

The Role of the Carcinoembryonic Antigen Receptor in Colorectal Cancer Progression

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Abstract

Clinical and experimental data suggest that carcinoembryonic antigen (CEA, CD66e, CEACAM-5) plays a key role in the formation of hepatic metastasis from colorectal and other types of epithelial cancers. The molecular events involved in CEA-induced metastasis have yet to be defined. Our group first cloned the gene (CEAR) for CEA-binding protein from the surface of fixed liver macrophages, (Kupffer cells). In this study to further elucidate the role of CEAR in colorectal cancer progression, its expression in colorectal cancer cells was suppressed by short hairpin RNAs (shRNAs) in CEA-overexpressing and CEA - negative MIP-101 colorectal cancer cell lines. The data show that targeted suppression of endogenous CEAR in tumor cells resulted in changes in cell invasiveness. RT-PCR data indicated reduced levels of E-cadherin, Snail, MMP-2, and Oct-4 in the clones with suppressed CEAR suggesting a role in the epithelial mesenchymal transition. The comparative analysis of tumorigenic activity to the liver of the cell lines with suppressed CEAR has also been conducted using an intrasplenic injection model in immuno-deficient mice. This data shows a decrease in tumor progression associated with CEAR suppression. In summary the results of this study revealed a novel role for CEAR gene in the regulation of colorectal cancer cell invasiveness and progression.

Keywords: Colorectal carcinoma; Carcinoembryonic antigen; Metastasis; CEAR; hnRNPM

Abbreviations CEA: Carcino Embryonic Antigen; CEAR: Carcino Embryonic Antigen Receptor; hnRNPM, Heterogeneous RNA-Binding Protein M; mRNA: messenger RNA; shRNA: short hairpin RNA; KC: Kupffer Cells; PAGE: Polyacrylamide Gel Electrophoresis; BP: Base Pair(s); FGFR2: Fibroblast Growth Factor Receptor 2; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction; MMP-2: Matrix-Metallo-Proteinase-2; CRC: Colorectal Carcinoma Cells; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; E-cadherin: Epithelial Cadherin; EMT: Epithelial Mesenchymal Transition; GLM: Generalized Linear Model

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and one of the most common causes of cancer-related deaths worldwide [1]. 20% to 25% of patients with this disease have evidence of distant metastases when first diagnosed and metastasis accounts for more than 90% of cancer patient's deaths [2].

Carcinoembryonic antigen (CEA, CEACAM5, or CD66e) is one of the most frequently used tumor markers for colorectal cancer and has been associated with enhanced metastatic potential in colorectal and other epithelial cancers, including breast, lung and gastric [3,4]. During cancer progression colorectal carcinoma cells (CRCs) produce and secrete increased amounts of CEA into the circulation. CEA is a large glycoprotein (~180 kD) and a member of a family of 29 related

genes that are part of the immunoglobulin-gene superfamily. Regulation of intercellular adhesion has been implicated as a major function of CEA [4,5]. CEA also plays a critical role in establishing and maintaining tissue architecture and function in the colon [6,7]. The tumorigenic effects of CEA include inhibiting cell differentiation, blocking cell polarization, distorting tissue architecture, and inhibiting anoikis (cell death due to the loss of cell-cell contacts) [7,8]. However, the molecular mechanism of CEA-induced metastasis is poorly defined and may involve several mechanisms. We identified a novel CEA binding protein (CEAR) on liver macrophages, (Kupffer cells), who's activity alters the liver microenvironment such that implantation and survival of tumor cells increases [9]. Previously this protein was identified as an isoform of the hnRNPM protein [10]. HnRNPM belongs to a large family of 20 heterogeneous nuclear RNA-binding proteins (hnRNPs A-U), also called "the histones of RNA" [11]. The hnRNP proteins share common structural domains and have central roles in regulating gene expression at both transcriptional and translational levels [11]. They also are involved in RNA splicing, telomere biogenesis, DNA repair and cell signaling [12]. Several recent reports show a role for individual hnRNPs in tumor development and progression in a variety of cancers [13-15].

HnRNPM (hnRNPM1-4, hnRNPM4) is the least studied of this group of proteins. It comprises of two N-terminal RNA-binding domains (RBD1 and RBD2), followed by a methionine/arginine/glycine-rich region and the C-terminal RNA binding domain RBD-3. RBD3 overlaps with the domain that binds CEA [16]. HnRNPM is a very abundant nuclear shuttling protein that has at least 4 protein isoforms [10]. However, only 2 mRNA transcripts have been

experimentally validated: the full-length protein (isoform 1) and the short isoform 2 that has a 39 amino acid deletion between the RNA binding domains 1 and 2 [10]. We initially identified isoform 2 as a CEA-binding protein in Kupffer cells though both isoforms are capable of binding CEA. [10]. HnRNPM is a multifunctional protein that is involved in mRNA processing [17], splicing [18], stress response [19], mRNA transport and stability of exosomes [20]. In human cells, hnRNPM plays a role in regulating FGFR2 alternative splicing and can affect the splicing of several other genes [21]. HnRNPM proteins can undergo several post-translational modifications, including phosphorylation, SUMOylation, ubiquitination and methylation, which regulate its activity [22]. In HEK 293 cells, HnRNPM was also identified in a complex with arginine methyltransferase PRMT8. Additionally, actin, tubulin, and heat-shock proteins were present in the PRMT8 complex [22]. Post-translational modifications may modulate hnRNPs activity by altering their localization, RNA binding specificity and interaction with other cellular factors. The cellular localization of hnRNPM isoforms can vary depending on the cells and conditions. Generally hnRNPM has a diffuse nuclear distribution, remains bound to the mRNA as it is transported through nuclear pores, associates with the ribosome or is sequestered in specific cellular compartments. While their localization is largely nuclear RNA binding proteins have also been found in the cytoplasm and on the cell surface [23,24]. Using isoform specific antibodies we showed that in macrophages and CRCs the full-length protein is mainly localized in the nucleus while the short isoform 2 is localized in the cytoplasm and on the cell surface [25].

