of a subsequent second neoplasm, a primary hepatocellular carcinoma.

Furthermore, an increase in lipase activity preceded, by an average of 2.6 months (4, 1.5, 3, and 2 months, respectively), the increase of specific tumor markers, such as CA 19.9 and α₁-FP.

Several possible mechanisms may explain this unexpected increase in lipase activity, such as the presence of an unusual lipase isoenzyme, a decrease in the clearance of lipase or rate of its inactivation in the circulation, the persistence of lipase in blood after complexation of lipase with a plasma protein (5), or the direct production of lipase from the neoplastic mass, as suggested by Donnelly et al. (6) in a patient with a retroperitoneal malignancy, and by Lopez-Soriano et al. (10).

Several authors claim that the nonspecificity of lipase assays for pancreatitis is due to the pancreas being sensitive to other abdominal disease; as a matter of fact, the possibility that tumors may produce humoral factors influencing pancreatic metabolism and/or lipolytic activity is supported by a recent study suggesting that tumor necrosis factor-α is involved in activating the lipid metabolic changes that develop in rats after transplantation of a fast-growing tumor (11).

Although we did not investigate which of the above mechanisms was involved in our patients, we consider intriguing the hypothesis of a tumor marker-like role for lipase; indeed, in our experience, lipase acted as an extremely precocious signal of malignancy in all patients, anticipating the increase of more common tumor markers, such as CA 19.9 and α₁-FP.

However, only prospective studies on an adequate number of patients could definitely assess the real role of lipase from this point of view.

References


An improved method for the detection of the thermolabile variant of methylenetetrahydrofolate reductase, Geneviève Van Amerongen,1 Florence Mathonnet,1,2 Catherine Boucly,1 Bertille Mathieu,1 Isabelle Vinatier,1 Jean-Yves Pel- tier,4 Nicole Catherine,2 Catherine Collet,1 and Philippe de Mazancourt1,4 (1 Laboratoire de biochimie et biologie moléculaire, Hôpital R. Poincaré, F92380 Garches, France; 2 Laboratoires d’hématoologie du centre hospitalier de Poissy-Saint Germain en Laye, F78303 Poissy, France; 3 Service de Médecine Interne, centre hospitalier de Poissy-Saint Germain en Laye, F78303 Poissy, France; 4 Faculté de Médecine Paris Ouest, CJF 9402, Université Paris, V, F92380 Garches, France; * author for correspondence: fax 331 47 10 79 23)

The thermolabile variant of the methylene tetrahydrofolate reductase (MTHFR) in the homozygous state has been shown to be responsible for mild hyperhomocystinemia, hypomethioninemia, and hyperhomocystinuria (1). This variant is responsible for an increased risk for recurrent early pregnancy loss and neural-tube defects (2, 3). The presence of hyperhomocystinemia is also predictive of both arterial and venous thromboembolic disease (4–7) and is a risk factor for coronary artery stenosis, independent of other risk factors such as age, smoking, hypercholesterolemia, and hypertension (8). Four to 6% of the Caucasian population (9) and 13–20% of the thrombosis-prone patients are homozygous for the thermolabile variant of MTHFR, which is caused by a C-to-T substitution at nucleotide 677 of the cDNA, resulting in the substitution of a valine for an alanine (8). A simple molecular diagnosis is of particular interest because this risk factor is quite common, the biochemical assay requires a methionine load, and folic supplementation is likely to prevent some of the complications (10, 11). Thus, the exploration has been recommended in the management of premature venous and arterial occlusive diseases (4). We report here an improvement of the method described previously to assess the thermolabile variant of MTHFR, based on multiplex amplification of MTHFR and an internal control.

We studied 30 healthy control volunteers and 30 patients with personal or familial history of thrombosis or phlebitis. Informed written consent was obtained in all cases.

DNA extraction was performed from frozen blood either by phenol-chloroform extraction according to McIndoe et al. (12) or with DNAzol (Life Technologies, Inc.) as recommended by the manufacturer. In most of the control subjects, DNA was extracted with DNAzol from the cell pellet of saliva after two washes in 9 g/L NaCl.

