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Advances in Brief

Molecular Detection of Genetic Alterations in the Serum of Colorectal Cancer Patients[1]

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Abstract

We have searched for the presence of genetic alterations in serum DNA obtained from 44 colorectal cancer patients. Microsatellite analysis using highly polymorphic markers revealed loss of heterozygosity and/or microsatellite instability in 35 of 44 (80%) primary tumors. No alterations were detected in the paired serum DNA. We next used an oligonucleotide-mediated mismatch ligation assay to detect tumor specific gene mutations in the serum. Among the 16 cases with a K-*ras* gene mutation in the tumor, the same mutation was detected in three paired serum samples. In the 10 cases with a *p53* mutation in the tumor, the identical mutation was detected in seven corresponding serum samples. Comparison of the molecular analysis with clinical diagnosis of these patients revealed that none of the seven Dukes' stage B patients with a K-*ras* mutation in their tumors demonstrated a mutation in the serum. In contrast, five of seven stage B patients with a *p53* mutation in the tumor demonstrated a mutation in the paired serum ($P = 0.01$, Fisher's exact test). Taken together, either a K-*ras* or *p53* mutation was detected in the serum in 40% of the 25 patients (95% confidence interval, 21–61%), whose primary tumors contained a mutation and in 23% of the 44 patients (95% confidence interval, 12–38%) with colorectal cancer. The frequent detection of *p53* mutation in the serum of patients with early stage tumors suggests a possible use of this approach for clinical prognosis and cancer monitoring of colorectal cancer patients.

Introduction

Colorectal cancer is one of the most common malignancies in the world and can usually be cured if diagnosed at an early stage. Methods to detect surgically resectable tumors could significantly reduce deaths from this disease. Several genetic changes, such as the activation of the K-*ras* oncogene, inactivation of *APC, p53* genes, and loss of other tumor suppressor genes on chromosome 18q are involved in the pathogenesis of colorectal cancer. Assays based on the molecular detection of these genetic changes have been shown as potential diagnostic tools for colorectal cancer (1–4). Furthermore, the identification of these genetic changes at sites away from the primary tumor may help to assess the extent of disease and overall tumor burden at initial diagnosis (5).

Previous studies have proposed that tumor DNA is released into the circulation and is enriched in plasma and serum (6, 7). Radioimmunoassays revealed that the serum of cancer patients contained approximately four times the amount of free DNA compared with normal control (8). Based on these observations, studies have shown that it is possible to detect tumor-specific DNA in the serum of head and neck cancer patients and in the plasma of lung cancer patients using microsatellite analysis (9, 10).

In the present study, we have examined 44 colorectal cancer patients using microsatellite analysis and a more sensitive mutation-specific mismatch ligation assay for genetic alterations in primary tumors and the paired serum samples. In the 25 cases with K-*ras* and/or *p53* gene mutations in the tumor, 10 cases exhibited the same alteration in the matched serum DNA using the mismatch ligation assay. Additionally, early stage tumors with *p53* mutations appeared to be more likely to demonstrate the identical mutation in the paired serum sample than those with a K-*ras* mutation. These results indicate that genetic alterations present in the tumors of colorectal cancer patients can be detected in the serum of the same patient, and that this approach can be potentially used for cancer prognosis and cancer monitoring for this deadly disease.

Materials and Methods

Sample Collection and DNA Preparation. Forty-four primary tumors and corresponding normal tissues were prepared from microdissected sections of formalin-fixed, paraffin-embedded tissues obtained from surgical resection of colorectal cancer patients. All tumor specimens contained more than 70% neoplastic cells. The pathological stagings at surgery for patients with either a K-*ras* or *p53* mutation are shown in Table 1. Serum samples were collected from the same patients prior to tumor resection and stored at −80°C. Normal, tumor, and serum DNA were prepared as described previously (11).

Microsatellite Analysis. Dinucleotide markers used for microsatellite analysis were *D18S55, D18S58, D18S61,* and *D18S69* on 18q (12), *CHRNB1* and *D17S786* on 17p (8), and *D8S133* and *D8S254* on 8p (13). These markers were chosen because they are highly polymorphic and are located at regions frequently lost in colorectal cancer. One of the two PCR primers for each marker was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. PCR amplification was performed as described (12) using paired normal, tumor, and serum DNA samples. The amplified products were separated in 8% denaturing urea-polyacrylamide-formamide gels followed by autoradiography (14). LOH[3] was scored if the ratio of one allele was significantly decreased (>30%) in tumor or serum DNA compared with normal DNA by independent visual observation of at least two investigators. Microsatellite instability was identified when novel band(s) were present in the tumor sample in addition to the constitutive allele(s) observed in the normal control.