In this study, we investigate the effects of CEAR/HNPNM gene silencing by shRNA on cell adhesion, invasion and the tumorigenic properties of colorectal cancer cells.

Experimental Procedures

Cell lines

The weakly metastatic MIP-101 and the highly metastatic CEA-producing CX-1 colorectal carcinoma cells have been described previously [6,26]. MIP-101 is a poorly differentiated human CRC cell line that does not produce CEA in monolayer culture. CEA-producing cells were generated via stable transfection of MIP101 cells with the full-length CEA cDNA [22]. All cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 300 µg/ml glutamine. CEA transfectants were maintained in complete RPMI 1640 medium containing 400 µg/ml G-418 (Invitrogen, CA, USA). Clones with down regulated CEAR were selected in complete RPMI 1640 medium containing 10 µg/ml puromycin.

Semi-quantitative RT-PCR of mRNA expression

Total RNA was extracted from the cells with TRIzol Reagent (Invitrogen) following the manufacturer's protocols. The yield of extracted RNA was determined with Nano Drop (PqLab, Erlangen, Germany). The relative mRNA expression levels of CEA, CEAR, SNAIL, OCT-4, INTEGRIN ALPHA-3, INTEGRIN BETA-5, MMP2 were determined using reverse transcriptase-polymerase chain reaction (RT-PCR). All primers were custom made by IDT (Coralville, IA, USA). Primers for CEA were forward 5'-caccactgccaagctcacta; reverse 5'-ctgggttcgggttccacat; β-actin, forward 5'-tgagcgcgctacagctt-3' and reverse 5'-tcctaatgtcaccggacgattt-3'; and human CEAR forward, 5'-gagcgaagaccactgaaag -3', and reverse 5'-

agaatgtctgctcgaccac -3'. The human CEAR primers were designed to detect two isoforms, to represent the wild type (full-length), and the deletion mutant with the expected PCR products of 321 and 204 bp. The e-cadherin forward and reverse primers are the following: F-(TGCCCAGAAAATGAAAAAGG), R-(GTGTATGTGGCAATGCGTTC). SNAIL, Integrin alpha-3, Integrin alpha-5, AND MMP-2 primer sequences are published elsewhere [6]. mRNA was extracted from CRCs using Dynabeads® mRNA Purification Kit mRNA isolation kit (Life technologies, USA), according to the manufacturer's protocol. Superscript™ III First Strand Synthesis system for RT-PCR (Invitrogen) was used to generate the cDNAs per manufacturer's protocol. Synthesized cDNAs were diluted in 50 µL of diethylpyrocarbonate-treated water, and 3 µL of each reaction was used in each 25-µL RT-PCR. DNA was amplified using the following parameters: 95°C for 1 minute, followed by 25 cycles of 95°C for 30 seconds, 52-60°C for 30 seconds, and 72°C for 1 minute. The gene expression was normalized using reference primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, forward 5'- gggaaaggatgaagtcggagt -3', and reverse 5'ttgaggctcaatgaagggtca -3'. PCR products were separated in a 2%–3% agarose gel and visualized by ethidium bromide staining. As a control for genomic DNA contamination PCR reactions that included cDNA synthesis, reagents except reverse transcriptase and water were set up in parallel. At least three independent experiments were performed per amplification.

Western blot analysis

Protein extracts were prepared from 50%–70% confluent cell monolayers that were washed with PBS (pH 7.4) at 4°C and then lysed at 4°C in TNE buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 40 mM β-glycerophosphate, 1 mM EGTA, 0.25% sodium deoxycholate, 1% NP40, 50 mM sodium fluoride, 20 mM sodium phosphate, 1 mM sodium orthovanadate, and protease inhibitors (2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, and 100 µg/ml pefabloc). Cellular debris was removed by centrifugation at 14,000 rpm for 15 minutes. Supernatants were assayed for total protein content using the BCA method (Pierce, Rockford, IL, USA). Ten to 30 micrograms of total protein were resolved on 4%–12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking the membranes with 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.4) with 0.5% Tween 20 (TBS-T) for 3 hours, the membranes were incubated with the primary antibodies overnight at 4°C. The primary antibodies used were a mouse monoclonal anti-hnRNPM1-4 (sc20001, Santa Cruz, CA, USA), anti-E-cadherin (sc8426, Santa Cruz, CA, USA), anti-β-catenin (sc65480, Santa Cruz, CA, USA), rabbit polyclonal anti-β-tubulin (sc5286, Santa Cruz, CA, USA), anti-CEA (C2331, Sigma-Aldrich), α-catenin (sc59890, Santa Cruz, CA, USA), GAPDH (sc-20357, Santa Cruz, CA, USA), β-actin (Sigma-Aldrich). Blots were visualized by enhanced chemoluminescence (Pierce, Rockford, IL, USA) using horseradish peroxidase-linked donkey anti-rabbit or anti-mouse IgG as the secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The proteins were quantified by scanning the images into Photoshop with analysis carried out using Image J version 1.30.6.

Generation of stable transfected cell lines with shRNA suppressed CEAR protein

For transfection MIP101, MIP101 clone 6, MIP101 clone 8, and CX1 cells were plated at 1×10^5 cells per well in six-well tissue culture clusters (Nunc cell culture products, USA). Cells were allowed to grow

for 24 hours and the control and shRNA hnRNPM vectors (OriGene Technologies Inc., cat #TR304071) were transfected in serum-free medium using the siRNA transfection reagent (Santa Cruz, CA, USA) according to the manufacturer's instructions. Six hours after transfection, cells were switched to RPMI media with 5% serum. Stable transfected clones were selected using 0.5–10 µg/ml puromycin concentrations depending on the cell line. All experiments were performed in triplicate (n=3), with each experiment in triplicate wells. Sequences were confirmed by NIH BLAST analysis to have no substantial homology to sequences in other vertebrate genes.

