

Plasma DNA as a Molecular Marker for Completeness of Resection and Recurrent Disease in Patients With Esophageal Cancer

Farzaneh Banki, MD; Rodney J. Mason, MD, PhD; Daniel Oh, MD; Jeffrey A. Hagen, MD; Steven R. DeMeester, MD; John C. Lipham, MD; Koji Tanaka, MD; Kathleen D. Danenberg, BS; Wael N. Yacoub, MD; Peter V. Danenberg, PhD; Tom R. DeMeester, MD

Objective: To identify a marker for completeness of resection and recurrent disease in patients with esophageal cancer.

Design: Case series.

Setting: Department of Surgery of the University of Southern California.

Patients: Forty-four healthy subjects and 45 patients with esophageal cancer prior to esophagectomy. Six patients were unresectable and 39 had a complete resection.

Main Outcome Measures: Plasma DNA levels were measured using polymerase chain reaction. Twenty resected patients had follow-up plasma DNA levels measured.

Results: Preoperatively, plasma DNA levels exceeded the normal level in 38 (84%) of 45 patients. Preoperatively, 12 patients received neoadjuvant therapy and 11 had plasma DNA levels higher than normal. All 6 unresectable patients had DNA levels higher than normal. At ini-

tial follow-up, the plasma DNA levels remained higher than normal in 2 (10%) of 20 patients, and systemic disease was subsequently detected in each. Plasma DNA levels dropped lower than or remained normal in 18 (90%) of 20. In 14 of 18 patients, there was no evidence of recurrent disease at a median of 12 months (range, 3-20 months); in 4 patients, the plasma DNA level rose higher than normal on follow-up and all developed subsequent systemic disease on computed tomographic or positron emission tomographic scan. Six of the 20 patients developed systemic disease during the follow-up (2 had persistently elevated plasma DNA levels, and 4 developed elevated plasma DNA levels at subsequent follow-ups). In 4 of these 6 patients, elevated plasma DNA levels were detected prior to imaging evidence of disease.

Conclusions: Plasma DNA levels are significantly elevated in patients with esophageal cancer and following complete resection should return to normal. Persistently elevated plasma DNA levels after resection or levels that rise on follow-up indicate residual or recurrent disease.

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Author Affiliations: Division of Thoracic Foregut Surgery, Department of Surgery, Keck School of Medicine, University of Southern California (Drs Banki, Mason, Oh, Hagen, S. R. DeMeester, Lipham, Yacoub, and T. R. DeMeester) and Response Genetics Inc (Drs Tanaka and P. V. Danenberg and Ms K. D. Danenberg), Los Angeles.

ESOPHAGEAL CANCER IS THE seventh leading cause of death among American men¹ and is the sixth leading cause of death from cancer worldwide.² The incidence in the United States is rising and in 2000 was 3.7 patients per 100 000 population.³

Esophagectomy remains the gold standard for the treatment of esophageal cancer; however, 50% of the patients are unable to undergo surgery because of locally advanced or systemic disease.¹ Among the patients who undergo primary surgery, the rate of recurrence in the first 5 years is reported to be 40% or higher.⁴⁻⁷ The lack of precise preoperative staging is a major difficulty. Staging accuracy rates for regional

lymph node metastases using endoscopic ultrasound varies from 70% to 80%⁸⁻¹⁰; computed tomographic (CT) scan, from 40% to 73%^{11,12}; and positron emission tomographic (PET) scan, from 48% to 76%.^{9,11,13} Accuracy rates for the preoperative detection of distant organ metastasis are reported to be 74%⁹ to 84%¹⁴ for PET, with substantially lower rates for CT scan.^{11,14,15} A significant predictor of survival is an R0 resection, which is removal of all existent disease. Determining if a resection has removed all existing disease is problematic since the spread of the disease to the regional nodes is sufficiently wide that unrecognized disease could be left behind. A need exists for a more specific marker to quantitate this disease.

Prior studies have shown that a plasma DNA level is present in healthy individuals.^{16,17} DNA levels are significantly higher in patients with malignancies such as breast,¹⁸ lung,¹⁹ liver,²⁰ and prostate.²¹ To our knowledge, there have been no studies evaluating plasma DNA levels in patients with esophageal cancer.

The purpose of our study was to measure the plasma DNA level in patients with esophageal cancer and assess its value as a marker for the extent of disease, the completeness of resection, and recurrence of disease after resection.