Mutation Analysis for the *p53* Gene. The PCR amplification of tumor DNA samples consisted of 40 cycles of 95°C for 30 s, 58°C for 1 min, and 70°C for 1 min for exons 5, 6, and 8, or 95°C for 30 s, 63°C for 1 min, and 70°C for 1 min for exon 7. The primers used were: exon 5, 5′-AGGAATTCACTTGTGCCCTGACTT and 5′-GAGGAATCAGAGGCCTGGG; exon 6, 5′-TGCCCCAGGCCTCTGATTC and 5′-CATCGAATTCCACTGACAACCACCCTT; exon 7, 5′-TGCTTGCCACAGGTCTCC and 5′-ATCGGTAAGAGGTGGGCC; and exon 8, 5′-GGACAGGTAGACCTGATTTCC and 5′-CCTCCACCGCTTCTTGTC. All PCR products were purified and sequenced directly using the AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA). The sequencing primers were 5′-AACCAGCCCTGTCGTCTC, 5′-GAGACCCAGTTGCAAACCA, 5′-GAGGCAAGCAGAGGCTGG, 5′-AGTGGTAATCTACTGGGA, and 5′-ACCTCGCTTAGTGCTCCCTG for exons 5–8, respectively.

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[3] The abbreviation used is: LOH, loss of heterozygosity.

Table 1 *K-ras and p53 gene mutations in the tumors and serum of colorectal cancer patients*

Case no.[a]	Tumor stage[b]	Gene alteration	Amino acid substitution	Mutation in serum
1	D	K-*ras*	Gly 12 Val	−
4	D	K-*ras*	Gly 12 Asp	+
7	B	K-*ras*	Gly 12 Asp	−
11	B	K-*ras*	Gly 12 Cys	−
15	B	K-*ras*	Gly 12 Ala	−
17	B	K-*ras*	Gly 12 Ala	−
19	C	K-*ras*	Gly 12 Ala	−
25	C	K-*ras*	Gly 12 Val	−
27	C	K-*ras*	Gly 12 Asp	−
29	C	K-*ras*	Gly 12 Asp	−
31	B	K-*ras*	Gly 12 Asp	−
37	B	K-*ras*	Gly 12 Asp	−
40	B	K-*ras*	Gly 12 Asp	−
41	C	K-*ras*	Gly 12 Val	−
45	C	K-*ras*	Gly 12 Ser	+
46	C	K-*ras*	Gly 12 Val	+
5	C	*p53*	Arg 248 Gln	−
6	B	*p53*	g to a (intron 5)[c]	+
15	B	*p53*	Arg 306 Term	−
16	B	*p53*	Arg 273 Cys	+
20	B	*p53*	Val 272 Met	−
21	C	*p53*	250-253 del	+
28	B	*p53*	Asn 200 Ser	+
33	B	*p53*	Arg 306 Term	+
34	C	*p53*	t to c (intron 6)[c]	+
44	B	*p53*	176 del	+

[a] All cases are listed by the mutations detected. The first 16 cases had K-*ras* gene mutation, whereas the latter 10 had *p53* mutation.
[b] Dukes classification.
[c] These mutations are located at the indicated introns.

Mismatch Ligation Assay. All possible alterations at K-*ras* codon positions 12a, 12b, and 13b were determined using a modified allele-specific ligation assay (15). The first exon of K-*ras* was amplified as described (4) and used as the template for three separate ligation assays (12a, 12b, and 13b) in all tumor samples. For each ligation assay, 50 ng of PCR product were mixed with 8 ng each of three mutation specific oligomers, 100 ng of blocking oligomer, and 8 ng of a common ^{32}P-labeled oligomer in a 20-μl reaction containing 150 mM NaCl, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM DTT, 1 mM ATP, and 3 μg of T4 gene 32 protein (Boehringer Mannheim). This mixture was denatured at 95°C for 5 min and allowed to cool at room temperature for 15 min, at which time 1 unit of T4 ligase was added. The ligation was carried out at 37°C for 1 h and terminated by heat inactivation at 68°C for 10 min. The [^{32}P]phosphate on the unligated 3′ oligomer was removed by the addition of 1 unit of alkaline phosphatase and subsequent incubation at 37°C for 30 min. The ligation products were separated on 12% denaturing polyacrylamide gel. The presence and the nature of mutations were determined based on the relative migration of the ligation products formed in control experiments using templates with known K-*ras* mutations. Oligonucleotide sequences used for the ligation assays were described previously (15).