Cell adhesion assays

Adhesion assays were performed in triplicate on collagen, laminin, and fibronectin, coated plates as described previously [23].

Cell invasion assays

Cell invasion assays were conducted using the Chemicon Cell Invasion Assay Kit (ECM550) per the manufacturer's instructions. Invasion chambers were rehydrated with serum-free RPMI for 2 hours, the media was removed, and $0.15\text{--}0.24 \times 10^6$ cells in serum-free media were added to the invasion chamber and media containing 10% serum was added to the lower chamber. Control wells were set up with identical numbers of cells in the same media without an insert in the well. After 24-hour incubation in a tissue culture incubator, the non-invading cells and EC Matrix gel was carefully removed from the invasion chamber. The invasive cells were stained with the reagent provided for 20 minutes and rinsed in water, and the chambers were allowed to air dry. The control cells had media removed from the wells and then stain added for 20 minutes; cells were removed and rinsed with water, dried, and then dissolved with acetic acid. Equal volumes of dissolved stain from invasion plates and control plates were transferred to 96-well plate for colorimetric reading at OD 550 nm. Media was used as blank control.

Tumor formation assay in mice

Male nude mice (BALB/C-nu/nu) were maintained in the Institute of Cytology in St. Petersburg. Experiment were performed in agreement with the animal protection legislation acts of the Russian Federation the Guidelines for the Care and Use of Laboratory Animals (NIH publications Nos. 80, and were approved as laboratory animals by the Institute of Cytology Ethical Board. 4 weeks old mice, body weight 15 g were anesthetized by intraperitoneal injection of avertin: 1 g/L 2,2,2-tribromoethanol in 2-methylbutanol, working dilution 1:50 in PBS. For modeling metastases formation in the liver, the cells were injected into the spleen by laparotomy under sterile conditions. The spleen was partially retrieved from the peritoneal cavity through the incision. Cell suspensions were injected into the spleen (MIP101 or its derivatives) in the culture medium (2.5×10^6 in 50 µl). Later the spleen was returned into the peritoneal cavity; the incision site was stitched with 0.08 mm surgical silk thread (Fine Science Tools, USA) and 5.0 mm wound clips (Perfect Ets, Bruneau, France)]. 90 days after the surgery the mice were sacrificed by cervical dislocation and then autopsied. Spleen and liver were fixed in 4.0 % formalin in PBS to confirm the presence of tumor by histology.

Statistics design and methodology

We tested 80 mice in 2 experiments for tumorigenic activity of cancer cells (10 mice for each of 4 cell lines in one experiment). 5 mice

died during 90 days of these experiments. R statistical software was used to perform statistical analysis [27,28]. First we calculated the logistic regression generalized linear model (GLM) to ensure good replication of two experiments. The exact 95% confidence intervals were obtained based on the binomial distribution of counts for each cell line. We calculated the exact 95% confidence intervals for odds ratios with respect to the baseline for the other three cell lines by the Fisher method. Finally, the logistic regression GLM was done with two factors: CEA and CEAR gene expressions (two way ANOVA) to analyze the effect of these factors on the tumorigenic potential of the cancer cells. Using the additive model we calculated the results of the analysis under the additive effects of assumptions. $P < 0.05$ were defined as significant.

Results

Silencing of CEAR expression with small hairpin RNA (shRNA) vectors

We used a poorly differentiated human CRC line- MIP101, which produces no CEA in a monolayer culture, and its CEA producing clones 6 and 8 obtained via stable transfection of MIP-101 cells with the full-length CEA cDNA [26]. These cell lines were transfected with empty vector and anti-hnRNPM shRNA vectors (OriGene, USA). After 48 hours, the media was changed and stable cell lines were selected using resistance to 10 µg/ml of puromycin. CEAR mRNA and protein expression levels were detected by RT-PCR and western blotting (Figure 1A-1C) in parental (MIP101 and MIP101 clone 6 and clone 8) and in the clones with shRNA suppressed endogenous CEAR (M-2, M-10, 6-7, 6-9, 8-9, 8-10). The data show that both major alternatively spliced CEAR mRNA, isoforms 1 and 2 (full length and truncated form, respectively), are present; however, the ratio between CEAR isoforms varies. The suppression of CEAR mRNA expression in selected stable shRNA clones is up to an 85% (Figure 1A, lanes 3, 4, 5; 7, 8, 9 and 12, 13, 14) versus control cell lines (lanes 2, 6 and 10) without modulating the amount of GAPDH that was used as a loading control. The data show the correlations between the loss of CEAR mRNA and protein levels (Figure 1B) in the selected shRNA clones. Western blots were scanned and the protein expression values were normalized to the GAPDH values. The CEAR protein expression values were plotted (Figure 1C). Up to 87% reduction in CEAR protein expression was seen in the selected shRNA clones versus the parental cell lines.

Genes regulated by CEAR

We tested the effect of CEAR silencing (Figure 1D and 1E) on the expression of CEA, the epithelial marker E-cadherin, EMT regulator Snail, stem cell marker Oct-4, and other genes/proteins, involved in extracellular matrix remodeling and invasion. We also examined matrix-metalloproteinase-2 or MMP-2, Integrin alpha 3, and Integrin alpha 5. During metastasis by sporadic adenocarcinomas, tumor cells at the invasive front lose their epithelial characteristics and take on the properties that are more typical of mesenchymal cells. Such transition from epithelial to mesenchymal phenotype (EMT) is a fundamental event in the metastatic cascade. The resulting phenotype increases migration by reducing cell/cell contact and, thus, activating tumor invasion and dissemination. This allows metastasis to proceed. Changes in several interconnected transduction pathways and a number of signaling molecules have been identified in this process [29]. RT-PCR data showed reduced levels of CEA, E-cadherin, Snail,

MMP-2, Integrin alpha 3, and Oct-4 in the clones with suppressed CEAR (Figure 1D and 1E, clones M-2, M-10 versus MIP101; 6-7, 6-9 versus clone 6; and 8-9, 8-10 versus clone 8).

