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Colon cancer secreted protein-2 (CCSP-2), a novel candidate serological marker of colon neoplasia

Baozhong Xin[1,2], Petra Platzer[1,2,5], Stephen P Fink[1,2,5], Lisa Reese[1,2], Arman Nosrati[1,2], James KV Willson[1,2], Keith Wilson[3,5] and Sanford Markowitz*[,1,2,4,5]

[1]*Department of Medicine and Ireland Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106, USA;* [2]*University Hospitals of Cleveland, Cleveland, OH 44106, USA;* [3]*Protein Design Labs, Fremont, CA 94555, USA;* [4]*Howard Hughes Medical Institute, Cleveland, OH 44106, USA*

Cancers of the colon and rectum are the second leading cause of cancer death among adult Americans. When detected at early stages, colon cancer is highly curable. Colonoscopy, an effective but invasive screening test, has been limited in its public acceptance. The goal of this study was to identify novel serum markers of colon cancers and precancerous colon adenomas as potential candidates for noninvasive detection of early colon neoplasms. Employing expression microarrays, we identified colon cancer secreted protein-2 (CCSP-2) as a novel transcript whose expression is generally absent in normal colon and other normal body tissues, but that is induced an average of 78-fold in Stage II, III, and IV colon cancers, as well as in colon adenomas and colon cancer cell lines. These findings were validated by real-time PCR analysis in an independent panel of colon cancer cases. Moreover, CCSP-2 was shown to encode a secreted protein that circulates stably and is detectable in the blood of mice bearing human cancer xenografts transfected with epitope-tagged CCSP-2. As a novel secreted protein that is markedly induced in colon adenomas and cancers, CCSP-2 is a novel candidate for development as a diagnostic serum marker of early stage colon cancer.
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Introduction

Cancers of the colon and rectum are the second leading cause of cancer incidence and of cancer death among adult Americans, with 135 000 new cases and 57 000 deaths in 2001, and with a 6% lifetime risk of developing the disease (Greenlee *et al*., 2001). These considerations have led to recommendations for mass screening of the average risk adult population for colon cancer starting at age 50 years (Bond, 2000; Pignone *et al*., 2002; Schoen, 2002; Smith *et al*., 2002). Despite these recommendations, only an estimated 25–35% of at-risk adults have undergone a colon cancer screening procedure (Vernon, 1997), highlighting the limitations of currently available colonoscopy-based screening. While serologic screening for colon cancer has theoretical appeal, serum CEA testing is unsuitable for screening an average risk population in which it would yield an estimated positive predictive value of only one in 100 (ASCO, 1996; Bast *et al*., 2001) for colon cancer, and would additionally fail to detect advanced colon adenomas that are direct colon cancer precursors.

The aim of this investigation was to use gene microarray technology to identify novel candidate serologic markers of colon adenomas and cancers. To this end, we generated comprehensive gene expression profiles of normal colon epithelium, colon adenomas, and colon cancers, and then analysed these data to define transcripts that were expressed by neoplastic but not by normal colonic epithelium, and whose sequences predicted for the encoding of secreted proteins.

Results

Identification of CCSP-2, a candidate marker of colon neoplasia

To identify novel candidate markers of colon neoplasia, we used GeneChip gene expression microarrays to compare patterns of gene expression in RNA samples extracted from colon cancers versus normal colon epithelium (Platzer *et al*., 2002). In all, 21 dissected colon epithelial strips from normal colonic mucosa were compared to a group of 72 primary and metastatic colon cancer resection specimens and to 36 colon cancer cell lines on DNA microarrays that measure gene expression of approximately 55 000 genes, EST clusters, and predicted exons (Platzer *et al*., 2002). Among all transcripts that showed a fivefold or greater increase in median expression level in colon cancer samples

*Correspondence: S Markowitz, Howard Hughes Medical Institute, Case Western Reserve University, Wolstein Research Building, 3rd Floor, Mailstop 7285, 10900 Euclid Avenue, Cleveland, OH 44106-7285, USA; E-mail: sxm10@cwru.edu
[5]These authors contributed equally to this work
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compared to normal colon epithelium, one transcript, an EST corresponding to GenBank Accession number AI357412, was noted to be of particular interest in that detectable expression of this transcript was absent in every one of the normal colonic epithelial samples, none of which showed expression above the microarray measurement threshold of 25. As shown in Figure 1, AI357412 expression was well detected on the microarrays in colon cancer primary tumors (median value 186), colon cancer hepatic metastases (median value 148), and colon cancer cell lines (median value 128), but was not detected above threshold in any normal colon epithelial sample. AI357412 expression was similarly elevated among early node-negative Stage II colon cancers as among more advanced node-positive Stage III cancers and as among Stage IV cancers with distant metastases (Figure 1). Moreover, AI357412 expression was also similarly elevated among a set of colon adenomas of 1 cm or greater in size, which are benign direct precursors of frank colon cancer (Figure 1). Thus, AI357412 expression is potentially a marker of early colon neoplasias for which clinical intervention can be curative.