The mutation-specific oligomers for the *p53* gene were designed individually according to each *p53* mutation and used in the ligation assay. For example, tumor 5 had a mutation in codon 248 (5′-CATGAACCGGAGGCCCAT to 5′-CATGAACCAGAGGCCCAT). To detect this alteration in the serum, the mutation-specific oligomer (5′-CATGAACCA), the adjacent ^{32}P-labeled oligomer (5′-GAGGCCCAT), and the blocking oligomer (5′-TGAACGGAGGC) were synthesized, and the mismatch ligation assay was performed exactly as described for the K-*ras* gene mutations. When a serum sample was found positive, the DNA extraction and PCR reaction were repeated at least once from the original serum sample to confirm the presence of the mutant allele. In all cases, the presence and the percentage of mutant DNA in the serum remained unchanged.

Results and Discussion

We tested the serum of 44 colorectal cancer patients for the presence of various types of genetic alterations. We first examined the allelic status of 18q, 17p, and 8p in the primary tumor samples because of their frequent loss in colorectal cancers. Subsequently, DNA from paired serum samples were examined to determine whether tumor-specific LOH or instability was present in the paired serum of these patients. Although LOH or a microsatellite shift of at least one locus was observed in 35 of 44 (80%) primary tumors, no loss of heterozygosity or microsatellite shifts were detected in the serum (Fig. 1). This negative outcome could be due to the following two reasons: (*a*) colorectal tumor DNA in the serum would be filtered by the liver and diminished or diluted in peripheral blood, because colorectal blood circulation passes through the portal vein before entering into the peripheral circulation; and (*b*) microsatellite analysis may not have enough sensitivity to detect small amounts of tumor DNA in the serum of a colorectal cancer patient (16).

To examine this second possibility, we proceeded with a more sensitive method to detect K-*ras* and *p53* gene mutations in the serum DNA. K-*ras* gene mutations are observed in about 50% of primary colorectal tumors of all stages and represent an ideal target for such an approach. *p53* mutations occur in up to 70% of advanced colorectal cancers and have been reported as a poor prognostic factor (17). K-*ras* status in all 44 tumors was determined using the ligation assay. Of the 16 cases having K-*ras* gene mutations in their tumors, the ligation assay was repeated using the paired tumor and serum samples, and the same mutation was observed in the serum in 3 cases (19%; Fig. 2*A*). Thirty-three tumors with sufficient DNA were sequenced for *p53* mutations in exons 5–8 of the gene. Of the 10 cases having a *p53* mutation in the tumor, the same mutation was found in the serum of 7 patients (Fig. 2*B*). In total, we identified either a K-*ras* or *p53* mutation in the serum in 10 of 25 patients whose tumor had a mutation. All alterations observed in the serum were confirmed at least once using independently extracted serum DNA samples. Furthermore, in the case of K-*ras*, although oligomers for all three possible mutations at a given codon position (*i.e.*, 12a, 12b, or 13b) were present in the same ligation reaction, only the mutation identical to the one present in the primary tumor was observed.

After completion of the mismatch ligation assay in all specimens, clinical-pathological data were correlated with the molecular analysis. The three patients with K-*ras* gene mutation in the serum had either Dukes' C or D disease. None of the seven stage B patients with a K-*ras* mutation in the tumor had a detectable mutation in the serum. In contrast, five of seven stage B patients with *p53* mutations demonstrated the identical *p53* mutation in the serum. In our study, only case 15 had both K-*ras* and *p53* gene alterations in the same tumor, but neither of these changes was detected in the serum. The results of the mutation-specific ligation assay for all 25 patients with a K-*ras* or *p53* mutation in the tumor are summarized in Table 1.