METHODS

STUDY POPULATION

The study population consisted of 44 healthy subjects, 26 men and 18 women with a median age of 33 years (range, 26-53 years), and 45 patients with esophageal cancer, 40 men and 5 women with a median age of 57 years (range, 46-88 years). Three patients had squamous cell carcinoma and 42 had adenocarcinoma. All patients were evaluated with CT, PET scan, and endoscopic ultrasound and considered to be resectable. Blood samples were obtained in all patients prior to surgery. Twelve patients received neoadjuvant therapy prior to obtaining their preoperative blood sample. Twenty resected patients had subsequent blood samples drawn at 3- to 6-month intervals after esophagectomy for a median follow-up of 12 months (range, 3-20 months). Plasma DNA levels were not obtained in the remaining 19 resected patients because 2 patients died during the hospitalization, 14 patients refused blood draws after surgery, and we were not able to contact 2 patients. Twenty-one patients were willing to participate in the study after hospital discharge and had follow-up blood draws; 1 of these 21 specimens was hemolyzed. Therefore, 20 (51%) of 39 patients had useable postoperative plasma DNA levels. All patients were clinically followed up with CT scan at 3-month intervals and yearly PET scans.

The study was approved by the investigational review board of the University of Southern California.

PLASMA SAMPLE COLLECTION

Ten-milliliter blood samples were withdrawn from a peripheral vein and placed in EDTA-containing tubes from 45 consecutive patients with resectable esophageal cancer diagnosed at the Department of Surgery of the University of Southern California. Similarly, blood was drawn from 44 healthy subjects. Twenty patients had follow-up blood draws after esophagectomy.

DNA ISOLATION AND QUANTIFICATION

DNA isolation and quantification were performed according to a specific protocol of Response Genetics Incorporated (Los Angeles, Calif). Blood samples were processed within 30 minutes of draw time. Whole blood was centrifuged at 1650 rpm at 20°C for 20 minutes. The plasma supernatant was micropipetted off the surface with special attention to prevent white blood cell contamination of the plasma by micropipetting only at the surface of the supernatant. The plasma samples were stored at -30°C.

Four hundred microliters of plasma from each sample were mixed with 400 µL of 1% sodium dodecyl sulfate, 1 µL of salmon sperm DNA, and 20 µL of proteinase kinase and then heated

at 58°C for 75 minutes for protein denaturing. DNA was isolated using 800 µL of solution containing phenol (pH 8), chloroform, and isoamyl alcohol (250:50:1), which was added to each specimen, vortexed, and placed on ice for 15 minutes. The samples were centrifuged at 13 000 rpm for 10 minutes at 4°C and the aqueous supernatant was precipitated using 5 µL of glycogen and 600 µL of isopropanol. The pellet was washed with 500 µL of 75% ethanol and centrifuged again at 13 000 rpm for 10 minutes at 4°C. The ethanol was removed and the pellets were air-dried for 15 minutes. The samples were resuspended in 60 µL of 5mM Tris buffer and stored at 4°C.

DNA quantification was performed using a fluorescence-based real-time polymerase chain reaction system (ABI PRISM 7900 Sequence Detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, Calif). The sample DNA concentration was determined by amplifying genomic DNA-specific β-actin. The forward primer, reverse primer, and probe sequences, respectively, for β-actin were as follows: AGGTTGGGGAAGTTTGTGTTTTG; CCACCAACCACACAATA; and 6FAM-TGGGGTGGTGATGGAGGAGTTTAMRA.

The polymerase chain reaction mixture consisted of 1200nM each primer, 200nM probe, 0.4 U of AmpliTaq Gold Polymerase, 200nM each deoxyadenosine triphosphate, 2-deoxycytidine-5-triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, 3.5mM magnesium chloride, and 1× TaqMan Buffer A containing a reference dye to a final volume of 20 µL (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. Final sample DNA concentration was calculated in nanograms per milliliter by referencing the amplification characteristics of serially diluted commercial genomic DNA with known concentrations.

STATISTIC ANALYSIS

Standard statistical descriptive techniques were used to analyze the healthy subjects' plasma DNA levels. The 95th percentile was calculated and used for the upper limit of normal values. Patient DNA was compared with the normal values using the Wilcoxon signed rank method. Differences in groups were analyzed using the nonparametric technique (Kruskal-Wallis) and a *P* value of .05 was used to determine statistical significance. Values reported are medians and interquartile ranges unless otherwise specified. Statistics were performed using Stata 9 (StataCorp, College Station, Tex).

RESULTS

Normal plasma DNA levels are shown in **Figure 1**. The 95th percentile in healthy subjects was 19 ng/mL. Prior to esophagectomy, patients with cancer had significantly higher levels of plasma DNA compared with healthy subjects (*P* = .001), and 38 (84%) of 45 patients exceeded the normal level.

