and 0.238 (interquartile range, 0.194-0.290), respectively ($P < 0.001$, Mann-Whitney test; Fig. 1A). Thus, NPC is associated with increased DNA integrity in plasma. The median plasma DNA integrity indices for patients with early (stages I and II) and late (stages III and IV) disease were 0.351 (interquartile range, 0.308-0.452) and 0.359 (interquartile range, 0.301-0.455), respectively (Fig. 1B). There was no significant difference between the plasma DNA integrity indices of the two groups ($P = 0.779$, Mann-Whitney test).

Lack of association between pretreatment plasma DNA integrity and EBV DNA levels. Circulating EBV DNA has

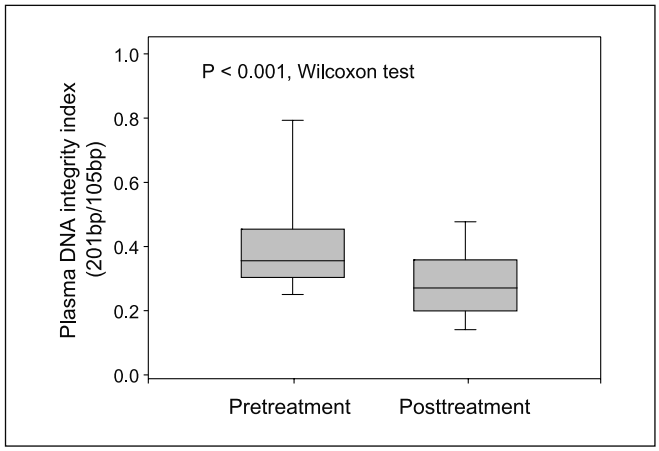

Fig. 2. Changes of plasma DNA integrity index (201 bp/105 bp) after curative-intent treatment in NPC patients.

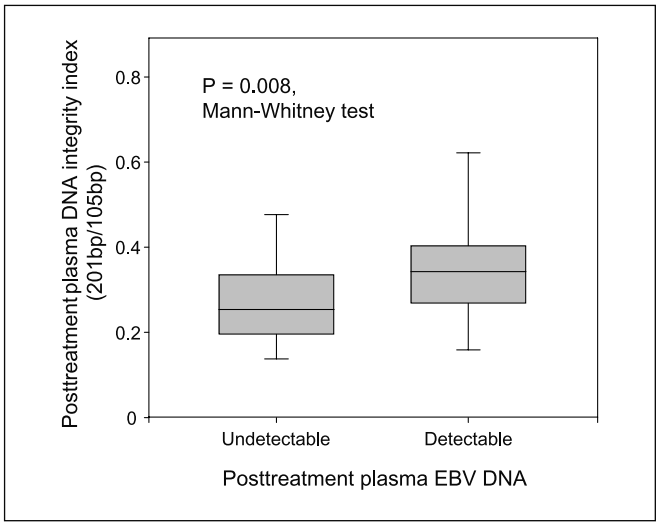

Fig. 3. Comparison of the posttreatment plasma DNA integrity index (201 bp/105 bp) between NPC patients with and without detectable plasma EBV DNA.

been shown to be derived from NPC tumor cells and its level could reflect the tumor load (24). In this regard, we have investigated if there is any correlation between the plasma genomic DNA integrity and plasma EBV DNA levels. EBV DNA was detectable in the plasma of 97 (92%) NPC patients before treatment and the median plasma EBV DNA concentration was 1,940 copies/mL. There was no significant relationship between the level of circulating EBV DNA and plasma DNA integrity index in the NPC patients before treatment (r = -0.049, P = 0.621, Spearman correlation).

Reduced plasma DNA integrity after treatment. Of the 105 patients, 74 (70.5%) showed a reduction in plasma DNA integrity index at 6 weeks after treatment. The median plasma DNA integrity of all patients dropped from 0.356 to 0.271 (P < 0.001, Wilcoxon test; Fig. 2).