AI357412 corresponds to a 340-bp EST sequence mapping to chromosome 10q25.3. To derive a full-length transcript corresponding to this EST, we identified by a BLAST search all ESTs in the dbEST database that mapped to within 50 kb of AI357412. Connection RT–PCR was used to identify ESTs linked to AI357412. Additional sequence was generated both by sequencing of these RT–PCR-amplified products and by sequencing of image clones corresponding to the linked ESTs. The 5′ and 3′ rapid amplification of cDNA end (RACE) PCR methods were used to complete the derivation of a transcript that corresponded to AI357412 and that contained the complete coding region of a novel gene. This gene, named colon cancer secreted protein-2 (CCSP-2), encodes an mRNA

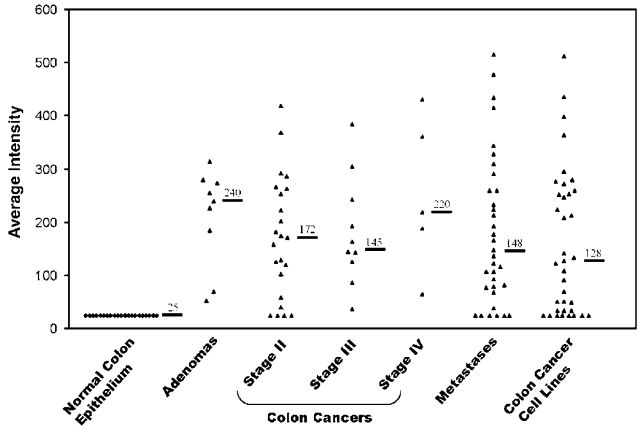

Figure 1 Expression of AI357412 on GeneChip microarrays. Shown for comparison are analysis of RNA samples from normal colon epithelium, colon adenomas, primary colon cancers of Stages II, III and IV, colon cancer hepatic metastases, and colon cancer cell lines. Horizontal bars denote median expression values within each group

consisting of at least 14 exons with an ATG start codon in exon 2 and a TGA stop codon in exon 14 (Figure 2a). The assembled CCSP-2 transcript contains an in-frame TAG stop codon 5′ to the presumptive ATG start codon (Figure 2b), and contains an open-reading frame encoding a 755 amino–acid (aa) protein with estimated molecular mass of about 83 kDa (Figure 2b). An analysis of the CCSP-2 protein sequence using the SignalP algorithm (Nielsen and Krogh, 1998) within the CBS prediction service (http://www.cbs.dtu.dk/services/) importantly identified a 23-aa predicted signal peptide, with a likely cleavage site between P23 and L24 (Figure 2b), suggesting that CCSP-2 may be a secreted protein, and hence a remote marker of colon cancers and adenomas. A further search for CCSP-2 protein motifs using the Pfam HMM search service (http://pfam.wustl.edu/hmmsearch.shtml) identified two epidermal growth factor (EGF) domains and three von Willebrand factor type A (vWA) domains within the protein (Figure 2b). BLAST alignment also showed that the CCSP-2 protein shared 80% identity to a predicted human protein (Accession number XM_291673), designated as 'similar to hypothetical protein 4832416E03', which was computationally predicted from the CCSP-2 locus (NCBI contig NT_030059) by the GenomeScan gene prediction method. Supplementary Figure S1 shows the genomic alignment of the cloned colon cancer CCSP-2 transcript as compared to the predicted structure of XM_291673. No sequences included within the predicted gene but excluded from CCSP-2 transcript were detectable by RT–PCR analysis of multiple colon cancer RNA samples. BLAST analysis of CCSP-2 also showed 75% homology to a mouse hypothetical protein 4832416E03 of unknown function (Accession number NP_766428).

CCSP-2 expression is commonly induced in colon cancer tissues and cell lines

As shown in Figure 3a, Northern analysis strongly corroborated that CCSP-2 is expressed by malignant but not normal colon tissues, detecting a single 5.5 kb CCSP-2 transcript with moderate to strong intensity in nine of 12 colon cancer cell lines, but in none of three normal colon epithelial tissue samples. To provide a more quantitative measurement of CCSP-2 induction, we extended this analysis by employing real-time PCR. Real-time PCR demonstrated only barely detectable CCSP-2 expression in eight of eight normal colon epithelial samples (mean value 7.3, range 1–17.5), whereas colon cancer cell lines showed an average 64-fold increased level of expression (mean value 468, range 4.5–1470), with 11 of 15 colon cancer cell lines showing a greater than 35-fold increase in expression (Figure 3b).

Nearly identical results were obtained by real-time PCR analysis of CCSP-2 expression in primary colon cancers versus matched normal colon mucosa from the same individual. CCSP-2 expression in primary colon cancer tumors showed a mean 78-fold increase versus matched normal mucosa (Figure 3c), with colon cancers