Of the 12 patients who received neoadjuvant therapy, 11 had plasma DNA levels higher than normal prior to surgery. Six patients were found to have systemic metastasis at the time of surgery and were not resected. Four had liver nodules, 1 had pulmonary nodules, and 1 had peritoneal implants. Plasma DNA levels were higher in unresectable than resectable patients, and both were higher than healthy subjects (**Figure 2**) (*P* < .001 and *P* < .001, respectively). No association was evident be-

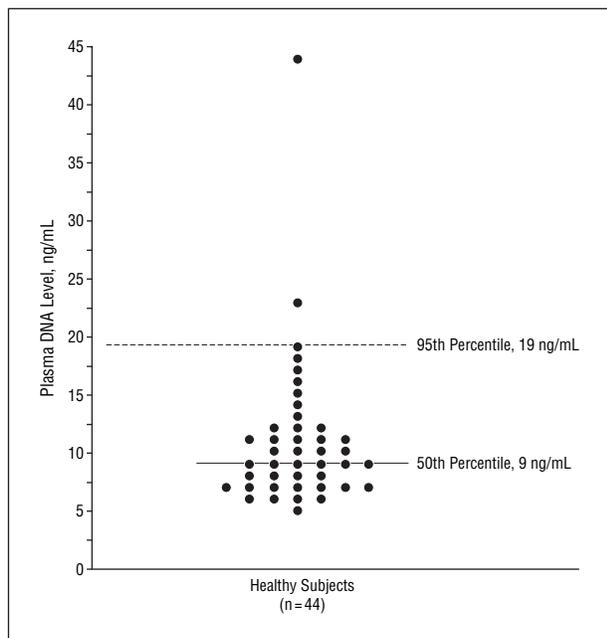


Figure 1. Scatterplot of plasma DNA levels in healthy subjects.

tween plasma DNA levels and tumor size, depth of the tumor penetration, and the number of lymph nodes involved.

At initial follow-up after esophagectomy, plasma DNA levels dropped lower than or remained normal in 18 (90%) of 20 patients (**Figure 3**). In 2 patients (10%), plasma DNA levels remained higher than normal at first follow-up after esophagectomy, and in both patients, evidence of disease was found in the liver on CT and PET scan on subsequent follow-up. Both patients received chemotherapy and subsequently died of disseminated esophageal cancer.

Of the 18 patients who had normal plasma DNA levels on the first follow-up after esophagectomy, the plasma DNA levels rose higher than normal in 4 at subsequent follow-up, and all 4 showed evidence of recurrent disease on CT or PET scan. One patient had enlarged left supraclavicular lymph nodes (plasma DNA level rose from 14 ng/mL on follow-up 1 to 30.3 ng/mL on follow-up 2), one had enlarged periaortic lymph nodes (plasma DNA level rose from 7 ng/mL on follow-up 1 to 66 ng/mL on follow-up 3), one had enlarged celiac lymph nodes (plasma DNA level rose from 9 ng/mL on follow-up 1 to 31.4 ng/mL on follow-up 4 and 52.2 ng/mL on follow-up 5), and one had multiple pulmonary nodules (plasma DNA level rose from 10 ng/mL on follow-up 1 to 35.9 ng/mL on follow-up 4 to 37.3 ng/mL on follow-up 5).

In the remaining 14 patients with normal plasma DNA levels, there was no evidence of recurrent disease on CT and/or PET scan after a median follow-up of 18 months (range, 6-25 months). Ten of these 14 patients subsequently had further plasma DNA level determinations, and in all 10, plasma DNA levels remained normal and all were free of disease on CT and PET scan at a median follow-up of 12 months (range, 3-20 years).

Overall, 12 of 45 patients developed systemic disease: 6 discovered at surgery, 2 at initial follow-up, and

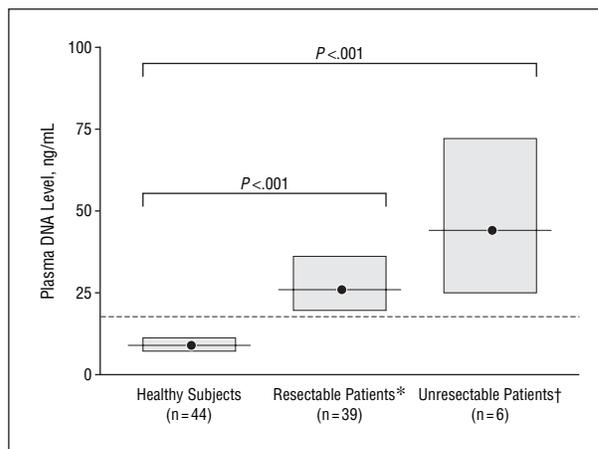


Figure 2. Plasma DNA levels of healthy subjects in comparison with patients with resectable and unresectable esophageal cancer. *Local/regional (resectable) disease; †Systemic metastases (unresectable) disease. Dotted line represents 95th percentile plasma DNA level of healthy subjects.