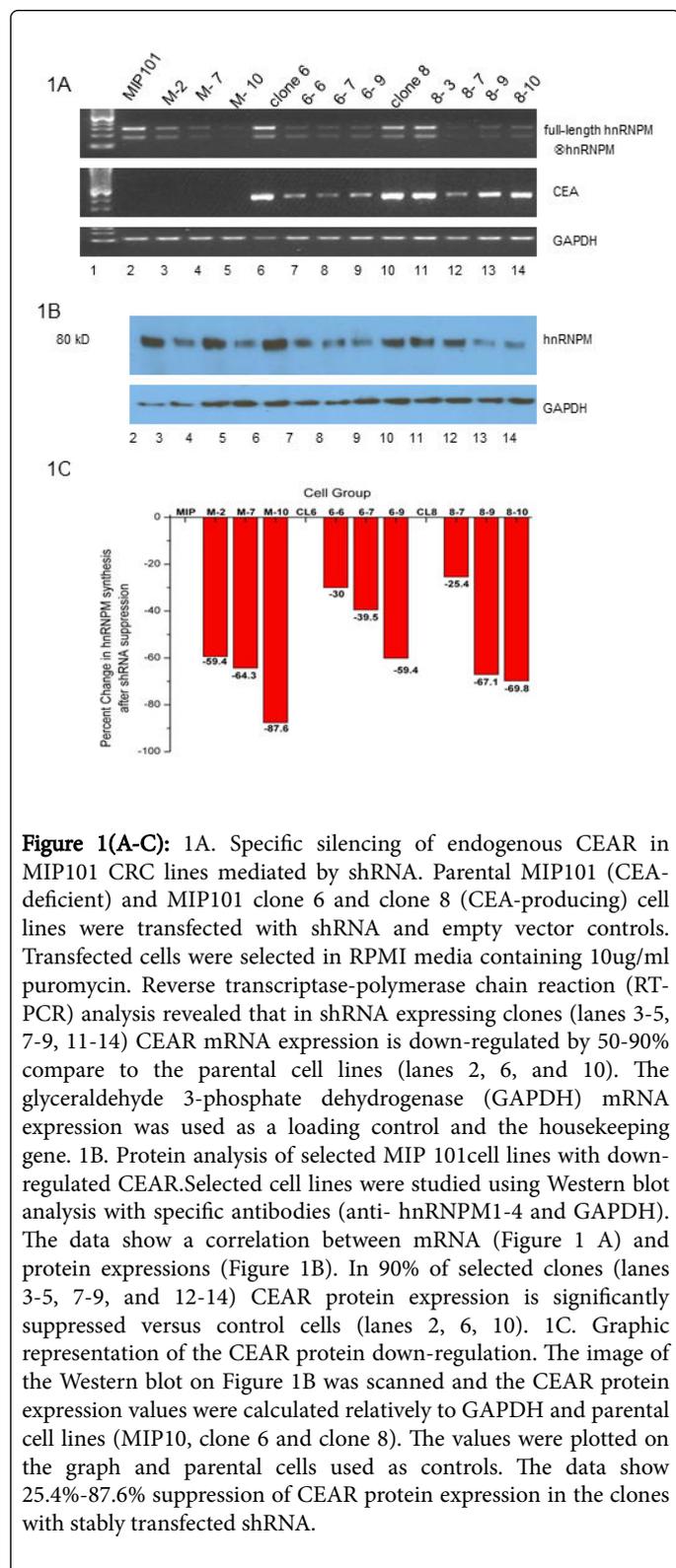


Figure 1(A-C): 1A. Specific silencing of endogenous CEAR in MIP101 CRC lines mediated by shRNA. Parental MIP101 (CEA-deficient) and MIP101 clone 6 and clone 8 (CEA-producing) cell lines were transfected with shRNA and empty vector controls. Transfected cells were selected in RPMI media containing 10ug/ml puromycin. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that in shRNA expressing clones (lanes 3-5, 7-9, 11-14) CEAR mRNA expression is down-regulated by 50-90% compare to the parental cell lines (lanes 2, 6, and 10). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was used as a loading control and the housekeeping gene. 1B. Protein analysis of selected MIP 101 cell lines with down-regulated CEAR. Selected cell lines were studied using Western blot analysis with specific antibodies (anti- hnRNPM1-4 and GAPDH). The data show a correlation between mRNA (Figure 1 A) and protein expressions (Figure 1B). In 90% of selected clones (lanes 3-5, 7-9, and 12-14) CEAR protein expression is significantly suppressed versus control cells (lanes 2, 6, 10). 1C. Graphic representation of the CEAR protein down-regulation. The image of the Western blot on Figure 1B was scanned and the CEAR protein expression values were calculated relatively to GAPDH and parental cell lines (MIP10, clone 6 and clone 8). The values were plotted on the graph and parental cells used as controls. The data show 25.4%-87.6% suppression of CEAR protein expression in the clones with stably transfected shRNA.

The level of Integrin alpha5 mRNA expression in the MIP101 clones is very low. We also show the expression of Integrin alpha 5 in colorectal cancer cell line CX1 where this gene is expressed at a high level. These changes in mRNA and protein expression are indicative of cell remodeling associated with EMT. Further the over expression of CEA in MIP cells was shown previously to cause morphological changes in the cells including a substantial increase in cell-cell adhesion [5,26]. This indicates a potential role for CEA in the EMT. Most genes affected by CEAR down-regulation are suppressed, a finding that suggests that CEAR may play a role in the regulation of CEA, EMT and stem cell factor OCT4.

