Methods for Detecting Circulating Cancer Stem Cells (CCSCs) as a Novel Approach for Diagnosis of Colon Cancer Relapse/Metastasis

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Abstract

Cancer stem cells (CSCs) are believed to be resistant to currently available therapies and maybe responsible for relapse of cancer in patients. Measuring circulating tumor cells (CTCs) in blood of patients has emerged as a non-invasive diagnostic procedure for screening patients who may be at high risk for developing metastatic cancers or relapse of the cancer disease. However, accurate detection of CTCs has remained a problem, since epithelial-cell-markers used to-date, are not always reliable for detecting CTCs, especially during epithelial-mesenchymal-transition. Since CSCs are required to initiate metastatic tumors, our goal was to optimize and standardize a method for identifying circulating CSCs (CCSCs) in patients, using established CSC markers. Here, we report for the first time the detection of CCSCs in blood of athymic nude mice, bearing metastatic tumors, and in the blood of patients positive for colonic adenocarcinomas. Using a simple and non-expensive method, we isolated a relatively pure population of CSCs (CD45−/CK19+), free of red blood cells and largely free of contaminating CD45+ white blood cells. Enriched CCSCs from patients with colon adenocarcinomas had a malignant phenotype and co-expressed CSC markers (DCLK1/LGR5) with CD44/Annexin A2. CSCs were not found in the blood of non-cancer patients, free of colonic growths. Enriched CCSCs from colon cancer patients grew primary spheroids, suggesting presence of tumor-initiating cells in the blood of these patients. In conclusion, we have developed a novel diagnostic assay for detecting CSCs in circulation, which may more accurately predict the risk of relapse or metastatic disease in patients. Since CSCs can potentially initiate metastatic growths, patients positive for CCSCs can be treated with inhibitory agents that selectively target CSCs, besides conventional treatments, to reduce the risk of relapse/metastatic disease for improving clinical outcomes.

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Introduction

Colon cancer is the third leading cause of cancer related deaths in the United States [1]. A subpopulation of cancer stem cells (CSCs), residing within the tumor bulk, are believed to be resistant to radiation and chemotherapeutic treatments, and are likely responsible for recurrence of the disease in cancer patients [2].

A number of non-invasive diagnostic assays/procedures are being developed in order to either diagnose patients with metastatic disease and/or to screen patients for relapse after treatment. A novel diagnostic tool, developed in recent years, consists of measuring the rare circulating tumor cells (CTCs) in the blood of patients for detecting early stage metastasis or recurrence of cancer in treated patients. This assay is based on the notion that metastatic cancer cells, going through epithelial-mesenchymal-transition (EMT), invade surrounding stroma and lymphatic/blood vessels by intravasation. The tumor cell emboli move through the vasculature to either lymph nodes or other organs and initiate tumor growth in secondary sites by extravasation [3,4]. Therefore, detection of CTCs in circulation is diagnostic for on-going metastasis and/or relapse [5].

However, developing reproducible and accurate methods for detecting and isolating CTCs has remained a challenge. Epithelial cell membrane markers used for identification and separation of the rare population of CTCs may provide erroneous results, since CTCs undergoing EMT during metastasis are down-regulated for the expression of epithelial cell markers [6]. The rare CTCs represent a minute percentage of total blood cells in circulation, ranging from only 1–100 CTCs/mL blood, amongst $>10^9$ red and white blood cells [7,8]. Thus, a sensitive bioassay is required for accurately detecting and isolating all CTCs/ CCSCs, while excluding contaminating white blood cells.

We now know that CSCs are critically required for initiating and maintaining the growth of primary and metastatic tumors [9–11]. Here, we present for the first time, a method for identifying circulating cancer stem cells (CCSCs) in the blood of colon cancer patients, using a combination of epithelial cell markers and cancer stem cell markers, such as DCLK1 [12–14], LGR5 [15,16] and CD44 [14,17]. Epithelial cell surface marker CK19 [18,19] and epithelial cell adhesion membrane protein, EpCAM [20], were used to confirm epithelial origin of the isolated CCSCs; combining surface markers has been shown to increase accuracy of detecting CTCs [18,19].

Progastrin (PG) peptide is expressed by a majority of human colon cancers (reviewed in 21). Cell surface Annexin A2 (CS-ANXA2), represents a non-conventional receptor for PG [22], and is required for mediating mitogenic/co-carcinogenic effects of PG on target cells [23]. Both PG and CS-ANXA2 regulate tumorigenic and metastatic potential of colon cancer cells [14,21–23]. CS-ANXA2 is increasingly expressed by aggressive epithelial tumor cells and is required for metastasis of many cancer cells [14,21–26]. Over-expression of PG by

embryonic epithelial cells transforms the cells, associated with a significant increase in the population of CSCs, co-expressing stem cell markers DCLK1/CD44 with CS-ANXA2 [14]. Co-expression of DCLK1 with CD44/CS-ANAX2 may dictate tumorigenic/metastatic potential of CSCs [14]. Therefore, PG and CS-ANXA2 were also used as novel markers of CCSCs, in combination with CSC and epithelial cell markers, in order to develop a more robust and accurate assay for identifying CCSCs.

Using the methods and markers described here, we report the development of relatively simple assay(s) for detecting CCSCs in the blood of colon cancer patients. CCSCs isolated from the blood of patients, positive for colonic adenocarcinomas (AdCAs), formed tumorospheres *in vitro*, confirming that the isolated CCSCs represent tumor initiating cells which can potentially grow into metastatic tumors *in vivo*. Tumorospheres generated from CCSCs can be used in the future to test efficacy of specific inhibitors, including inhibitors of stem cell markers, for eliminating CCSCs, and hence metastatic tumor growths, in order to improve clinical outcomes for the patients, as an approach towards personalized medicine.

MATERIALS AND METHODS

Reagents used

Antibodies used include: anti-CD44 (Cell Signaling Technology, Danvers, MA); anti-DCLK1, anti-CD45, anti-EpCAM, anti-CK19 and anti-GPCR GPR49 (Lgr5) (Abcam, Cambridge, MA); anti-AnxA2 (BD Biosciences, Carlsbad, CA), and anti-β-actin (total) (Sigma, St Louis, MO). Anti-PG antibody was generated in our laboratory as described [27]. Alexa Fluor-594 and Alexa Fluor-488 coupled secondary IgG were from Invitrogen (Carlsbad, CA). Three kits from Stem Cell Technologies were used: 1) RosetteSep™ Human CD45 Depletion Cocktail (#1522), 2) RosetteSep™ Human Circulating Epithelial Tumor Cell Enrichment Cocktail (#15127), and 3) EasySep™ Human Whole Blood CD45 Depletion Kit (#18289), (Vancouver, Canada).

