

# Mutations in the Carcinoembryonic Antigen Gene in Colorectal Cancer Patients: Implications on Liver Metastasis<sup>1</sup>

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## Abstract

Carcinoembryonic antigen (CEA) expression is used clinically to monitor patients with colorectal and other cancers. A subset of patients have extraordinarily high CEA levels that cannot be attributed solely to tumor load. We have shown mutations in the region of CEA (PELPK motif) responsible for its hepatic clearance in three of eight patients with high CEA levels. We used denaturing high-performance liquid chromatography to provide evidence of polymorphism in these patients. These mutations were scored by DNA cycle sequencing and shown to be heterozygous. The patients with mutations in the PELPK motif showed remarkably reduced circulatory clearance rates in an animal model. A patient without mutation in the region showed normal clearance rates. Mutations in PELPK may affect structural stability and binding affinity to the Kupffer cell receptor in the liver. These studies have implications for the role of CEA as a facilitator of hepatic metastasis.

## Introduction

CEA<sup>3</sup> is a glycoprotein belonging to a large gene family that includes the pregnancy-specific glycoproteins. All of these proteins are members of the much larger immunoglobulin supergene family. CEA itself has a molecular mass of 180–200 kDa, depending on the extent of its glycosylation (1, 2). The protein consists of a series of immunoglobulin-like domains, a 108-amino acid V domain at the NH<sub>2</sub> terminus, and three pairs of C2-like domains, each of 178 amino acids. There is a small hydrophobic COOH-terminal domain (26 amino acids) that is modified to give a glycosylphosphatidylinositol-linked membrane anchor. Its structural features also allow up to 27 carbohydrate chains of the N-linked type. The complete CEA gene has been cloned and includes a promoter region that appears to confer cell type-specific expression (3). Because the serum level of CEA is used clinically in the evaluation of colorectal cancer patients, there has been an interest in determining what controls CEA concentration in the circulation. Input of CEA into the circulation is controlled by production rates of the tumor, its location and stage, size, differentiation, vascularity, and the presence or absence of distant metastases. However, the serum level is also controlled by the rate of CEA elimination. Elevated levels of serum CEA are found in patients with benign liver disease including cirrhosis, biliary obstruction, and hepatitis (4–6), and the highest CEA levels are usually associated with patients with liver metastases (7). Because some patients tend to have extraordinarily high serum CEA (8), often with a low tumor load (9) and in the absence of liver metastasis, we were interested in determining the cause.

CEA injected into experimental animals accumulates in the liver and is largely confined to the resident macrophages (Kupffer cells; Ref. 10). The use of proteolytic (pepsin) digests and examination of the Kupffer cell binding properties and sequences of the isolated peptides showed that CEA binds to the cells via the hinge region between its N domain and its first (A1) immunoglobulin loop domain. Use of overlapping synthetic peptides based on the known sequences in this region narrowed the binding domain to five amino acids (PELPK), located at amino acids 108–112 in the sequence (11, 12). Molecular modeling studies have suggested that this region is exposed on the surface of the molecule (13).<sup>4</sup> Studies with isolated Kupffer cells also showed that the critical amino acids involved in binding were the LP part of the sequence (14). We were interested in looking at DNA coding for the PELPK binding sequence itself to see whether patients with high serum levels may have alterations in CEA structure that can affect clearance rates. We report on three patients with high CEA levels who have mutations in the CEA gene at the region coding for the PELPK sequence. This is the first reported observation of mutations in the CEA coding sequence. These observations also have implications in colorectal cancer metastasis to the liver because CEA appears to be a facilitator and acts by its ability to activate Kupffer cells (15). Activation of Kupffer cells results in a release of cytokines, which influences retention and survival of tumor cells in the liver (16–18). The inability of CEA to bind to Kupffer cells may thus reduce the incidence of hepatic metastasis, even in the presence of extraordinary high CEA levels.

