UNIVERSITY OF CALIFORNIA, SAN DIEGO

Expression of Stem Cell Markers in Patient Needle Biopsy Samples of Early Prostate Cancer

A thesis submitted in partial satisfaction of the
requirements for the degree
Master of Science

in

Biology

by

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2016

The thesis of Maggie Yangyang Jiang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2016

DEDICATION

To my mom for supporting me, and my boyfriend for enlightening me.

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ACKNOWLEDGEMENTS

Thank you Dr. Martin Haas for introducing me to research and guiding me with my experiments. I would not have learned so much without your knowledge and patience. Thank you Stacie Hao for always being there when I needed help with my project and thesis. Thank you Tammy Lee for assisting me with anything I needed during my experiments. Thank you Paulina Sun for teaching me the technique of orthotopic xenograft.

Thank you Dr. Dan Donoghue for helping me to have the opportunity of becoming a student researcher. Thank you Dr. P.A. George Fortes and Dr. Li-Fan Lu for being my committee members and allowing me to have a nice ending to my research experience.

Thank you mom for always checking up on me and making sure I am doing alright. Thank you Denny for advising me when I felt confused and upset.

ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

University of California, San Diego, 2016

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Prostate cancer is the second most common cause of cancer death for U.S. men. While androgen deprivation therapy is effective for treating many patients, some patients' prostate cancer nevertheless progress into a lethal form which does not depend on androgen. The mechanism behind androgen-independent prostate cancer remains unknown; however, previous data suggests cancer stem cells are a potential cause. To confirm the presence of cancer stem cells in prostate cancer and to explore the timing of prostate cancer stem cell detection, patient needle biopsy samples confirmed as containing prostate

cancer were stained with six stem cell markers.

Stem cell markers CD133, LGR5, ALDH7A1, CD44, Nanog and Oct4 were found to react with the exact structures used by pathologists to diagnose prostate cancer in H&E staining procedures. The six markers are positively expressed in all available patient needle biopsy samples with the exception of CD44. CD44 is not expressed in one of the samples. The expression of six stem cell markers confirms that the adenocarcinoma structures in prostate cancer are composed of stem cell-like cells. The stem cell nature of cancer cells in early prostate cancer provides insight to a new treatment, differentiation therapy, to target the disease.

1 Introduction

1.1 Current diagnosis and treatments for prostate cancer

Prostate cancer (PrCa) is the cancer with the highest incidence (1 in 6 U.S. men) and the second highest cause of cancer death (1 in 38 U.S. men) in the U.S. [1]. One method of detection screens for the level of prostate specific antigen (PSA) in blood. Though the reliability of this test is debatable, a PSA level of more than 10ng/ml is considered indicative of prostate cancer [2]. Another method used is the digital rectal exam which can detect abnormal growth in the prostate. To further diagnose PrCa after these initial tests, needle biopsies are required to obtain tissues for histological examination. Tissue samples are stained with H&E and analyzed with immunohistochemistry, then prostate glands and cells are assessed for irregular features and arrangements. In addition, markers specific to the prostate and cell invasion into surrounding areas are also examined. These characteristics combined are used to evaluate the severity of the cancer and predict patient prognosis [3].

Currently there are several treatment options available to PrCa while the tumors are confined within the prostate. For individuals with a less severe disease, short life expectancies, or other health complications, watchful waiting is usually conducted. To eliminate the local tumors, either radiotherapy or radical prostatectomy is performed.

Androgen deprivation therapy is then used to prevent proliferation of the remaining tumor cells. The androgen-dependent cells that make up the bulk of the tumor are highly dependent on testosterone, and deprivation of the hormone prevents their proliferation by inducing apoptosis [4]. Many patients are effectively helped by these treatments while the tumor continues to be local. If the tumor cells have spread or metastasized outside the prostate, androgen deprivation therapy may still be conducted to attain PrCa remission in about 80 to 90% of patients [5, 6]. However, some patients relapse into androgen-independent PrCa and hormonal therapy ceases to be effective. Removal of the tumor is also unable to inhibit the progression of the disease, and the survival time for patients at this stage is about 16 months [7]. Currently, androgen-independent PrCa remains untreatable. Although chemotherapy is available, progression-free survival is typically only two to six months. [8].

1.2 Progression of Prostate Cancer

Prostate cancer is a disease of the epithelial cells that make up the prostatic glands, which include the flat basal cells that form a single layer at the base of the gland, and the columnar luminal cells that form an inner layer on top of the basal cells [4]. The pathobiology of PrCa suggests that PrCa develops sequentially starting with prostatic intraepithelial neoplasia (PIN), followed by locally invasive adenocarcinoma, then metastatic androgen-dependent PrCa, and finally androgen-independent PrCa [9, 10, 11].