Growth of primary and metastatic tumors from HCT-116 cells in athymic (SCID/nude) mice

Sub-confluent HCT-116 cells in culture were processed for inoculating athymic nude mice as described previously [14]. To induce the growth of sub-dermal xenografts (primary tumors), 5×10^6 HCT-116 cells/100μL phosphate buffered saline (PBS) were inoculated on the right and left flanks of female mice (Harland Sprague Dolly). For developing metastatic tumors, athymic female nude mice (5–6wks old) were subjected to intrasplenic inoculations with 2×10^6 HCT-116 cells/50–100μL PBS as described previously [14]. Mice receiving intrasplenic inoculations were subjected to splenectomy after 24h of inoculation to avoid splenic/peritoneal growths. Mice in group I were inoculated with heat inactivated colon cancer cells and served as the control group, with no tumor growths. Mice in group II were inoculated subcutaneously to develop primary tumor xenografts. Mice in group III were inoculated intrasplenically in order to develop metastatic tumors in the liver and lung, as described previously [14]. Approximately 4wks post-inoculation, when sub-dermal xenografts were clearly visible in mice from group II (~750 mm^3 in size), mice in all three groups were anesthetized for blood collection as per our approved IACUC protocols, and euthanatized by CO_2 asphyxiation. The size of sub-dermal tumors (group II) and total

number of metastatic lesions in the liver of each mouse in group III was similar to numbers previously reported [14]. ~1mL blood was collected from 1–2 mice in a lithium heparin tube, representing one sample/group. Three samples of blood were collected per group from 5 mice/group and processed for isolating CTCs as described below.

Procurement of blood samples from patients

'Normal' blood samples were obtained from consented UTMB patients scheduled for screening colonoscopy, aged 50 or older, irrespective of gender and ethnicity, as per our approved IRB Protocol. Blood was also obtained from consented patients scheduled for surgical removal of their colonic adenocarcinomas and lymph nodes at UTMB. 20–30mL of blood was collected at the time of intravenous line placement, as per our approved IRB Protocol. Consent was obtained from alert patients, who had not received any medication as yet. Clinicopathological information of patients enrolled in this study is provided in Supplementary Fig 1. Blood samples were collected from 7 patients with CRCs and 7 patients with 'normal' colons, with no adenocarcinomas or adenomas, but only hyperplastic polyps. Blood samples, in each case, were divided into 3 samples, and processed separately as triplicate samples. Samples in triplicate from all patients were subjected to CCSC analysis. Samples from 2–4 patients were also used for comparing the different isolation kits, recovery analysis and spheroid growths as indicated in the legend of the figures.

Generation of GFP expressing HCT-116 colon cancer cells (HCT-116-GFP)

Preparation of lentiviral plasmids expressing eGFP—Plasmids encoding eGFP in lentivirus packaging plasmid was generated by amplifying the gene by PCR. The plasmid pEGFP-C1 (Clontech; Mountain View, CA) was used as a template. Primers used contained the sequences for flanking restriction endonucleases (*Bam*HI and *Apa*I), and the product was ligated into the lentivirus packaging plasmid, pLenti6 (Invitrogen; Carlsbad, CA), to create pLenti6-GFP constructs, which was confirmed by DNA sequencing in the recombinant core facility at UTMB.

Generation of stable clones of HCT-116-GFP cells—HCT-116 cells were obtained in late 1980's from Dr. Michael Brattain's laboratory, and have been maintained in our laboratory since then. The HCT-116 cells were recently authenticated by BioSynthesis DNA Identity Center in 2012. The authenticated HCT-116 cells were grown in DMEM medium supplemented with 2.0 mM L-Glutamine (Invitrogen; Carlsbad, CA), penicillin/ streptomycin (Invitrogen; Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT). Sub-confluent HCT-116 cells were transfected with pLenti6-GFP plasmid. Stable clones of HCT-116 cells, expressing GFP, were generated by drug selection, as previously described [14]. The drug-resistant GFP expressing HCT-116 colonies were retrieved and expanded. The clones were further FACSorted to isolate HCT-116 cells expressing high levels of GFP, which were then maintained in drug selection medium in culture.

Recovery of colon cancer cells from human blood, spiked with HCT-116-GFP cells—HCT-116-GFP clones, growing in culture, were collected into centrifuge tubes by scraping. An aliquot was used for confirming viability of cells to be >99% by trypan blue

exclusion method using Cellometer™ Auto T4 (Nexcelom Bioscience, Lawrence, MA). Blood from patients who were either negative for colonic tumors, other than small hyperplastic polyps (termed normal samples) or who were positive for colonic adenocarcinomas, were spiked with 0–500 HCT-116-GFP cells/mL blood, within 2–4h of collecting the samples. The spiked blood was gently mixed manually and subjected to negative selection for RBCs using the HetaSep kit™ (as described in the legend of Supplementary Fig 2). Blood free of RBCs was subjected to further negative selection for CD45+ WBCs using the EasySep kit™, as per the manufacturer's recommendations. Human blood samples thus processed for negative selection of RBCs and WBCs were cytospun at 200g for 5min onto Superfrost®/Plus B microscope slides (Fisher Scientific, Pittsburgh, PA) using a CytoSpin III cytocentrifuge (Cheshire, England), followed by processing the cells for IF staining for the indicated markers; DAPI (4',6-diamidino-2-phenylindole) (Sigma, St Louis, MO) was used as a counterstain for identifying nucleated (epithelial/WBC) cells. HCT-116-GFP+ cells were further confirmed by measuring green fluorescent GFP-expressing cells, which allowed us to calculate % recovery of the spiked cells from the patient blood samples.

Isolation and characterization of Circulating Tumor Cells from the blood of mice and humans

Mice—Blood samples collected from mice were centrifuged at 200g for 5min, giving rise to three layers: 1) pelleted red blood cells, 2) buffy coat containing epithelial cells, and 3) supernatant containing WBCs. Buffy coat and supernatant were analyzed together for total number of CTCs/ml, by subjecting the samples to negative selection using the EasySep kit™, in order to eliminate WBCs from the samples. The elute was cytospun onto microscope slides using cyto-centrifuge and slides processed for immunostaining with mouse antibodies against CD45 and stem cell markers; nucleated cells were stained with DAPI.

Determination of % cells expressing CD45 in the three layers of mouse blood by FACS analysis—Cells present in the three layers of blood after centrifugation at 200g were subjected to FACS analysis to identify the layer in which the majority of WBCs, positive for CD45, were located. Cells within the indicated layers were carefully re-suspended at 1×10^6 cells/ml of PBS with 2% BSA at room temperature for 1h each. The cell suspensions were then incubated with anti-CD45−antibody (1:100) tagged to fluorophore Alexa-488 (DyLighd™ 488 NHS-Ester Die Light[RM] 488 NHS-Ester) for 2h at 4°C on a gently rocking platform. Cells were then pelleted at 200g and washed 3X with PBS containing 2% BSA to remove unbound primary antibody, followed by FACS analysis using FACSariaII (Carlsbad, CA) in the core facility at UTMB Health, as previously described [14].

Isolation and characterization of circulating tumor cells (CTCs) from human blood—Blood (~30ml) collected from patients was subjected to negative selection for RBCs and WBCs using 3 separate kits from stem cell technologies, as per the manufacturer's recommendations. The combined use of HetaSep™ and EasySep™ Kit gave the best results, and was used to analyze the blood from all patients. Blood plasma samples

negatively selected for RBCs and WBCs were processed for IF staining with the indicated antibodies, and co-stained with DAPI. Images of IF staining were acquired using an epifluorescent microscope and cells were analyzed using Metamorph, V6.0 software (Molecular Devices), as described previously [14,28].