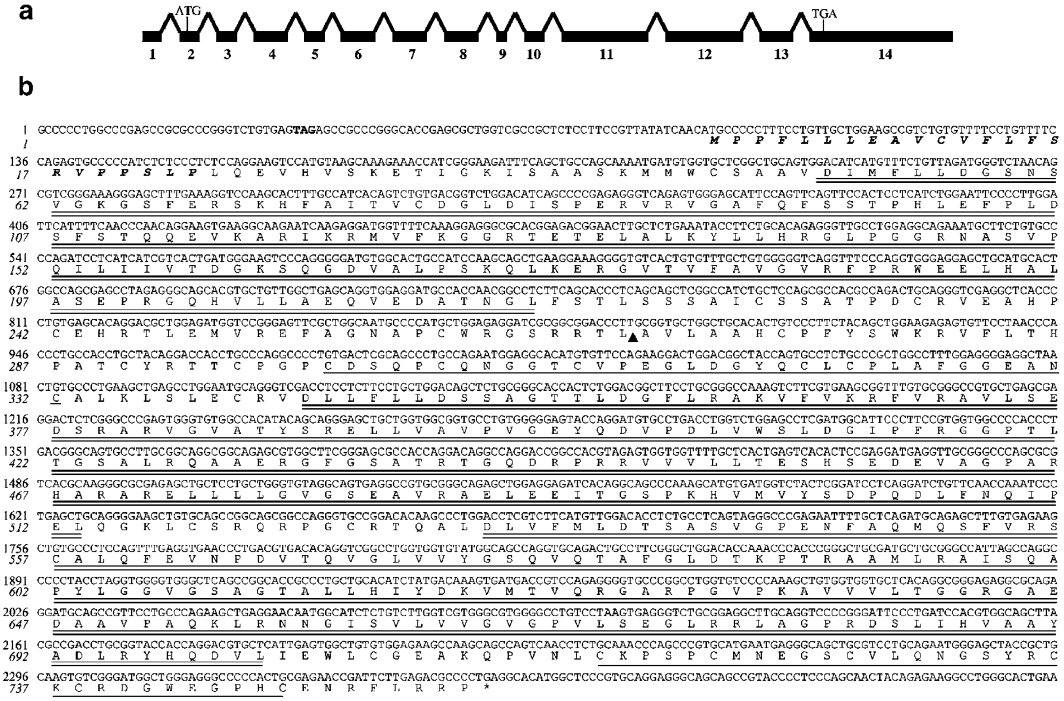

Figure 2 Structure of the *CCSP-2* gene. (**a**) Numbered black boxes denote the 14 *CCSP-2* gene exons, with locations of initiator ATG and termination TGA designated. (**b**) Nucleotide and deduced aa sequence of complete CCSP-2 coding region. The CCSP-2 nucleotide sequence is provided in the upper reading frame and numbered in roman type. The deduced aa sequence is provided underneath the nucleotide sequence, and is numbered in italic. The in-frame stop codon 5′ of the start codon is indicated in boldface. The putative signal peptide is shown boldface italics. The cleavage site producing the 55 kDa CCSP-2 peptide is indicated by an arrow. The predicted EGF and vWA domains are indicated by an underline and a double underline, respectively

showing a mean expression value of 367 (range 1–1176) versus a mean value in normal mucosa of 7 (range 1–20). In half of colon cancer cases, CCSP-2 induction in the tumor exceeded 46-fold, with 79% of cases showing at least an eightfold or greater induction (Figure 3c). The 28 colon cancers examined by real-time-PCR constituted a 'validation set' of samples completely independent of those that had been previously characterized on the GeneChip expression microarrays.

CCSP-2 encodes a secreted protein

As discussed, analysis of the 755-aa CCSP-2 sequence predicted that aa 1–23 comprise a signal peptide, and that cancer cells accordingly would secrete the CCSP-2 protein. To test this prediction, we constructed a eukaryotic expression vector expressing CCSP-2 fused to a V5-His epitope tag at the carboxyl-terminus. Two colon cancer cell lines, Vaco-400 and SW480, were transiently transfected with the CCSP-2-V5-His-tagged expression plasmid. At 48 h post-transfection, the cell culture media were harvested and any secreted CCSP-2 protein was collected by immunoprecipitation with an anti-V5 antibody. Expression of CCSP-2 protein was then visualized by Western blot analysis, with an anti-V5 antibody used to compare CCSP-2 protein levels detected in 10% of the conditioned culture medium versus that detected in a lysate from 10% of the corresponding transfected cell pellet. As shown in Figure 4, an 85 kDa V5-tagged CCSP-2 protein band was well detected in the cell culture medium from both Vaco-400 and SW480 CCSP-2-transfected cells, but was not detected in medium from cells transfected with an empty expression vector. CCSP-2 protein was additionally detected in the cell pellets from CCSP-2-transfected SW480 and Vaco-400, but the cell pellet-associated CCSP-2 was of much lesser amount than corresponding CCSP-2 detected in the cell culture medium. Thus, as predicted, the expressed CCSP-2 protein is mainly secreted by colon cancer cells, and accumulates stably in cell culture medium.

CCSP-2 secretion into blood of xenografted mice

To determine whether secretion of CCSP-2 by a tumor is sufficient to enable CCSP-2 protein to gain access to the circulation and serve as a detectable tumor marker in blood, we employed as a model system athymic mice xenografted with tumors expressing the CCSP-2-V5-tagged protein. We anticipated that ambient immunoglobulin levels in mouse serum would pose a technical challenge to our previous assay for detecting V5-tagged CCSP-2 using a mouse monoclonal antibody. Accordingly, we developed a 'direct' detection assay in which immunoprecipitation of V5-tagged CCSP-2 was

