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Mutations in *K-ras* Codon 12 Detected in Plasma DNA Are Not an Indicator of Disease in Patients with Non-Small Cell Lung Cancer

To the Editor:

The demonstration that cell-free circulating DNA detected in the plasma of cancer patients is genetically identical to that of the primary tumor has generated substantial interest, leading to >200 publications (http://www.ncbi.nlm.nih.gov). Recently, Wang et al. *(1)* reported in this journal that the method chosen for DNA isolation might contribute significantly to mutation detection (in their case, *K-ras* mutations in the plasma of patients with colorectal cancer). Briefly, they recommended the use of a modified guanidine/Promega resin method (G/R) to isolate DNA, affirming that this method enhances assay sensitivity. We used the same approach to detect *K-ras* mutations in the plasma of patients with non-small cell lung cancer (NSCLC) and compared the Qiagen vs the G/R method for isolation of circulating DNA. We purified DNA from plasma samples and cancer tissues from 12 patients. The DNA in 2 aliquots of the plasma from each patient was isolated by the Qiagen method *(1, 2)* and by the G/R method according to Wang et al. *(1)*. Additionally, DNA was isolated by the Qiagen method from matched plasma and tissue samples (n = 10 for each) and from 76 plasma-only samples (36 from cancer patients and 40 from cancer-free volunteers) according to Kopreski et al. *(2)*. There was no difference in the number of *K-ras* mutations detected in the plasma samples collected from patients (n =15; 41.7%) and from volunteers (n = 12; 30.0%; odds ratio = 1.6; $P = 0.21$). In addition, when we evaluated the presence of *K-ras* mutations in the matched plasma and neoplastic tissue samples, we observed no correlation. Finally, when we compared the results (*K-ras* status) for DNA samples isolated from plasma by the 2 different methods with the results obtained for the DNA isolated from tissue samples (12 patients), we observed *K-ras* codon 12 mutations in 2 different tissue samples, whereas we detected no mutations in plasma DNA isolated with the Qiagen method and 2 mutations different from those identified in the corresponding tumor tissue in the plasma DNA isolated with the G/R method. The correlation between controls and cases was not significant ($\chi^2 = 0.7$; $P = 0.5$). The correlations between results obtained for tissue DNA and for plasma DNA isolated by the Qiagen or G/R method also were not significant ($P = 0.4$ and $P = 1.0$, respectively, nonparametric test for cases vs samples), nor was the correlation between the G/R and Qiagen isolation methods ($P = 0.2$).

These data show no relationship between *K-ras* mutations found in DNA from plasma and tumor tissue from NSCLC patients. Our results do not support the suggestion that *K-ras* mutations detected in plasma DNA are markers for tumor detection.

Ramirez et al. *(3)* analyzed *K-ras* mutations (codon 12) in tumor and paired serum DNA of 51 NSCLC patients undergoing surgery and detected mutations in 9 tumors and 12 serum samples. As suggested by Gautschi and Ziegler *(4)*, serum may contain not only DNA of tumor origin, but also a variable fraction of DNA derived from in vivo- and in vitro-damaged hematopoietic cells. Indeed, lymphocytes stimulated with phytohemagglutinin or antigen may release DNA *(5)*. Furthermore, human leukocytes stimulated by neutrophil-derived hydroxyl radicals may cause activation of *K-ras* codon 12 *(6)*. In addition, inhaled particles in exposed individuals can generate reactive oxygen species that can activate *K-ras (7)*. Moreover, *K-ras* mutations are detected in neogenetic lesions of subpleural fibrotic lesions, including ciliated bronchial epithelium and metaplastic epithelium *(8)*. Because chronic inflammation may be present in patients without cancer as well as those with cancer *(9)*, the analysis of *K-ras* mutations in plasma may be influenced by such factors. Finally, as reported recently by Keohavong et al. *(10)*, *K-ras* mutations are frequently found in histologically normal tissues near tumors, suggesting that such mutations may represent an early event in the development of lung cancer. *K-ras* mutations thus may be present before clinically detectable tumors. As a final point, it is important to note that *K-ras* mutations have been detected in patients with ulcerative colitis, Crohn disease *(11, 12)*, and *Helicobacter pylori*-associated chronic gastritis *(13)*. We observed *K-ras* mutation in 2 volunteers diagnosed with chronic gastritis, in 1 patient with kidney failure, and in 2 who were heavy smokers.

In light of these observations, plasma DNA assays for the detection of mutations in codon 12 of *K-ras* do not provide a reliable method to screen populations for the somatic mutations frequently found in neoplasms. Further confirmatory studies are required.

References

1. Wang M, Block TM, Steel L, Brenner DE, Su YH. Preferential isolation of fragmented DNA enhances the detection of circulating mutated k-ras DNA. Clin Chem 2004;50:211–3.
2. Kopreski MS, Benko FA, Borys DJ, Khan A, McGarrity TJ, Gocke CD. Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. J Natl Cancer Inst 2000;92:918–23.
3. Ramirez JL, Sarries C, de Castro PL, Roig B, Queralt C, Escuin D, et al. Methylation patterns and K-ras mutations in tumor and paired serum of resected nonsmall-cell lung cancer patients. Cancer Lett 2003;193:207–16.
4. Gautschi O, Ziegler A. Comment on "Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-call lung cancer patients" (*Cancer Letters* 2003; 193:207–216). Cancer Lett 2004;204:1.
5. Rogers JC, Boldt D, Kornfeld S, Skinner A,

Valeri CR. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. Proc Natl Acad Sci U S A 1972; 69:1685–9.