During PIN, the epithelial cells become abnormally shaped, and they pile up and disrupt the normal epithelial border. Afterwards, the cells proliferate, break the epithelial lining, and invade surrounding stroma and tissues. They also invade organs near the prostate such as the bladder and the rectum. When PrCa becomes metastatic, the tumor

cells spread to other parts of the body. About 90% of PrCas metastasizes to bone [12].

During the androgen-dependent stage, the cells that make up the bulk of the tumor are dependent on testosterone to grow. However, it is common for the disease to advance to a stage where the tumor cells are unresponsive to testosterone, and they proliferate even in the absence of the hormone. This stage is known as androgen-independent PrCa, and the five-year survival rate for patients with androgen-independent PrCa is about 30% [13]. There are currently no methods to determine which patients will develop this form of PrCa, and there is also no widely accepted explanation of the mechanism underlying androgen-independent PrCa.

1.3 Cancer stem cell hypothesis and experimental objective

Previously, our group has shown that PrCa cells obtained from prostatectomy cases, a stage where all PrCa cells still reside in the prostate, can be propagated in short-term culture giving rise to epithelial cells that possess characteristics of cancer stem cells [14]. The cells derived from prostatectomy samples proliferated in anchorage-independent culture conditions, expressed telomerase reverse transcriptase, and were positive for stem cell markers CD44, CD133, $\alpha 2\beta 1$, CK5/14 and ALDH7A1[4, 15, 16, 17]. The cells were grown in the absence of androgen, and generated human PrCas when transplanted orthotopically into the anterior prostates of severe combined immuno deficient (SCID) mice. The cancers generated in SCID mice were histologically indistinguishable from the cancers of the patients donating prostatectomy-derived cells [14]. These findings gave rise to the idea that a group of androgen-independent cancer stem cells exist in human prostate carcinomas, and they could be the precursors to the lethal androgen-independent PrCa.

If prostatectomy tissue harbors PrCa stem cells in ˜50% of collected clinical samples [14], what is the earliest phase PrCa stem cells are detectable? In the sequence of PrCa development, the stem cell phase of the disease might be present at a stage prior to prostatectomy. Knowing the timing may lead to early targeting of this cell-of-consequence in PrCa and bring about a novel way of treating this cancer to circumvent the current burn, cut and poison treatments of this disease. Additionally, early elimination of these cancer stem cells may preclude the development of the lethal phase of androgen-independent PrCa.

To approach the questions around the exact timing of PrCa stem cell detection, prostate tissue obtained during needle biopsy of the prostate were examined. Practically, needle biopsy prostate tissue is the earliest tissue available for examination. I stained the biopsy sections by immunofluorescence with antibodies specific for six widely accepted human stem cell specific markers: CD133, CD44, ALDH7A1, LGR5, Nanog and Oct4.

The six antibodies were chosen based on evidence of their association with stem cells and tumorigenicity in the literature. Prostate cell populations that are positive for CD133 and CD44 markers are shown to have a high proliferative potential and can also regenerate prostatic glands in immunodeficient mice [18, 19]. In addition, tumor derived PrCa cells that were positive for CD133 and CD44 have the ability to self-renew and also grow indefinitely [15].

A high expression of ALDH has been linked to PrCa stem cells: $ALDH^+$ PrCa cells initiated tumors in mice that resemble the parental PrCa cells [20, 21]. In the prostate, the isoform ALDH7A1 predominates [21]. Knocking down ALDH7A1 leads to a decrease in $CD44^+$ stem cell subpopulation in PrCa cell lines [22].

In the prostate, it was found that single $LGR5^+$ cells generated prostatic structures, and regressed prostates were not able to fully regenerate with $LGR5^-$ cells [23]. Although not many studies have been done to show the tumorigenicity of $LGR5^+$ PrCa cells,

$LGR5^+$ gastric cancer cells have shown to form tumor spheres, and depletion of $LGR5^+$ gastric cells resulted in inhibition of the cancer cell growth in vitro and in vivo [24]. LGR5 has also been identified as a potential marker for cancer stem cells in colon cancer and breast cancer [25, 26].