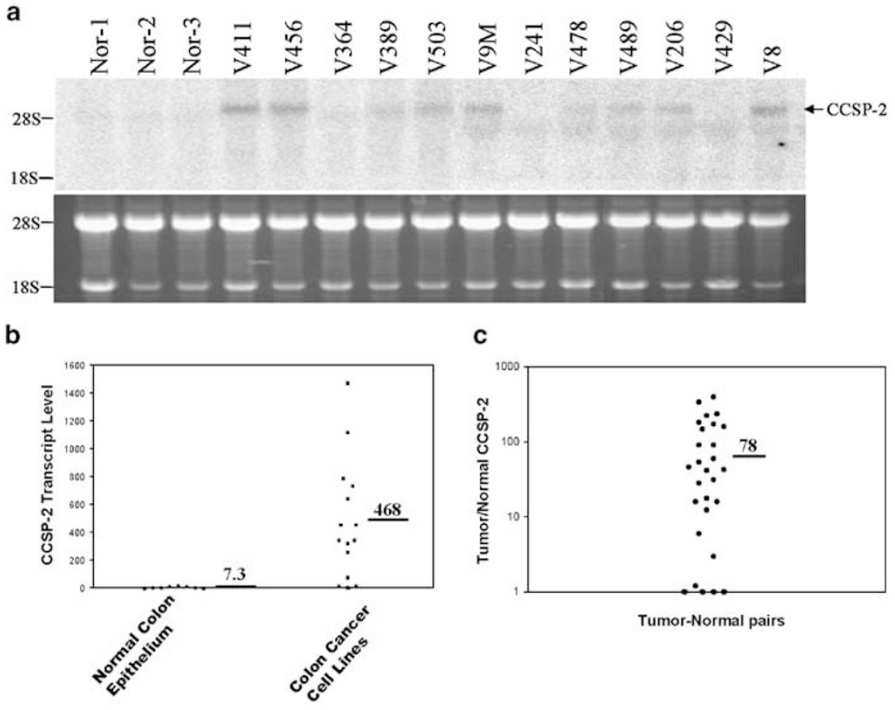

Figure 3 CCSP-2 mRNA expression in normal colon epithelium and colon cancer samples. (**a**) Northern blot analysis of CCSP-2 expression in three normal colon epithelium samples (Nor-1, -2, and -3) and 12 colon cancer cell lines (designated by the prefix V) (upper panel). Lower panel shows the ethidium bromide staining of 28S and 18S ribosomal RNA subunits for each of the corresponding samples. (**b**) Real-time PCR measurement of CCSP-2 transcript expression in eight normal colon epithelial samples versus 15 colon cancer cell lines. CCSP-2 values are normalized against expression of the house-keeping gene B2M. Black bars indicate the mean value for each group. (**c**) Real-time PCR measurement of the ratio of CCSP-2 expression in colon cancer versus matched normal colon mucosa as determined in 28 patients. Horizontal bar indicates the mean value for CCSP-2 induction of 78

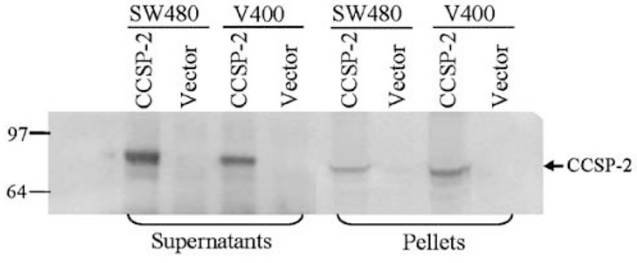

Figure 4 Secretion of CCSP-2 protein. Shown is detection by Western blot assay of CCSP-2 protein in lysates of CCSP-2-transfected cells (pellet) versus in the immunoprecipitates from corresponding cell culture media (supernatant). CCSP-2 lanes designate samples from cells transfected with an expression vector encoding V5-His epitope-tagged CCSP-2. Vector denotes samples from cells transfected with a control empty expression vector. Immunoprecipitation and Western blot assays were performed using a mouse monoclonal antibody directed against the V5 epitope-tag. Results are shown for transiently transfected SW480 and Vaco-400 (V400) colon cancer cell lines. An arrowhead denotes the 85 kDa CCSP-2 protein detected predominantly in the cell culture medium of the CCSP-2-transfected cells. Not displayed is contaminating IgG that obscures the 50 kDa region on the Western blot. Molecular weight markers are indicated at left

performed using a mouse anti-V5 antibody that was directly conjugated to agarose beads. The immunoprecipitated CCSP-2-V5 protein was then detected on Western analysis using a primary anti-V5 mouse monoclonal antibody that was directly conjugated to horseradish peroxidase.

To proceed further, transfectants of the Vaco-364 colon cancer cell line were selected for stable expression of the CCSP-2-V5-His-tagged fusion protein. Assay of these clones by the direct CCSP-2-V5 detection assay demonstrated that each clone secreted the 85 kDa CCSP-2 protein into the cell culture medium (Figure 5a, lane 2), and that control cells transfected with an empty expression vector correctly gave no reactivity in the assay. Moreover, the direct assay also detected a second 55 kDa CCSP-2-derived secreted protein present in cell culture medium from stable transfectants. In the initial assay for the CCSP-2-V5 protein, detection of this 55 kDa product was obscured by contaminating signal from IgG heavy chain that is inherent to standard serial immunoprecipitation and Western blot methods (data not shown). Mass spectrometry confirmed the identification of the 85 kDa secreted protein as full-length CCSP-2, and identified the smaller 55 kDa protein as representing the carboxyl-terminal CCSP-2 peptide, commencing at A268 and likely generated by proteolytic cleavage between L267 and A268 (Figure 2b).