Association between plasma DNA integrity and posttreatment EBV DNA. EBV DNA was detectable in the plasma of 17 (16%) NPC patients after treatment and their median EBV DNA level was 47 copies/mL. The median plasma DNA integrity indices for patients with and without detectable plasma EBV DNA after treatment were 0.355 and 0.254, respectively (P = 0.008, Mann-Whitney test; Fig. 3). In other words, patients with detectable EBV DNA in plasma after treatment had significantly higher plasma genomic DNA integrity index when compared with patients who had complete clearance of circulating EBV DNA.

Persistence of plasma DNA integrity aberrations is associated with inferior disease-free survival. For the 31 patients who did not show a reduction in plasma DNA integrity after treatment, 12 (38.7%) of them developed disease recurrence after a median follow-up interval of 552 days. For the 74 patients who showed a reduction in plasma DNA integrity, 11 (14.9%) of them developed disease recurrence after a median follow-up interval of 650 days. Using log-rank analysis, patients with persistent plasma DNA integrity aberrations had inferior disease-free survival probability than those who had reduction in plasma DNA integrity index after treatment (P < 0.001; Fig. 4A). Using Cox proportional hazard analysis, the prognostic value of plasma DNA integrity was shown to be independent of the pretreatment EBV DNA level, the posttreatment detectibility of EBV DNA, and the disease stage of the patient. The odds ratio for the prediction of disease-free survival for patients without reduction in plasma DNA integrity was 2.97 (P = 0.024, Cox regression; Table 1) when compared with patients with reduction in plasma DNA integrity. Figure 4B shows the survival curves for NPC patients according to the detectibility of EBV DNA and the changes in plasma DNA integrity. Patients with undetectable plasma EBV DNA and reduction in DNA integrity had the highest probability of survival whereas patients with detectable plasma EBV DNA and persistent increase in DNA integrity had the poorest survival probability.

Discussion

In this study, we have shown that the size of circulating DNA was significantly increased in NPC patients when compared with the healthy control subjects. More importantly, we further showed that these cancer-associated changes are potentially reversible after curative-intent treatment. In the 105 NPC patients, 70% of them showed a reduction in plasma DNA integrity index after treatment, and

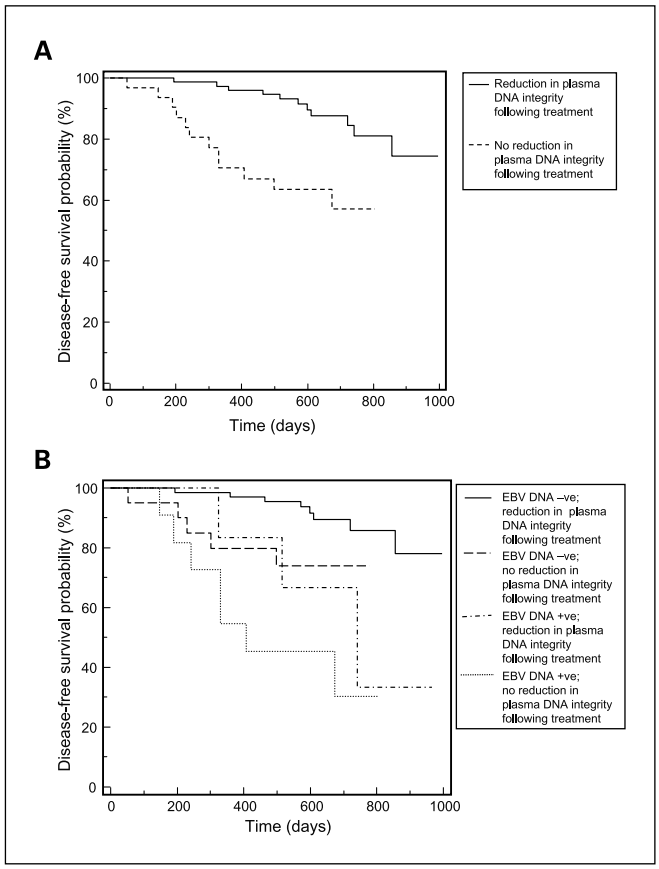

Fig. 4. Probabilities of disease-free survival for NPC patients with and without a reduction in plasma DNA integrity index (201 bp/105 bp) following treatment (*A*), and probabilities of disease-free survival for NPC patients according to the detectibility of plasma EBV DNA and reduction in plasma DNA integrity index (201 bp/105 bp) following treatment (*B*).

