Soluble Normal and Mutated DNA Sequences from Single-Copy Genes in Human Blood[1]

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Abstract

Healthy individuals have soluble (extracellular) DNA in their blood, and increased amounts are present in cancer patients. Here we report the detection of specific sequences of the cystic fibrosis and K-*ras* genes in plasma DNA from normal donors by amplification with the polymerase chain reaction. In addition, mutated K-*ras* sequences are identified by polymerase chain reaction utilizing allele-specific primers in the plasma or serum from three patients with pancreatic carcinoma that contain mutated K-*ras* genes. The mutations are confirmed by direct sequencing. These results indicate that sequences of single-copy genes can be identified in normal plasma and that the sequences of mutated oncogenes can be detected and identified with allele-specific amplification by polymerase chain reaction in plasma or serum from patients with malignant tumors containing identical mutated genes. Mutated oncogenes in plasma and serum may represent tumor markers that could be useful for diagnosis, determining response to treatment, and predicting prognosis.

Introduction

Plasma from healthy donors usually contains approximately 10 ng DNA/ml (1–3), which is equivalent in amount to the DNA in more than 1600 diploid cells (4). In patients with multiple forms of cancer, soluble DNA in serum is significantly increased (5, 6). In a study of 65 patients with pancreatic carcinoma, more than 90% had DNA levels exceeding 100 ng/ml of serum (6). However, cellular DNA can be released during clotting of whole blood causing assays of DNA in serum to yield falsely high results (7, 8). Thus, levels of DNA in serum may not reflect conditions *in vivo*. In a recent study, plasma DNA levels were elevated above 80 ng/ml in 71% of a group of 45 patients with primary pulmonary carcinoma. The mean level in the healthy controls was 31 ± 18 ng/ml and none had more than 80 ng DNA/ml (9).

The increased soluble DNA in the blood of patients with cancer (5, 6, 9) is presumed to be derived largely from the tumors (10), probably through apoptosis and necrosis, but there may also be an increase in DNA derived from non-neoplastic cells. Nevertheless, we hypothesized that if a patient had a tumor containing a mutated gene, this mutated sequence would be represented in the soluble DNA in the circulating blood. The K-*ras* gene is mutated in one of three different codons in more than 10% of human cancers, including such common forms as those developing in the colon, lung, and pancreas (11). In the last site, approximately 75% of cases of adenocarcinoma contain mutations in the K-*ras* gene at position 1 or 2 of codon 12 (12–15). Thus, it seemed advisable to focus on the mutated K-*ras* gene in patients who had adenocarcinoma of the pancreas as a way to efficiently test our hypothesis.

In this study we demonstrate the occurrence of specific gene sequences in the plasma from normal donors and the presence of mutated K-*ras* sequences in the plasma of patients with pancreatic adenocarcinoma that contain the identical mutation of the K-*ras* gene. The latter is demonstrated with ASA[3] by PCR and confirmed by sequencing of the tumor and plasma DNA. Portions of these data have been reported previously in abstract form (16).

Materials and Methods

Specimen Preparation. Blood was collected preferentially in vacutainer tubes containing 15% EDTA (K3) for plasma extraction. Occasionally, only clotted blood specimens were available. Tubes were immediately centrifuged at 4° for 30 min at 1000 × *g*. Plasma or serum was removed, recentrifuged for 30 min at 1000 × *g* and stored at −70°.

DNA Extraction. Specimens were deproteinized by adding an equal volume of 20% NaCl, boiling for 3–4 min, cooling to room temperature, and centrifuging at 3000 × *g* for 30 min (17). The supernatant was removed and dialysed against three changes of 10 mM Tris (pH 7.5)-1 mM EDTA for 18–24 hours at 4°. DNA was phenol extracted using standard techniques and subsequently precipitated with NaCl to 0.3 M, 20 μg glycogen and 2.5 volumes of 100% EtOH at −20°.

PCR Amplification. The usual amplification reaction was carried out in a total volume of 40μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 50 μg/ml BSA, 200 μM each dNTP, 40 pmols each primer, and 2.5 units Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT). After initial

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[3] The abbreviations used are: ASA, allele-specific amplification; PCR, polymerase chain reaction.