Cells from three independent CCSP-2-V5-His expressing Vaco-364 clones and from a control pool of

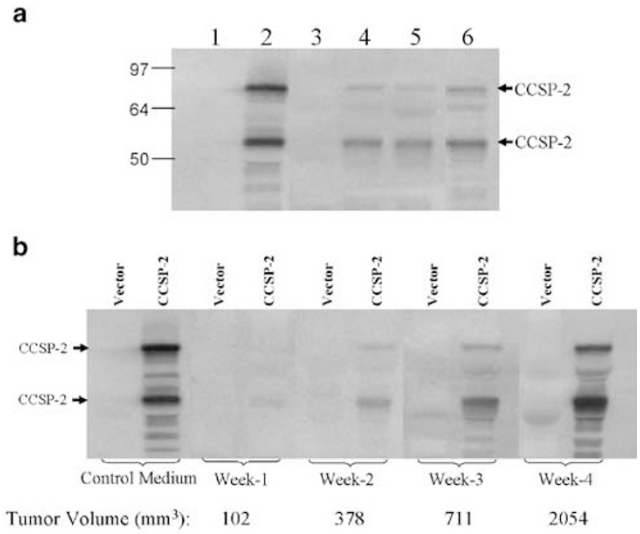

Figure 5 CCSP-2 protein detected in the blood of xenografted mice. (**a**) Detection of V5 epitope-tagged CCSP-2 protein by a direct serial immunoprecipitation and Western blot assay directed against the V5 epitope tag. Lane 1: culture medium from Vaco-364 cells transfected with an empty expression vector; lane 2: culture medium from a Vaco-364 stable transfectant expressing the CCSP-2-V5-tagged protein; lane 3: plasma harvested from an athymic mouse bearing a xenograft from control empty vector-transfected Vaco-364 cells. Lanes 4, 5, 6, plasma harvested from three athymic mice bearing xenografts from three different Vaco-364 clones stably expressing the V5 epitope-tagged CCSP-2 protein. Arrowheads denote positions of the 85 kDa CCSP-2 protein, and of a 55 kDa CCSP-2-derived peptide detected in the supernate of CCSP-2-V5-transfected cells and in the blood of mice bearing CCSP-2-V5 expressing xenografts. (**b**) Detection of V5 epitope-tagged CCSP-2 protein by direct serial immunoprecipitation and Western blot assay directed against the V5 epitope-tag in mice bearing xenografts from HeLa cells transfected with an expression vector for the CCSP-2-V5-tagged protein (CCSP-2) versus with a control empty vector (Vector). Control medium denotes HeLa conditioned cell culture medium. Additional lanes denote assay of plasma prepared from mice bearing tumors of volumes shown at 1, 2, 3, and 4 weeks after xenograft inoculation. Arrowheads denote positions of the secreted 85 kDa CCSP-2 protein and the 55 kDa CCSP-2-derived peptides. A nonspecific reactivity of 48 kDa is faintly detected in plasma from some of the mice in both the CCSP-2 expressing and vector control groups

Vaco-364 cells transfected with an empty expression vector were subcutaneously injected into the flanks of different groups of athymic nude mice. Mice were killed when xenograft tumor volume exceeded a volume $\geqslant 400\,mm^3$. Plasma samples were then collected and analysed by the direct CCSP-2-V5 detection assay. As demonstrated in representative samples in Figure 5a, tagged CCSP-2 protein was detected in plasma from every mouse bearing a CCSP-2-V5-His expressing xenograft and from no control mouse bearing xenografts from empty vector-transfected cells. Thus, tumor-derived CCSP-2 gains access to the mouse blood, circulates as a stable product, and can be detected as a serological tumor marker by immunoassay. While both 85 and 55 kDa CCSP-2 proteins were detected in the circulation, the 55 kDa cleavage product predominated.

To examine how early CCSP-2 protein can be detected as a serological marker of a mouse xenograft, a time-course study was performed in mice xenografted with HeLa cells stably transfected with either a CCSP-2-V5-His expression vector or an empty expression vector. As shown in Figure 5b, the 55 kDa CCSP-2 protein was detected in the blood of mice bearing CCSP-2 expressing tumors as early as 1 week after injection, when tumor volume was only $102\,mm^3$. Both the 85 and 55 kDa CCSP-2 proteins could be detected in mice 2 weeks after injection bearing tumors of $378\,mm^3$.

Scant expression of CCSP-2 in normal human body tissues

To identify other potentially competing sources of CCSP-2 expression, we analysed a multitissue Northern blot representing 31 different normal human tissues. A weak CCSP-2 signal was detected in the stomach and a borderline detectable signal was noted in the prostate (Figure 6a). The level of CCSP-2 probe hybridization detected in $2\,\mu g$ of polyadenylated RNA from the stomach was significantly less than that seen in the accompanying control Northern blots bearing $10\,\mu g$ of total colon cancer cell line RNA.

