

Lung Cancer-related Genes in the Blood

Makoto Sonobe, MD, Fumihiko Tanaka, MD, and Hiromi Wada, MD

Tumor-related genes can be found circulating in the blood of cancer patients. These genes may be derived from circulating cancer cells or from the patient's primary tumor directly by a process referred to as "gene shedding." Selective and sensitive detection of tumor-related genes in the blood of cancer patients has been made possible by the advent of polymerase chain reaction-based technology that can detect mutations, polymorphisms, microsatellite instability, loss of heterozygosity, and promoter hypermethylation. Several reports have documented the clinical potential of using circulating tumor-related genes as a molecular marker for the early detection of lung cancer, and as a prognostic tool in these patients; larger, prospective studies will be needed to test the feasibility of this approach. Certainly, such an approach in lung cancer patients would be attractive since it is noninvasive and employs relatively easy and rapid methodologies. (Ann Thorac Cardiovasc Surg 2004; 10: 213–7)

Key words: lung cancer, blood, circulating gene, "shed" gene, PCR

Introduction

It has been reported that both free DNA and RNA are present in the blood of cancer patients, and that their concentration increases in patients whose cancer has metastasized.¹⁻³⁾ The increased presence of circulating DNA and RNA is not in and of itself specific to cancer patients, since it is also found in patients with inflammation, infection, or autoimmune disease such as systemic lupus erythematosus.⁴⁾ Recent advances in biochemical technology have made it possible to selectively detect very small amounts of tumor-related genes in the blood including tumor-specific genes, tumor-specific gene alterations, and tumor-specific gene expression. Many of the increasing number of reports in this area have suggested that the ability to selectively detect tumor-related genes in the blood may be useful in the management of cancer patients.

From Department of Thoracic Surgery, Kyoto University Hospital, Kyoto, Japan

Received March 30, 2004; accepted for publication April 30, 2004. Address reprint requests to Makoto Sonobe, MD: Department of Thoracic Surgery, Kyoto University Hospital, Shogoin Kawaracho 54, Sakyo-ku, Kyoto City, Kyoto 606-8507, Japan.

In this article, we review which tumor-specific genes have been found in circulating blood and discuss the methods used for detecting those genes. We also discuss the clinical potential of this technique in diagnosing lung cancer, providing information necessary in formulating a follow-up strategy, and predicting the patients' prognosis.

Origin of Genes in the Blood

Microsatellite instability (MI), which was demonstrable in small^{5,6)} and non-small⁶⁻⁸⁾ cell lung carcinoma tumors, was also found in the circulating DNA of patients with these neoplasms. Allan et al. reported that the loss of allelic heterozygosity (LOH) in circulating genes in patients with lung cancer corresponded to the LOH seen in their primary tumor.⁹⁾ Usadel et al. reported that promoter hypermethylation of the APC1A gene was detectable in 47% of the serum/plasma samples of 89 patients with lung cancer that had promoter hypermethylation of the APC1A gene, but not in the sera of 50 healthy controls.¹⁰⁾ Ramirez et al. similarly demonstrated that the patterns of promoter hypermethylation in the serum free DNA of 51 lung cancer patients correlated highly with the patterns seen in the lung cancer cells themselves.¹¹⁾ However, tumor-related alterations in circulating genes in cancer patients