## Materials and Methods

**CEA Purification.** CEA was purified from exudate ascitic fluids of five of eight colorectal cancer patients by perchloric acid extraction and gel filtration on Sepharose 4B and Sephadex G-200 (10). Immunoreactivities were compared with the Roche standard CEA (Roche, Indianapolis, IN) immunoassay. The purified CEA proteins were examined by 7.5% SDS-PAGE, all showing a single diffuse band corresponding to a molecular mass of ~180 kDa (data not shown).

**Analysis of CEA Clearance Rates.** Rates of clearance of CEAs from the circulation of rats were determined as follows. Male Sprague Dawley rats were anesthetized with 50 mg/kg of sodium pentobarbital given i.p. Sufficient CEA (~20 µg for a 250-g rat), unpurified as native CEA in the patient's extracted serum, was injected into the femoral vein of rats to yield an initial concentration of 15 ng/20 µl of rat blood. Injections were of 0.5 ml of sample/animal. Blood (20 µl) was collected from the tail vein at 2-min intervals for 10 min and then at 5-min intervals to 1 h. The blood samples were pipetted promptly into 1 ml of 0.037 M EDTA buffer (pH 7.6) containing 0.02% of BSA. The samples were assayed for CEA content in duplicate using the Roche immunoassay as described previously. Clearance rates of purified <sup>125</sup>I-labeled CEA were measured in the same way, except that the <sup>125</sup>I content of the blood samples was measured directly in an autogamma counter (Packard). Clearance values could not be obtained for all patients in the study because those with relatively low CEA levels (<2000 ng/ml) did not have sufficient CEA for detection in the rat blood samples.

Received 12/29/00; accepted 2/15/01.

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<sup>1</sup> Supported by NIH Grant CA74941.

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<sup>3</sup> The abbreviations used are: CEA, carcinoembryonic antigen; DHPLC, denaturing high-performance liquid chromatography; wt, wild type; TEAA, triethylamine acetate.

<sup>4</sup> P. A. Bates, personal communication.

**DNA Preparation and PCR.** Tumor cells were collected from 500  $\mu$ l of frozen ascites material from each patient by centrifugation at  $14,000 \times g$ . Genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. Hot-Start PCR was performed using 200 ng of genomic DNA from the ascites material, and final concentrations of 2.5 mM  $MgCl_2$ , 200  $\mu$ M of each dATP, dCTP, dGTP, and dTTP (Life Technologies, Grand Island, New York), 250 nM of each primer,  $1 \times$  GeneAmp PCR Gold buffer, and 0.5 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California) were combined to a final reaction volume of 50  $\mu$ l. PCR cycling was as follows. An initial denaturing cycle at 95°C for 5 min was used, followed by 30 cycles of 94°C, 60°C, and 72°C for 30 s each, and a final extension cycle at 72°C for 5 min. Oligonucleotide primers were designed using the Primer3 program (MIT, Boston, MA) to generate an amplicon of 319 bp from a region straddling intron 2 and the hinge of the A1 domain of CEA. The forward and reverse sequences were 5'-TGGAAGGTGCCAACTCTGAT-3' and 5'-CTGGGTTCTGGGTTTCACAT-3', respectively. A second set of PCR was performed using primers straddling most of the N domain and the upstream region of intron 2 (5'-GTGCTGGTGTGTGCTGTC-3' and 5'-TTGGGTCGACACTAGTACGC-3') to generate a 384-bp amplicon. PCR conditions were the same as before.