Nanog and Oct4 are known as pluripotent embryonic stem cell markers [27, 28, 29], and they are also two of the four Yamanaka genes that reprogram differentiated cells into pluripotent stem cells [30]. Knocking down Nanog in cultured PrCa cells inhibited their clonogenic growth. When these Nanog knocked out cancer cells were transplanted into SCID mice, tumor development also decreased [31]. Oct4 was found to be upregulated in PrCa cells that are resistant to chemotherapeutic drugs [32]. When these drug-resistant cancer cells were inoculated in an amount as low as ten cells, tumor formation occurred in SCID mice.

All six markers have been shown to identify stem cells and cells that are capable of generating tumors. If these six antibodies react with structures routinely used for prostate cancer diagnosis in needle biopsies, then this experiment will show that prostate cancer stem cells are present as early as the needle biopsy stage of diagnosis.

2 Materials and Methods

2.1 Sample Acquisition

All eight patient needle biopsy historical samples were anonymously coded and obtained according to UCSD HRPP-040689. Histological analysis was done by clinical pathologists to confirm the presence of prostate cancer in all of the samples except for patient sample I1.

2.2 Indirect Immunofluorescent Staining

5 μm sections were cut from paraffin-embedded blocks containing patients' needle biopsy samples. Sections were deparaffinized sequentially with xylene, 100% ethanol, 95% ethanol and 70% ethanol. After washing the sections with DI H_2O, antigen retrieval was performed by boiling the sections in a mixture of 0.1M sodium citrate, 0.1M citric acid, and DI H_2O in a microwave with high-heat setting. When the sections cooled down, they were washed with DI H_2O followed by PBS 1x. Sections were permeabilized with 0.2% Tween 20 in 5% donkey serum, and washed with PBS 1x. Afterwards they were blocked with 5% donkey serum in PBS 1x, and washed with PBS 1x. Sections were then incubated with primary antibodies overnight at 4 °C. Isotype controls were included as well.

The primary antibodies used include rabbit anti-CD133 (Santa Cruz Biotech sc-30220) at 8μg/ml, mouse anti-CD44 (BD Pharmingen 550392) at 10μg/ml, rabbit anti-ALDH7A1 (Abgent AJ1002A) at 4μg/ml, rabbit anti-LGR5 (Santa Cruz Biotech sc-135238) at 8μg/ml, goat anti-Nanog (R&D Systems AF1997) at 8μg/ml, and rabbit anti-Oct4 (Santa Cruz Biotech sc-9081) at 4μg/ml. Sections were washed with PBS 1x, and then incubated with appropriate secondary antibodies at room temperature for an hour in dark environment. The secondary antibodies used include Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch 111-545-003) at 15μg/ml, Alexa Fluor 488 donkey anti-goat (Jackson ImmunoResearch 705-545-003) at 15μg/ml, and Alexa Fluor 488 donkey anti-mouse (Jackson ImmunoResearch 715-545-15) at 7.5μg/ml.

After washing with PBS 1x in dark, counterstain ProLong Gold Antifade Reagent with DAPI (Invitrogen P36935) was applied onto the sections. Sections were left to dry overnight and then observed under a fluorescence microscope, Nikon Eclipse E800. Pictures of stained sections were taken under 20x objective with an exposure time of 3 seconds.

3 Results

3.1 H&E stains show prostate cancer in patient needle biopsy samples

H&E stains of needle biopsy samples for all eight patients were provided by clinical pathologists Dr. Stephen Baird and Dr. Sepi Mahouti. All samples except for sample I1 contain both cancer and hyperplastic glands; sample I1 only contains hyperplastic tissues (Figure 1).

The cancer glands in seven out of the eight patient samples have a single layer of nuclei and there is a lack of polarity of nuclei within the cells. In contrast, the hyperplastic glands in sample I1 show double layers of nuclei and there is a basal polarization of nuclei within cells. In addition, the hyperplastic glands have complex invaginations, and their size appear to be bigger than the size of cancer glands.