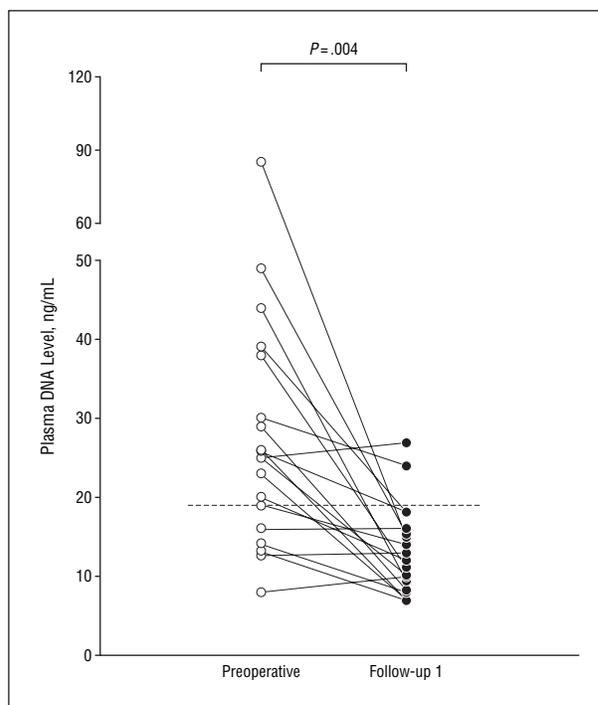


Figure 3. Plasma DNA levels before and at the initial follow-up after esophagectomy in 20 resected patients.

4 at subsequent follow-up. All had elevated plasma DNA levels. Of the 20 patients who had sequential plasma DNA levels, 6 developed systemic disease during the follow-up (2 had persistently elevated plasma DNA levels at initial follow-up, and 4 developed elevated plasma DNA levels at subsequent follow-ups). In 4 of these 6 patients, elevated plasma DNA levels were detected prior to imaging evidence of disease.

In summary, all the patients who developed systemic disease had elevated DNA levels and all patients with elevated plasma DNA levels on follow-up had recurrence of persistent disease. All patients with a normal plasma DNA level on follow-up were free of disease.

Previous studies have shown that plasma DNA levels can be measured in healthy subjects. Steinman¹⁶ reported a plasma DNA level between 10 and 30 ng/mL in healthy subjects and Leon et al¹⁷ reported a 93rd percentile value in healthy subjects of 25 ng/mL. These values are similar to our 95th percentile value of 19 ng/mL.

Evidence is emerging that plasma DNA levels higher than normal are a common event in solid organ malignancies; Silva et al¹⁸ reported a mean plasma DNA level in breast cancer of 135 ng/mL, Sozzi et al¹⁹ reported a median level of 24.3 ng/mL in lung cancer, Ren et al²⁰ reported a mean level of 47.1 ng/mL in liver cancer, and Boddy et al,²¹ a median level of 27.1 ng/mL in prostate cancer. Our study shows that elevated DNA levels also occur in esophageal cancer. Consequently, plasma DNA levels may provide a means to quantify the extent of tumor spread, the completeness of resection, and the recurrence of tumor following complete resection of solid organ malignancies.

Despite the common finding of elevated plasma DNA levels in solid organ cancers, we were unable to find an association between the plasma DNA levels with tumor size, depth of tumor penetration, or the number of involved lymph nodes removed. Similarly, Leon et al¹⁷ showed no association of DNA levels with tumor size and location but did show, as we did, significantly higher DNA levels in patients with metastasis. The conclusion from the earlier-mentioned studies is that plasma DNA levels are elevated in patients with solid organ malignancies and are higher when systemic metastasis occurs.

Contrary to our study and all the earlier-mentioned studies, Herrera et al²² found no difference between plasma DNA levels of asymptomatic volunteers and patients with gastroesophageal reflux disease vs patients with esophageal cancer. They had a similar population (with 32 adenocarcinoma and 6 squamous) and used the same human β -actin gene as our study. An explanation for the discrepancy in their results and ours may be due to the dissimilarity between their control group size and composition. The number of asymptomatic volunteers in their study was 11 compared with 44 in our study. Furthermore, 51 subjects in their control group were patients with gastroesophageal reflux disease, whereas our control group contained no patients and comprised entirely asymptomatic volunteers. In our study, all the blood samples were collected prospectively, whereas in the Herrera et al study,²² 20 of the 58 samples analyzed were from banked blood. None of the patients in their study received neoadjuvant therapy prior to initial blood draws; meanwhile, 12 of our patients had completed neoadjuvant therapy at the time of initial blood draw.

We found that patients with esophageal cancer had significantly higher levels of plasma DNA and that after complete resection of the tumor, the plasma DNA level returned to normal. Failure to do so would indicate that the resection was incomplete. Indeed, the 10% of patients who had persistently elevated plasma DNA levels on their first follow-up visit after esophagectomy had residual disease identified.