Real-time PCR was used to more precisely compare CCSP-2 expression in five samples of normal human stomach versus 28 samples of colon cancer and paired normal colon mucosa. While normal stomach was verified to express CCSP-2 at a level (mean value 37, range 9–73) that exceeds that of normal colon epithelium (mean value 7, range 1–20), mean expression by the stomach remained at only 10% of the corresponding level seen in colon cancers (mean value 367, range 1–1176) (Figure 6b).

Discussion

We have identified a novel human gene, *CCSP-2*, whose expression is dramatically upregulated in colon adenomas and cancers, and that encodes a secreted protein that in animal models is able to access the blood and stably circulate as a detectable tumor marker. Detection and removal of premalignant colon adenomas has been shown to prevent effectively colon cancer (Winawer *et al*., 1993). The finding of dramatic elevation of CCSP-2 expression in colon adenomas and in curable Stage I and II colon cancers suggests that this protein is a highly attractive candidate as a serological marker for early detection of human colon neoplasia, and that development of assays to detect this protein in human serum would be of substantial interest.

Recent efforts to develop noninvasive testing for colon cancers have focused on detection of mutant DNA markers in stool (Ahlquist *et al*., 2000; Dong *et al*., 2001; Traverso *et al*., 2002a, b). This approach is of substantial interest, although its ability to detect premalignant colon adenomas remains unknown. We and others have shown that tumor-derived mutant and aberrantly methylated DNA can be detected in the serum of some colon cancer patients (Hibi *et al*., 1998; Grady *et al*., 2001), but this approach currently

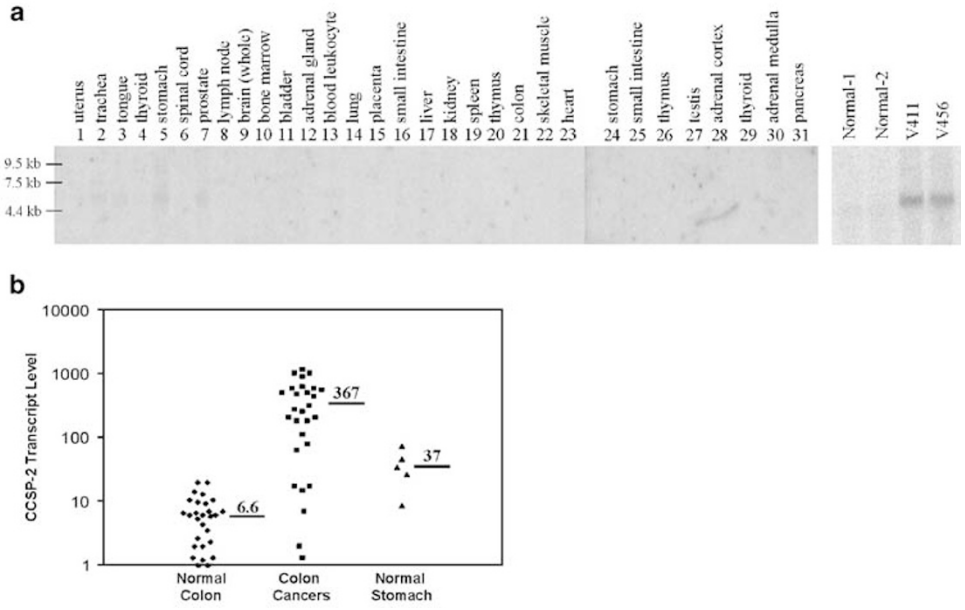

Figure 6 Comparison of CCSP-2 expression in colon cancer versus normal human tissues. (**a**, left panel) Northern analysis of CCSP-2 transcript expression in 31 normal human tissue samples (2 μg of polyadenylated RNA per sample) compared to a control blot (right panel) containing 10 μg of total RNA from two normal colon mucosa ('Normal') samples and from two colon cancer cell lines (V411 and V456). (**b**) Real-time PCR analysis of CCSP-2 expression in normal colon mucosa, paired colon cancer tissues, and normal stomach. CCSP-2 values are normalized against expression of the house-keeping gene B2M. Horizontal black bars indicate the mean value for each group of samples

identifies only a minority of such cases. The identification of CCSP-2 suggests that identifying abnormal protein components in blood, either singly or as a panel, also remains an avenue of interest for development of noninvasive screening of colon neoplasias.

The function of CCSP-2 in human colon cancers remains obscure. CCSP-2 showed no evidence of being an oncogene, as we did not detect CCSP-2 mutations in colon cancers, and as the *CCSP-2* gene failed to either induce focus-forming activity in NIH3T3 cells or to induce tumorigenicity in nontumorigenic human cell lines. There was also no evidence for amplification of the *CCSP-2* gene or rearrangement of the gene promoter. Thus, the basis for induction of CCSP-2 expression in colon adenomas and cancers remains unclear. Nonetheless, the empiric finding that CCSP-2 is a secreted protein whose expression is dramatically upregulated in colon adenomas and early colon cancers nominates this protein as a highly interesting candidate serological marker of early human colon neoplasia, for which future studies will certainly be warranted.

Materials and methods

Cell lines and tissues

Vaco cell lines were established and maintained as described (Markowitz *et al*., 1995). HeLa-S3 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All normal colon, primary colon cancer, and liver metastasis tissues were obtained from the archives of University Hospitals of Cleveland (Cleveland, OH, USA) under an IRB-approved protocol.