6. Jackson JH, Vollenweider M, Hill J, Rodriguez H, Schwabacher AW, Mitra G, et al. Stimulated human leukocytes cause activating mutations in the K-ras protooncogene. Oncogene 1997; 14:2803–8.
7. Knaapen AM, Borm PJ, Albrecht C, Schins RP. Inhaled particles and lung cancer. Part A: mechanisms. Int J Cancer 2004;109:799–809.
8. Maeshima AM, Maeshima A, Kawashima O, Nakajima T. K-ras gene point mutation in neogenetic lesions of subpleural fibrotic lesions: either an early genetic event in lung cancer development or a non-specific genetic change during the inflammatory reparative process. Pathol Int 1999;49:411–8.
9. Ballaz S, Mulshine JL. The potential contributions of chronic inflammation to lung carcinogenesis. Clin Lung Cancer 2003;5:46–62.
10. Keohavong P, Mady HH, Gao WM, Siegfried JM, Luketich JD, Melhem MF. Topographic analysis of K-ras mutations in histologically normal lung tissues and tumours of lung cancer patients. Br J Cancer 2001;85:235–41.
11. Heinzlmann M, Lang SM, Neynaber S, Reinshagen M, Emmrich J, Stratakis DF, et al. Screening for p53 and K-ras mutations in whole-gut lavage in chronic inflammatory bowel disease. Eur J Gastroenterol Hepatol 2002;14:1061–6.
12. Borchers R, Heinzlmann M, Zahn R, Witter K, Martin K, Loeschke K, et al. K-ras mutations in sera of patients with colorectal neoplasias and long-standing inflammatory bowel disease. Scand J Gastroenterol 2002;37:715–8.
13. Hiyama T, Haruma K, Kitadai Y, Masuda H, Miyamoto M, Tanaka S, et al. K-ras mutation in *Helicobacter pylori*-associated chronic gastritis in patients with and without gastric cancer. Int J Cancer 2002;97:562–6.

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Denaturing HPLC-Based Assay for Detection of *ATRX* Gene Mutations

To the Editor:

In 2003, we described *(1)* a broad-range denaturing gradient gel electrophoresis method for mutation scanning of the entire open reading frame and canonical splice sites of the *ATRX* gene (OMIM 300032), a zinc finger transcriptional regulator undergoing X inactivation and probably involved in chromatin remodeling, DNA methylation, and gene expression in mammalian development *(2, 3)*. Mutations affecting the *ATRX* gene lead to the α-thalassemia/mental retardation syndrome (ATR-X syndrome; OMIM 301040).

We now propose a simpler, rapid mutational approach based on denaturing HPLC (DHPLC) *(4)*, with which we were able to confirm all of the nucleotide variations described in our first report *(1)* and to detect 5 other mutations in 7 of 15 unrelated Italian patients with a clinical suspicion of ATR-X syndrome. Segregation of the syndrome was sporadic in all but 2 individuals. X-inactivation status at the human androgen receptor locus was tested in all patients' mothers as described previously *(5)*.

PCR primers (Table 1 of the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol51/issue7/) were designed to amplify all 35 exons and the consensus splicing sites of the *ATRX* gene (Entrez Gene ID 546). A total of 44 reactions were performed at a single annealing temperature (57 °C) with Optimase DNA polymerase in 1× buffer (both from Transgenomic); exon 9 was amplified as 10 overlapping fragments. Heteroduplex formation was obtained by mixing together, denaturing, and gradually reannealing equimolar quantities of PCR products for patients and controls. DHPLC analysis was performed with the WAVE™ 3500HT System (Transgenomic). Each crude PCR product (50 μL) was eluted by a 2.5-min run at 3 different analysis temperatures, and the buffer gradients were chosen as suggested by the Navigator software (Transgenomic; Table 1 of the online Data Supplement). Wild-type controls were included in each run. For the heterozygous elution profiles, genomic DNA was reamplified with the DHPLC primers and sequenced using Big-Dye sequencing chemistry (Applied Biosystems). No false-positive results were reported. In the 8 patients negative by DHPLC analysis, direct sequencing failed to detect any deleterious variations. The DHPLC results are summarized in Table 1 of the online Data Supplement.

The new p.L195P (c.584T>C) mutation identified in this study was not found in 200 apparently healthy females; moreover, this amino acid belongs to the zinc finger domain, was conserved during evolution (data not shown), and is segregated in a 3-generation pedigree with a classic ATR-X phenotype and a skewed X-inactivation status in carrier females.

The chromatograms for all mutations found in our patients, including those described in our first report *(1)*, are shown in Fig. 1.

Taking into account the time needed to set up and run the PCR