Recent evidence suggests that microsatellite shifts (low level instability) or LOH can be detected in the circulating tumor DNA from the serum

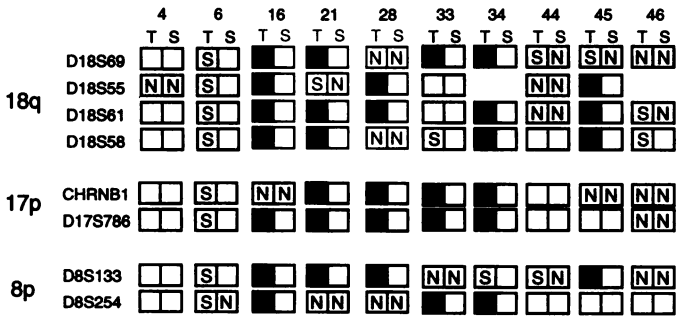

Fig. 1. Microsatellite analysis of paired tumor and serum samples. DNA was extracted from the tumor (*T*) and paired serum (*S*) of 44 colorectal cancer patients and analyzed as described in "Materials and Methods." Case numbers are indicated at the *top*. *Left*, microsatellite markers and their chromosomal location. *Open box*, retention; *closed box*, LOH; *S*, microsatellite shift (instability); *N*, not informative.

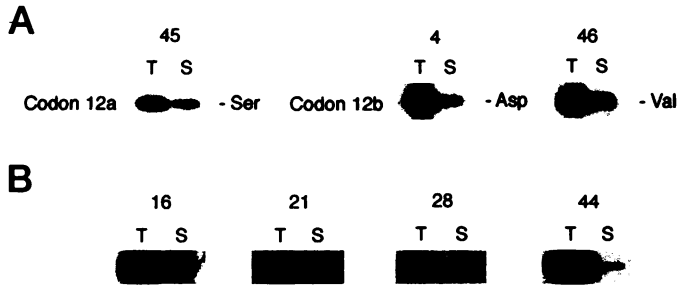

Fig. 2. Representative autoradiographs of the mismatch ligation assay. Examples of ligation assays detecting K-*ras* (*A*) and *p53* (*B*) mutations in the paired serum (*S*) and tumor (*T*) DNA samples from patients with colorectal cancers.

Table 2 *Genetic markers for the detection of colorectal tumor in the serum*

Genetic alteration	Occurrence in tumor	Serum positive (%)
LOH	31/44 (70%)	0/31 (0%)
Microsatellite instability	15/44 (34%)	0/15 (0%)
K-*ras* mutation	16/44 (36%)	3/16 (19%)
p53 mutation	10/33 (33%)[a]	7/10 (70%)[b]

[a] Sufficient DNA samples were available for complete sequence analysis in 33 cases only.

[b] $P = 0.014$. Comparison of K-*ras* mutation *versus p53* mutation serum-positive cases by Fisher's exact test.

of head and neck cancer patients, and the plasma of small cell lung cancer patients (9, 10). Using reverse transcription-PCR, it has also been shown that detection of circulating tumor cells from the blood of colorectal cancer patients may have prognostic value or be related to stage at diagnosis (18, 19). In this study, we tested four different genetic markers for the detection of colorectal cancer in the DNA from serum (Table 2). Although LOH and microsatellite shifts could not be detected, K-*ras* or *p53* mutations were detected in the serum of 40% of patients with a mutation in the tumor using the mismatch ligation assay. Interestingly, the serum-positive rate for tumors with a *p53* mutation was significantly higher than that with a K-*ras* mutation ($P = 0.014$ by Fisher's exact test, Table 2). Most of this difference can be explained by the *p53* mutant tumors, which displayed mutant serum DNA in patients with early stage disease. It is conceivable that colorectal tumors with *p53* mutations may have increased access to the peripheral blood due to the loss of the wild-type *p53*-induced apoptosis pathway. This result coincides with a report that colorectal tumors with *p53* mutation are also more likely to metastasize (3).

Our results have two potential clinical applications: (*a*) patients at high risk for colorectal cancer could be screened for the presence of tumor cells in the serum by analysis of K-*ras* or *p53* gene mutations. Based on the method described in this study, such screenings are limited by the large number of different mutations identified in the *p53* gene; and (*b*) a second application would be in the follow-up of patients diagnosed of colorectal cancers. In such patients, mutations of the *p53* gene could first be identified using GeneChip technology[4] and then used as targets for the mismatch ligation assay.

Colorectal cancer is among the most common and fatal cancers in the world. Thus far, the only noninvasive test for this disease is the testing of fecal occult blood. However, the appearance of hemoglobin in stool is not specific for neoplasia. It has been shown that DNA fragments from colorectal tumor cells could be amplified from the stool using PCR for the detection of K-*ras* mutations (4), but it remains difficult to reproducibly PCR amplify tumor DNA from the stool. In this preliminary study, we detected tumor-associated DNA alterations in the serum of 23% colorectal cancer patients (10 of 44) by using either K-*ras* or *p53* mutation as a target. The clinical sensitivity of this assay can be potentially improved by incorporating other common genetic targets such as *APC*. Advances in technologies to permit rapid detection of an array of specific mutations would enhance the utility of this approach. Previous studies in cancer patients have suggested that those with genetic alterations in serum or plasma are more likely to develop metastases and die of their disease (9, 18). Further studies are needed to determine the clinical relevance of identifying specific genetic alterations in the serum of colorectal cancer patients for the prognosis and monitoring of the disease.