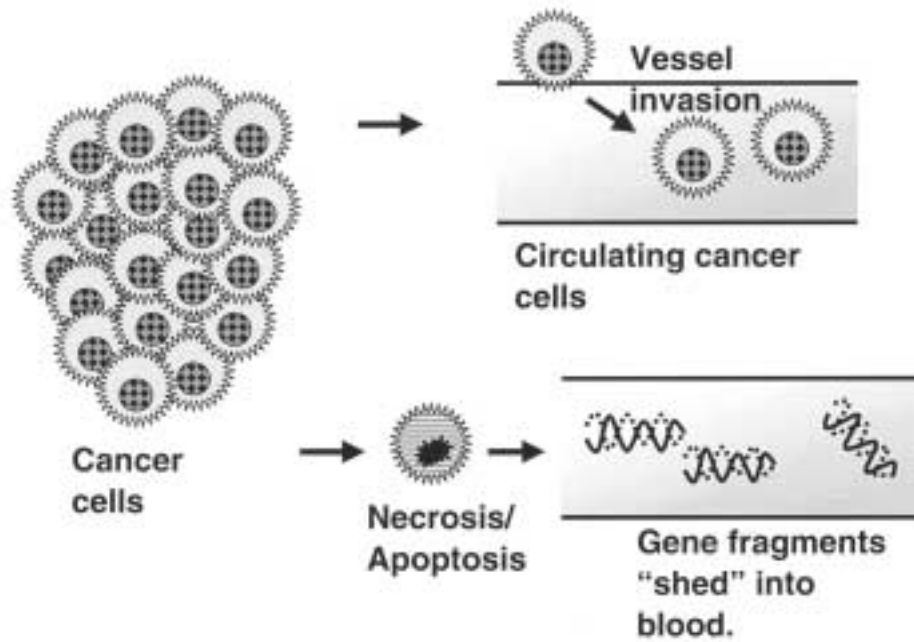


Fig. 1. Two theories as to the source of circulating tumor-related genes.

have not always corresponded to the modification seen in the original tumor.^{12,13} Smokers exhibit the same DNA methylation in their normal and precancerous tissues as seen in lung cancer tissue, suggesting that smoking may directly induce the methylation of circulating DNA.¹⁴ Nevertheless, alterations in circulating genes appear to reflect the presence of cancer, having likely originated, at least in the case of lung cancer, from the original tumor.

The presence of cancer-related genes in the blood can most readily be explained in one of two ways¹⁵ (Fig. 1). It may be that these genes originated in circulating cancer cells. Though there is no direct evidence supporting this view in patients with lung cancer, the mRNAs of several genes (e.g., the epidermal growth factor receptor and carcinoembryonic antigen genes), which are rarely detected in healthy persons, have been detected in the blood of lung cancer patients using the reverse transcription polymerase chain reaction (PCR) method.¹⁶⁻¹⁹ These data suggest that circulating lung cancer cells were the source of these mRNAs. Free DNA may also be derived from circulating cancer cells.

Alternatively, tumors may shed their genes into the blood. Some of the cells in primary lung tumors undergo apoptotic or necrotic cell death. As a result, genomic DNA from these cells may enter the circulation, though there is no direct evidence of such “gene shedding.” However, Silva et al. did report that the plasma of 44% of breast

cancer patients that was collected before mastectomy had some tumor-related DNA alterations, while only 19.5% of patients showed these alterations in plasma that was collected after mastectomy.²⁰ This reduced rate of DNA alterations after mastectomy indirectly supports the “gene shedding” hypothesis.

Detection of Circulating Tumor-related Genes in the Blood

Several PCR assays, described below, can be used to detect tumor-related genes in the blood.

Detection of mutated genes

Several methods have been used to detect cancer-related gene mutations such as in K-ras codons 12 and 13 and p53. Gene mutations such as point mutations, deletions, and insertions can be detected by single-strand conformation polymorphism analysis,²¹ the mutant-enriched PCR method which applies the principle of restriction fragment length polymorphisms,²² or mutation allele specific PCR.²³

Detection of MI and LOH

Microsatellites are stretches of variable length DNA that consist of mono-, di-, tri-, tetra-, penta-, or hexanucleotide repeats. While rarely mutated in normal cells,

microsatellites are often mutated in tumor cells where such mutations are referred to as MI. Such instability is a common genetic alteration in lung cancer.²⁴⁻²⁶ LOH refers to the deletion of one of two copies of chromosomal allelic DNA sequences. In lung cancer, LOH in regions of chromosomes 3, 5, 9, 13, or 17 is frequently observed.²⁷⁻³⁶

Electrophoresis of the PCR products of microsatellite markers revealed MI in one or more new bands, as well as LOH as revealed by the significant reduction in the density of the allelic band. Chen et al. were the first to report the presence of MI⁵⁾ and Sanchez-Cespedes et al. were the first to report LOH analysis in the circulating DNA of lung cancer patients.⁷⁾

