Introduction

The origin of prostate cancer (PrCa) is still a matter of debate in the scientific community [1]. The three specific lineages (basal, luminal and neuroendocrine) of the normal prostatic epithelium [2] result from the directional differentiation of normal stem cells (NSCs) [3]. The vast majority of PrCa cells has a luminal phenotype, but unlike normal luminal cells that are terminally differentiated and have limited proliferative capacity, PrCa cells still retain the ability to proliferate, resembling “basal” characteristics [4]. To date, two major theories have been proposed to explain the initiation of PrCa [5]. First, PrCa may arise from niches of NSCs that are localized in the basal layer of prostate glands [2, 6]. Under physiological conditions, these low-proliferating NSCs can give rise to a second population of more rapidly cycling cells, known as transit amplifying cells [7], which differentiate into mature, secretory cells [2]. However, during tumorigenesis genes able to tightly control cell proliferation or DNA damage repair in NSCs can accumulate “transforming” mutations, resulting in the conversion to cancer stem cells (CSCs) that show growth advantages over their normal counterparts [8, 9]. A second theory postulates that PrCa can originate from normal cells in the luminal/transit amplifying layer, that re-acquire abilities for self-renewal, initiating the uncontrolled tumor growth [10].

CSCs, also designated cancer stem/progenitor cells [11], are defined as cells with the special ability to generate tumors when injected in animal hosts [12] through processes of self-renewal and differentiation into the multiple...
lineages of cancer cells that comprise a tumor [13]. In the tumor, CSCs form a distinct niche which is resistant to conventional treatments, such as radiotherapy [14, 15], and can be responsible for relapse and metastatic dissemination of the disease. Novel therapies able to kill both differentiating/differentiated cancer cells and CSCs are absolutely needed in order to improve survival and quality of life of cancer patients and avoid the use of invasive approaches, such as radical prostatectomy for PrCa patients. Surface receptors expressed at high levels in cancer cells and CSCs would be ideal targets for treatments with specific antibodies or small-molecule inhibitors. Several candidate markers (such as CD24, CD44, CD133, CD166, Trop-1, and some integrins) have been tested to date, alone or in combination with each other, but profiles elaborated for CSCs are still weakly useful, since strongly dependent on the tumor histotype [16]

The transmembrane protein CD133, also known as Prominin-1, is one of the most important markers of hematopoietic [17] and neural stem cells [18], and it has also been used to identify prostate NSCs [19, 20]. In addition, epithelial cells from human prostate sorted in two populations, CD133+ and CD133-, show a gene expression signature of putative stem/progenitor cells, with similarities to the gene expression profile of hormone-refractory prostate cancer cells, and transit amplifying cells, respectively [21]. CD133 has been successfully used for the identification of CSC niches in glioblastoma [22], colon cancer [23] and other solid carcinomas [24-26], suggesting a strong relevance of this protein for the activity of CSCs. On the other hand, both CD133+ and CD133- populations isolated from metastatic colon cancers [27] and lung adenocarcinoma cell lines [28] were recently shown to have comparable abilities to generate tumors when implanted into immunodeficient mice. Thus, the role of CD133 in CSCs still remains controversial [29].

The stem compartment is localized in the basal layer of epithelial tissues, and the interaction with the basement membrane appears critical for the maintenance of stem-activity [30, 31]. In cancer, this type of interaction can stimulate the necessary early steps of the metastatic cascade [32]. A fundamental role in the activity of stem cells can be hypothesized for integrin receptors, which are critical regulators of extracellular matrix (ECM) interactions [33, 34]. A conditional knock-out of β1 integrins from basal cells of the mammary gland was demonstrated to severely affect the normal development of the mammary epithelium [35]. In addition, ablation of β1 in a transgenic mouse model of breast cancer resulted in dramatically impaired initiation and maintenance of mammary tumor growth in vivo [36], suggesting that β1 play a critical role in the very early steps of tumorigenesis. Recently, α2β1 integrin was proposed as a marker of stem compartments in the normal prostate [37]. Moreover, Keratinocyte Growth Factor was found to induce terminal differentiation of the α2β1n transit amplifying cells, with concomitant suppression of α2β1 [38]. This evidence suggests that β1 integrins (and in particular α2β1 in the prostate) can be essential for the maintenance of a functional stem niche. A CD44+ /α2β1hi/CD133+ population isolated from PrCa patients was shown to have enriched tumorigenic properties in vitro [39]. However, these cells were not investigated for their ability to reproduce the entire tumor in vivo, partially supporting the use of the α2β1hi/CD133+ profile as a marker of the PrCa stem niche. The standard form of CD44 (CD44s) was reported to be localized in benign epithelium, mostly in basal cells but also somewhat in secretory cells, and its expression was undetectable in cancer except for the neuroendocrine cells [40]. In addition, CD44s was also found in lymphocytes and nerves surrounding the prostatic epithelium, raising doubts on its utility for the targeting of stem niches in vivo [40]. However, a splice variant isoform, CD44v7-10, emerges in cancer [41, 42] and is currently under investigation as a more specific PrCa stem cell marker.