**DHPLC and DNA Sequencing.** DHPLC analyses of the 319-bp amplicons from the CEA-A1 domain (GC content, 53.5%) were performed using the WAVE system (TransGenomic, Crewe, Cheshire, United Kingdom). PCR products were run both unmixed and mixed 1:1 with a reference (wt) DNA, which was obtained from a colorectal cancer cell line, MIP101. Samples were denatured at 95°C for 5 min and cooled down to 65°C over 40 min. The samples were kept at 4°C until volume between 8 and 10  $\mu$ l of crude PCR product was loaded onto a preheated reverse phase column based on nonporous poly(styrene-divinyl-benzene) particles (SNASep column; TransGenomic, Crewe) using an automatic sample injector model L7200. PCR products were eluted from the column using an acetonitrile gradient in a 0.1 M TEAA buffer (pH 7.0) at a constant flow rate of 0.9 ml/min. The gradient was created by mixing eluents A and B, which were delivered using the solvent delivery system model L7100. Eluent A was 0.1 M TEAA, 0.1 mM  $Na_3EDTA$ . Eluent B was 25% acetonitrile in 0.1 M TEAA. The temperature at which heteroduplex detection occurred was deduced from the melting profile of the specific DNA fragment using a newly developed algorithm (WaveMaker; TransGenomic, Crewe). Gradient information and melting temperatures used to analyze the fragment are 54–64% buffer B at 60°C, 53–63% buffer B at 63°C; in each case, the method started at 5% less buffer B than the gradient for 0.1 s. The fragments were analyzed over 5 min gradient with a total run time of 7.8 min/sample. The linear acetonitrile gradient was adjusted so the peaks were eluted between 3 and 5 min. Regeneration of the column was achieved by washing with 100% buffer B for 2 min. The eluted DNA fragments were detected by UV absorbance at 260 nm. DNA sequencing was performed using the Big-Dye Terminator Cycle Sequencing Ready Reaction kit and the ABIPRISM 377 system (Applied Biosystems, Foster City, CA). Sequencing was repeated three times for each patient sample from three separate PCR reactions in both forward and reverse strands.

## Results and Discussion

Because many human genetic diseases contain heterozygous mutations, we decided to investigate this possibility in colorectal cancer patients expressing very high levels of circulatory CEA. The rationale behind this is that the presence of heterozygous mutations in the liver binding sequence, PELPK, at the hinge region between the N and the A1 immunoglobulin domain could explain the presence of high levels of plasma CEA (*e.g.*, they are theoretically expressing both mutated and nonmutated CEAs in similar levels), while simultaneously, the low level of Kupffer cell uptake of nonmutated CEA would account for the absence of liver metastasis in most of these patients. We have therefore examined DNA from tumor cells in peritoneal exudate ascitic fluid samples from a total of eight patients ranging from moderately high (>250 ng/ml) to very high (>10,000 ng/ml) serum CEA levels (4).

For circulatory clearance analysis, patients were selected on the basis of both high CEA levels and the occurrence of ascites or pleural

effusion (in one case, patient 4). CEA levels can be used to distinguish transudate from exudate ascites (18). In the case of these patients, the exudate has higher CEA levels in the ascites than in serum. These ascites were, therefore, not attributable to portal hypertension from liver metastasis but were an exudate attributable to the presence of peritoneal tumor.

Plasma CEA level and its clearance from the circulation are expressed in half-life ( $T_{1/2}$ ), and ranged from 7 to 136 min (Table 1). The rates of CEA clearance for four patients were plotted (Fig. 1). Patients 2 and 7 showed similarly slow clearance rates, whereas patient 3 showed intermediate rates. Patient 4 presented near normal clearance (10).

The DNAs of interest were initially amplified by conventional PCR from tumor cells collected from ascitic fluid. The N and first immunoglobulin loop (A1) domains were amplified in two stages as intron 2 cuts through the coding triplet for the first Pro (P) in the PELPK sequence. The DHPLC technique (19–22) was used to screen for the presence of mutations in the PELPK region of CEA. DHPLC exploits the differential retention of homo- and heteroduplex DNA molecules on a reusable matrix of alkylated nonporous particles under partial heat denaturation by ion-pair reversed phase liquid chromatography. After conventional PCR, the amplicons were denatured to allow the formation of heteroduplex species. A total of three samples from the eight patients tested showed polymorphic-mutated chromatograms when compared with a wt sample. Sample 7 showed a distinctive chromatogram compared with the wt, when the sample was mixed 1:1 ratio with wt PCR products (Fig. 2A). This result is seen at both temperatures tested, 60°C and 63°C, based on the biphasic melting curve (Fig. 2C), suggesting homozygosity. No other sample differed from the wt chromatogram in this fashion. However, by DNA sequencing, we observed that the PELPK sequence mutations on patient 7 were heterozygous. When samples alone are compared with the wt chromatogram, we found some more differences. Samples 2 and 3 also showed a different profile when compared with the wt, suggesting that the mutations present in these samples are heterozygous because this pattern only appears when they are not mixed in a 1:1 ratio with the wt PCR product (Fig. 2B).