The development of an elevated plasma DNA level during follow-up indicated recurrent disease. This occurred in 22% of our patients who were followed up sequentially with plasma DNA levels and recurrent disease was found in all. In the majority of these patients, the elevated plasma DNA level preceded imaging evidence of disease.

Taken together, these findings suggest that the plasma DNA level is a valuable tool for the surgeon. It can raise concerns about possible occult systemic metastasis in patients with high levels prior to surgery. A return of the plasma DNA level to normal after esophagectomy indicates an R0 resection, whereas persistent elevated levels suggest incomplete resection. A rise in the plasma DNA level higher than normal during follow-up in patients who previously had normal levels after surgery suggests recurrent disease. In these patients, there may be a need for chemotherapy despite normal postoperative CT or PET scan results.

The origin of the plasma DNA is unknown. Tissue and cell injury do take place under normal physiological and pathological conditions and it can be expected that some intracellular material such as DNA may be released into the circulation under both conditions. Indeed, DNA can be detected in blood samples from patients with nonmalignant disease, such as systemic lupus erythematosus and rheumatoid arthritis, or after surgical trauma. Further, Anker et al²³ showed that human blood lymphocytes in culture spontaneously release DNA without previous stimulation. They hypothesized that, in healthy volunteers, circulating DNA is released from lymphocytes and other nucleated cells.²⁴

It is not known why patients with cancer have elevated levels of plasma DNA. Several hypotheses have been mentioned in the literature, including one proposed by Schwarzenbach et al²⁵ that postulates that a large proportion of the plasma DNA in patients with cancer is derived from tumor cells. Derivation of plasma DNA directly from tumor, however, is only part of the equation, since Stroun et al²⁶ showed that in a portion of the plasma DNA no tumor-related markers could be found. Consequently, a portion of plasma DNA presumably originates from nonmalignant host cells. Plasma DNA could be released from necrotic tumor cells. Support for this hypothesis is the observation that higher DNA levels have been found in patients with larger tumors.²⁷ Opposed to this hypothesis is the observation that the plasma DNA level decreases in response to radiation therapy.¹⁷ One would expect that effective therapy would increase the plasma DNA level because of the destruction of tumor cells. Circulating DNA in the plasma of patients with cancer could derive directly from micrometastasis shed from the tumor directly into the circulation, but Giacona et al²⁸ showed that in pancreatic cancer, thousands of tumor cells need to be present in 1 mL of plasma for the plasma DNA level to be detectable, which far exceeds the usual number of circulating micrometastasis found in patients with cancer. Finally, if DNA is released from the host cells in response to the pressure of the tumor on the surrounding host tissue, one would expect that larger tumors would have higher plasma DNA levels. Our and several other reported studies do not support this

hypothesis in that all have not found an association between the size of tumor and the plasma DNA level.

In summary, analysis of circulating DNA in plasma is a promising noninvasive diagnostic tool. It requires only a limited blood sample and a relatively simple blood test based on a single marker and is potentially applicable to large-scale trials. It requires specialized technical skills for isolation and extraction of DNA, and careful attention must be given during the isolation of the plasma not to include the nucleated cells, which could give an erroneous result. Hemolyzed specimens should not be used since hemolysis results in cell death and may falsely increase plasma DNA levels. We used one centrifugation system, but a 2-step centrifugation including microcentrifugation has been used by other investigators.²⁹ The fact that we had follow-up in only 20 patients (51% of resected patients) may result in a selection bias and decrease the power of the study, but we believe that this tumor marker has the potential to enable health care professionals to identify the group of patients with residual disease or early recurrent disease after esophagectomy. A larger number of patients, longer follow-up, and standardization and refinement of DNA isolation and extraction techniques would be necessary to consider this molecular marker as a standard diagnostic tool in the diagnosis and treatment of patients with esophageal cancer.

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Correspondence: Tom R. DeMeester, MD, Department of Surgery, Keck School of Medicine, University of Southern California, Los Angeles, 1950 San Pablo, 514, Los Angeles, CA 90033 (demeester@surgery.usc.edu).

Author Contributions: *Study concept and design:* Banki, Mason, K. D. Danenberg, P. V. Danenberg, and DeMeester. *Acquisition of data:* Banki, Oh, Hagen, DeMeester, Lipham, Tanaka, Yacoub, and DeMeester. *Analysis and interpretation of data:* Banki, Mason, Hagen, Tanaka, K. D. Danenberg, P. V. Danenberg, and DeMeester. *Drafting of the manuscript:* Banki. *Critical revision of the manuscript for important intellectual content:* Mason, Hagen, DeMeester, Lipham, Tanaka, K. D. Danenberg, P. V. Danenberg, and DeMeester. *Statistical analysis:* Mason and Hagen. *Obtained funding:* K. D. Danenberg. *Administrative, technical, and material support:* Banki, Tanaka, and DeMeester.