The transmembrane protein Trop-2, a homologue of Trop-1/EpCAM which is expressed in tissues of epithelial origin and has an important role in the early stages of tumor progression [43, 44], is frequently up-regulated in many human carcinomas as compared with their normal counterparts, suggesting an involvement of this protein in tumor growth [45, 46]. CSCs have been demonstrated to re-express several embryonic genes such as OCT4, NANOG, SOX2 [47]; thus, regulatory programs related to early embryonic development can be re-activated during cancer initiation. This evidence suggests a potential role of Trop-2, which is expressed at high levels during early embryonic development...
in the trophoblast [43], in CSCs activity. Recently, Trop-2 was proposed as a novel marker for the identification of stem cells in murine normal prostate [48]. Lin-Sca1+CD49fhi normal murine prostate cells showed low or high stem-like activity in vitro and in vivo, based on low or high expression levels of Trop-2, respectively [48]. However, Trop-2 expression has never been studied in human prostate.

In this study, we have investigated the expression pattern of CD133, α2β1 integrin and Trop-2 in human PrCa specimens by immunohistochemistry (IHC) in order to identify niches of cells with putative CSC-like activity. Our findings demonstrate that the combination of CD133 and Trop-2 is useful to mark putative CSC-containing compartments in human prostate.

Materials and methods

PrCa specimens

Tissue specimens from radical prostatectomies were provided by: the Cooperative Human Tissue Network (CHTN; other investigators may have received samples from these same tissues) and the Department of Pathology, University of Massachusetts Medical School. The samples were unidentifiable, discarded human tissues processed according to institution-approved protocols. Specimens were processed for IHC as described below.

Antibodies

Mouse monoclonal antibodies (Ab) to p63 (clone 4A4) and α2β1 integrin (clone HAS-3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat polyclonal Ab to Trop-2 was purchased from R&D Systems. Rabbit polyclonal Ab to CD133 was purchased from Abcam. Mouse IgG (Vector Labs, Burlingame, CA), rabbit IgG (Vector Labs) and goat IgG (Santa Cruz Biotechnology) were used as negative controls.

CD133 immunohistochemical staining

Tissue specimens were processed as described [49]. Briefly, 4 μm sections were cut from formalin-fixed, paraffin-embedded specimens. Sections were deparaffinized by three changes in xylene for 10 minutes each, and then rehydrated in an ethanol series of 100%, 95%, 70%, 50%, ddH2O for 2 minutes each. Removal of endogenous peroxidase activity was performed by incubation in 3% hydrogen peroxide for 5 minutes. Antigen retrieval was performed by microwave for 8 minutes in a buffer containing 1 M urea, pH 8. Similar results were also obtained by steam heat in buffer containing 10 mM sodium citrate, pH 6. Blocking was performed by incubation in PBS supplemented with 5% normal goat serum and 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) at room temperature (RT) for 30 minutes. Tissue sections were incubated with the Ab to CD133 diluted in PBS+0.5% BSA at 4°C, overnight. After three washes with PBS+0.05% Tween-20, sections were incubated with an alkaline phosphatase-conjugated goat anti-rabbit (AP-GAR, Invitrogen, Carlsbad, CA) at RT for 1 hour. Signal from AP-GAR was visualized by Fast-Red staining (PicTure Staining Kit, Invitrogen) following manufacturer’s instructions. Finally, the sections were counterstained with Mayer’s hematoxylin and mounted using an aqueous-based mounting medium (Clearmount, Invitrogen). Sections were examined on an Olympus BX41 microscope equipped with an Olympus DP12 camera.