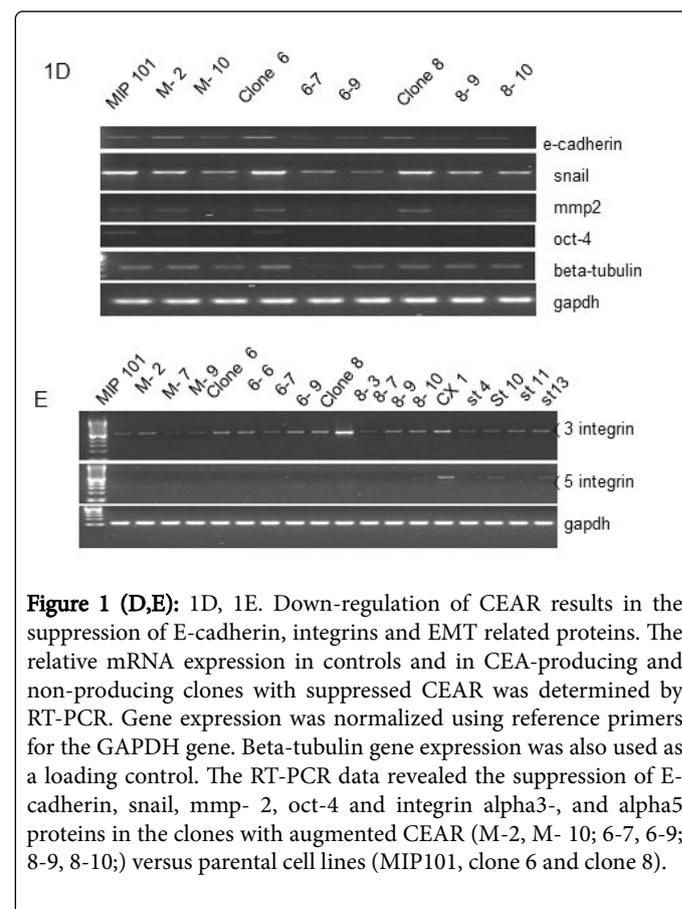


Figure 1 (D,E): 1D, 1E. Down-regulation of CEAR results in the suppression of E-cadherin, integrins and EMT related proteins. The relative mRNA expression in controls and in CEA-producing and non-producing clones with suppressed CEAR was determined by RT-PCR. Gene expression was normalized using reference primers for the GAPDH gene. Beta-tubulin gene expression was also used as a loading control. The RT-PCR data revealed the suppression of E-cadherin, snail, mmp- 2, oct-4 and integrin alpha3-, and alpha5 proteins in the clones with augmented CEAR (M-2, M- 10; 6-7, 6-9; 8-9, 8-10); versus parental cell lines (MIP101, clone 6 and clone 8).

Impairment of cell adhesion to matrix proteins is associated with CEA expression

To evaluate the role of CEA and CEAR in cell adhesion, we conducted adhesion assays using parental MIP101 cells, its CEA-producing clones (6 and 8) and clones with shRNA-suppressed CEAR (M2, M10, 6-7, 6-9, 8-9, and 8-10). Single cell suspensions were plated in RPMI media with 3.0% FBS on plates with and without laminin, collagen, or fibronectin coatings as described by Zoboralski et al [27,29]. After 75 minutes, non-adherent cells were washed away and adherent cells were stained and counted. The analysis (Figure 2) revealed a dramatic reduction in cell adhesion to laminin in CEA producing cells (MIP 101 clones 6 and 8) relative to non-CEA producing (MIP101) ($P < 0.001$) cells. The effects of CEA on cell adhesion were observed only in response to laminin and absent in control (non-coated), collagen, and fibronectin plates (not shown). The suppression of CEAR did not have any effect on non-CEA-producing

MIP101 clones (M-2 and M10 versus MIP101). In contrast, in CEA-producing clones (6-7, 6-9, and 8-9), the adhesion to laminin was significantly increased compared to parental clone 6 and clone 8 cells ($P < 0.001$). A previous study by Danaker et al. also showed that CEA producing colorectal cancer cells had reduced adherence to laminin and a reduced capacity to invade through Matrigel though the role of CEAR was not examined [30]. These data indicate that CEAR works as a suppressor of cell adhesion in concert with CEA and laminin receptors may be involved in response to CEA in MIP101 cells.

CEAR is involved in CRC invasion

The initiation of the metastatic cascade requires an increase in cell invasiveness of carcinoma cells. Invasion assays were conducted with the Chemicon Cell Invasion Assay Kit following the manufacturer's instructions (Figure 2). After 24 hours, the amounts of invading cells were evaluated by colorimetric assay. There was no difference in the invasion rates between parental MIP101 and CEA-producing clone 6 and clone 8 cells. Targeted suppression of CEAR impaired the invasiveness of the highly invasive non-CEA producing MIP101 cells (M-2 and M-10 versus MIP101) (Figure 2). In contrast, down-regulation of CEAR in CEA-producing clones showed a significant increase in CRC invasion; specifically, CEA-producing clone 6 derivatives with suppressed CEAR (6-7 and 6-9 versus clone 6) showed a significant increase in cell invasiveness ($P < 0.001$). The values in clone 8 derivatives (8-9 and 8-10) versus parental clone 8 did not change significantly.