Methylation-specific PCR (MSP) for detecting gene promoter hypermethylation

Many human cancers, including lung cancer, exhibit promoter hypermethylation in several tumor suppressor genes. CpG islands i.e., the clustering of CpG dinucleotides in small regions of DNA, are found in promoter regions of almost half of the genes in the genome, including tumor-suppressor genes. Hypermethylation of normally unmethylated cytosines in CpG islands in promoter regions leads to the loss of transcription of the gene which has been referred to as "gene silencing."³⁷⁾ Promoter hypermethylation of several tumor-suppressor genes including p16ink4a, O6-methylguanine-DNA methyltransferase, death-associated protein kinase, and E-cadherin, among others, is common in lung cancer.³⁸⁾

Promoter hypermethylation can be detected using MSP, which was first introduced by Herman et al.³⁹⁾ In this technique, sample DNA is initially treated with sodium bisulfite to convert unmethylated, but not methylated, cytosines to uracil. PCR with primers specific for methylated DNA can amplify methylated DNA separately from unmethylated DNA, and conversely. This method can detect one methylated copy in 1,000 unmethylated copies, which is a sufficiently high sensitivity for the detection of small amounts of tumor-related genes in blood. Esteller et al.⁴⁰⁾ first employed this technique to detect aberrant promoter hypermethylation in tumor suppressor genes in serum DNA from non-small cell lung cancer patients.

Clinical Implications

Detection of tumor-related genes in the blood is a promising new approach that has the advantages of easy access, minimal invasiveness, and rapid analysis,^{41,42)} though

several problems do exist.

Early detection of lung cancer

Whether the detection of tumor-related genes in the blood can be used to diagnose lung cancer at an early stage has not been fully evaluated. By analyzing microsatellite alterations, 40% to 100% of patients with small cell carcinoma were shown to have altered circulating DNA,^{5,6,12)} while 28% to 77% of patients with non-small cell carcinoma showed such anomalies,^{6,7,12,43)} Promoter hypermethylation was reported to be present in the circulating DNA in nearly 40% of patients who had lung cancer that displayed promoter hypermethylation.^{10,11,44)} p53 or K-ras mutations were detected in up to 73% of the circulating DNA in lung cancer patients.⁴⁵⁻⁴⁷⁾ The frequency of these gene alterations was independent of lung cancer stage suggesting that, even in early stage lung cancer, nearly 40% of patients demonstrate gene alterations in their blood. Thus, detection of alterations in circulating genes may be useful in diagnosing early stage lung cancer, though its low sensitivity rules out its use alone as a screening tool. However, it could be quite useful when used in combination with several markers such as p53 mutations, FHIT LOH, and 3p LOH, as suggested by Andriani et al.⁴⁷⁾

Prediction of prognosis and use as follow-up marker of lung cancer recurrence

Usadel et al. reported a poor prognosis in lung cancer patients who had high levels of methylated APC genes as detected using quantitative MSP.¹⁰⁾ Gonzalez et al. similarly reported a poor prognosis in small cell lung cancer patients who had both a p53 mutation and MI in their plasma DNA.⁴⁸⁾ Finally, Ramirez et al. reported a poor prognosis in cancer patients who had K-ras mutations in their serum DNA but no correlation between the patients' methylated serum DNA and their prognosis.^{11,49)}

While it seems reasonable to speculate that the prognosis of patients who display tumor-related genes in their blood that originated from the primary tumor would be poor, no evidence currently exists to support this view. The significance of "shed" genes on tumor development also remains unclear. Certainly, these are areas that require further investigation.

Gene analysis of blood collected after curative therapy for lung cancer should contribute to the evaluation of this tool as a predictor of prognosis. Usadel et al. reported that lung cancer recurrence could be detected by increased levels of plasma APC methylation though their data were

preliminary.¹⁰⁾ Sozzi et al. showed that high concentrations of DNA in plasma were predictive of lung cancer recurrence.⁴³⁾ Finally, Silva et al. reported that the persistence of tumor-related DNA in plasma after complete resection of the original breast tumor correlated with histological parameters that were associated with poor outcome.²⁰⁾ These data suggest that quantifying the concentration of tumor-related genes in the blood may be useful as a follow-up tool for the care of lung cancer patients. Large, prospective studies will need to be undertaken to determine the clinical value of blood gene analysis after curative therapy.

