K-Ras Point Mutations in the Blood Plasma DNA of Patients with Colorectal Tumors

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INTRODUCTION

Oncogene mutations are found with varying frequency among several tumor types and sometimes play an important role in their development (5,2). The identification of these mutations could be very important for the early detection of several cancers (4, 21). Normally, this is done by investigating biopsy specimens which sometimes entails surgery or at least an invasive test. An easily accessible human material is blood plasma in which increased levels of DNA have been found in patients suffering from various malignant diseases (12, 16). The suggestion that an increased amount of DNA might originate from tumor cells was supported by the finding that some biophysical characteristics of cancer cell DNA (3) were also detected in the DNA extracted from the plasma of cancer patients (17). We have therefore investigated the possibility of finding activated oncogenes in the plasma of cancer patients which could be useful for the diagnosis and monitoring of various kind of malignancies. Among the different modifications observed in oncogenes, point mutations of the ras genes are particularly significant. For instance, up to 50% of the colon adenocarcinoma tumors harbor a mutation in the ras genes, most of them taking place in codon l2 of K-ras (6, 9). These mutations usually occur during the transition from

adenoma I to adenoma II, before loss or mutation of the p53 gene (11), that is, relatively early in the evolution of the tumor. For this reason we have screened the blood plasma DNA in search of mutations in codon 12 of the K-ras gene among 15 patients with intestinal cancer (13 undergoing ablative surgery shortly after blood collection).

MATERIALS AND METHODS

Materials. Concanavalin A-Sepharose (Pharmacia Biotech AG, Dübendorf, Switzerland). PCR Thermal Cycler 480 and Taq 1 polymerase (Perkin Elmer Cetus, Kuesnacht, Switzerland). Oligonucleotide primers and probes (Oncogene Science, Uniondale, N.Y.). Mutation-specific oligonucleotides (Microsynth, Windisch, Switzerland). Polyacrylamide (Fluka, Buchs, Switzerland). Zeta probe membranes (Bio-Rad, Hercules, CA). 3'end labeling kit and ^{32}P-ddATP(Amersham, England).NuSieve 3:1 agarose (FMC, Rockland ME)

Patients and sources of DNA. Blood samples (20 to 30 ml) were collected on heparin from 15 informed and consenting patients with different stages of colorectal adenocarcinoma. The patients were not receiving any kind of cancer drug during this period. The DNA was extracted from the blood cells and plasma, and for 13 of the patients, also from paraffin-embedded tumor samples. Blood was also taken from 10 healthy controls where an amount of 400 ml had to be collected for plasma DNA isolation.

DNA extraction. The tumor (10) and blood cell DNA (1) were extracted as previously described. The plasma DNA proved to be more delicate to extract (16, 17). In brief, the plasma was subjected to phenol, ether and chloroform treatments. After dialysis against 1 x SSC (0.15 M Sodium chloride, 0.015 M trisodium citrate), the material was passed through a Concanavalin A-Sepharose column to remove the polysaccharides and then centrifuged in a Cs_2SO_4 gradient. After this centrifugation the DNA often formed two bands, one band in a normal position, the other higher, the DNA of this lighter fraction being still strongly attached to proteins. In these cases the DNA from each band was investigated separately.

***Ras* gene amplification.** Purified DNA (10-100 ng) was subjected to PCR amplification of the first exon of the K-ras gene in a volume of 100 ul. The primers were 5'-GACTGAATATAAACTTGTGGTAGT-3' and
5'-CTATTGTTGGATCATATTCGTCC-3'. The amplifications were performed in a buffer containing 50 mM KCl, 10 mM Tris-HCl at pH 8.3, 200

mM of each nucleotide, 1.8 mM $MgCl_2$, 0.2 uM of each primer and 2.5 units of AmpliTaq DNA polymerase. Thirty five cycles were performed for tumor and blood cell DNA and 45 cycles for plasma DNA (94° C for l min, 59°C for l,5 min, 72°C for l min. The last cycle was followed by a 7 min extension at 72°C). The amplification products were analysed by electrophoresis in 8% polyacrylamide gel.