In vivo experiments in mice

Tumorigenic potential of CRC cells with different levels of CEA and CEAR expression was studied by the intra-splenic injection of 10 immuno-deficient mice per cell line. Three months later primary (spleen) and secondary (liver) tumors were analyzed in animals after autopsy. This experiment was performed in two independent replications; total for both experiments 80 mice were included [28]. The results are presented in Figure 2 and Table 1. The CEA producing MIP101 clone 8 cell line was characterized by the highest frequency of primary tumors (89,5%) whereas the frequency of tumor for parental MIP101 cells was 36,8% (Table 1). In MIP101 clone 8-7 cells with suppressed expression of CEA receptor the frequency of primary tumors was 17,6% (Table 1) while in MIP101 clone 7 cells expressing neither CEAR nor CEA generated 1 tumor in 20 mice (5%). The cells with CEAR downregulation (MIP101 clone 7 and clone 8-7) exhibited significantly reduced tumorigenic potential as compared to clone 8 cells (Table 2). The results of the statistical analysis in the additive logistic regression model presented in Table 3 confirms that both factors, CEA and CEAR expression have a significant effect on the tumor formation *in vivo* ($P = 0.001$ and $P < 0.001$ respectively). The effect of the interaction of CEA and CEAR is not significant ($P = 0.394$). Most of the clones generated primary tumors at the splenic injection site. At the histological level, no notable differences were observed in tumors generated by various cell lines. To verify the human origin of tumor cells in mice genomic DNA was isolated from the liver and spleen of control and experimental animals and assayed by real-time PCR for the presence of human specific repeats. These organs were fixed for histological staining and analysis. In most cases we did not find secondary tumors either by anatomic assessment or PCR assay. A single example of metastasis to the liver was observed after injection of MIP101 clone 8 cells those, presumably, were the most metastatic cells.

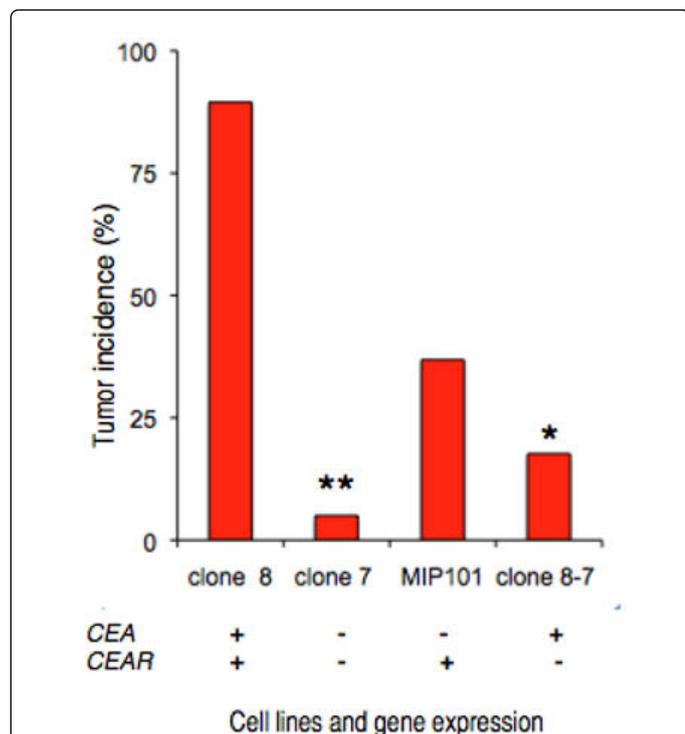


Figure 2: Tumorigenic potential of CRC cells with different levels of CEA and CEAR. Tumor incidence *in vivo* after cells injection into the spleen of nude mice was evaluated for 4 cell lines: MIP101 (non-CEA producing with normal CEAR level, n=19 mice), clone 8 (CEA producing with normal CEAR level, n=19 mice), clone 7 (non-CEA producing with downregulated CEAR, n=20 mice) and clone 8-7 (CEA producing with downregulated CEAR, n=17 mice). The cells with CEAR down-regulation (clone 7 and clone 8-7) exhibited significantly reduced tumorigenic potential as compared to MIP101 clone 8 cells. * $P < 0.05$, ** $P < 0.01$ (GLM two-way ANOVA model).

Cell lines	CEA expression	CEAR expression	Mice tested in both the experiments (total/missed)	Amount of mice with spleen tumors/Total no. of mice classified for tumor		Tumors in the spleen (proportion) average	Confidence interval for proportions (Fisher method)	
				Experiment			lower	upper
				no. 1	no. 2			
MIP101	-	+	20/1	4/10	3/9	0.368	0.162	0.616

clone 7	-	-	20/0	0/10	1/10	0.05	0.001	0.249
clone 8	+	+	20/1	9/10	8/9	0.896	0.669	0.987
clone 8-7	+	-	20/3	2/10	1/7	0.176	0.038	0.434

Table 1: The experimental data and the 95% confidence intervals for proportions of tumor incidence for colorectal cancer cell lines with different expression of CEA and CEAR *in vivo*. Two replicate experiments were performed with each cell line. It has been shown that the effect of experiment is not significant (P=0.946). This result confirmed good replication of two experiments and motivate to unify data from both experiments.

Cell line	Odds ratios	Lower 95%CL	Upper 95%CL
MIP101 clone 8	1	-	-
MIP101 clone 8-7	0.0298	0.0022	0.2159
MIP101 (parental)	0.0746	0.0065	0.4596
MIP101 clone 7	0.0084	0.00015	0.0911

Table 2: Tumorigenic potential of cell lines with different levels of CEA and CEAR. Odds ratios and the 95% confidence intervals with respect to the baseline level MIP101 clone 8 obtained by Fisher method. The logistic regression generalized linear model displays the significant influence of the genetic factors on the tumorigenic activity of cancer cells (P<0.001).