Trop-2 immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue specimens were processed as described above, with the following modifications. Blocking solution was PBS supplemented with 5% normal rabbit serum and 1% BSA. Sections were incubated with the Ab to Trop-2 at RT for 1 hour. After three washes, incubation with a biotinylated rabbit anti-goat IgG (bio-RAG, Vector Labs) was performed at RT for 1 hour. Streptavidin conjugated to horseradish peroxidase (HRP-streptavidin, Vector Labs) was then added to sections and incubation was performed at RT for 30 minutes. The immunoperoxidase reaction was visualized using the DAB Substrate-Chromogen System (Zymed®, Invitrogen) following the manufacturer’s instructions. Finally, the sections were counterstained with Mayer’s hematoxylin, de-hydrated in an ethanol series of 50%, 70%, 95%, 100%, 2 minutes each, cleared by three changes in xylene, 1 minute each, and mounted for visualization using a synthetic, toluene-based resin (Permount, Fisher Scientific, Pittsburgh, PA).

Trop-2/CD133 double immunohistochemical staining
Double staining on formalin-fixed, paraffin-embedded tissue specimens was performed using the PicTure Double Staining Kit (Invitrogen), following the manufacturer’s instructions with some modifications. Briefly, blocking was performed by incubation with PBS+5%BSA at RT for 45 minutes. Incubation with the Ab to Trop-2 was performed at RT for 1 hour, followed by incubation with the Ab to CD133 at 4°C overnight. Sections were incubated with a HRP-conjugated donkey anti-goat (HRP-DAG) at RT for 1 hour. After five washes with PBS+0.05% Tween-20, sections were incubated with an AP-GAR at RT for 1 hour. The signal from Trop-2 was detected by HRP staining (brown color), and the signal from CD133 was detected by Fast-Red staining (red color). Stained tissue sections were examined as described before. Goat IgG and rabbit IgG were used as negative controls.

α2β1 integrin immunohistochemical staining

IHC staining was performed on 5µm sections prepared from snap-frozen prostatic tumors. Sections were fixed in acetone at -20°C, 10 minutes. Blocking was performed by incubation with PBS supplemented with 0.5% casein and 0.05% thimerosal at RT for 15 minutes. Incubation with the Ab to α2 integrin was performed at 4°C overnight. After three washes in PBS, sections were incubated with a biotinylated horse anti-mouse IgG (bio-HAM, Vector Labs) at RT for 1 hour. HRP-streptavidin was used for signal amplification and signal was developed using the DAB Substrate-Chromogen System. Finally, the sections were mounted for visualization using the Clearmount medium.

p63 immunohistochemical staining

Tissue samples were processed as described above, with the following modifications. Antigen retrieval was performed by microwave for 15 minutes in a buffer containing 10 mM sodium citrate, pH 6. Blocking solution was PBS supplemented with 50% normal horse serum and 1% BSA. Sections were incubated with the Ab to p63 at 4°C overnight. Then, incubation with a bio-HAM was performed at RT for 1 hour.

Results

In this study, we performed a semi-quantitative immunohistochemical analysis of expression and localization of three surface receptors: CD133, Trop-2 and α2 integrin (which is known to form complexes with β1 integrin subunit, and is from here designated as α2β1) in human PrCa specimens in order to identify niches containing epithelial cells with putative CSC-like activity.

In nine out of ten specimens from patients with localized PrCa, CD133 was found to be expressed in isolated cells or small clusters in both benign glands and malignant lesions (Figure 1), with a pattern expected for niches containing putative CSCs.

Then, we investigated the expression of Trop-2 in sixteen human PrCa specimens. The role of Trop-2 has been previously demonstrated in murine normal prostate development [48], but its expression has never been investigated in human prostate. In all the cases analyzed here, Trop-2 was found to be localized in epithelial cells of benign and malignant areas with undetectable expression in the surrounding stroma (Figure 2). Its expression levels in benign prostate were higher in the basal layer as compared
with the luminal layer and were significantly increased in PrCa (Figure 2).