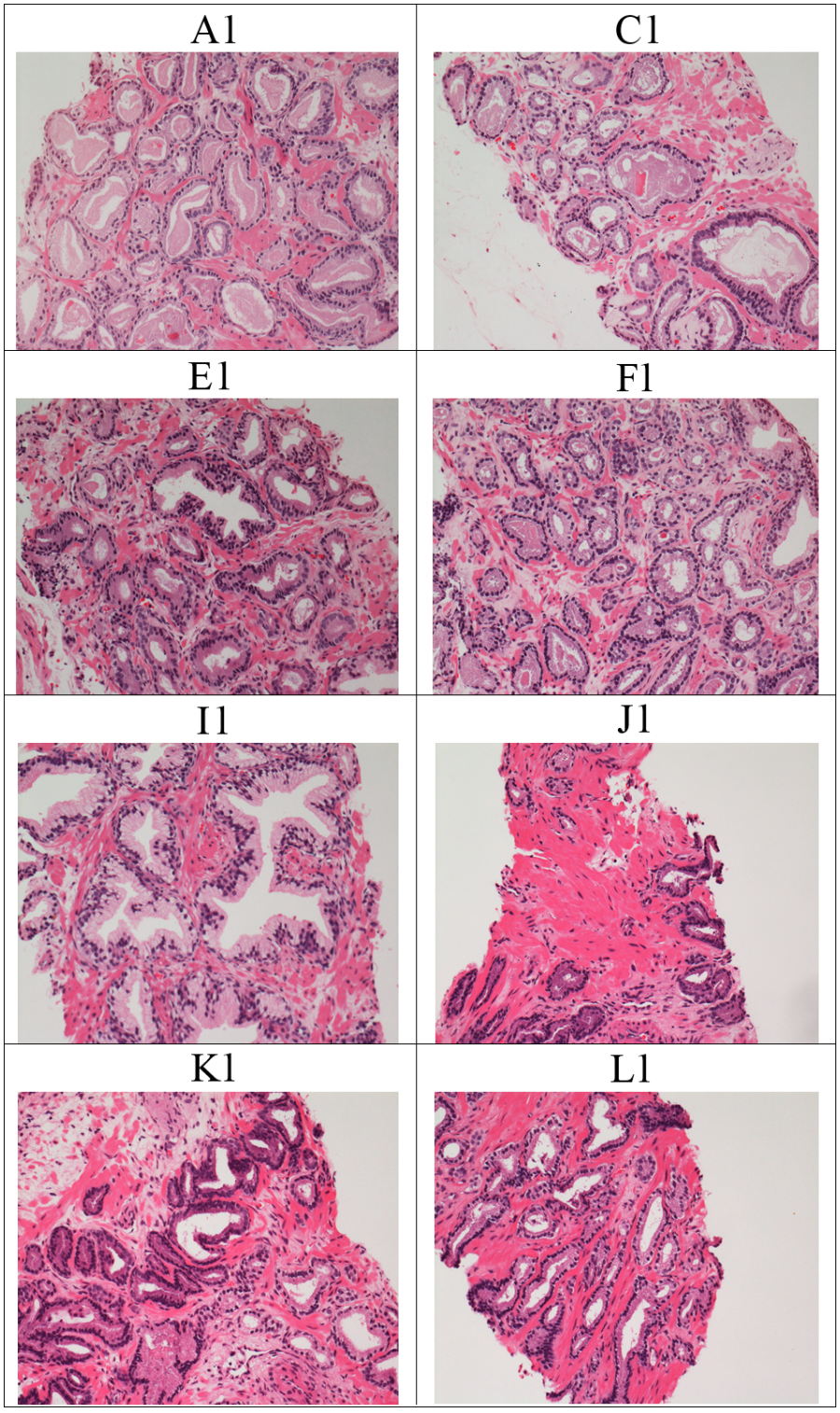

Figure 1: **H&E stains of patient needle biopsy samples.** H&E stains of the needle biopsy samples were used by clinical pathologists to confirm the presence of prostate cancer. Pictures were taken under 20x objective, and pictures were provided by Dr. Sepi Mahouti, M.D. and Dr. Stephen Baird, M.D. All samples contain adenocarcinoma and hyperplasia except for sample I1; sample I1 only contains hyperplasia.

3.2 Immunofluorescent stains show stem cell marker expression in prostate cancer

The six stem cell specific antibodies (CD133, LGR5, ALDH7A1, CD44, Nanog and Oct4) are reactive on the same structures used by pathologists as diagnosis of prostate cancer in H&E stained sections (figure 2, 3, 4). The stem cell markers show expression in all of the available needle biopsy samples with the exception of CD44. Figure 2 shows that CD44 is not detectable in one sample, K1. Images of negative controls show no reactivity.

Figure 2 and 3 show that ALDH7A1 and Nanog have the highest expression, and CD44 has the lowest expression among all markers. In samples C1 and E1, CD44 is expressed in a perinuclear fashion with an emphasis in the basal area (figure 2).

Staining result of the hyperplasia sample I1 is also included (figure 2, 4). CD44 is not expressed, and CD133 has a low expression in the hyperplastic glands. In comparison, CD133 has a high expression in cancer samples (figure 4).