Growth of CTCs as primary spheroids *in vitro*—In a separate set of experiments, CTCs isolated from the blood of patients positive for colonic adenocarcinomas, were subjected to negative selection for RBCs/WBCs, and plated to grow primary spheroids in low-attachment plates using the serum free spheroid assay buffer as described previously [14,28]. Blood samples collected from patients free of colonic growths, were similarly processed. The spheroids were imaged daily at 4x and 40x magnification using a white light microscope (Nikon Instruments, Inc, Melville, NY). At day 25, spheroids were processed for Western Blot (WB) [28]. Blots were cut into horizontal strips containing either the target or the loading control protein (β-actin) and processed for detection of antigen-antibody complexes by chemiluminescence [14,28]. Membrane-strips containing target/loading control proteins were simultaneously exposed to autoradiographic films. The loading-control, β-actin, was measured in corresponding samples containing equivalent-protein. Relative band density on scanned autoradiograms was analyzed using Image J program (rsbweb.nih.gov/ij/download), and expressed as a ratio of the target protein to β-actin in the corresponding sample.

Statistical analysis of data—Quantitative analysis of data is presented as mean±SEM of values obtained from the indicated number of samples in each experiment. To test for significant differences between values obtained from normal vs CRC samples, nonparametric student T-test and/or Mann-Whitney test was employed using GraphPad Prism software, Inc (La Jolla, CA); *P* values were considered statistically significant if less than 0.05.

RESULTS

Detection of CCSCs in blood of athymic nude mice bearing metastatic colon cancers

Athymic nude mice (5 mice/group), were inoculated with HCT-116 cells as described under Methods. Blood collected from all 3 groups, was centrifuged and FACSsorted as diagrammatically presented in Fig 1A. Population of CD45+/− FACSorted cells in supernatant+buffy coat and in RBC pellet are shown as a forward scatter plot in Fig 1B; average percentages of CD45+ cells in the fractions is presented in Fig 1A. Majority of CD45$^+$ (>98%) and CD45− (>99%) cells were present in the supernatant+buffy coat and RBC pellet layers, respectively. A small % of cells in the supernatant+buffy coat fraction were CD45− (1.1%), which likely represents CTCs, as reported by others [29,30]. CD45− cells from supernatant+buffy coat layers were cytospun on slides and processed for IF staining for cancer stem cell (CSC) markers (DCLK1/CD44/Lgr5) and ANXA2 (Figs 1C). ~1.5–3% of CD45− cells in the buffy coat+supernatant layers of plasma from Group III mice expressed DCLK1, CD44, Lgr5 and ANXA2 (Fig 1C). In contrast, <0.5–1% of CD45− cells in plasma of mice in groups I and II were positive for indicated markers (Fig 1C). A slightly higher % of CD45− cells (~0.7–1%) in groups I/II, expressed CD44 and ANXA2,

compared to stem cell markers DCLK1/Lgr5 (Fig 1C). The remaining CD45− cells (>97%), likely represent CTCs, which are not circulating cancer stem cells (CCSCs). Some of the ANXA2+/CD44+ cells may also represent contaminating CD45+ cells in these fractions, since negative selection for WBCs is not 100% efficient. CD45+ cells are known to express CD44 and ANXA2, as previously reported [31–33]. We have previously reported co-expression of stem cell marker DCLK1 with CD44/ANXA2 by human colon cancer cells as a marker of transformation/metastatic potential [14]. CD45− cells in the plasma of group III mice were found to similarly co-express DCLK1/ANXA2 and DCLK1/CD44 (Fig 1C, right panels), confirming that CSCs in circulation maintain the malignant phenotype of CSCs, reported in primary tumors [14].

Recovery of colon cancer cells from human plasma—The efficiency of EasySep™ human whole blood CD45 depletion kit, for recovering colon cancer epithelial cells from human plasma samples was analyzed by spiking the samples from normal and colon cancer patients with HCT-116-GFP cells. Majority of the HCT-116-GFP cells (>97%) were recovered from the spiked plasma samples using the kit (Supplementary Fig 2). Number of nucleated cells (epithelial+CD45+) recovered from spiked plasma was higher than the number of colon cancer cells used for spiking. The latter increase was due to contamination with CD45+ WBCs (Supplementary Fig 2i–ii); however, none of the GFP+ cells co-stained for CD45 (Supplementary Fig 2i–ii).

Percent contamination of processed plasma samples with CD45+ cells in the final elute—On an average, 82±10 CD45+ cells were present/mL sample from cancer patients, while 70±10 CD45+ cells were present/mL sample from normal patients (Fig 2A), suggesting that a minute population of CD45+ WBCs remains as a contaminant in the final elute. Since CD44 is also expressed by CD45+ WBCs [31], on an average, 50 and 85 CD44+ cells/mL were present in the blood of normal and AdCA patients, respectively (Fig 2B). Interestingly, ~98% of CD44+ cells in the blood from normal patients co-stained with CD45, suggesting that almost all the CD44+ cells present in the blood of normal patients were WBCs (Fig 2C). However, only ~70% of CD44+ cells co-stained with CD45 in the blood of cancer patients (Fig 2C), suggesting that ~30% of CD44+ cells circulating in the blood of colon cancer patients, represents CCSCs. Numerically, an average of 9 cells/mL were CD44+/CD45− cells in AdCA patients, while 0 cells/mL were CD44+/CD45− in normal patients (Fig 2D).

CCSCs in the blood of normal and cancer patients—The number of cells positive for DCLK1, CD44, Lgr5, ANXA2, PG, EpCAM, and CK19 were significantly higher in the blood of AdCA patients compared to that in the blood of normal patients (Fig 3A). Besides CD44, a minute subpopulation of leukocytes also expressed DCLK1/ANXA2 (Fig 3B), suggesting for the first time that CD45+ cells can potentially express DCLK1. A subpopulation of CD45+ peripheral blood monocytes co-express ANXA2 [32,33] and CD44 [31]. However, majority of DCLK1/ANXA2 positive cells in the final elute of plasma samples from AdCA patients were negative for CD45, suggesting that these markers are largely expressed by epithelial CCSCs in CRC patients. Comparative tables, enumerating

average number of CTCs/ml sample from normal vs cancer patients, with or without contaminating CD45, are presented in Supplementary Figs 3A–B.

Representative IF images of CTCs in the final elute of plasma samples from AdCA patients, stained for the indicated markers, are shown in Fig 4A. Number of cells/mL sample which were positive for the indicated markers in the absence of CD45 staining are presented in Fig 4B. After excluding CD45+ cells, normal samples were almost completely devoid of CTCs. However, plasma samples from AdCA patients were positive for a significant number of CTCs, which stained for the indicated epithelial and stem cell markers (Fig 4B). Majority of CTCs were positive for CD44 (~10 cells/mL) while only a minority were positive for EpCAM (1.3 cells/mL), providing further evidence that majority of CTCs, including CCSCs, in cancer patients are down-regulated for the expression of the adhesion protein, EpCAM. Majority of CD44+/CD45− cells were positive for epithelial cell marker CK19 (~7 per 10 CD44+ cells/mL) suggesting that CK19 is a better marker for epithelial tumor cells; but CK19 may also be under expressed by a sub-population of CTCs [29]. On an average, ~5,10,4,5,4 and 7 CTCs/mL blood from AdCA patients expressed DCLK1, CD44, Lgr5, ANXA2, PG and CK19, respectively (Fig 4B).