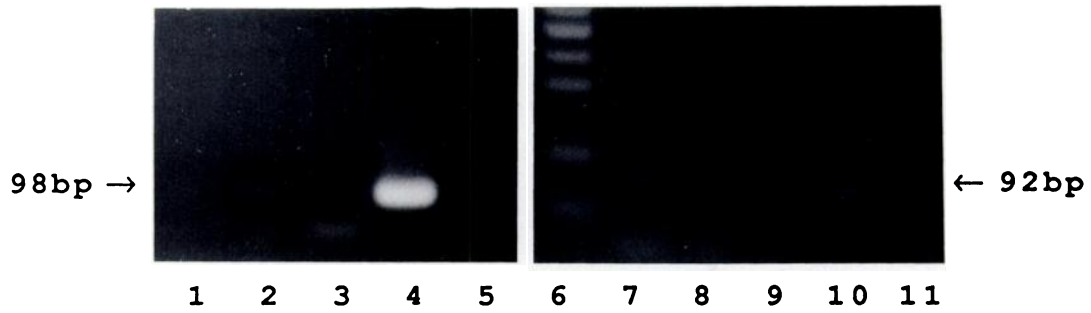

Fig. 1. Detection of cystic fibrosis (*lanes 1–5*) and K-*ras* (*lanes 7–11*) gene sequences in plasma DNA from healthy donors after PCR amplification with C16B-C16D and L1-L2 pairs of primers respectively. *Lanes 1–3*, plasma DNA from donors A, B, and C. *Lane 4*, human placental DNA. *Lane 5*, reagent control. *Lane 6*, size markers. *Lanes 7–9*, plasma DNA from donors A, B, and C. *Lane 10*, human placental DNA. *Lane 11*, reagent control.

DNA denaturation for 4 min at 94°, amplification was performed for 31 cycles (1 min at 94°, 48 s at 68° and 30 s at 72°). This was followed by a final extension for 7 min at 72°. Aliquots of DNA extracted from 1 ml of normal plasma were amplified by PCR utilizing the oligonucleotide primers C16B and C16D, which span a 98 base pair fragment of the cystic fibrosis gene (18), and the oligonucleotide primers L1 (3′) and L2 (5′), which span a 92 base pair fragment of the K-*ras* gene. The latter amplification was as described (19).

Allele-Specific Amplification. DNA from 0.5 ml of patients' plasma was amplified by PCR, utilizing a series of primer combinations. The initial amplification was with oligonucleotide primers CP1 (A-3′) and CP2 (B-3′) (20) that amplify a 275 base pair fragment including the first exon of K-*ras*. The reaction mixture was as above except for 25 μM each dNTP, 50 pmols each primer, and 1.5 units Taq polymerase. DNA was initially denatured at 95° for 4 min followed by 30 cycles of amplification (1 min at 95°, 2 min at 58°, and 1 min at 72°). 1:10,000 of the amplified product was used in each of the next series of allele-specific amplifications.

For use in identifying mutations by ASA, two sets of four oligonucleotide primers representing the wild-type sequence and each of the three possible mutations in the first and second nucleotide position of codon 12 were synthesized (20). For identification of mutations at position 1, one set of 4 oligonucleotide primers, complementary to the antisense strand (P1 primers), was prepared. The P1 primers are identical in the 17 nucleotides upstream from position 1 of codon 12 and differ only at the last nucleotide at the 3′ end corresponding to position 1. The wild-type primer sequence is 5′-TTGTGGTAGTTGGAGCTG-3′. To detect mutations at position 1 of codon 12, primers with the 3′ end nucleotide altered from G to T, C, or A were utilized. These P1 primers, in conjunction with a single, common, downstream antisense oligonucleotide primer (L1), amplify a 64-base pair fragment.

Similarly, a second set of 4 oligonucleotide primers, complimentary to the sense strand, were synthesized for analyzing position 2 of codon 12 (P2 primers). These primers differ only at the 3′ end nucleotide corresponding to the second position of this codon and matching the 16 downstream nucleotides. The sequence of the wild-type primer is 5′-ACTCTTGCCTACGCCAC-3′. To detect mutations in the second position of codon 12, primers were utilized with the 3′ end nucleotide of the wild-type primer altered from C to T, G, or A. These primers, in conjunction with a single, common, upstream primer (L2), amplify a 62-base pair fragment (3.0% formamide was added to the reactions involving the P2T primer to eliminate false positive results). The PCR reaction conditions were the same as for DNA from normal plasma, amplified with the spanning K-*ras* primers, except that a 5 μM concentration of each dNTP was used. Using the above primer combinations and stringent PCR conditions, only the primer pair with a 3′ nucleotide complementary to the nucleotide existing in the patient's plasma DNA, at position one or two of codon 12, will generate a DNA fragment of the appropriate size. This arrangement of primers eliminates the influence of mutations at one position of codon 12 during the evaluation of the other position (20).