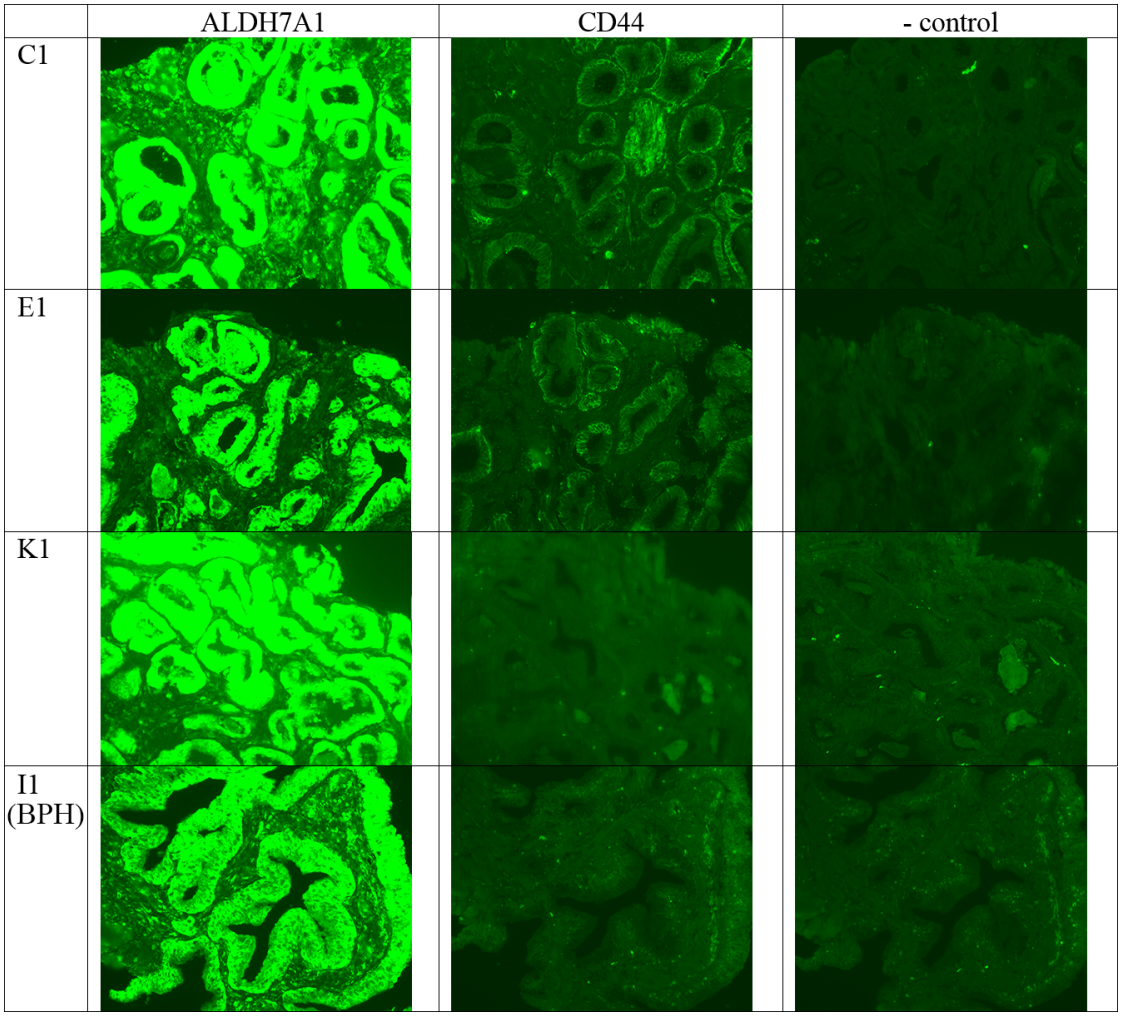

Figure 2: **Immunofluorescent stains of patient needle biopsy samples with ALDH7A1 and CD44.** All patient samples except for sample I1 contain prostate cancer. Patient sample I1 only contains BPH, benign prostatic hyperplasia. Pictures were taken with an exposure time of 3 seconds.