CCSCs in blood samples from AdCA patients co-express markers for stem cells (DCLK1/Lgr5) with CD44/ANXA2, representing a malignant phenotype—
After negative selection of plasma samples for RBCs/WBCs, remaining cells in the final elute were co-stained with antibodies against specific markers. IF images from representative AdCA samples are presented in Figs 5A–C. Total number of cells which co-stained with the indicated markers/mL of sample from several patients is presented as bar graphs in Fig 5D. A comparative table enumerating average number of CTCs/mL co-expressing the specific markers from normal vs cancer patients is presented in Supplementary Fig 3C. Normal blood samples were largely negative for cells which co-stained with the indicated markers. However, <0.5 cells co-stained with DCLK1 and ANXA2/CD44 in normal plasma samples suggesting lingering contamination with non-epithelial cells, as described above. Plasma samples from AdCA patients, on the other hand, were positive for a significant number of cells (~1–6/mL) that co-stained with the indicated markers. Co-staining of cells with stem cell markers and EpCAM continued to be the least robust marker. Interestingly a significant number of stem cells (DCLK1+) and ANXA2+ cells, co-stained with colon cancer cell marker, PG, providing a unique marker for colon cancer stem cells in circulation. To confirm epithelial nature of CCSCs, cells were co-stained with CK19, a well-established epithelial marker for CTC recognition [18,34] (Fig 5C; Supplementary Fig 4).

CCSCs likely represent tumor initiating cells—Cells in the final elute were seeded in low attachment plates to grow as spheroids, to test stemness of the cells (as an indirect indicator of tumor initiating potential). By day 14, epithelial cells enriched from the blood of AdCA patients started growing as distinct spheroids while cells remaining in the elute of normal patients did not form spheroidal structures (Fig 6A). On day 25 of culture all cells were collected from normal and AdCA plates, pelleted by centrifugation, and processed for Western Blot analysis. The spheroidal cells from AdCA patients were positive for Lgr5/

DCLK1, while normal samples were negative (Figs 6Bi,ii), confirming that normal blood lacks CCSCs, while AdCA samples are positive for cancer stem cells which likely have the potential for initiating metastatic tumors *in vivo* [35].

Discussion

Presence of CTCs in CRC patients has been reported to be associated with poor prognosis for survival/recurrence, irrespective of chemo/radiation therapy [36–38]. Several CTC assays have been developed in the past, all of which use some combination of negative selection (removal of red and white blood cells) and positive selection (cells positive for epithelial and/or intracellular stem cell marker, such as ALDHA1) [4,8,18,39]. CD133 has also been used for positive selection [39]. However, stem cell markers, DCLK1/Lgr5, have not been used previously for detecting circulating CSCs. Since DCLK1 has emerged as a specific marker of colon/pancreatic cancer stem cells [13,14,28,40,41], we report for the first time detection of DCLK1+ CCSCs in the blood of CRC patients. In addition we used the metastatic marker, AnexinA2. Thus the markers used in this study, for detecting CCSCs, are expected to be more valuable for diagnosis/prognosis of recurrence/relapse of the disease and for perhaps detecting even primary CRCs with metastatic potential.

Several laboratories have tested novel techniques to negatively select for leukocytes (WBCs) in the peripheral blood; however due to the rarity of CTCs in the blood and the inability to entirely remove the abundant WBCs from blood specimens, this approach has remained challenging [42]. Size based purification [43], high speed microscopic scanning of nucleated tumor cells [44] and *in vivo* multi-photon imaging of circulating tumor cells [45] also present technical challenges resulting in low CTC purity. We first optimized and standardized a relatively simple assay for measuring circulating CSCs (CCSCs). Our results suggest that, negative selection for RBCs (using HetaSep™ solution) and WBCs (using the EasySep kit™), followed by positive identification of CCSCs by immunostaining may represent a relatively simple and inexpensive method. Although the optimal assay used by us yielded the presence of < 0.0015% CD45+ cells after negative selection, we were able to discount them by using a combination of leukocyte and CSC/epithelial cell markers.

ANXA2 [32,33] and CD44 [31,46] have been previously reported to be expressed by peripheral blood monocytes; however, expression of DCLK1 by CD45+ cells has not been reported, and represents a new and novel finding of the current study. Majority of cells expressing CSCs/ANXA2, were CD45– (Fig 3B). Therefore, in order to distinguish rare CCSCs from white blood cells, negative selection by staining with anti-CD45 antibody needs to be added as a final step for most CTC assays, in order to eliminate false positive results. We also analyzed isolated cells for EpCAM/CK19 staining to confirm epithelial nature of isolated CCSCs. EpCAM is commonly used as a marker for enumerating CTCs, and is generally absent in normal blood cells [4,47]. EpCAM has been previously reported to be expressed by many epithelial cancers, including lung, gastric, breast and colon [48,49]. However, our results showed that EpCAM was not a robust marker for detecting CTCs in the blood from CRC patients (Figs 4–5). Several recently developed CTC enrichment technologies, such as microfluidic-based CTC capture [4] and independent density gradient centrifugation [50], have utilized EpCAM as a marker of CTCs. However, the reliability of

using EpCAM as a robust marker has been questioned, since this adhesion protein is down-regulated during invasion and dissemination [51]. The metastatic epithelial tumor cell likely looses adhesion due to down-regulation of EpCAM, and undergoes epithelial-mesenchymal transition [52]. EpCAM antibodies were reported to be insufficient for detecting subtypes of breast cancer cells [53], as confirmed in our studies with CRC patients (Figs 3A,4B). Thus antibodies against several cell surface antigens needs to be used for accurate detection of all epithelial CTCs. The latter point is especially important, since epithelial cells are not known to be present in the circulation of individuals, who are free of cancerous growths. Normally, epithelial cells from the tissues of origin are eliminated after cell death with the help of macrophages and/or sloughed off into the lumen in the case of glandular cells lining the GI tract. Thus the presence of epithelial cells in the circulation, in itself, reflects presence of cancerous tumors which have acquired invasive properties. However, in order to avoid false negative results, one needs to carefully choose the epithelial cell marker for identifying these rare cells in the circulation, which are generally positive for EMT. We used CK19 as an additional marker to confirm epithelial nature of CCSCs (Fig 5). Cytokeratin 19 is a well-established marker of epithelial cells, including primary tumor cells, and is known to be absent from mesenchymal and normal blood cells [54–57]. Presence of CK19+ cells in the blood has been shown to be associated with poor clinical outcome for patients with many types of epithelial cancers, including breast cancers [58]. Importantly, almost all the CCSCs isolated by us, expressed CK19 (Fig 5C), confirming that CCSCs, positive for one or more stem cell markers (such as DCLK1, LGR5, CD44), as isolated and enumerated by IF staining, were of epithelial origin. The current findings from <10 patients, however, needs to be validated by analyzing blood samples from a larger cohort of patients.

Cell surface associated ANXA2 (CS-ANXA2) is known to play a critical role in metastasis of breast and lung cancers (discussed in 59); down-regulation of ANXA2 with shRNA-ANXA2-nanoparticles significantly inhibited formation of metastatic lesions from lung cancer stem cells [26]. Embryonic stem cells, induced to overexpress progastrin (a potent autocrine growth factor for colon cancer cells, [21]), became transformed and developed metastatic potential and were found to over-express CS-ANXA2, unlike the non-transformed embryonic stem cells [14]. Down-regulation of ANXA2 reduced metastatic potential of the transformed epithelial embryonic cells [14]. Since CS-ANXA2 is required for metastasis of cancer cells [discussed in 26,59], we examined possible expression of ANXA2 by CCSCs, and report for the first time co-expression of ANXA2 by CCSCs in the blood of nude mice bearing metastatic tumors and in the blood of CRC patients (Figs 1C,5).