References

- 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**: 646–50.
- Shapiro B, Chakrabarty M, Cohn E, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer* 1983; **51**: 2116–20.
- 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**: 318–22.
- Raptis L, Menard HA. Quantitation and characterization of plasma DNA in normals and patients with systemic lupus erythematosus. *J Clin Invest* 1980; **66**: 1391–9.
- Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* 1996; **2**: 1033–5.
- Bruhn N, Beinert T, Oehm C, et al. Detection of microsatellite alterations in the DNA isolated from tumor cells and from plasma DNA of patients with lung cancer. *Ann NY Acad Sci* 2000; **906**: 72–82.
- Sanchez-Céspedes M, Monzo M, Rosell R, et al. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. *Ann Oncol* 1998; **9**: 113–6.
- Sozzi G, Musso K, Ratcliffe C, Goldstraw P, Pierotti MA, Pastorino U. Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect for early diagnosis. *Clin Cancer Res* 1999; **5**: 2689–92.
- Allan JM, Hardie LJ, Briggs JA, et al. Genetic alterations in bronchial mucosa and plasma DNA from individuals at high risk of lung cancer. *Int J Cancer* 2001; **91**: 359–65.
- Usadel H, Brabender J, Danenberg KD, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. *Cancer Res* 2002; **62**: 371–5.
- Ramirez JL, Taron M, Balana C, et al. Serum DNA as a tool for cancer patient management. *Rocz Akad Med Bialymst* 2003; **48**: 34–41.
- Beau-Faller M, Gaub MP, Schneider A, et al. Plasma DNA microsatellite panel as sensitive and tumor-specific marker in lung cancer patients. *Int J Cancer* 2003; **105**: 361–70.
- Beau-Faller M, Weber JC, Schneider A, et al. Genetic heterogeneity in lung and colorectal carcinoma as revealed by microsatellite analysis in plasma or tumor tissue DNA. *Cancer* 2003; **97**: 2308–17.
- Toyooka S, Maruyama R, Toyooka KO, et al. Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer. *Int J Cancer* 2003; **103**: 153–60.
- Stroun M, Maurice P, Vasioukhin V, et al. The origin and mechanism of circulating DNA. *Ann NY Acad Sci* 2000; **906**: 161–8.
- Kurusu Y, Yamashita J, Ogawa M. Detection of circulating tumor cells by reverse transcriptase-polymerase chain reaction in patients with resectable non-small-cell lung cancer. *Surgery* 1999; **126**: 820–6.
- Bessho A, Tabata M, Kiura K, et al. Detection of occult tumor cells in peripheral blood from patients with small cell lung cancer by reverse transcriptase-polymerase chain reaction. *Anticancer Res* 2000; **20**: 1149–54.
- De Luca A, Pignata S, Casamassimi A, et al. Detection of circulating tumor cells in carcinoma patients by a novel epidermal growth factor receptor reverse transcription-PCR assay. *Clin Cancer Res* 2000; **6**: 1439–44.
- Clarke LE, Leitzel K, Smith J, Ali SM, Lipton A. Epidermal growth factor receptor mRNA in peripheral blood of patients with pancreatic, lung, and colon carcinomas detected by RT-PCR. *Int J Oncol* 2003; **22**: 425–30.
- Silva JM, Garcia JM, Dominguez G, et al. Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. *Ann Surg Oncol* 2002; **9**: 71–6.
- Suzuki Y, Orita M, Shiraiishi M, Hayashi K, Sekiya T. Detection of ras gene mutations in human lung cancers by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene* 1990; **5**: 1037–43.
- Kahn SM, Jiang W, Culbertson TA, et al. Rapid and sensitive nonradioactive detection of mutant K-ras genes via 'enriched' PCR amplification. *Oncogene* 1991; **6**: 1079–83.
- Takeda S, Ichii S, Nakamura Y. Detection of K-ras mutation in sputum by mutant-allele-specific amplification (MASA). *Hum Mutat* 1993; **2**: 112–7.
- Lawes DA, SenGupta S, Boulos PB. The clinical importance and prognostic implications of microsatellite instability in sporadic cancer. *Eur J Surg Oncol* 2003; **29**: 201–12.
- Shridhar V, Siegfried J, Hunt J, del Mar Alonso M, Smith DI. Genetic instability of microsatellite se-