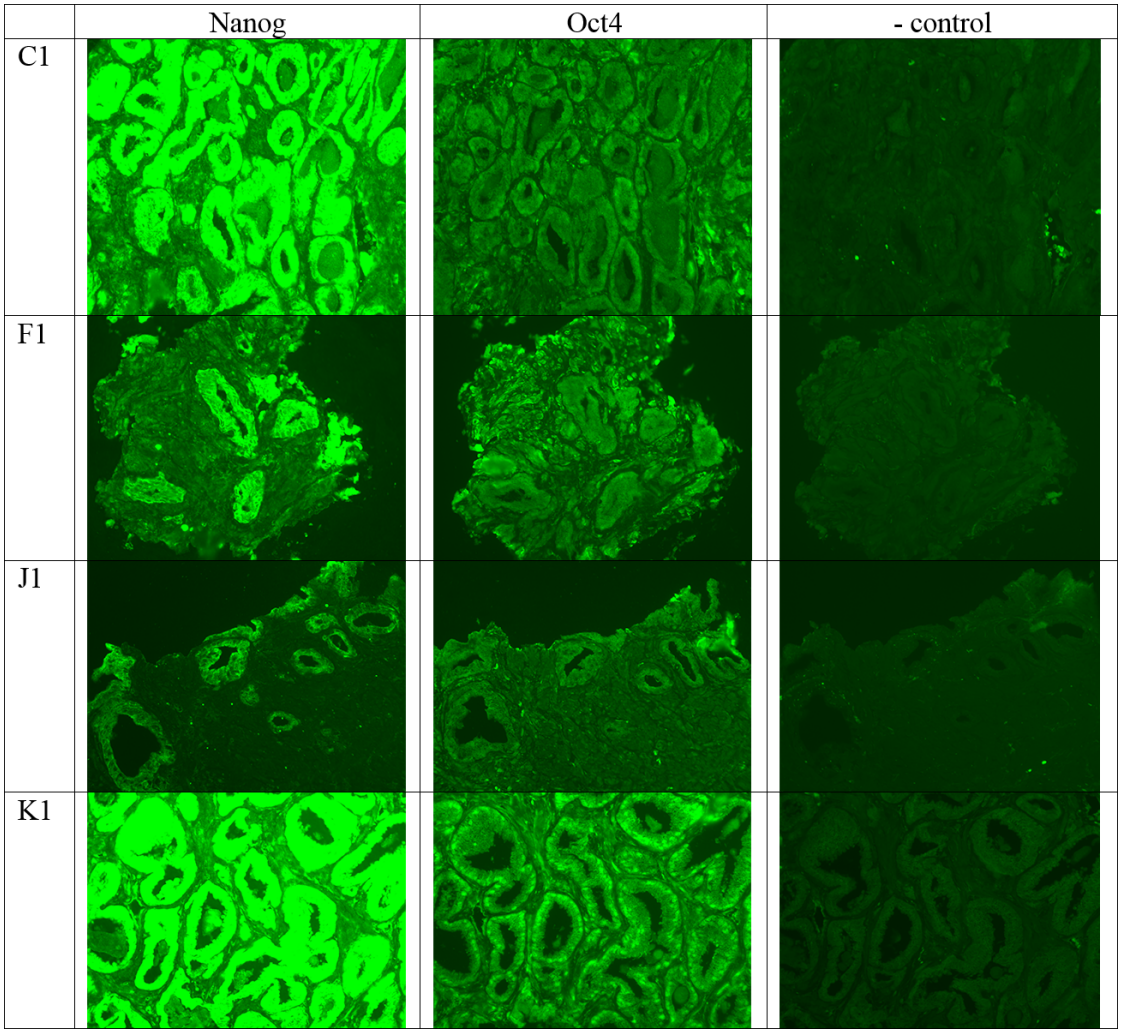

Figure 3: **Immunofluorescent stains of patient needle biopsy samples with Nanog and Oct4.** All patient samples contain prostate cancer. Pictures were taken with an exposure time of 3 seconds.