Genetic Factor	Estimate	Std. error	Z-value	P-value
(Intercept)*	1.8869	0.6261	3.014	0.00258
CEA expression	-2.3143	0.7287	-3.176	0.00149
CEAR expression	-3.2368	0.7764	-4.169	0.0000306

Table 3: The influence of CEA and CEAR expression on the tumorigenic activity cell lines *in vivo*. Logistic regression additive generalized linear model (GLM) and two way ANOVA results show significant effect of both factors. We fit the GLM with two factors CEA and CEAR expression under baselines CEA+ and CEAR+ corresponding to the genotype of the cell line clone 8. The corresponding 95% confidence intervals for odds ratios ([0.0196, 0.3673] for CEA and [0.0069, 0.1550] for CEAR) display real magnitude of the effect. *Intercept is corresponding to the baseline level clone 8 (CEA + CEAR +).

Discussion

CEA production occurs in many types of epithelial cancers and can be observed in 9-15% of testicular, ovarian, pancreatic, gastric and thyroid cancers, as well as in 50-90% metastasizing colorectal and breast cancers [10,31]. To study metastasis it is especially important to have an adequate biological model to elucidate the genes that are involved in metastasis *in vitro* and *in vivo*. A very promising field is the analysis of genetically modified same origin cell lines that differ in metastasis-related genes and due to this have different tumorigenic and metastatic potential. Such a model has been developed in this study - a collection of colorectal cell lines with different expression of metastasis related genes CEA and CEAR (Figure 1A-1C & Table 1). This study first demonstrates that silencing of CEAR gene expression has multiple

effects on colorectal cancer cells and is closely associated with the changes in the cancer cell adhesion molecules, proteins associated with EMT invasiveness and metastasis to the liver. The RT-PCR analysis revealed that suppression of CEAR leads to the down-regulation of CEA, extracellular matrix remodeling and stem cell genes and proteins: Snail, MMP-2, Integrin alpha 3, and Integrin alpha 5, Oct-4, including the epithelial cell marker E-cadherin (Figure 1D and 1E). These perturbations correlate with changes in cell adhesion and invasiveness. CEA-producing cell lines also exhibit significant reduction in adherence to laminin (Figure 3A), a major glycoprotein found in the basement membranes [27,30]. Analysis of cell invasiveness revealed that CEA production alone is not sufficient to significantly change the invasive properties of CRCs. Down-regulation of endogenous CEAR in MIP101 cells decreases cell invasiveness, which implicates CEAR as a promoter of invasion. In contrast, down-regulation of CEAR in the CEA-producing clones showed a statistically significant increase in CRC invasion for example in clone 6 derivatives (clones 6-7, 6-9) (p<0.005). Our findings also support the hypothesis that the level of invasiveness correlates with fascin expression as it is up-regulated in clones 6-7 and 6-9 compared to MIP101 clone 6 cell line (Figure 3B). Previously we have shown the disruption of the function of E-cadherin adherens junction complexes by CEA production in colorectal cancer cells leading to EMT and an increase in the amount of nuclear beta-catenin [7]. The cell-cell adhesion molecule and tumor suppressor E-cadherin regulates cell polarity, architecture, differentiation, proliferation and migration through its intimate association to the actin cytoskeletal network [32,33]. By 2-D electrophoresis and MALDI-TOF mass spectrometry. We found that CEAR interacts with the actin-binding protein alpha-actinin 4 (ACTN4) in colorectal cancer cells [34]. We therefore hypothesize that an acquisition of invasive phenotype early in metastasis depends on the functionality of the CEA/CEAR complex and its cooperation with the surface and intracellular protein networks. These findings imply that the CEAR/ ACTN4 complex can be involved in the regulation of cancer cell migration. Cell migration and invasion are highly complex processes those require the integrated activities of cytoskeleton reorganization and cell-matrix interactions. Cancer cell migration is typically regulated by integrins, matrix-degrading enzymes and cell-cell adhesion complexes [32]. During migration, cells attach to the matrix via focal adhesion complexes, while stress fibers anchor to focal adhesion complexes at their ends and generate forces to move and reshape the cell [33,35,36]. It has been shown that alpha-actinin proteins facilitate focal adhesion formation and link focal adhesion complexes to the cytoskeleton and suggested that alpha-actinin 4 is the mechano-transducer that mediates the effects of pressure on cancer cell proliferation and adhesion [37].

Recently in support of our studies, new evidence has emerged regarding CEA/CEAR signaling pathway activation in endothelial cells

[38]. Endothelial cells do not synthesize CEA, however, soluble CEA, produced by cancer cells, can bind with CEAR on the endothelial cell surface. It was shown that CEA-induced activation of endothelial cells is dependent on integrin beta-3 signals that activate the focal adhesion kinase and c-Src kinase. Their downstream MAP-ERK kinase/extracellular signals regulate kinase and phosphoinositide 3-kinase (PI3K)/Akt effector pathways. Interestingly, while interference with VEGF signaling had no effect on CEA-induced endothelial cell activation, down-regulation of CEAR in endothelial cells attenuated CEA-induced signaling and tumor angiogenesis [38]. Corroborating these results clinically, it was found that tumor micro-vascularization was higher in patients with colorectal cancer exhibiting higher serum levels of soluble CEA. It has been also shown that upregulation of hnRNPM/CEAR is associated with the more aggressive types of colon [39] and breast cancers [40]. Interaction of CEA with CEAR in macrophages can also result in the secretion of pro-angiogenic factors such as IL-6 [9].