Chromatograms of both forward and reverse DNA sequences in the PELPK region of three patients are shown (Fig. 3). Point mutations were detected in the PELPK region of patients 2, 3, and 7 only in the forward strand (Fig. 3), thus indicating heterozygosity. The three patients with PELPK mutations either had no evidence of metastasis to the liver or developed it late in their disease progression. In addition, point mutations were detected in two other sites close to but outside the PELPK region in all patients analyzed (Fig. 4A). These mutations, however, appear to have no correlation with CEA clearance rates (see patient 4). No other mutations from the wt were detected for the area sequenced. CEA peptide sequences (Fig. 4B)

Table 1 Plasma CEA levels and circulatory CEA clearance rates in colorectal cancer patients

Patients 2, 3, 6, and 7 produced enough plasma CEA to allow three repetitions of CEA clearance in rats. Patient 4 produced intermediate levels of CEA that allowed for a single clearance experiment. Patients 5, 9, and 10 did not produce enough CEA for the performance of clearance experiments.

| Patient no. | CEA level (ng/ml) | CEA clearance (min to $T_{1/2}$ ) |
|-------------|-------------------|-----------------------------------|
| 2           | 16,800            | 124 ( $\pm$ 10)                   |
| 3           | 12,700            | 60 ( $\pm$ 9)                     |
| 4           | 9,165             | 7                                 |
| 5           | 1,360             |                                   |
| 6           | 60,000            | 18 ( $\pm$ 2)                     |
| 7           | 115,000           | 136 ( $\pm$ 32)                   |
| 9           | 252               |                                   |
| 10          | 353               |                                   |

were inferred directly from the DNA sequences. Patient 3 has changes in amino acid sequence where an Arg (R) replaces the first Pro (P) and a Ser (S) replaces the second Pro (P). Patients 2 and 7, in addition to these two changes, have a Gly (G) replacing a Glu (E). In all cases, the two Ser (S) after the PELPK sequence are replaced by Phe (F), this

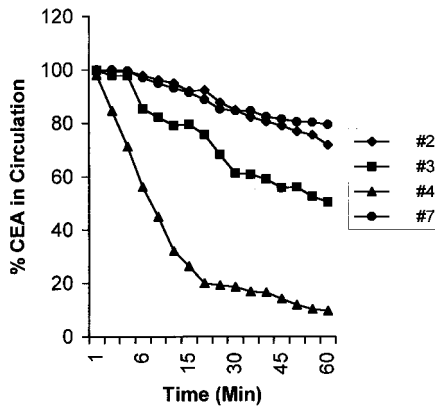


Fig. 1. Clearance rates for CEA from four patients (see text) from the circulation of the rat. Half-lives for the CEAs are given in Table 1.