[4] Steve Ahrendt, personal communication.

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References

1. Bos, J. L., Fearon, E. R., Hamilton, S. R., Vries, M. V., Boom, J. H., and Vogelstein, B. Prevalence of *ras* gene mutations in human colorectal cancers. Nature (Lond.), *327:* 293–297, 1987.
2. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., Tuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and *p53* gene mutations in colorectal carcinomas. Science (Washington DC), *244:* 217–221, 1989.
3. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. N. Engl. J. Med., *319:* 525–532, 1988.
4. Sidransky, D., Tokino, T., Hamilton, S. R., Kinzler, K. W., Levin, B., Frost, P., and Vogelstein, B. Identification of *ras* oncogene mutations in the stool of patients with curable colorectal tumors. Science (Washington DC), *256:* 102–105, 1992.
5. Hayashi, N., Ito, I., Yanagisawa, A., Kato, Y., Nakamori, S., Imaoka, S., Watanabe, H., Ogawa, M., and Nakamura, Y. Genetic diagnosis of lymph-node metastasis in colorectal cancer. Lancet, *345:* 1257–1259, 1995.
6. Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., and Beljanski, M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology (Basel), *46:* 318–322, 1989.
7. Leon, S. A., Shapiro, B., Sklaroff, D. M., and Yaros, M. J. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res., *37:* 646–650, 1977.
8. Shapiro, B., Chakrabarty, M., Cohn, E. M., and Leon, S. A. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer (Phila.), *51:* 2116–2120, 1983.
9. Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat. Med., *2:* 1035–1037, 1996.
10. Chen, X. Q., Stroun, M., Magnenat, J. L., Nicod, L. P., Kurt, A. M., Lyautey, J., Lederrey, C., and Anker, P. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat. Med., *2:* 1033–1035, 1996.
11. Goelz, S. E., Hamilton, S. R., and Vogelstein, B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissues. Biochem. Biophys. Res. Commun., *130:* 118–126, 1985.
12. Jen, J., Kim, H., Piantadosi, S., Liu, Z-F., Levitt, R. C., Sistonen, P., Kinzler, K. W., Vogelstein, B., and Hamilton, S. R. Allelic loss of chromosome 18q and prognosis in colorectal cancer. N. Engl. J. Med., *331:* 213–221, 1994.
13. Yaremco, M. L., Recant, W. M., and Westbrook, C. A. Loss of heterozygosity from the short arm of chromosome 8 is an early event in breast cancers. Genes Chromosomes Cancer, *13:* 186–191, 1995.
14. Litt, M., Hauge, X., and Sharma, V. Shadow bands seen when typing polymorphic dinucleotide repeats: some causes and cures. Biotechniques, *15:* 280–284, 1993.
15. Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Molecular determinants of dysplasia in colorectal lesions. Cancer Res., *54:* 5523–5526, 1994.
16. Mao, L., Lee, D. J., Tockman, M. S., Erozan, Y. S., Askin, F., and Sidransky, D. Microsatellite alterations as clonal markers for the detection of human cancer. Proc. Natl. Acad. Sci. USA, *91:* 9871–9875, 1994.
17. Kern, S. E., Fearon, E. R., Tersmette, K. W., Enterline, J. P., Leppert, M., Nakamura, Y., White, R., Vogelstein, B., and Hamilton, S. R. Clinical and pathological associations with allelic loss in colorectal carcinoma. J. Am. Med. Assoc., *261:* 3099–3103, 1989.
18. Hardingham, J. E., Kotasek, D., Sage, R. E., Eaton, M. C., Pascoe, V. H., and Dobrovic, A. Detection of circulating tumor cells in colorectal cancer by immunobead-PCR is a sensitive prognostic marker for relapse of disease. Mol. Med., *1:* 789–794, 1995.
19. Denis, M. G., Lipart, C., LeBorgne, J., LeHur, P-A., Galmiche, J-P., Denis, M., Ruud, E., Truchaud, A., and Lustenberger, P. Detection of disseminated tumor cells in peripheral blood of colorectal cancer patients. Int. J. Cancer, *74:* 540–544, 1997.

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