Amplification of MTHFR was adapted from the method described by Froost et al. (1) as follows. Initial denaturation step was for 4 min at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C, and elongation for 90 s at 72 °C. The final elongation step was for 12 min at 72 °C. Primers used were: primer A, 5′-TGA AGG AAG TGT CCG CGG GA; primer B, 5′-AGG ACG GTG CCG TGA GAG TG; primer C, 5′-CTC.
CCT TCA CTT TCA GAA CTA CA; and primer D, 5’ GAC CTC TCA GTT TTC ACC TTT A for MTHFR (1) and fibrinogen Ao exon III coamplification (HUMFIBRA, positions 1723 and 2252). Each primer was used at 0.25 μmol/L in a 25-μL final volume in the presence of standard PCR master mix (Boehringer-Mannheim). Final concentrations were 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L each deoxynucleotide triphosphate, 0.5 mL/L Brij 35, and 0.625 U Taq DNA polymerase. Amplifications were performed with a Perkin-Elmer 2400 thermocycler. Amplicons were then digested for at least 2 h (usually 4 h) with HindIII (Euromedex; 1 IU/10 μL ampiclon) in the buffer supplied by the manufacturer, and the products were visualized by ethidium bromide staining (0.5 mg/L) of a 3% agarose gel.

When using only the A and B primers as in the reference method, the elongation time in the amplification can be shortened to 60 s, and the final elongation step at 72 °C to 7 min. However, in the adaptation we describe above, shortening the elongation steps resulted in insufficient amplification of the fibrinogen fragment and poor signal ratio for MTHFR and fibrinogen amplicons, hampering the detection of fibrinogen digestion fragments.

When coamplified with the primers A, B, C, and D as described above (90 s at 72 °C for the elongation steps and 12 min for the final elongation), DNA samples generated two DNA fragments of 552 and 198 bp for the fibrinogen Aα and MTHFR fragments, respectively (Fig. 1, lane 1). Digestion of the fibrinogen fragments for 2–4 h at 37 °C with 2 U HindIII generated three fragments of 56, 136, and 360 bp for the Aα fragments. The 56-bp fragment was not seen under our experimental conditions. As can be seen from Fig. 1, lanes 2 to 4, the 136- and 360-bp fragments did not overlap with the MTHFR amplification and digestion products. Digestion of the MTHFR fragment generated a 175-bp fragment when an allele coding for the thermolabile variant of MTHFR was present (Fig. 1, lanes 3 and 4). For homozygous patients for the thermolabile variant of MTHFR, the 198-bp fragment was absent (Fig. 1, lane 4). For heterozygous patients, both the 198- and 175-bp fragments were present (Fig. 1, lane 3), and for unaffected patients, the 175-bp fragment was absent (Fig. 1, lane 2).

For strategies based on digestion of unique sites, incomplete digestion of PCR products from homozygous patients leads to a pattern similar to heterozygous patients, and failure of digestion of PCR product from heterozygous patients leads to a pattern similar to unaffected patients. Some nonorganic DNA extraction procedures carryover marked amounts of enzyme inhibitors (13). The addition of homozygous, heterozygous, and unaffected controls in the series does not avoid tube-to-tube variability in inhibitor contamination. A first possibility is to amplify a larger DNA fragment including a second site, as reported for the inclusion of a second MstI site downstream of the sickle cell mutation (14). For rare restriction sites, this is sometimes impossible. With the method we describe here, the control fragment carries HinfI sites generating fragments that do not interfere with the detection of the MTHFR fragments. Should any incomplete digestion occur, the digestion of the control fragment would leave part of the 552-bp fragment, avoiding misdiagnosis due to incomplete digestion. This method is simple, reliable, can be set up in any laboratory, and requires only another set of primers and prolongation of the elongation steps in the PCR.

Fig. 1. Electrophoresis of PCR products.

DNA amplification and HindIII digestion of a 198-bp fragment of the MTHFR gene (accession no. U0 9806) encompassing nucleotide 677, coamplified with a 552-bp fragment of exon III of Fibrinogen Aα. Lane 1, control (no enzyme); lane 2, unaffected patient; lane 3, heterozygous patient for the C677T mutation; lane 4, homozygous patient for the C677T mutation; lane 5, negative control (no DNA); lane 6, DNA ladder.

Lipoprotein(a) [Lp(a)] is an LDL-like particle whose apo B100 is disulfide-linked to an apolipoprotein A [apo(a)]. Apo(a) is a glycoprotein containing 23% carbohydrate by weight (1). Apo(a) possesses a high degree of polymorphism because of a variable number (11–52) of the repetitive structural unit called “kringle” IV (K IV; M₄, ~17 kDa), which leads to a wide range of molecular weights, from almost 200 kDa to 3000 kDa (2). The apo(a) K IV shares a strong sequence homology with plasminogen K IV, and apo(a) has been shown to inhibit fibrinolysis in vitro (3).