Progastrin (PG) peptides are expressed by majority of CRCs [21] and high expression of PG by adenocarcinomas from patients was reported to be prognostic for poor survival [60]. PG expression by CD133+ colon cancer stem cells was recently reported [61]. In the current studies we report for the first time, co-expression of PG by a significant population of CCSCs (Fig 5). PG expression by CCSCs was found to heavily co-localize with CS-ANXA2 (Figs 5B,D); co-localization and endocytosis of CS-ANXA2, bound to PG, was required for measuring biological effects of PG [59]. Thus expression of PG in CCSCs, suggests potential autocrine activity of PG in CCSCs, which may be required for seeding and initiating the growth of CCSCs as metastatic lesions in peripheral organs. PG may thus not only support mitotic growth of colon cancer cells, by up-regulating CSC populations, as

previously reported [23], but may also support dissemination and metastatic growth of colon CTCs, providing a CRC specific CTC marker.

To date, 'Cell Search System' is the only automated CTC detection bioassay, which has been approved by the FDA [8, 62]. The system utilizes a combination of well-established epithelial cell markers such as EpCAM and cytokeratins; however reproducibility between laboratories has remained an issue [52–55]. Laboratories using Cell Search systems for detecting CTCs in colorectal patients, have reported presence of <3CTCs/7.5mL blood [8]; these results are consistent with our findings of <1CTC/mL blood of CRC patients, when EpCAM alone is used as a marker (Figs 3–4). However, detection of CTCs with CSC markers (CD44/DCLK1) along with CK19, with or without ANXA2/Progastrin, resulted in isolating a significantly higher number of CCSCs (>4–5/mL blood) (Figs 4–5). The latter results re-emphasize the unreliability of using EpCAM alone as a marker for CTC detection, since it can potentially result in false negative results. Use of EMT markers, significantly increased the numbers of CTCs measured in cancer patients [56]. However, EMT markers alone may also give erroneous results, since many cells other than CSCs also express EMT markers during dissemination. Therefore, use of one or more CSC markers along with epithelial/EMT/metastatic markers will likely provide the most accurate assessment of the risk for recurrence and metastatic disease. Addition of PG may provide a CRC specific marker and help diagnose patients for the type of cancer as well. Criterion, such as specificity and sensitivity, are generally important for diagnostic assays [63]. However, these criteria do not apply to all types of tests, especially cell-based assays, as is the case with CTCs. The latter point is well discussed in a rebuttal letter to the editor in response to the article by Parikh, et al [63]. As discussed above, the presence of CTCs (irrespective of number/ml blood), in itself, is diagnostic for the presence of cancerous growths and/or metastatic disease. However, to avoid false positives, we have suggested the manual inspection of the cells to eliminate false positive results due to CD45 staining. To avoid false negative results, the use of more robust markers are described above, including CK19. In order to further increase the value of the assay, we have suggested the analysis of the cells for several stem cell markers and markers such as ANXA2/PG, based on the results of our investigations.

Therefore, based on the results of our study, we report a novel approach for detecting cancer stem cells in circulation, using a combination of CSC and epithelial cell markers. Since, CSCs are believed to be responsible for tumor-initiation and metastases; we can potentially screen patients for relapse or presence of metastatic disease, by measuring CCSCs in the blood of patients, and improve the automated bioassays by including one or more CSC markers. We recently reported that targeted disruption of DCLK1 along with other non-toxic dietary agents can potentially eliminate CSCs and the possibility of relapse [28]. Thus based on the results of CCSC assays described in here, personalized treatment regimens can be developed for targeting and eliminating circulating CSCs, to significantly reduce the possibility of relapse and improve clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

Glossary

|	
AdCA	Adenocarcinoma
ANXA2	Annexin A2
CCSCs	circulating cancer stem cells
CD44	Cluster of differentiation 44
CD45	Cluster of differentiation 45
CK19	cytokeratin 19
CRC	colorectal cancer cells
CS-ANXA2	cell surface Annexin A2
CSCs	cancer stem cells
CTCs	Circulating Tumor Cells
CCSCs	circulating cancer stem cells
DCLK1	doublecortin-CAM-kinase-like1
EMT	epithelial-mesenchymal-transition
EpCAM	Epithelial cell adhesion molecule
FACSorting	Fluorescence-activated cell sorting
Fig	Figure
Gp	Group
HCT-116-GFP	human colorectal cancer cell line induced to express green fluorescent protein
IF	Immunofluorescence
Lgr5	Leucine-rich repeat-containing G protein coupled receptor 5
PG	Progastrin
Sup	Supplementary
vs	versus
WB	western blot

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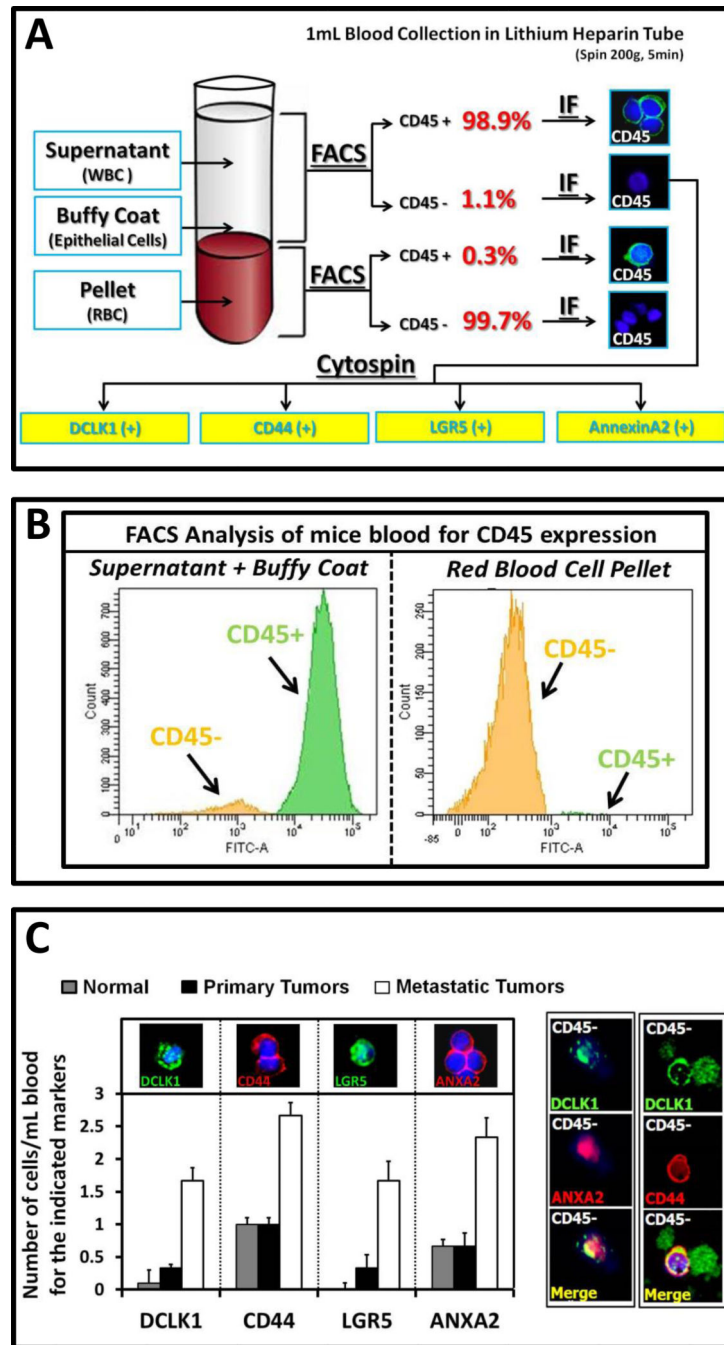
Figure 1. Detection of metastatic circulating tumor cells in blood of athymic nude mice
A) Diagnostic scheme for isolating nucleated epithelial cells from the blood of mice. Three samples of blood/mouse group were collected and analyzed by FACSorting for percent cells positive or negative for CD45 in the supernatant/buffy coat and red blood cell layers. CD45– cells isolated from the supernatant/buffy coat layer are stained by IF staining for the indicated markers. **B)** Representative data from FACSorting of the cells into CD45+/– populations, showing forward scatter plots for the two fractions of mouse plasma from GroupIII mice. **C)** Bar graphs depicting percent cells positive for the specific markers/mL

