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 initial 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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Prior studies have shown that a plasma DNA level is present in healthy individuals. DNA levels are significantly higher in patients with malignancies such as breast, lung, liver, and prostate. 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.
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 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. Steinman\(^6\) reported a plasma DNA level between 10 and 30 ng/mL in healthy subjects and Leon et al\(^7\) 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 al\(^8\) reported a mean plasma DNA level in breast cancer of 135 ng/mL, Sozzi et al\(^9\) reported a median level of 24.3 ng/mL in lung cancer, Ren et al\(^10\) reported a mean level of 47.1 ng/mL in liver cancer, and Boddy et al\(^11\), 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 al\(^7\) showed no association of DNA levels with tumor size and location but did show, as did we, 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 al\(^22\) 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 \(\beta\)-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 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 al\(^23\) 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 al\(^25\) 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 al\(^26\) 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 al\(^28\) 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
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. The fact that we had follow-up in only 20 patients (51% of selected 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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REFERENCES

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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 pre-treatment 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 post-operative 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 change 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 such as 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 chemotheraphy, 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. For 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 post-operative chemotheraphy. 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 on DNA content of mononuclear phagocytes, but we are working on refining the cell population 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.

**Jefrey 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 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

**Mark Talamonti, MD, Chicago, Ill:** The authors present data examining the relative presence of circulating plasma DNA between 44 normal healthy volunteers and 43 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.

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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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