Mutation detection. Two different methods were used for each sample : Hybridization of the PCR products with mutation specific oligonucleotide probes (20) and a more sensitive technique based on PCR amplification with point mutation specific primers also called PCR amplification for specific alleles (PASA)(14).

According to the first method the PCR products, in equal quantities, were spotted on Zeta-probe membranes and hybridized with oligunucleotides specific for wild-type or mutant K-ras. (20). The oligonucleotide probes were end labeled with ^{32}P ddATP. To discriminate between perfect and mismatched hybrids, the final washing of the membrane was done in a solution containing 3 M tetramethylammonium chloride, 50 mM Tris-HCl at pH 8.0, 0.2 mM EDTA, 0.l % SDS at 58°C for l h.

In the more sensitive technique (14), the DNA was subjected to PCR amplification with primers complementary to the normal GLY or to the mutated ALA, VAL, SER, ASP or CYS sequences. The mutation-specific primers have 3'-ends complementary to specific point mutations. Taq I polymerase enzyme lacks a 3'-exonuclease activity and is therefore unable to amplify DNA if the single base mismatch is located at the 3'-end of the primer . Each PCR was done in a volume of 40 ul of a solution containing 50 mM KCl, l0 mM Tris-HCl at pH 8.3, 2 mM of each nucleotide, 0.7 mM $MgCl_2$, 0.2 mM of each primer and l unit of AmpliTaq DNA polymerase. Thirty five cycles were performed (94°C for l min, annealing at 55o to 62°C for 2 min, extension 72°C for l min). The last cycle was followed by 7 min extension at 72°C. Every reaction began with the hot-start technique. The primers were

5'-ACTTGTGGTAGTTGGAGCTGG-3' for the wild type K-ras (annealing 55°C), 5'-ACTTGTGGTAGTTGGAGCTGC-3' for the ALA l2 mutant (annealing 62°C), 5'-ACTTGTGGTAGTTGGAGCTGT-3' for the VAL l2 mutant (annealing 61°C), 5'-ACTTGTGGTAGTTGGAGCTA-3' for the SER l2 mutant (annealing 59°C), 5'-ACTTGTGGTAGTTGGAGCTGA-3' for the ASP l2 mutant (annealing 60°C), 5'-ACTTGTGGTAGTTGGAGCTT-3' for the CYS l2 mutant (annealing 59°C), and in each case the antisense primer 5'-CTATTGTTGGATCATATTCGTCC-3'. After amplification the reaction products were analysed by electrophoresis in 8% polyacrylamide gel. In some cases, the amplification products were run on a 4% NuSieve 3:1 agarose (FMC), blotted on a Zeta probe membrane and hybridized with oligonucleotides specific for wild type or mutant K-*ras* (20).

For each sample all the specified probes and primers were used.

RESULTS

Using the selective dot-blot hybridization with oligonucleotide probes, we detected a Valine mutation (Figure 1) in one of the two plasma specimens for which we had neither tumor nor blood cell sample (patient 15). Out of the 13 tumors analysed, six (46%) presented mutations (GLY to VAL, CYS or ALA) while with the same technique these mutations could not be revealed in the corresponding plasma DNA. Indeed, a PCR amplification followed by dot-blot hybridization allows a point mutation to be usually clearly identified only if 10 % (8), at most in our hands 2 % (Figure 1), of the total amplified DNA carries a point mutation.