Direct Sequencing of PCR Products. DNA was extracted from plasma or serum, and a special strategy was used to remove wild-type alleles prior to sequencing (21, 22). The initial PCR utilized primer A, which substituted a C residue at the first position of codon 11, and primer B (22). Amplification of the normal allele created a *Bst*N1 restriction enzyme cleavage site (CCTGG), overlapping the first two nucleotides of codon 12, which is not present if there is a mutation at either of the first two positions. Following digestion with BstN1, a second PCR utilizing primer F and primer C amplified the mutated K-*ras* sequence that was not cut by *Bst*N1 [22]. The *Bst*N1 digestion was repeated, the product was gel-purified (Mermaid, BIO101) and sequenced using a modified version of the Sequenase method (23) with a ^{32}P end-labeled primer F (5′-GGACGACGAATTCACTGA-3′). PCR-generated DNA (0.5 pmoles) plus 1.0 pmole of the labeled sequencing primer and 5× Sequenase buffer were boiled for 3 min, then snap cooled for 5 min on ice/dry ice. Then 1.0 μl DTT, 1.0 μl Mn, 2.0 μl Sequenase (diluted 1:8), and dH_2O to 15.5 μl were added to the reaction and kept on ice for 5 min. A portion of the reaction mix (3.5 μl) was transferred to each of four prewarmed tubes at 45° containing the termination ddNTPs, incubated at 45° for 5 min, and 4.0 μl Stop Solution was added.

DNA was extracted from two or three 10 μm sections of formalin-fixed, paraffin-embedded tumor tissue by a previously described method (22). Aliquots of 10 μl were used for PCR. Amplification, *Bst*N1 digestion, and sequencing were performed as described above for plasma or serum.

Results

Plasma was promptly separated from blood, and following deproteinization (17), DNA was extracted and further char-

Table 1 Patients with pancreatic adenocarcinoma

Patient No.	Age	Sex	Plasma DNA mutation[a] (ASA)	Plasma DNA sequence	Tumor DNA sequence	Clinical status[b]
1 (LC696)	74	F	CGT	G/C GT	N/A[c]	large primary tumor and extensive metastases
2 (HWO49)	69	M	GAT	GG/AT[d]	GG/AT	small primary tumor with bone metastases
3 (HP385)	57	M	GTT[d]	GG/TT[d]	GG/TT	moderately large primary tumor with small metastases

[a] 12th codon, K-ras.
[b] At time specimen was obtained.
[c] No tumor available.
[d] Serum specimen.

acterized (24). Aliquots of the DNA from five healthy donors and four patients with carcinoma of the pancreas were nick-translated and electrophoresed in 0.8% agarose, blotted, and exposed to film (24). A characteristic ladder of DNA nucleosomal oligomers appeared on the autoradiograph indicating DNA fragments of 200–800 base pairs in size.[4] The labeling occurred without the usual addition of DNase since the plasma DNA is apparently composed of double-stranded fragments with single-stranded regions (3). The appearance and size of the autoradiographic signal indicated that the labeled DNA was not derived from white blood cell breakdown during the plasma separation step since all newly released DNA would be double stranded and unlabeled by nick translation without the use of DNase. Moreover, if DNA from leukocyte breakdown were labeled, it would be much larger (i.e., >20 kilobases).

Aliquots of DNA extracted from normal plasma were utilized in PCRs with oligonucleotide primers C16B and C16D, which span a specific region of 98 base pairs of the cystic fibrosis gene (18), and L1 and L2, which span a 92 base pair fragment, including part of the first exon of the K-*ras* gene (19). In both instances, when the amplified reaction products were electrophoresed in 2.5% agarose and stained with ethidium bromide, bands of the appropriate size (i.e., 98 and 92 base pairs respectively) were produced (Fig. 1). These results, utilizing pairs of primers for randomly selected genes, indicate that many single-copy gene sequences can be detected in normal plasma by utilizing the appropriate primers with PCR.