blood, collected from all 3 groups. Images of CD45− cells stained by IF for DCLK1/ ANXA2/CD44/LGR5 markers are shown above the bar graphs and co-expression is depicted as merged images on the right. Mean±SEM of data obtained from ~10 slides/3 samples/group. *=P< 0.05 versus normal values.
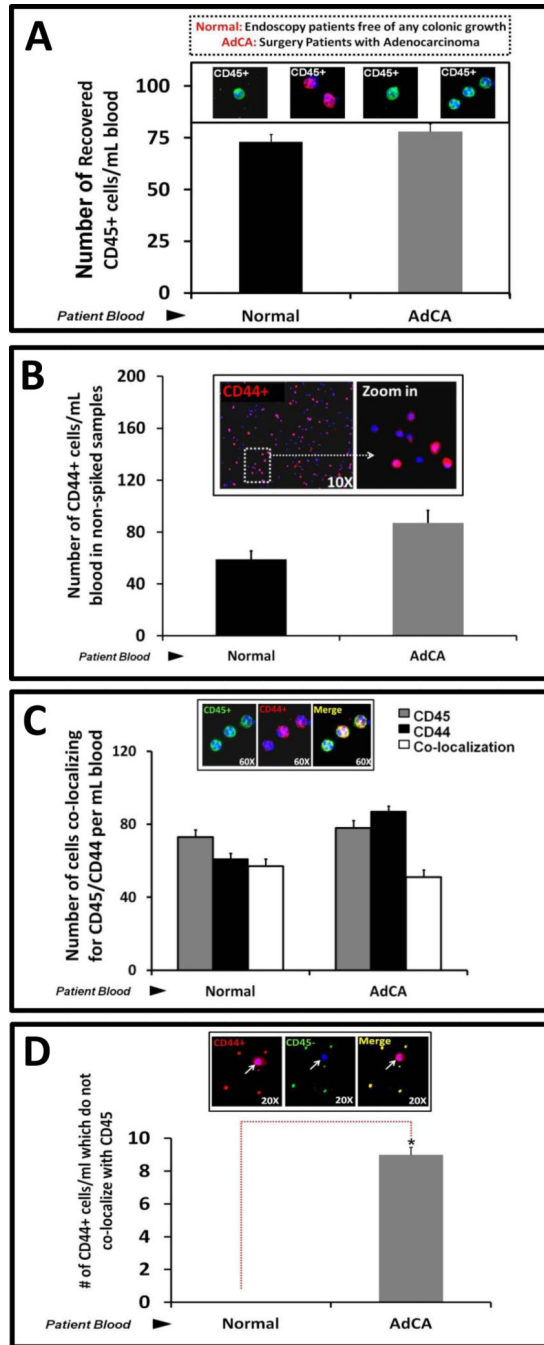
Figure 2. Presence of CD44+/CD45– cells in the blood of patients with colon-adenocarcinomas
Bar graphs illustrating: **A)** Number of recovered CD45+ cells/mL in the blood of patients, who were either free of colonic growths (normal), or positive for Adenocarcinomas (AdCA); IF images of remaining CD45+ cells collected in the final elution are shown above the bar graphs. **B)** Number of CD44+ cells/mL in blood of normal vs AdCA patients. **C)** Number of cells co-localizing for CD45/CD44 per mL of blood. Representative IF staining of circulating cells stained with indicated markers are shown above bar graphs, after negative selection of the samples. **D)** Number of CD44+ cells/mL, which do not co-localize with
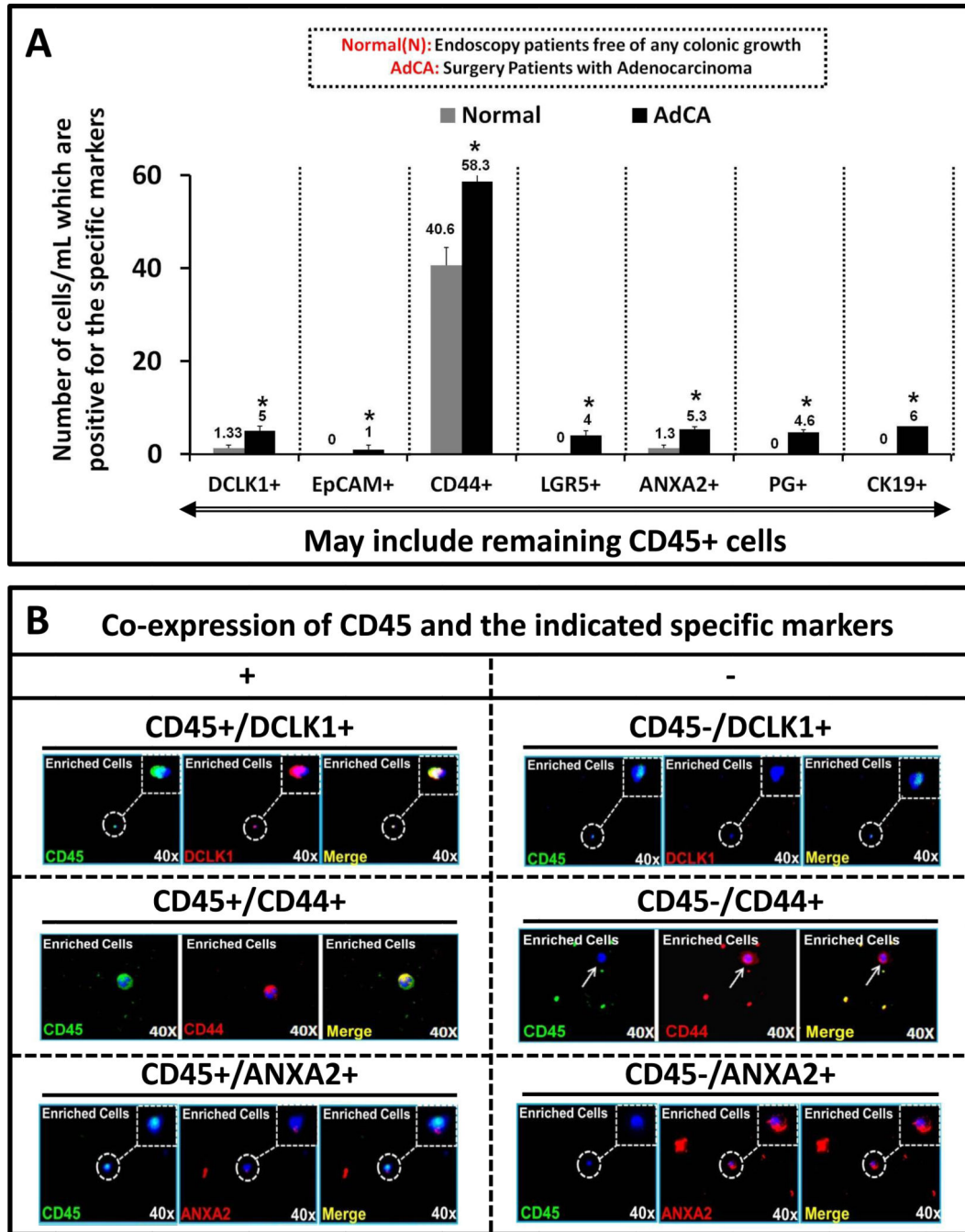
CD45 in blood of normal vs AdCA patients; representative IF images are shown above bar graphs. Each bar graph=mean ±SEM of data from 5–7 patients analyzed in triplicate as described in Methods. *=P< 0.05 versus normal values.