Analysis performed by double-staining IHC detected cells co-expressing both Trop-2 and CD133 at high levels in benign and malignant lesions (Figure 3A, arrows). As shown in Figure 3B (high magnification box), cells co-expressing both Trop-2 and CD133 (double staining, arrow)

were clearly distinguishable from cells stained for CD133 or Trop-2 (single staining, arrowheads), suggesting that the use of these markers may help the identification of putative CSC niches in the prostatic epithelium.

We also analyzed the expression pattern of α2β1 integrin in six human PrCa specimens. α2β1 integrin has been proposed to play an important role for human prostate NSCs in vitro [37] even though its role in vivo is still unclear. In all the cases analyzed, α2β1 was found to be expressed in the prostatic epithelium (Figure 4, arrows) as well as in the surrounding stroma (Figure 4, arrowheads). Moreover, α2β1 was expressed at comparable levels in both basal and luminal layers of the glands. Thus, this pattern raises doubts on the use of α2β1 as a marker for isolation of CSCs in human PrCa specimens.

Six specimens from patients with PrCa were also analyzed for expression of p63. This protein is a homologue of the tumor suppressor protein p53, and was previously reported to be expressed in the basal layer of benign prostate glands and down-regulated in PrCa [50, 51]. In all the cases analyzed, we found strong nuclear localization of p63 in basal epithelial cells of the benign glands (Figure 5A, and arrow in Figure 5B), whereas in malignant areas its expression
was undetectable (Figure 5B). These findings support p63 role as a marker to be used uniquely for the differential diagnosis of human PrCa by IHC.

Altogether, the present analysis suggests the combined use of CD133 and Trop-2 surface receptors for the isolation and characterization of putative CSCs in human prostate.

Discussion

In this study, we demonstrate for the first time that in human PrCa clusters of cells with putative CSC-like activity co-express CD133 and Trop-2. This novel markers’ combination is proposed for prospective identification of putative CSCs in human prostate. At variance with previous reports [37], the wide expression pattern of α2β1 in both basal and luminal prostatic epithelium as well as in the surrounding stroma does not support the use of this protein, alone or in combination with other markers, for identification of CSCs in human prostate.

CSCs represent ideal targets for design of novel anti-cancer drugs, particularly in those cases where conventional treatments, such as radiation therapy [14, 15], are ineffective. Therefore, novel therapies able to target both cancer cells and CSCs are needed for prostate as well as for other types of cancer. Although proteins with selective expression in normal versus cancer (such as p63) were already proposed for diagnosis of PrCa [51] and for identification of CSCs [50], in this study we sought to analyze markers expressed on the cell surface as potential targets for therapeutic treatments, like antibody-mediated therapies. Given the co-expression of CD133 and Trop-2 in clusters of putative CSCs, this novel combination of surface receptors could be used to develop specific compounds for targeted therapy.
Trop-2 appears to be significantly up-regulated in PrCa as compared with benign luminal cells, which are known to give rise to PrCa. Since Trop-2 is found to be expressed in the basal layer of benign glands, it may not be excluded that the CD133+/Trop-2+ profile can identify prostatic NSCs. In this event, we speculate that cancer-specific aberrations of post-translational modifications may allow discrimination between NSCs and CSCs. Cancer-related modifications of N-linked glycosylation patterns are not infrequent, as seen for several proteins [52, 53], including Prostate Specific Antigen (PSA) and the heat-shock protein gp96 in PrCa [54-57]. Moreover, altered N-glycosylation of β1 integrins was also reported to deregulate the invasive potential of human fibrosarcoma and colon carcinoma cells [58, 59]. In support of this hypothesis, CD133 was recently demonstrated to undergo differential glycosylation in colon CSCs as compared with differentiated cancer cells [60]. This phenomenon could explain some discordant observations that have been reported when the CD133 protein and mRNA expression patterns were compared [61]. As recently proposed by Kemper et al., differential glycosylation of the extracellular domain of CD133 can shield the epitope from detection by IHC using the antibody AC133 [60]. However, in the present study an antibody to the C-terminal, intracellular domain of CD133 was used, avoiding potential masking of the differentially glycosylated variants. Trop-2 has been found to be differentially glycosylated in transformed vs normal keratinocytes [62], suggesting potential post-translational mechanisms of regulation of this molecule in cancer. Thus, aberrant glycosylation of CD133 or Trop-2 in prostate CSCs may affect their folding and stability, resulting in incorrect protein assembly and abnormal signaling networks, thus promoting cancer growth.

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