We sought to detect a mutated K-*ras* gene in plasma or serum where there may be a high background of normal allelic DNA. Previous investigations utilizing PCR with allele-specific oligonucleotide primers and cellular DNA had indicated that this is possible (20, 25–27). PCR, using primers with 3′-ends complementary to specific genetic point mutations, allows the rapid detection of such mutations in genomic DNA even with a high background of DNA containing normal alleles. This is possible because Taq DNA polymerase utilized in the PCR lacks 3′-exonuclease activity and is therefore unable to repair single-base mismatches at the 3′-end of primers [28]. Thus, if synthetic oligonucleotides, complementary to a given genetic sequence containing a specific point mutation are used in PCR studies, there should be significantly less enzymatic amplification of homologous, nonmutated genes if the base, complementary to the mutation, is located at the 3′-end of the primers. Therefore, such primers, under the appropriate conditions, should be mutation specific because they should allow preferential enzymatic amplification of genetic sequences containing the specific point mutation. The sensitivity of ASA by PCR was such that mutations could be detected in DNA samples mixed with 10^{-5}-fold excess of normal, nonmutated DNA with primers which had mismatches at the penultimate and ultimate 3′ nucleotide (29) or even if the mismatch involved only the ultimate 3′ nucleotide.[5]

Aliquots of DNA extracted from 0.5 ml of plasma from a normal donor were amplified in a PCR with allele-specific primers for position 1 and 2 of codon 12 of the wild-type K-*ras* gene (20). The characteristic amplification products of 64 and 62 base pairs were obtained (data not shown). Plasma and serum were collected from patients with a histopathological diagnosis of adenocarcinoma of the pancreas, and specimens from three individuals were investigated in detail (Table 1). DNA was isolated as described for normal plasma. To minimize the amount of blood drawn from individual patients, the isolated DNA from plasma or serum was initially amplified by PCR utilizing the common primers for ASA (i.e., CP1 and CP2) (20). These primers amplified a 275 base pair fragment that includes exon 1 of the K-*ras* gene. Aliquots of 1:10,000 of the amplified product were placed in a series of eight tubes with allele-specific primers for the wild-type K-*ras* and each of the six possible mutations in position 1 and 2 of codon 12. If such a mutation is present, the primer pair with a 3′ nucleotide complementary to the nucleotide in the patient's plasma DNA at position 1 or 2 of codon 12 will preferentially generate a DNA fragment of the appropriate size. One would also expect to see a band in the lanes containing the amplification product from the tubes that included the allele-specific primer for position 1 and 2 of codon 12 of the wild-type K-*ras* gene, since the patients with cancer would also be expected to have normal DNA in their plasma (Fig. 2). The ASA indicated mutations in the 12th codon of the K-*ras* gene as follows: Patient 1, GGT→CGT; Patient 2, GGT→GAT; and Patient 3, GGT→GTT (Table 1).

In control series, normal genomic DNA was amplified by PCR utilizing the common primers for ASA and the product was subsequently amplified with the allele-specific primers for the wild-type K-*ras* and each of the six possible mutations in position 1 and 2 of codon 12. Following electrophoresis, bands were observed only in the lanes containing the amplification products from the tubes that included the allele-specific primers for the wild-type K-*ras* gene. In each of the three patients, the mutation in codon 12 of K-*ras* that was indicated by ASA was confirmed on both strands by direct sequencing of the PCR products amplified

[4] K. A. Iczkowski, D. M. Pribish, and G. D. Sorenson, unpublished observations.

[5] L. Dubeau, personal communication.

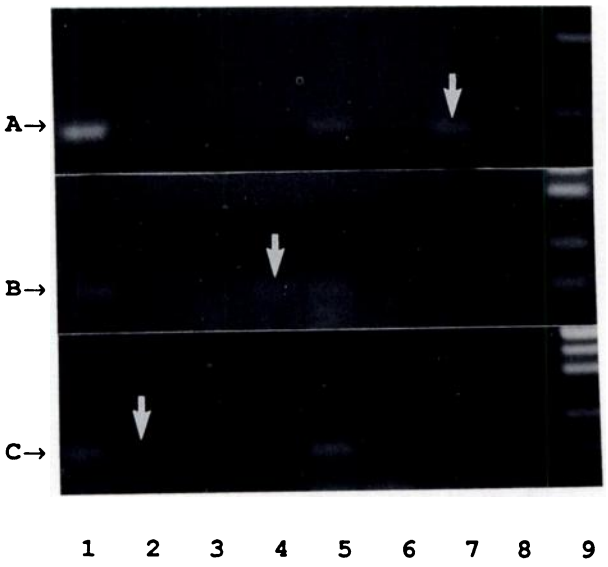

Fig. 2. Detection of wild-type and mutated gene sequences by PCR analysis utilizing allele-specific primers. A common primer L1 or L2 was paired with each of the allele-specific primers for position 1 and 2 of codon 12 of the K-*ras* gene and used in a PCR with DNA from patients A, B, or C. *Lanes 1–4*, amplified products with primers for position 2 containing a 3′ nucleotide of C (wild-type), A, G, or T. *Lanes 5–8*, amplified products from primers for position 1 containing a 3′ nucleotide of G (wild-type), A, C, or T. *Lane 9*, size markers. *Arrows*, mutated sequence.

from DNA extracted from the patient's tumor and also in isolated plasma or serum DNA. (Table 1 and Fig. 3).