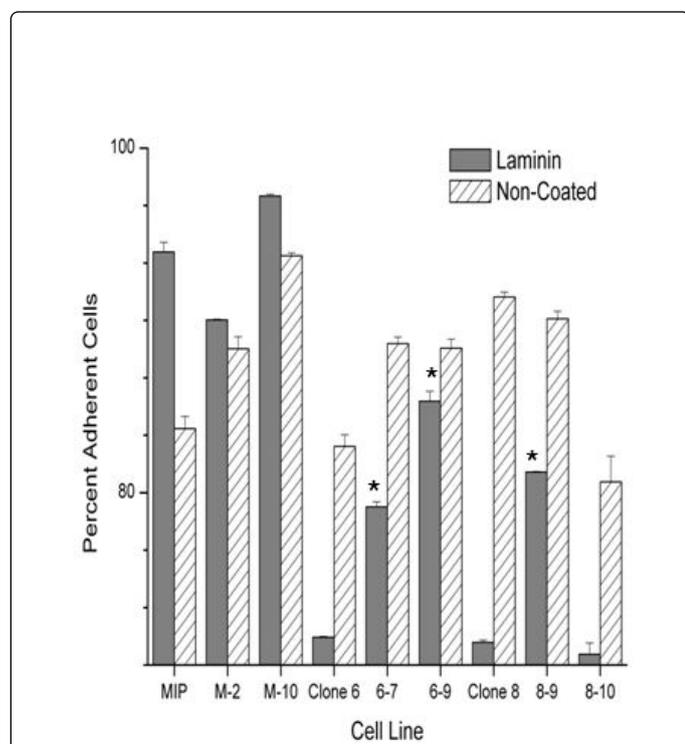


Figure 3A: The effect of CEAR silencing on cell adhesion and invasion. 2A. Cell adhesion to laminin is impaired in CEA-producing MIP-101 clone 6 and clone 8 cell lines. Cells were grown in RPMI media with 10% FBS, plated on the laminin and non-coated plastic six-well plates. After 75 minutes non-adherent cells were washed out and adherent cells were stained, and counted. The analysis revealed that CEA-producing (clone 6 and clone 8) cells have significantly reduced level of laminin adhesion versus non-CEA-producing MIP101 cells. This effect occurs only on the laminin-coated plates and is absent in the non-coated control plates. The CEAR suppression did not change the cell adhesion of M-2 and M-10 versus MIP 101 cells and increased cell adhesion in clones 6-7, 6-9 and 8-9 in comparison to parental clone 6 and clone 8 cells ($P > 0.001$).

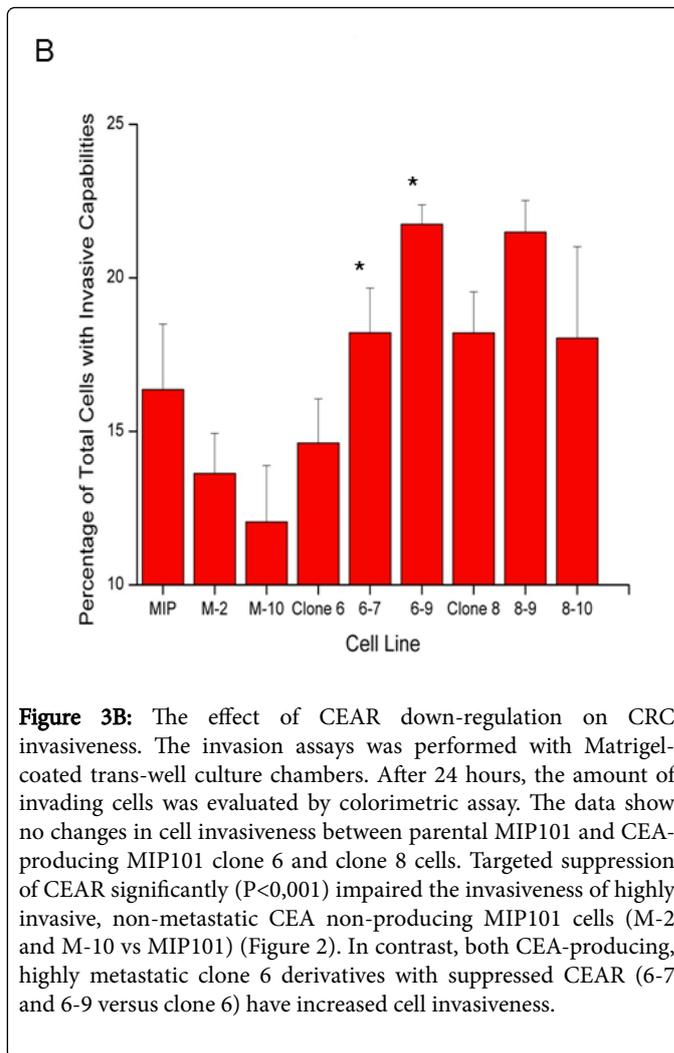


Figure 3B: The effect of CEAR down-regulation on CRC invasiveness. The invasion assays was performed with Matrigel-coated trans-well culture chambers. After 24 hours, the amount of invading cells was evaluated by colorimetric assay. The data show no changes in cell invasiveness between parental MIP101 and CEA-producing MIP101 clone 6 and clone 8 cells. Targeted suppression of CEAR significantly ($P < 0.001$) impaired the invasiveness of highly invasive, non-metastatic CEA non-producing MIP101 cells (M-2 and M-10 vs MIP101) (Figure 2). In contrast, both CEA-producing, highly metastatic clone 6 derivatives with suppressed CEAR (6-7 and 6-9 versus clone 6) have increased cell invasiveness.

Together, these results elucidate a novel function for CEA/CEAR signaling in tumor angiogenesis. Currently anti-VEGF/VEGFR systemic therapies are the only method of therapy for several types of human cancers. However, it is not always effective for CEA producing malignancies. In colorectal cancer the addition of Bevacizumab (Avastin) to conventional chemotherapy (FOLFOX or FOLFIRI) did prolong survival over chemotherapy alone [41]. Uncovering the underlying molecular mechanisms of the effect of CEA/CEAR in angiogenesis may lead to the expansion of new therapeutic anti-angiogenic factors. Overall CEA and its receptor are multifunctional proteins that are involved in multiple processes in cancer cells that can influence both invasion and implantation of tumor cells at distant metastatic sites. This study adds another potential function to CEA, that of involvement in the epithelial mezenchymal transition. This transition is essential to the development of metastases [42,43] and CEA/CEAR complex may represent new therapeutic target.

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