A high Lp(a) plasma concentration is an independent risk factor for cerebro- and cardiovascular atherosclerosis; furthermore, low molecular mass isoforms are more frequent in patients with high Lp(a) concentrations (4). In addition, the antifibrinolytic effect of Lp(a) is inversely correlated with the size of these isoforms (5). Accurately determining the number of apo(a) Ks is, therefore, important.

The molecular weight determination of apo(a) is complicated by its anomalous mobility in SDS gels, which differs from the mobility of most commercially available molecular weight markers (1). In addition, because of the relatively small size of a K, electrophoretic techniques for apo(a) phenotyping must provide high resolution.

The aims of our study were to develop an accurate and simple method for apo(a) phenotyping by measuring the number of Ks, to validate this method by comparison with a genotyping method, and to determine, through this technique, the frequency of occurrence of the common apo(a) isoforms in healthy subjects.

We mixed EDTA-treated plasma samples from 238 healthy Caucasian subjects with antiproteases (1 μmol/L di-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, Calbiochem 520 222; 0.5 mmol/L aminoethyl-benzenesulfonyl fluoride from Sigma diluted in isopropanol; 100 000 U/L aprotinin, Bayer Pharma 92807) and stored them frozen at −80 °C until they were phenotyped.

Plasma Lp(a) was measured by an immunonephelometric assay (Beckman).

Apo(a) genotyping was performed on 15 samples by pulsed-field gel electrophoresis of KpnI-digested genomic DNA as described by Lackner et al. (6). Electrophoresis was carried out according to a method modified from Kamboh et al. (7) and using 1.5% agarose gels containing sodium dodecyl sulfate (SDS). A 1.5-mm thick gel slab containing ultra pure Seakem LE® dissolved in 90 mmol/L Tris, 90 mmol/L boric acid, 2 mmol/L Na₂EDTA, and 1 g/L SDS, pH 8.5, was cast in a 14 × 16-cm vertical cell (LKB). A commercially available calibrator (Immuno®) was prepared by mixing different plasmas; the calibrator contained five apo(a) isoforms (35, 27, 23, 19, and 14 Ks). For the first sample run, the sample was applied undiluted so that faint isoforms in heterozygous subjects could be detected. When necessary, the sample was reanalyzed after the apo(a) mass load onto the gel was adjusted to ~25 ng. Before electrophoresis, 20 μL of plasma was mixed with 60 μL of reducing buffer (1:2:10, by volume, of β-mercaptoethanol, 5 g/L bromphenol blue in 50 mL/L glycerol, and 50 g/L SDS); the mixture was then heated for 1 min at 100 °C. The mixture (3 μL) was loaded into 6 mm-wide wells cast into the gel. Gels were run at 12 °C for 2 h at a constant power of 10 W and an initial voltage of 250 V. Proteins were then pressure-transferred for 2 h to a 0.2-μm nitrocellulose (NC) membrane. The NC membrane was incubated for 15 min in a 10 g/L bovine serum albumin solution to block any remaining protein binding sites and then immersed overnight in monospecific polyclonal rabbit anti-human apo(a) antiserum from Beckman (1 mL antiserum/L buffered milk). This step can be shortened to 2 h by incubating the NC membrane in the anti-apo(a) antiserum diluted to 5 mL/L buffered milk. After the filter was washed, it was incubated with a goat anti-rabbit IgG conjugated with peroxidase (Sigma A4914; 0.5 mL/L buffered nonfat milk). After the filter was washed extensively, the apo(a) banding pattern was visualized through chemical staining for peroxidase with diaminobenzidine.

In our method, the dye in the samples runs off the gel during electrophoresis; therefore, we measure the absolute distance of migration. A standard curve was thus generated by plotting the distance of migration of the standard bands against the log of the K number, using least-square regression analysis.

The detection limit of the method was determined by analyzing a sample with a known Lp(a) concentration at different dilutions [100, 10, and 1 ng apo(a)].

Statistical analyses were performed with Statview IV Software (Alsyd 38240). The relationship between the number of Ks and the Lp(a) concentrations was estimated using polynomial regression. Lp(a) concentrations in men and women were compared using an ANOVA in which log Lp(a) concentrations were entered because of the skewness of the Lp(a) concentration distribution. Lp(a) concentration frequency distributions in men and women