- quences in many non-small cell lung carcinomas. *Cancer Res* 1994; **54**: 2084–7.
26. Merlo A, Mabry M, Gabrielson E, Vollmer R, Baylin SB, Sidransky D. Frequent microsatellite instability in primary small cell lung cancer. *Cancer Res* 1994; **54**: 2098–101.
 27. Neuville EM, Stewart MP, Swift A, et al. Allelotype of non-small cell lung cancer. *Int J Oncol* 1996; **9**: 533–9.
 28. Pylkkanen L, Karjalainen A, Anttila S, Vainio H, Husgafvel-Pursiainen K. No evidence of microsatellite instability but frequent loss of heterozygosity in primary resected lung cancer. *Environ Mol Mutagen* 1997; **30**: 217–23.
 29. Endo C, Sagawa M, Sato M, et al. Sequential loss of heterozygosity in the progression of squamous cell carcinoma of the lung. *Br J Cancer* 1998; **78**: 612–5.
 30. Virmani AK, Fong KM, Kodagoda D, et al. Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosomes Cancer* 1998; **21**: 308–19.
 31. Kohno H, Hiroshima K, Toyozaki T, Fujisawa T, Ohwada H. p53 mutations and allelic loss of chromosome 3p, 9p of preneoplastic lesions in patients with non-small cell lung carcinoma. *Cancer* 1999; **85**: 341–7.
 32. Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D. Microsatellite alterations as clonal markers for the detection of human cancer. *Proc Natl Acad Sci U S A* 1994; **91**: 9871–5.
 33. Tomizawa Y, Adachi J, Kohno T, et al. Prognostic significance of allelic imbalances on chromosome 9p in stage I non-small cell lung carcinoma. *Clin Cancer Res* 1999; **5**: 1139–46.
 34. Michelland S, Gazzeri S, Brambilla E, Robert-Nicoud M. Comparison of chromosomal imbalances in neuroendocrine and non-small-cell lung carcinomas. *Cancer Genet Cytogenet* 1999; **114**: 22–30.
 35. Zhou X, Kemp BL, Khuri FR, et al. Prognostic implication of microsatellite alteration profiles in early-stage non-small cell lung cancer. *Clin Cancer Res* 2000; **6**: 559–65.
 36. Sasatomi E, Finkelstein SD, Woods JD, et al. Comparison of accumulated allele loss between primary tumor and lymph node metastasis in stage II non-small cell lung carcinoma: implications for the timing of lymph node metastasis and prognostic value. *Cancer Res* 2002; **62**: 2681–9.
 37. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003; **349**: 2042–54.
 38. Tso JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. *Oncogene* 2002; **21**: 5450–61.
 39. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996; **93**: 9821–6.
 40. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999; **59**: 67–70.
 41. Mao L. Recent advances in the molecular diagnosis of lung cancer. *Oncogene* 2002; **21**: 6960–9.
 42. Hu YC, Sidransky D, Ahrendt SA. Molecular detection approaches for smoking associated tumors. *Oncogene* 2002; **21**: 7289–97.
 43. Sozzi G, Conte D, Mariani L, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res* 2001; **61**: 4675–8.
 44. Bearzatto A, Conte D, Frattini M, et al. p16 (INK4A) hypermethylation detected by fluorescent methylation-specific PCR in plasmas from non-small cell lung cancer. *Clin Cancer Res* 2002; **8**: 3782–7.
 45. Silva JM, Gonzalez R, Dominguez G, et al. TP53 gene mutations in plasma DNA of cancer patients. *Genes Chromosomes Cancer* 1999; **24**: 160–1.
 46. Kovalchuk O, Naumnik W, Serwicka A, Chyczewska E, Niklinski J, Chyczewski L. K-ras codon 12 mutations may be detected in serum of patients suffering from adeno- and large cell lung carcinoma. A preliminary report. *Folia Histochem Cytobiol* 2001; **39** (Suppl 2): 70–2.
 47. Andriani F, Conte D, Mastrangelo T, et al. Detecting lung cancer in plasma with the use of multiple genetic markers. *Int J Cancer* 2004; **108**: 91–6.
 48. Gonzalez R, Silva JM, Sanchez A, et al. Microsatellite alterations and TP53 mutations in plasma DNA of small-cell lung cancer patients: follow-up study and prognostic significance. *Ann Oncol* 2000; **11**: 1097–104.
 49. Ramirez JL, Sarries C, de Castro PL, et al. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. *Cancer Lett* 2003; **193**: 207–16.