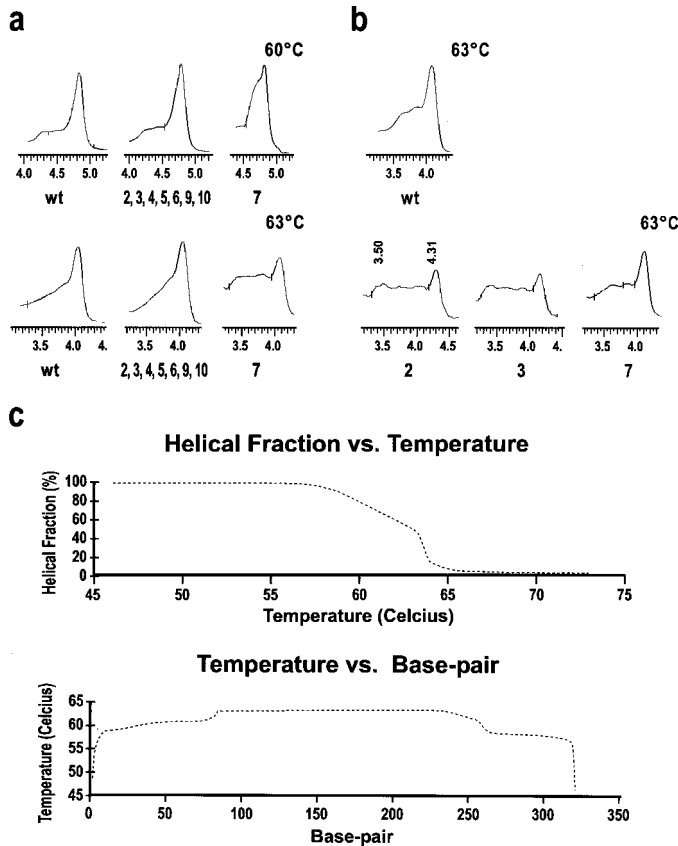


Fig. 2. DHPLC chromatograms of CEA A1 domain PCR fragments of eight patients plus the wt. *Y axis*, peak intensity expressed in mV (values not shown); *X axis*, run times in min. *a*, DHPLC analysis of patients' samples mixed 1:1 with the wt and performed at both 60°C and 63°C. Only patient 7 showed an altered profile when compared with the wt curve. *b*, DHPLC analysis of unmixed samples of three patients showing altered profiles that confirmed heterozygosis. *c*, the top melting curve shows the estimated helical fraction (i.e., the fraction of double stranded DNA in the denaturing amplicon) at different temperatures. The melting temperature used for DHPLC analysis is calculated based on the point at this graphic where the PCR amplicon is ~75% denatured (in this case, 60°C). The second graphic (bottom) displays the temperature required for melting along the amplicon sequence and its subdomains. The graphic indicates the temperatures required for melting of the double-stranded PCR product as an indirect function of the GC content. This graphic suggested the use of 63°C to resolve the middle part of the fragment.