Figure 3. A sub-population of Leukocytes is also positive for DCLK1, ANXA2 and CD44
A) Blood from normal and AdCA patients were processed with the EasySep™ kit, catalog #18289 Cells collected after final elution, were cytospun onto glass slides, followed by IF staining for indicated markers. Based on the staining results, number of cells positive for the indicated markers was determined/mL blood, as shown in the bar graphs. **B)** Representative IF images showing co-expression of DCLK1/CD45, CD44/CD45 and ANXA2/CD45. Images were taken at 10x magnification and a single cell image was magnified and is presented in an inset. (n=7 normal samples and n=7 AdCA samples).
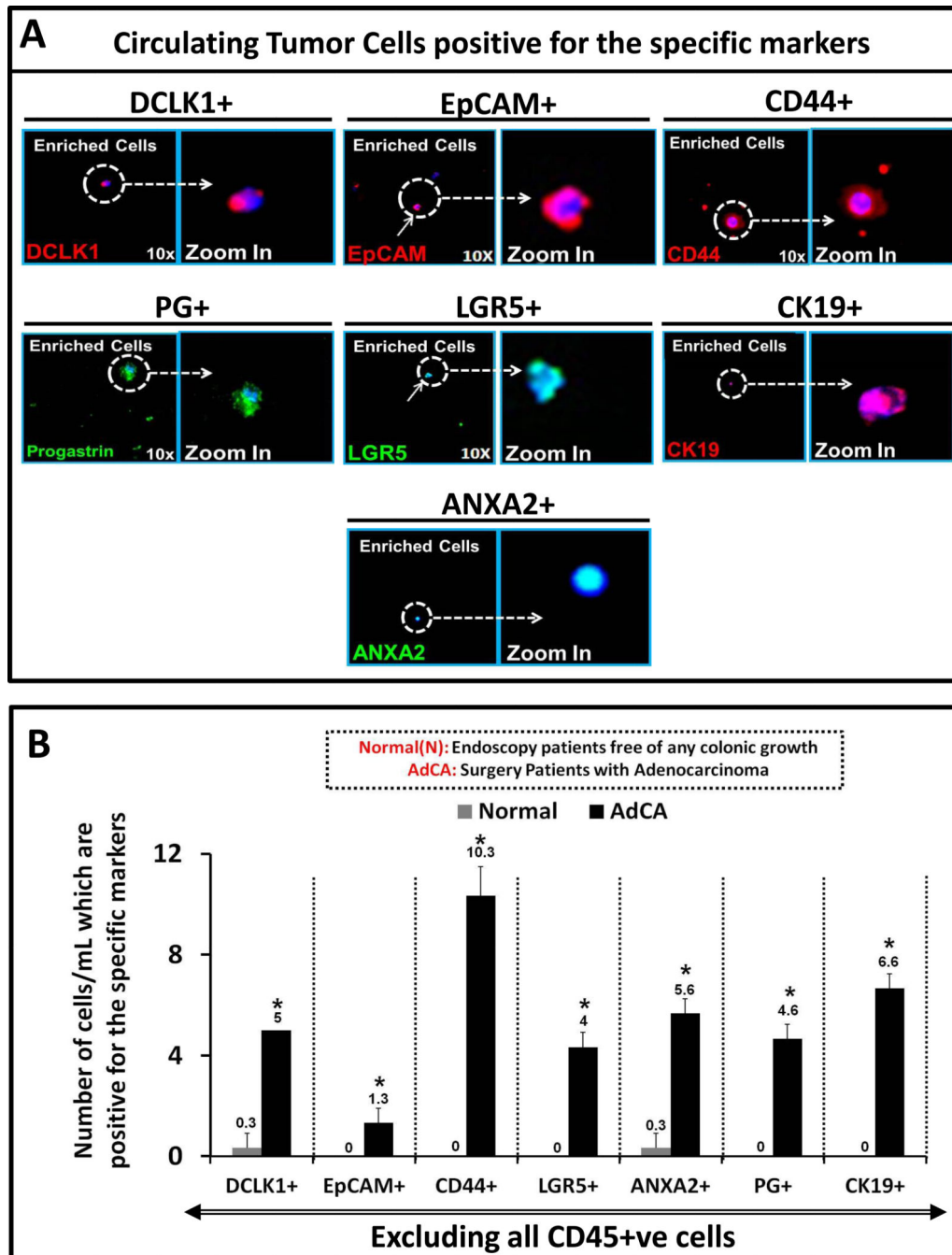
Figure 4. Circulating cancer stem cells (CCSCs) in the blood of patients
A) Representative IF images of circulating cancer stem cells, positive for indicated markers. Images were digitally enhanced, as shown by dotted arrows. **B)** Bar graphs illustrating number of cells/mL, positive for indicated markers, excluding CD45+ cells. (n=7 normal samples and n=7 AdCA samples).
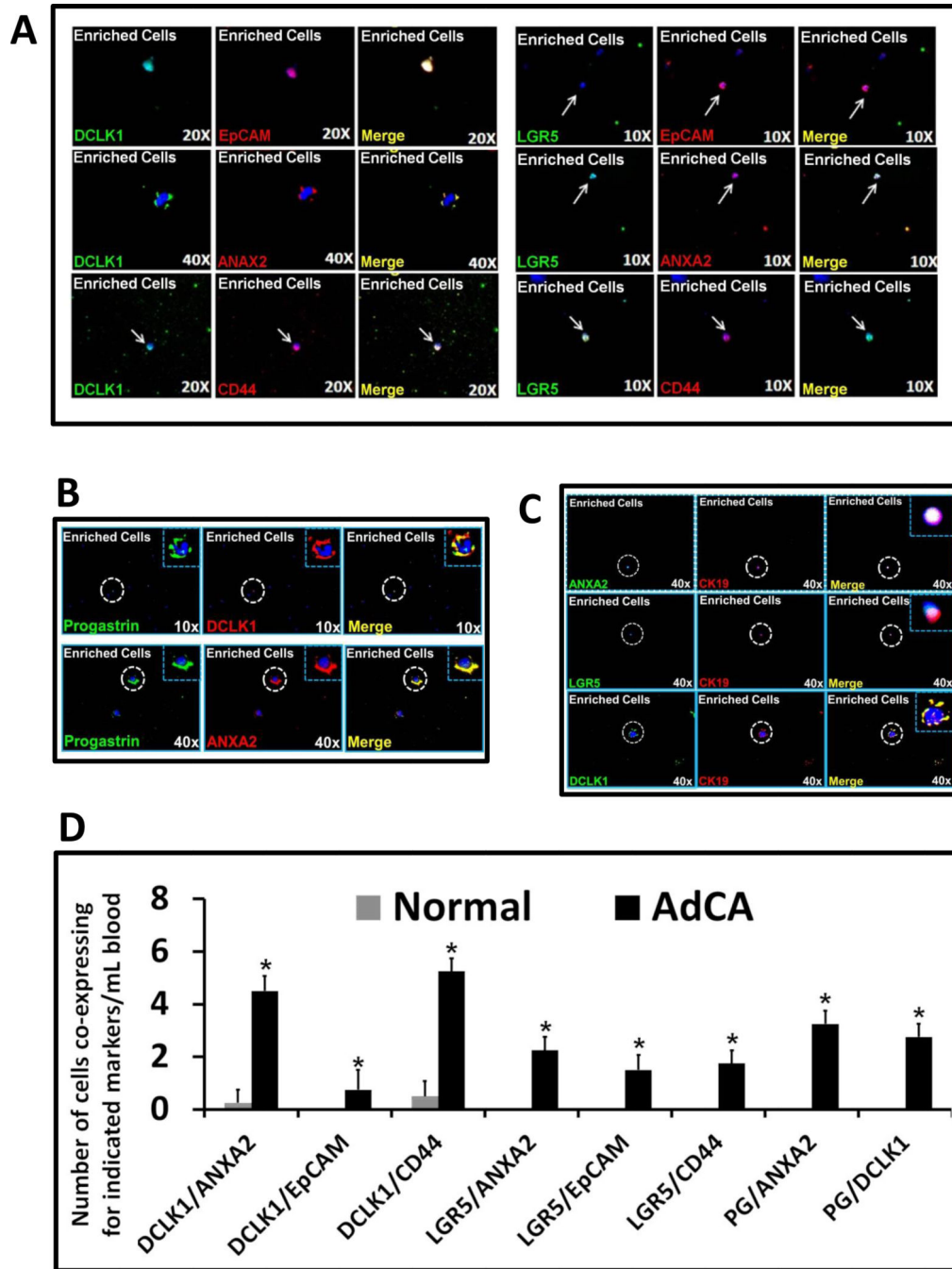
Figure 5. Co-expression of CSC markers (DCLK1/LGR5) with AnxA2/PG/CD44/EpCAM/CK19 by CCSCs in the blood of colon cancer patients
A) Co-expression (IF staining) of DCLK1+/LGR5+ cells with either EpCAM, CD44 or ANAX2 in AdCA samples; white arrows depict stained cells. **B) C**o-expression of DCLK1/PG or ANXA2/PG in CCSCs. **C) C**CSCs co-expressing CK19 with either ANXA2, DCLK1 or LGR5. Merged images are enhanced in the inset. **D)** Bar graphs illustrating number of CCSC cells co-expressing indicated markers/mL blood. (n=7 normal samples and n=7 AdCA samples).