Discussion

The ASA by PCR has given reproducible results consistently under the described conditions. Specificity has been achieved by optimizing the PCR protocol. Limiting dNTP concentrations is an important and possibly the most important single step in achieving specificity in annealing and extending the allele-specific primers (30). In our ASA the concentration of each dNTP was decreased to 5 μM. However, as specificity is increased by limiting dNTP concentration, the amount of product formed is restricted. Thus, in some instances, the product band may be relatively faint. Specificity is also increased by not having excessive template in the reaction (27). Thus, typically a dilution of 1:10,000 was made of the original amplified product prior to the ASA. Under the general conditions described, only the allele-specific primer for a G→A mutation at position 2 (i.e., with a 3′ terminal T) gave false positive reactions with wild-type DNA. This was eliminated by adding 3% formamide to that reaction mixture.

A priori, the presence of mutated K-*ras* sequences in blood is presumed to be a highly specific indication for the presence of a premalignant, or much more likely, a malignant tumor of any of multiple common types. It is likely that the mutated sequences could also be quantitated and that blood levels may reflect tumor extent (mass). Similar methods could be applied to the evaluation of other oncogenes. With this in mind, studies are currently under way to extend this investigation. The analysis of a variety of individual mutated oncogenes in plasma or serum may provide useful, sensitive, and specific assays for detecting malignant tumors, evaluating the efficacy of treatment, and for detecting tumor reoccurrence.

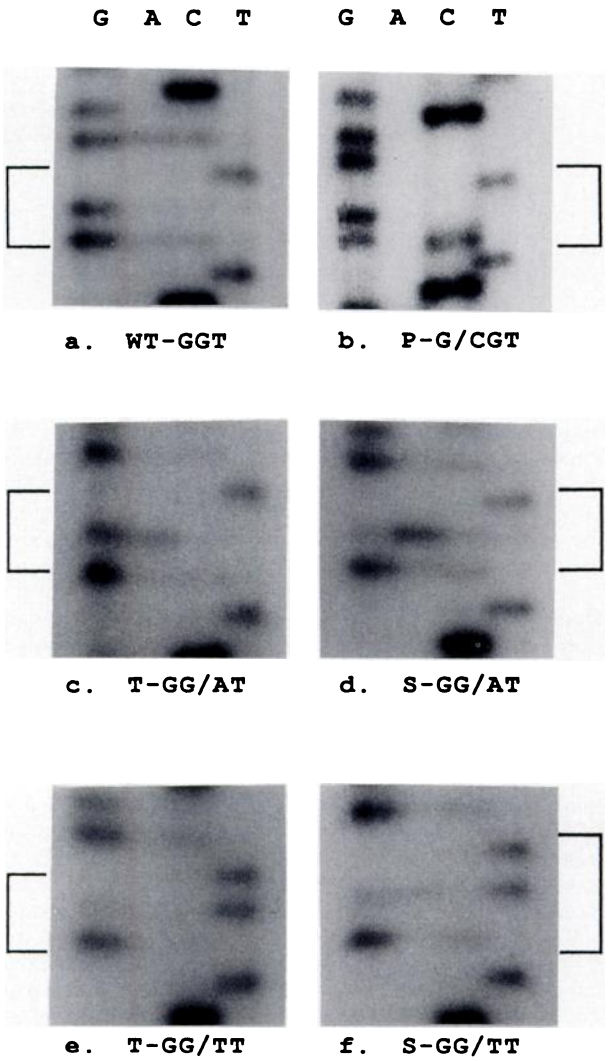

Fig. 3. Nucleotide sequences of codon 12 of the K-*ras* gene in DNA from plasma or serum and from the respective tumors of patients with pancreatic adenocarcinoma. (*a*) Wild-type (*WT*) sequence of normal human DNA (GGT). (*b*) Mutated sequence in plasma (*P*) DNA from patient 1 (GGT→CGT). (*c and d*) Mutated sequences in tumor (*T*) and serum (*S*) DNA respectively from patient 2 (GGT→GAT). (*e and f*) Mutated sequences in tumor (*T*) and serum (*S*) DNA, respectively, from patient 3 (GGT→GTT). (All specimens contain more or less "normal" DNA. Therefore, the wild type sequence may be seen as well as the mutation). *Brackets*, 12th codon.

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