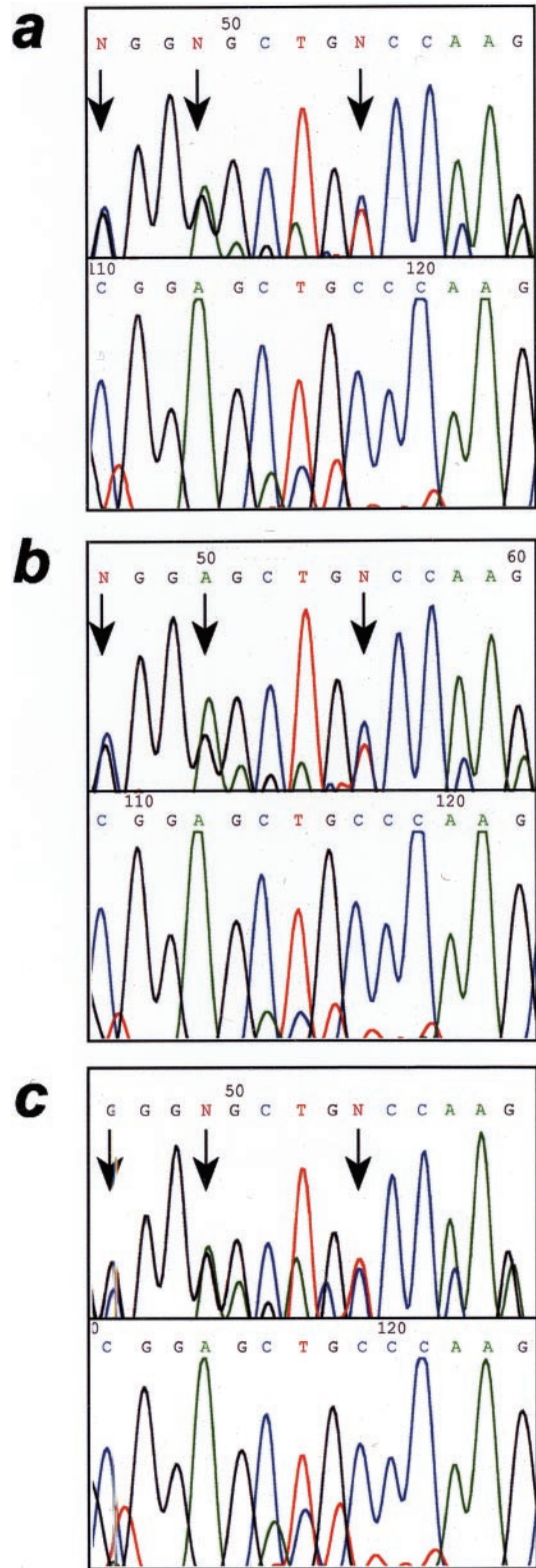


Fig. 3. *a*, patient 2; *b*, patient 3; *c*, patient 7. Chromatograms of DNA sequences of three patients at the PELPK region (arrows) showing heterozygous mutations in the forward strands (top) but not in the reverse sequences (bottom).

results from a single point mutation in the codon from TCC to TTC in both instances.

Samples derived from tumors, however, will often deviate from a 1:1 ratio of mutant to the wt sequences (19, 23). The amount by which this ratio is skewed depends on multiple factors, among them are the



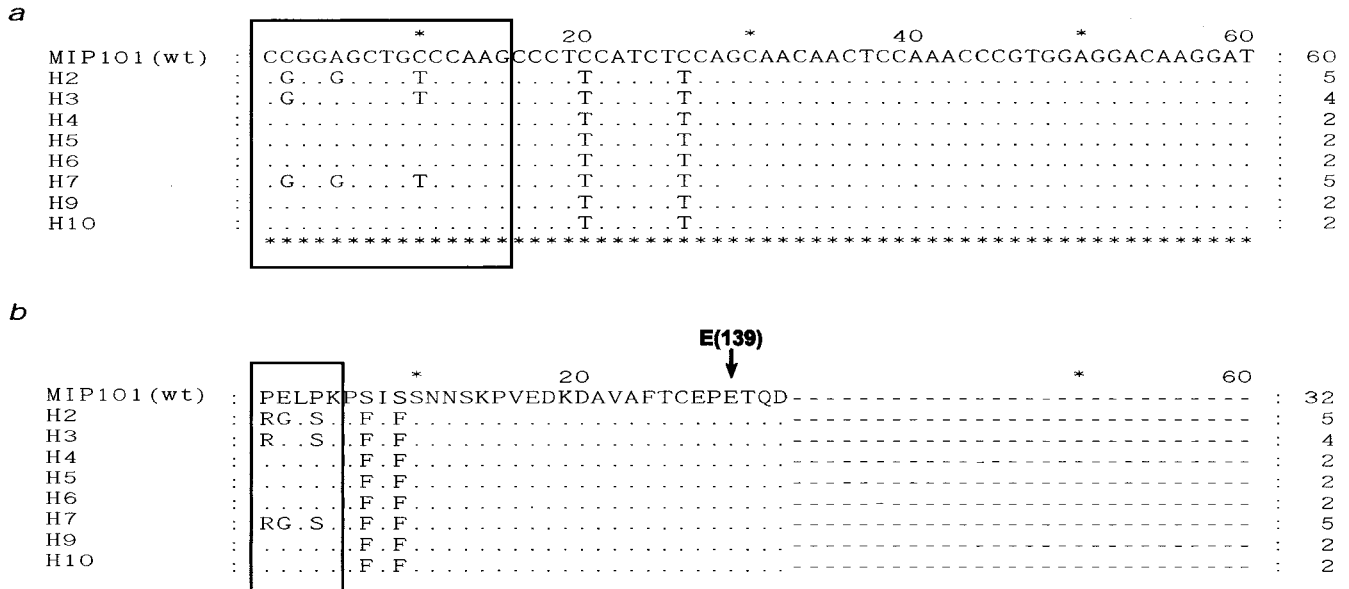


Fig. 4. Partial DNA sequences and inferred peptide sequences of the CEA A1 domain amplicons. *a*, aligned DNA sequences of the 319-bp PCR fragment. *Boxed area*, PELPK-coding region. *b*, aligned peptide sequences were inferred from the DNA sequences above. *Boxed area*, PELPK region. *Arrow*, Glu(139) that presents potential electrostatic interaction with the mutated amino acids of the link region.