DNA expression microarray analysis

As described previously (Platzer *et al*., 2002), we designed custom expression monitoring microarrays using Affymetrix GeneChip technology (Lipshutz *et al*., 1999). Preparation of samples, hybridization to GeneChip expression microarrays, and data analysis were all performed as described previously (Platzer *et al*., 2002).

Amplification and sequencing of CCSP-2

Full-length CCSP-2 was PCR amplified from cDNA using forward primer 5′-GCC GCT CTC CTT CCG TTA TAT C-3′ and reverse primer 5′-CCT CCT CCC TCA GTG AGA ATA GTG-3′. Human CCSP-2 mRNA and gene sequence GenBank Accession numbers as deposited by our group are, respectively, AY572972 and AY572973. The coding sequence of the colonic CCSP-2 agrees with the recently described sequence of AMACO, a transcript found as transiently expressed in the developing kidney (GenBank entry NM-198496) (Sengle *et al*., 2003).

RACE PCR

The 5′ RACE was performed using the 5′/3′ RACE kit (Roche, Indianapolis, IN, USA). The gene-specific primers used for 5′ RACE were as follows: SP1, 5′-TGG AAT GCT CCC ACT CTG AC-3′; SP2, 5′-TTT CCC GAC GCT GTT AGA C-3′; and SP3, ATG GGG GCA CTC TGG AAA ACA G-3′.

Construction of a CCSP-2 expression vector

The complete coding sequence of CCSP-2 was amplified by RT–PCR using forward primer 5′-AAC ATG CCC CCT TTC CTG TTG CTG-3′ and reverse primer 5′-GGG GCG TCT CAA GAA TCG GTT CTC-3′, and cloned into the eukaryotic expression vector pcDNA3.1/V5/His-TOPO (Invitrogen) with the V5-His epitope tag located at the C-terminus of the protein.

Northern blot analysis

Northern analysis was performed as previously described (Brunschwig *et al*., 2003) using a probe spanning exons 6–12 of the CCSP-2 transcript.

CCSP-2 real-time PCR

Primers and a fluorogenic hybridization probe were designed using Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). CCSP-2 was amplified using 400 nM of forward primer 5′-TGG AAG AGA GTG TTC CTA ACC-3′ and reverse primer 5′-GTC CAG TCC TTC TGG AAC A-3′, and detected using fluorogenic hybridization probe 5′-/56-FAM/CCA CCT GCT ACA GGA CCA CCT G/3BHQ_2/-3′. Each PCR was carried out in triplicate in a 25 μl volume using TaqMan Assay Mastermix (Applied Biosystems, Foster City, CA, USA) for 8 min at 95°C, followed by 50 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. Beta-2-microglobulin (B2M) was amplified using 0.2 × of the human B2M TaqMan primer/probe kit (Perkin-Elmer Biosciences, Foster City, CA, USA) and 1 × IQ Supermix (Bio-Rad, Hercules, CA, USA). The PCR cycling conditions for B2M were 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The level of CCSP-2 expression was determined as the ratio of CCSP-2 : B2M = $2\exp-(CT_{CCSP-2}-CT\ B2M)$.

Transfection and detection of CCSP-2 from cell lysates and cell culture media

Cells were seeded at 0.8×10^6 cells/100 mm dish (SW480) or 1.0×10^6 cells/100 mm dish (Vaco-400) and transfected the next day with 2 μg of V5/His-tagged CCSP-2 expression vector using 12 μl of Fugene (Roche, Indianapolis, IN, USA) as per the manufacturer's protocols. A measure of 1 ml of cell culture medium was collected 48 h after transfection, centrifuged for 5 min at 2000 *g*, and precleared with 30 μl of protein G–agarose (Upstate, Lake Placid, NY, USA). The V5/His-tagged CCSP-2 was then immunoprecipitated using 1 : 333 dilution of anti-V5 mouse monoclonal antibody (Invitrogen, Carlsbad, CA, USA). Samples were washed five times with RIPA buffer, incubated in 30 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) at 95°C for 5 min and loaded onto a 4–12% Bis–Tris SDS–PAGE (Invitrogen, Carlsbad, CA, USA). Companion total cell lysates were prepared by incubating cells at 4°C for 30 min in the RIPA buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 1 mM EDTA) supplemented with Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Lysates were centrifuged for 10 min at 14 000 r.p.m. at 4°C, and the clarified supernates were then also loaded onto SDS–polyacrylamide gels. After SDS–PAGE, proteins were transferred onto Immobilon™-P PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked for 1 h with 5% nonfat milk, and incubated overnight at 4°C with a 1 : 1500 dilution of mouse anti-V5 antibody (Invitrogen, Carlsbad, CA, USA). The following day, the blots were washed 3 × for 10 min with PBS/0.2% Tween-20, and then incubated with donkey anti-mouse horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Enhanced Chemiluminescence Plus (Amersham Biosciences, Piscataway, NJ, USA) and a STORM 840 phosphoimager were used to detect protein bands.