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REFERENCES

1. Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med*. 2003;349:2241-2252.
2. Pisani P, Parkin DM, Bray F, Ferlay J. Erratum: estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer*. 83, 18-29 (1999). *Int J Cancer*. 1999; 83:870-873.
3. Bollschweiler E, Wolfgarten E, Gutschow C, Holscher AH. Demographic variations in the rising incidence of esophageal adenocarcinoma in white males. *Cancer*. 2001;92:549-555.
4. Hagen JA, DeMeester SR, Peters JH, Chandrasoma P, DeMeester TR. Curative resection for esophageal adenocarcinoma: analysis of 100 en bloc esophagectomies. *Ann Surg*. 2001;234:520-530, discussion 530-531.
5. Altorki N, Skinner D. Should en bloc esophagectomy be the standard of care for esophageal carcinoma? *Ann Surg*. 2001;234:581-587.
6. Visbal AL, Allen MS, Miller DL, Deschamps C, Trastek VF, Pairlero PC. Ivor Lewis esophagogastrectomy for esophageal cancer. *Ann Thorac Surg*. 2001;71:1803-1808.
7. Nakagawa S, Kanda T, Kosugi S, Ohashi M, Suzuki T, Hatakeyama K. Recurrence pattern of squamous cell carcinoma of the thoracic esophagus after extended radical esophagectomy with three-field lymphadenectomy. *J Am Coll Surg*. 2004;198:205-211.
8. Heidemann J, Schilling MK, Schmassmann A, Maurer CA, Buchler MW. Accuracy of endoscopic ultrasonography in preoperative staging of esophageal carcinoma. *Dig Surg*. 2000;17:219-224.
9. Räsänen JV, Sihvo EI, Knuuti MJ, et al. Prospective analysis of accuracy of positron emission tomography, computed tomography, and endoscopic ultrasonography in staging of adenocarcinoma of the esophagus and the esophagogastric junction. *Ann Surg Oncol*. 2003;10:954-960.
10. Wu LF, Wang BZ, Feng JL, et al. Preoperative TN staging of esophageal cancer: comparison of miniprobe ultrasonography, spiral CT and MRI. *World J Gastroenterol*. 2003;9:219-224.
11. Flanagan FL, Dehdashti F, Siegel BA, et al. Staging of esophageal cancer with 18F-fluorodeoxyglucose positron emission tomography. *AJR Am J Roentgenol*. 1997;168:417-424.
12. Skehan SJ, Brown AL, Thompson M, Young JE, Coates G, Nahmias C. Imaging features of primary and recurrent esophageal cancer at FDG PET. *Radiographics*. 2000;20:713-723.
13. Lerut T, Flamen P, Ectors N, et al. Histopathologic validation of lymph node staging with FDG-PET scan in cancer of the esophagus and gastroesophageal junction: a prospective study based on primary surgery with extensive lymphadenectomy. *Ann Surg*. 2000;232:743-752.
14. Luketich JD, Friedman DM, Weigel TL, et al. Evaluation of distant metastases in esophageal cancer: 100 consecutive positron emission tomography scans. *Ann Thorac Surg*. 1999;68:1133-1136, discussion 1136-1137.
15. Rankin SC, Taylor H, Cook GJ, Mason R. Computed tomography and positron emission tomography in the pre-operative staging of oesophageal carcinoma. *Clin Radiol*. 1998;53:659-665.
16. Steinman CR. Free DNA in serum and plasma from normal adults. *J Clin Invest*. 1975;56:512-515.
17. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res*. 1977;37:646-650.
18. Silva JM, Silva J, Sanchez A, et al. Tumor DNA in plasma at diagnosis of breast cancer patients is a valuable predictor of disease-free survival. *Clin Cancer Res*. 2002;8:3761-3766.
19. Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol*. 2003;21:3902-3908.
20. Ren N, Ye QH, Qin LX, Zhang BH, Liu YK, Tang ZY. Circulating DNA level is negatively associated with the long-term survival of hepatocellular carcinoma patients. *World J Gastroenterol*. 2006;12:3911-3914.
21. Boddy JL, Gal S, Malone PR, Harris AL, Wainscoat JS. Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. *Clin Cancer Res*. 2005;11:1394-1399.
22. Herrera LJ, Raja S, Gooding WE, et al. Quantitative analysis of circulating plasma DNA as a tumor marker in thoracic malignancies. *Clin Chem*. 2005;51:113-118.
23. Anker P, Stroun M, Maurice P. Spontaneous release of DNA by living cells [proceedings]. *Riv Istochim Norm Patol*. 1976;20:111.
24. Anker P, Stroun M, Maurice PA. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Res*. 1975;35:2375-2382.
25. Schwarzenbach H, Muller V, Stahmann N, Pantel K. Detection and characterization of circulating microsatellite-DNA in blood of patients with breast cancer. *Ann N Y Acad Sci*. 2004;1022:25-32.
26. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology*. 1989; 46:318-322.
27. Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer*. 1983; 51:2116-2120.
28. 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.
29. Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM, Lo YM. Effects of blood-

processing protocols on fetal and total DNA quantification in maternal plasma. *Clin Chem.* 2001;47:1607-1613.