relative amounts of tumor and normal cells in the sample and the presence of multiple mutant species in the sample. As for cycle sequencing, dye-terminator chemistry can result in uneven peak heights and, consequently, produce an inaccurate heterozygous diagnosis, specially when scoring the reverse sequence (24). Furthermore, it was shown that dye-terminator sequencing using AmpliTaq DNA polymerase produces larger C peaks after a G but minimizes the G peaks following A (25). This effect could also be seen in the forward sequences of three patients that showed heterozygous mutations (Fig. 3).

We have known for some time that patients with very high CEA serum levels are unable to clear CEA from the circulation at the normal rate (8, 9). The reason for this is unclear, but it was speculated earlier that association with another protein may block the binding site and thus retard Kupffer cell uptake (26). However, our previous studies have shown that the sequence PELPK and especially an intact LP (Leu, 110; Pro, 111) sequence is necessary for Kupffer cell binding (14). A similar ligand binding area between two immunoglobulin-like domains is found in the fibroblast growth factor receptor (27). This linking region is one of the main points of contact with the growth factor, and the sequence is similar to that seen in the CEA linker. This appears to support the idea that links between immunoglobulin domains can be involved in protein-protein interactions. Dr. Paul Bates<sup>4</sup> has looked at the effect of the RELSK and the RGLSK mutations in his computer simulation of the CEA structure and found that, when he graphically mutates these residues in the link region, they both make an electrostatic interaction with Glu (E, 139), the first E within the sequence ETQD. This is because the electrostatic interaction between Glu-139 and the link mutations restricts the free movement between the N and A1 domains, and this may be needed for docking to the Kupffer cell receptor. The changes in these regions of the molecule have a profound effect on the biological behavior of CEA. For instance, CEA purified from patient 7 clears from the circulation of the rat with a  $T_{1/2}$  of  $136 \pm 32$  min, compared with a wt CEA with a  $T_{1/2}$  of  $3.7 \pm 0.8$  min (10). Similar effects are seen in patients 2 and 3. The replacement of Ser-114 with the bulky hydrophobic amino acid Phe (F) seen in these patients could also result in conformational changes and interfere with binding. Patients 4 and 6

did not have the mutations in the PELPK sequence but had the mutation at Ser-114. They had a reduced rate of clearance, but not to the extent of the PELPK mutations. The buildup of the mutated molecule in the blood and ascitic fluids is presumably attributable to its inability to be cleared by the liver (Kupffer cell). Thus, these patients have very high CEA levels without Kupffer cell activation. Because the production of cytokines by the activated cells is a factor in the development of hepatic metastasis (15–17), this could explain the lack of hepatic tumor in patient 7, even in the presence of very high CEA levels. The mutations reported here may affect binding to the Kupffer cell receptor by various mechanisms, including decreased mobility between immunoglobulin domains, altered dimerization, and alterations in the affinity of ligand-binding recognition. Similar effects have been reported for the *fibroblast growth factor receptor 2* (28), *vascular endothelial growth factor receptor/placenta growth factor Fit-1* (29), and *rhoGDI* (30). Future studies will determine the effect of these mutations on prognosis in colon cancer patients.

#### Acknowledgments

We thank Dr. Gloria Ribas-Despuig (UK-HGMP, Cambridge, United Kingdom) for the DHPLC analysis and Dr. Paul A. Bates (Imperial Cancer Research Fund, London, United Kingdom) for helpful comments on CEA molecule modeling.

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