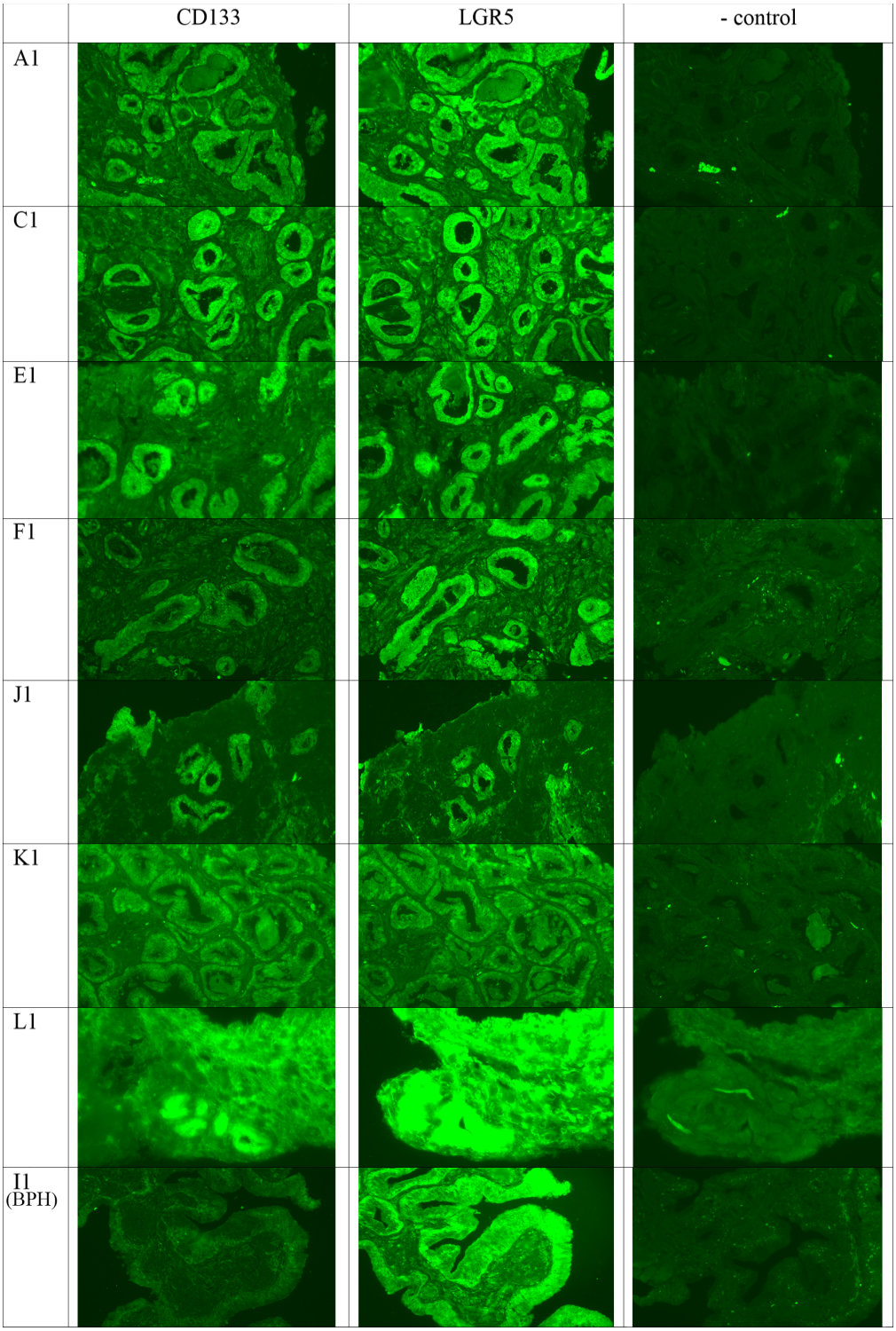

Figure 4: **Immunofluorescent stains of patient needle biopsy samples with CD133 and LGR5.** All patient samples except for sample I1 contain prostate cancer. Patient sample I1 only contains BPH, benign prostatic hyperplasia. Pictures were taken with an exposure time of 3 seconds.

3.3 Immunofluorescent stains show different stem cell marker expression for prostate adenocarcinoma and hyperplasia

The stain of hyperplastic glands from patient sample I1 suggests a difference may exist in stem cell expression between adenocarcinoma and benign prostatic hyperplasia. Expression of all six stem cell markers were compared between adenocarcinoma and hyperplasia, and a summary is listed in table 1.

Table 1: **Differences in stem cell marker expression between adenocarcinoma and hyperplasia.** Expression of stem cell markers in the nucleus and the cytoplasm are evaluated. - corresponds to negative expression, + corresponds to low expression, ++ corresponds to high expression, and +++ corresponds to very high expression.