Selection of stable CCSP-2 expressing clones

Vaco-364 cells were seeded at 20 000 cells/100 mm dish, and transfected the next day with 2 μg of pcDNA3.1/CCSP-2-V5/His plasmid construct using Fugene transfection reagent (Roche, Indianapolis, IN, USA). At 2 days after transfection, the cells were selected with 0.5 mg/ml geneticin (G418, Gibco). Individual stable clones were isolated and assayed for production of V5-His-tagged CCSP-2. Stable transfectants derived from an empty pcDNA3.1-V5-His vector were pooled. HeLa-S3 cells were transfected with V5-His-tagged CCSP-2 using TransIT®-HeLa Monster transfection reagent (Mirus, Madison, WI, USA) and stable clones selected in 1 mg/ml geneticin.

Detection of CCSP-2 in mouse plasma

Athymic female nude mice, 4–6 weeks of age were injected subcutaneously on each flank with 5×10^6 Vaco-364 or HeLa cells stably transfected with the V5-His epitope-tagged CCSP-2 vector or with a control empty vector. After killing, blood from exsanguinated mice was collected in tubes containing 100 mM EDTA and centrifuged for 10 min at 2000 *g* at 4°C. A measure of 100 μl of mouse plasma was incubated with 30 μl of agarose-conjugated anti-V5 antibody beads (Sigma-Aldrich, St Louis, MO, USA) overnight at 4°C with rocking. The next day, the beads were washed three times with RIPA; 30 μl of loading dye was added; and the entire sample was loaded onto a 4–12% Bis–Tris, SDS–PAGE gel. Western blotting was performed using a mouse anti-V5-horseradish peroxidase-conjugated antibody (Invitrogen, Carlsbad, CA, USA). All animal handling was in accord with institutional guidelines for animal care and research.

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References

Ahlquist DA, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, Thibodeau SN and Shuber AP. (2000). *Gastroenterology*, **119,** 1219–1227.
ASCO (1996). *J. Clin. Oncol.*, **14,** 2843–2877.
Bast Jr RC, Ravdin P, Hayes DF, Bates S, Fritsche Jr H, Jessup JM, Kemeny N, Locker GY, Mennel RG and Somerfield MR. (2001). *J. Clin. Oncol.*, **19,** 1865–1878.
Bond JH. (2000). *Med. Clin. N Am.*, **84,** 1163–1182, viii.

Brunschwig E, Wilson K, Mack D, Dawson DM, Lawrence E, Wilson JKV, Lu S, Nosrati A, Swinler S, Beard L, Lutterbaugh J, Willis J, Platzer P and Markowitz S. (2003). *Cancer Res.*, **63,** 1568–1575.

Dong SM, Traverso G, Johnson C, Geng L, Favis R, Boynton K, Hibi K, Goodman SN, D'Allessio M, Paty P, Hamilton SR, Sidransky D, Barany F, Levin B, Shuber A, Kinzler KW, Vogelstein B and Jen J. (2001). *J. Natl. Cancer Inst.*, **93,** 858–865.

Grady WM, Rajput A, Lutterbaugh JD and Markowitz SD. (2001). *Cancer Res.*, **61,** 900–902.

Greenlee RT, Hill-Harmon MB, Murray T and Thun M. (2001). *CA Cancer J. Clin.*, **51,** 15–36.

Hibi K, Robinson CR, Booker S, Wu L, Hamilton SR, Sidransky D and Jen J. (1998). *Cancer Res.*, **58,** 1405–1407.

Lipshutz RJ, Fodor SP, Gingeras TR and Lockhart DJ. (1999). *Nat. Genet.*, **21,** 20–24.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan R, Zborowska E, Kinzler K, Vogelstein B, Brattain M and Willson JKV. (1995). *Science*, **268,** 1336–1338.

Nielsen H and Krogh A. (1998). *Proc. Int. Conf. Intell. Syst. Mol. Biol.*, **6,** 122–130.

Pignone M, Rich M, Teutsch SM, Berg AO and Lohr KN. (2002). *Ann. Intern. Med.*, **137,** 132–141.

Platzer P, Upender MB, Wilson K, Willis J, Lutterbaugh J, Nosrati A, Willson JK, Mack D, Ried T and Markowitz S. (2002). *Cancer Res.*, **62,** 1134–1138.

Schoen RE. (2002). *Nat. Rev. Cancer*, **2,** 65–70.

Sengle G, Kobbe B, Morgelin M, Paulsson M and Wagener R. (2003). *J. Biol. Chem.*, **278,** 50240–50249.

Smith RA, Cokkinides V, von Eschenbach AC, Levin B, Cohen C, Runowicz CD, Sener S, Saslow D and Eyre HJ. (2002). *CA Cancer J. Clin.*, **52,** 8–22.

Traverso G, Shuber A, Levin B, Johnson C, Olsson L, Schoetz Jr DJ, Hamilton SR, Boynton K, Kinzler KW and Vogelstein B. (2002a). *N. Engl. J. Med.*, **346,** 311–320.

Traverso G, Shuber A, Olsson L, Levin B, Johnson C, Hamilton SR, Boynton K, Kinzler KW and Vogelstein B. (2002b). *Lancet*, **359,** 403–404.

Vernon SW. (1997). *J. Natl. Cancer Inst.*, **89,** 1406–1422.

Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF, Ackroyd F, Shike M, Kurtz RC, Hornsby-Lewis L, Gerdes H, Stewart E and The National Polyp Study Workgroup. (1993). *N. Engl. J. Med.*, **329,** 1977–1981.

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