Table 1. Cox proportional hazard analysis for the prediction of disease-free survival in NPC patients after treatment

Variable	*P*	OR (95% CI)
Stage	0.123	1.7 (0.9-3.3)
Pretreatment plasma EBV DNA level	0.354	1.0 (1.0-1.0)
Posttreatment detectibility of plasma EBV DNA	0.011	3.4 (1.3-8.7)
Reduction in plasma DNA integrity	0.024	2.9 (1.2-7.6)

Abbreviation: OR, odds ratio; 95% CI, 95% confidence interval.

the median integrity index of the 105 NPC patients dropped from 0.356 before treatment to 0.271 at 6 weeks after treatment. The reversibility of plasma DNA integrity aberrations after treatment suggests that this parameter might be potentially useful as a surrogate marker for residual disease. In this regard, we showed that patients with detectable plasma EBV DNA after treatment had significantly higher plasma DNA integrity. Whereas the presence of plasma EBV DNA is predictive of the presence of residual disease (24, 25), this observation suggests that the persistent increase in plasma DNA integrity may also be associated with incomplete eradication of tumor cells. Being consistent with this finding, we showed that patients with a reduction in plasma DNA integrity after treatment had significantly better disease-free survival probability when compared with those showing persistent aberrations in plasma DNA integrity after treatment. Furthermore, the prognostic value of plasma DNA integrity changes was independent of the stage of the patient, the pretreatment EBV DNA level, and the detectibility of EBV DNA after treatment. Therefore, the presence of plasma EBV DNA and the persistence of DNA integrity aberrations can be used synergistically for stratifying the risk of disease recurrence. Patients with detectable EBV DNA and persistent aberrations in plasma DNA integrity had the worst prognosis whereas patients with undetectable EBV DNA and a reduction in plasma DNA integrity after treatment had the best chance of disease-free survival (Fig. 4B). The lack of association between the pretreatment plasma EBV DNA and integrity index suggests that the integrity index may be more useful for the detection of cancer rather than reflecting the tumor load.

The precise mechanism of plasma DNA lengthening in cancer patients has not been completely understood. In healthy subjects, the main source of cell-free circulating DNA is apoptotic cells (26). During apoptosis, genomic DNA would undergo enzymatic cleavage to produce DNA fragments of ~180 bp (26). The increased integrity of circulating DNA in cancer patients has been postulated to be a consequence of pathologic processes other than apoptosis, including necrosis, autophagy, and mitotic catastrophe (16, 27, 28) of tumor cells. Interestingly, the integrity of circulating RNA is also altered in malignant diseases. In contrast to the increased integrity of circulating DNA, the integrity of circulating RNA is reduced in cancer patients (14). Being consistent with the findings in this study, the cancer associated changes in RNA integrity are also reversible after treatment. Further investigation into the mechanisms associated with the alterations in circulating nucleic acids integrity would definitely improve our understanding of the underlying pathologic process and facilitate the development of more powerful molecular tumor markers.