30. Jahr S, Hentz H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantifications and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* 2001;61:1659-1665.

DISCUSSION

Mark Talamonti, MD, Chicago, Ill: The authors present data examining the relative presence of circulating plasma DNA between 44 normal healthy volunteers and 45 patients with various stages of esophageal cancer. After establishing statistically significant differences in the median plasma levels of circulating DNA between normals, patients with resectable disease, and patients with advanced disease, the authors then test the utility of this biomarker in the staging of esophageal cancer as a measure of completeness of resection and as a marker for recurrence after esophagectomy. Their data suggest the quantity of free plasma circulating DNA after resection may have prognostic significance. The fact that the assay is relatively straightforward, inexpensive, and can be done on a small sample of blood makes this a potentially useful biomarker and worthy of investigation. I have 3 questions for the authors.

First, plasma DNA levels were generally sensitive in distinguishing between normals, patients with resectable tumors, and patients with metastatic or advanced disease, yet little was mentioned of any correlation with tumor size, tumor stage, or nodal status. While being able to predict residual or recurrent disease is impressive, its value is somewhat limited by the fact that we do not yet have effective salvage therapy for these patients. More valuable would be the ability of the test to provide pretreatment molecular staging, since many of these patients are now being considered for neoadjuvant clinical trials. So was there any correlation between preoperative DNA levels and postoperative pathologic tumor and nodal staging in patients who were not treated preoperatively with chemoradiation? Which also leads to my second question: 2 recent longitudinal studies from the MD Anderson Cancer Center and a second from the Memorial Sloan-Kettering Cancer Center have demonstrated that the extent of pathologic downstaging to neoadjuvant therapy is highly significant and an independent predictor of both disease-free survival and overall survival. Twelve of your patients received neoadjuvant therapy, and following treatment, 11 of the 12 still had levels above normal prior to surgery. In these 12 patients, to what degree did plasma DNA levels change before and after neoadjuvant therapy and did this delta correlate with the extent of downstaging between your pretreatment clinical tumor and nodal stage and the final pathologic tumor and nodal stage? If so, then this test might become a useful predictor of survival as a molecular measurement of treatment response to neoadjuvant therapy.

Similarly, some of the patients in your series developed recurrent disease or were found to have metastatic disease at operative staging. I imagine that some of these patients then went on to systemic chemotherapy treatments under the direction of your medical oncologist. Did you see any treatment response to plasma DNA levels after the patients were started on systemic chemotherapy?

Finally, my last question has to do with the remarkable association between persistently elevated plasma DNA levels after resection and residual disease or levels that rose on follow-up and the ultimate manifestation of recurrent disease. Now, circulating free plasma DNA levels have been examined in a variety of tumor types and in different tumor types than esophageal cancer, and when larger numbers of patients have been examined, the statistical correlations seen in the current study have not always been consistent or reproducible. In response to these variations, other investigators have suggested the need

to more specifically characterize the DNA in the plasma. For example, the quantity of a gene-specific DNA level or hypermethylated DNA level may be a more accurate prognostic marker than overall free plasma DNA levels. I would like to know if the authors have any plans or data using something like this to improve the accuracy of this marker and perhaps to hint at mechanisms and future therapeutic targets responsible for elevated plasma DNA levels.

Dr Banki: To answer question 1, there was no association between the plasma DNA level, tumor size, number of lymph nodes involved, and depth of tumor penetration. As Dr Talamonti mentioned, 12 of our patients had preoperative chemotherapy, and we included all those 12 patients in our study. In all those 12 patients, plasma DNA level was measured after chemotherapy was already given. Of the 12 patients who had preoperative chemotherapy, 11 had elevated plasma DNA levels. Seventy-five percent of these 12 patients had tumor penetration of T3 and above vs 7 patients who did not receive preop chemotherapy and all 7 had normal DNA levels. Four of these 7 without preop chemotherapy had a T3 lesion. So overall, we did not find any correlation with DNA level and depth of tumor. And this confirms the data from other studies, which showed no correlation with DNA level tumor size and depth of penetration.