A

Primary Spheroids grown from Enriched Circulating Epithelial Cells

B
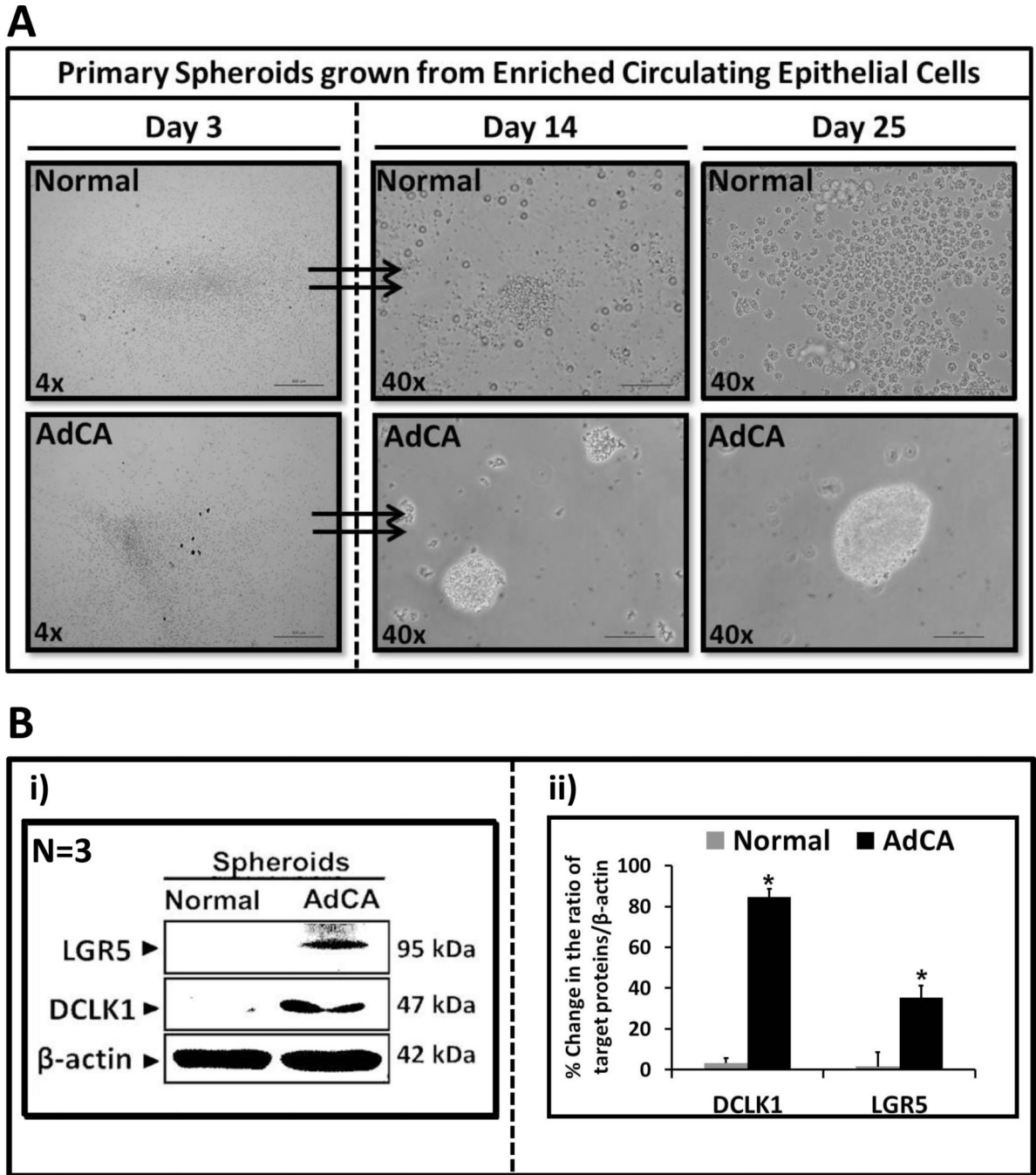
Figure 6. Formation of spheroids, *in vitro*, from circulating tumor cells, isolated from AdCA patients

A) Representative Images of spheroids formed from enriched circulating epithelial cells, isolated from blood of normal and AdCA patients. Images were taken at 4x and 40x magnification at days 3, 14 and 25 after seeding the cells. **Bi)** Western blot analysis demonstrating increased expression of LGR5 and DCLK1 in spheroids from CTCs isolated from a representative AdCA patient, compared to that obtained from a representative normal

patient. **Bii)** Bar graphs demonstrating percent change in ratio of target proteins to β-actin. (n=3 normal patient samples; n=3 AdCA patient samples).