In summary, our findings suggest that plasma DNA integrity is increased in NPC patients and this cancer-associated change is potentially reversible after successful treatment. The persistent aberrations of plasma DNA integrity are associated with significantly increased probability of disease recurrence. Therefore, plasma DNA integrity analysis may be potentially useful for the noninvasive detection, monitoring, and prognostication of NPC and possibly other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Chan KCA, Lo YMD. Circulating tumour-derived nucleic acids in cancer patients: potential applications as tumour markers. Br J Cancer 2007;96:681–5.
2. Hagiwara N, Mechanic LE, Trivers GE, et al. Quantitative detection of p53 mutations in plasma DNA from tobacco smokers. Cancer Res 2006;66:8309–17.
3. Shinozaki M, O'Day SJ, Kitago M, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. Clin Cancer Res 2007;13:2068–74.
4. Jiao L, Zhu J, Hassan MM, Evans DB, Abbruzzese JL, Li D. K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. Pancreas 2007;34:55–62.
5. Schwarzenbach H, Chun FK, Lange I, et al. Detection of tumor-specific DNA in blood and bone marrow plasma from patients with prostate cancer. Int J Cancer 2007;120:1465–71.
6. Hoque MO, Feng Q, Toure P, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J Clin Oncol 2006;24:4262–9.
7. Wong IH, Lo YMD, Zhang J, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res 1999;59:71–3.
8. Chan KCA, Lo YMD. Circulating EBV DNA as a tumor marker for nasopharyngeal carcinoma. Semin Cancer Biol 2002;12:489–96.
9. Leung SF, Zee B, Ma BB, et al. Plasma Epstein-Barr viral deoxyribonucleic acid quantitation complements tumor-node-metastasis staging prognostication in nasopharyngeal carcinoma. J Clin Oncol 2006;24:5414–8.
10. Lo YMD, Chan LYS, Lo KW, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res 1999;59:1188–91.
11. Lo YMD, Chan LYS, Chan ATC, et al. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. Cancer Res 1999;59:5452–5.
12. Lo YMD, Chan ATC, Chan LYS, et al. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. Cancer Res 2000;60:6878–81.
13. Wang BG, Huang HY, Chen YC, et al. Increased plasma DNA integrity in cancer patients. Cancer Res 2003;63:3966–8.
14. Wong BCK, Chan KCA, Chan ATC, et al. Reduced plasma RNA integrity in nasopharyngeal carcinoma patients. Clin Cancer Res 2006;12:2512–6.
15. Umetani N, Kim J, Hiramatsu S, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. Clin Chem 2006;52:1062–9.
16. Umetani N, Giuliano AE, Hiramatsu SH, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol 2006;24:4270–6.

17. Jiang WW, Zahurak M, Goldenberg D, et al. Increased plasma DNA integrity index in head and neck cancer patients. Int J Cancer 2006;119:2673–6.

18. Greene FL, Page DL, Fleming ID, et al., editors. AJCC cancer staging manual. 6th ed. New York: Springer-Verlag; 2002. p. 59–68.

19. Sobin LH, Wittekind C, editors. UICC TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss; 2002. p. 36–42.

20. Chan KCA, Yeung SW, Lui WB, Rainer TH, Lo YMD. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. Clin Chem 2005;51:781–4.

21. Chan KCA, Zhang J, Chan ATC, et al. Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. Cancer Res 2003;63:2028–32.

22. Chan KCA, Zhang J, Hui AB, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004;50:88–92.

23. To EW, Chan KCA, Leung SF, et al. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. Clin Cancer Res 2003;9:3254–9.

24. Chan KCA, Chan ATC, Leung SF, et al. Investigation into the origin and tumoral mass correlation of plasma Epstein-Barr virus DNA in nasopharyngeal carcinoma. Clin Chem 2005;51:2192–5.

25. Chan ATC, Lo YMD, Zee B, et al. Plasma Epstein-Barr virus DNA and residual disease after radiotherapy for undifferentiated nasopharyngeal carcinoma. J Natl Cancer Inst 2002;94:1614–9.

26. Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM, Sorenson GD. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas 1998;17:89–97.

27. Jin Z, El-Deiry WS. Overview of cell death signaling pathways. Cancer Biol Ther 2005;4:139–63.

28. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.