As far as question number 2, we did not have access to the 12 patients who were referred to us after chemotherapy from other centers in the United States, so we did not have the pretreatment plasma DNA levels in those patients. But we are refining our study right now by measuring plasma DNA level in patients who have positive lymph nodes and are receiving postoperative chemotherapy. We have found that in patients who are responding to chemotherapy, plasma DNA level decreases, and in the patients who are not responding to chemotherapy, the plasma DNA increases. An example of those are the 2 patients who had disseminated esophageal cancer; despite chemotherapy, both patients had elevated plasma DNA at subsequent follow-up.

To answer the third question, we were planning to first evaluate the role of plasma DNA level in esophageal cancer and to better understand this phenomenon. Our goal was to identify a molecular marker that is easy to measure and to measure the global DNA increase in plasma in response to tumor. There have been studies, such as one by Jahr et al³⁰ mentioned in the paper that measured the DNA methylation, that have measured the loss of heterozygosity and microsatellite instability as tumor-specific DNA, but our focus was to measure the global DNA and begin to understand this phenomenon.

Jeffrey H. Peters, MD, Rochester, NY: Nicely done, Farzaneh, and really remarkable data. I can think of half a dozen ways to use it clinically if it turns out to be true. If plasma DNA doesn't correlate with tumor size or nodal status, what is it that is responsible for the findings? Is it the biology of the tumors? Is it the burden of the tumors? Or is it just the fact that the patient may harbor systemic disease that is causing the plasma DNA to go up? Do you have any insight into these issues?

Dr Banki: The plasma DNA doesn't seem to correlate with TN [tumor/node] status but correlates with M [metastasis] status, so in the presence of systemic metastatic disease, the plasma DNA level increases. The cause of this increase remains unknown. In the paper, we mentioned some theories that can explain the cause of this increase, but they are all hypotheses.

One of them is, as you mentioned, the pressure of a tumor on the surrounding tissue can cause cell death and increase the plasma DNA level, but in our study, there was no correlation with T level. Another theory is the host response to tumor, which may result in an increase in plasma DNA. Another is the release of tumor cells directly into the blood. Some studies have shown that one needs thousands and thousands of tumor cells in the blood for the DNA to be detectable. The mechanism of

increase for plasma DNA level in the presence of tumor remains unknown. It seems that in the presence of systemic disease the plasma DNA is elevated, but the absence of any correlation with tumor size, depth of penetration, and lymph node involvement remains unclear.

Wayne H. Schwesinger, MD, San Antonio, Tex: Do you have large enough numbers to feel confident that you have no correlation with size?

Dr Banki: We had 45 patients involved in the study. But there are other different studies with larger populations including different types of cancer that have shown the same result, namely that the plasma DNA correlates with metastatic or systemic disease but does not necessarily correlate with the size of the tumor or depth of penetration.

Stephen G. Jolley, MD, Anchorage, Alaska: You touched a little bit on, perhaps speculation, a fairly significant false-negative information for your DNA testing. Do you have any patients who have systemic disease who have normal serum DNA levels?

Dr Banki: All the patients with systemic disease had abnormal DNA levels, including those 6 patients initially and 6 patients postoperatively. In those 6 patients who had normal plasma DNA level, none of them had systemic disease.

Dr Jolley: My second question—I know your numbers are kind of small—do you see any difference in the serum DNA levels between the adenocarcinomas and the squamous cell carcinomas?

Dr Banki: We considered the group as a whole; we did not distinguish between adenocarcinoma or squamous carcinoma, because we had only 3 squamous patients. And I don't think that the number of 3 vs 42 would be enough to confer any statistical difference or relevance.

David W. Easter, MD, San Diego, Calif: It looks like systemic burden correlates with plasma DNA levels. Did systemic therapy correlate or confound your results?

Dr Banki: For the preoperative patient who had chemotherapy, we measured plasma DNA level after chemotherapy was given. But postoperatively, the patients who were treated with chemotherapy and who responded to chemotherapy showed a decrease in plasma DNA and those who did not respond to chemotherapy showed an increase in plasma DNA, including those patients who died of disseminated esophageal cancer.

Dr Easter: So does treatment systemically increase plasma DNA levels?

Dr Banki: There have been some studies that show that effective radiation therapy decreases the plasma DNA level. One may think that effective chemoradiation therapy causes tumor cell death and therefore should increase plasma DNA level. We addressed that in the paper. There are studies that show chemotherapy decreases the plasma DNA level because the tumor burden decreases. So this is the controversy that still needs to be answered.

Robert Sticca, MD, Grand Forks, ND: Have you considered looking at this in patients with unresectable metastatic disease as an indicator of response to chemotherapy?

Dr Banki: Yes, we are refining our study by measuring the plasma DNA level in patients with metastatic esophageal cancer who are receiving definitive chemotherapy and in patients with positive lymph nodes who are receiving adjuvant therapy. Our goal is to use the plasma DNA level as a marker for response to chemotherapy as well as a prognostic marker.

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