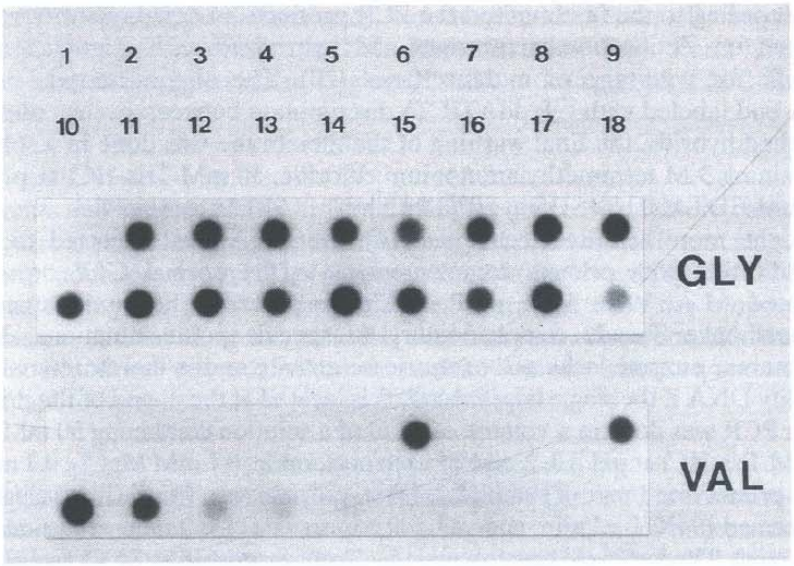

FIG. 1. Detection of point mutations in codon 12 of the K-ras gene by dot-blot hybridization. The DNA was amplified, spotted in equal quantities onto Zeta probe membranes and hybridized with oligonucleotides specific for wild type or mutant K-ras as described in "Material and Methods". Well No.1. Negative control (no DNA in PCR); No. 2. DNA from human placenta; No.3.,4.,5. DNA from three different samples of normal blood plasma; No. 6. DNA from the tumor of patient 4; No.7.8. DNA from the blood plasma of patient 4, L and H respectively (plasma DNA was often found in two bands after Cs_2SO_4 centrifugation : L, low density DNA; H, high density DNA);No. 9. DNA from the blood plasma of patient 15: No. 10 to 18. Mixture of normal human placenta DNA and mutant DNA from cell line SW 480 (which harbors a Valine mutation of codon 12 of the K-ras gene on both alleles) in varying proportions which were used as a template for PCR amplification with 50%, 25%, 10%, 5%, 2%, 1%, 0.5%, 0.25%, 0.1% of mutant DNA, respectively..

To improve the sensitivity we used the technique of PCR amplification using mutation-specific primers with 3'-ends complementary to the point

mutation. This method enables identification of mutations in a DNA sample mixed with a 10^4 to 10^5 fold excess of normal non-mutated DNA (18). With this assay the blood plasma of five out of the six mutation positive patients presented in a reproducible way the same mutation as identified in the tumor (Table 1) while the other possible point mutations of codon 12 did not appear.

Table 1. *Patients with colorectal adenocarcinomas studied for ras gene mutations in plasma DNA*

Patients	Age/Sex	Tumor location	Tumor stage	Tumor size (cm3)	Tumor mutation	Mutant ras gene in plasma
1	64/M	Rectum	B	4 x 4 x 0,8	ALA	ALA
2	59/M	Colon (relapse)	D	peritoneal nodules	CYS	CYS
4	59/F	Colon	C	6 x 6 x 2,5	VAL	VAL
7	59/M	Rectum	A	3,5 x 3,5 x 1,2	VAL	VAL
12	65/M	Rectum (relapse)	D	perirectal infiltrations	VAL	not detected
13	74/F	Colon (relapse)	D	peritoneal nodules	VAL	VAL

Seven negative tumors are not shown on the table, nor the 10 controls of healthy donors. Adenocarcinomas were classified according to Duke (Cohen et al.,1989) : A, confined to muscularis propria; B, extension to muscularis propria, but confined to colon; C, metastatic to regional lymph nodes; D, metastatic tumor outside regional lymph nodes.

Interestingly enough, when we investigated with the same sensitive technique the DNA present in the peripheral nucleated blood cells, the mutation observed in the plasma DNA could not be detected (Figure 2, patients 1 and 2). As controls we used DNA from human placenta, normal lymphocyte DNA and ten different samples of plasma DNA from healthy donors which were all found to be negative.

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