		Adenocarcinoma	Benign Prostatic Hyperplasia
Nucleus	CD133	++	++
	LGR5	++	++
	CD44	+	+
	ALDH7A1	+	-
	Nanog	+/-	-
	Oct4	++	-
Cytoplasm	CD133	++	-
	LGR5	++	++
	CD44	-	-
	ALDH7A1	+++	+++
	Nanog	+++	+++
	Oct4	+	++

Among the six stem cell antibodies, Nanog reactivity in the nucleus of adenocarcinoma was inconsistent between patient needle biopsy samples. However, CD133, Oct4 and ALDH7A1 show a consistent difference in staining patterns between adenocarcinoma and hyperplasia. CD133 appears to stain both cytoplasm and nucleus of carcinoma cells, but only the nucleus of hyperplastic cells. Oct4 stains both cytoplasm and nucleus of

carcinoma cells, but only the cytoplasm of hyperplastic cells. ALDH7A1 shows weak nuclear staining and strong cytoplasmic staining in adenocarcinoma cells, but shows no detectable nuclear staining in hyperplastic cells. These differences are shown in figure 5 below.

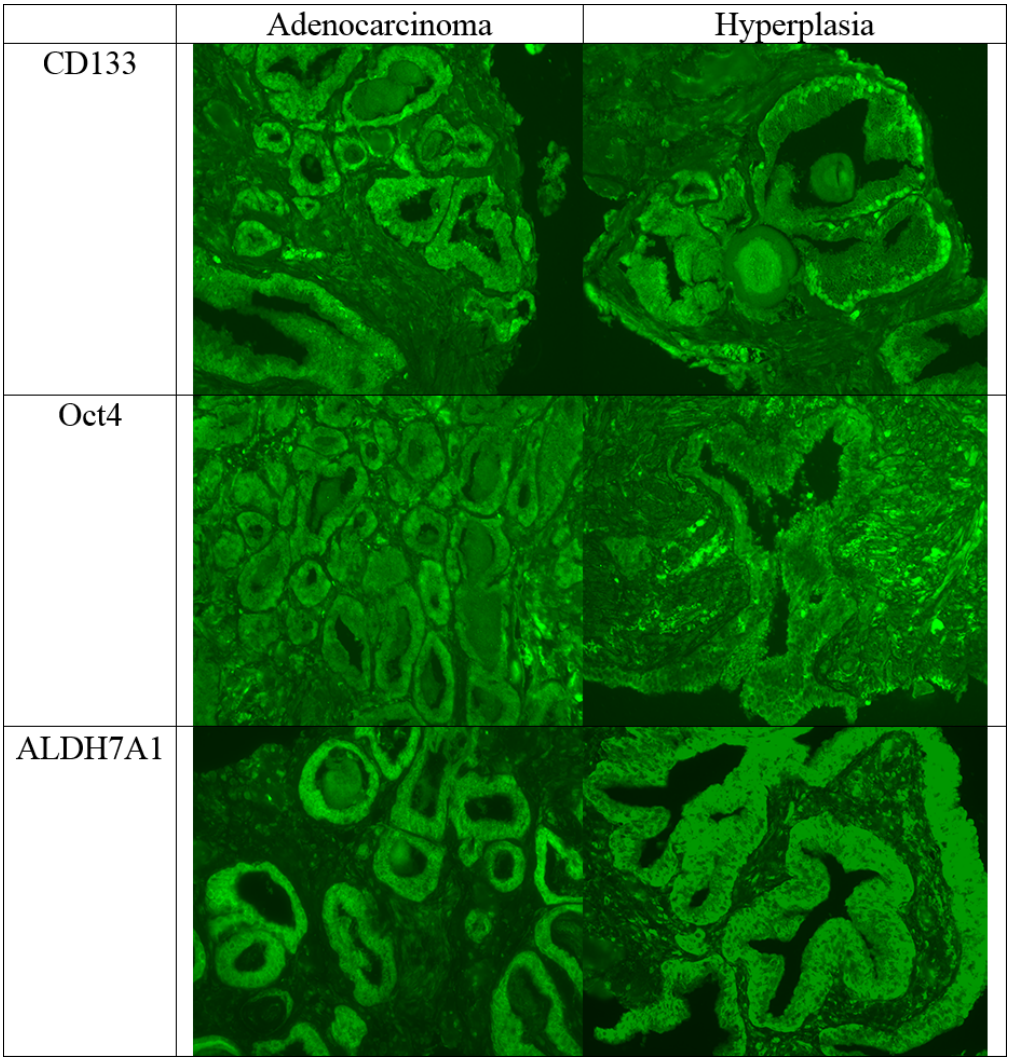

Figure 5: **Differences in nuclear and cytoplasmic stains of adenocarcinoma and hyperplasia.** Adenocarcinoma and hyperplasia show differences in staining for CD133, Oct4, and ALDH7A1. Images of CD133 come from patient sample A1, images of Oct4 come from patient sample C1 (left) and J1 (right), and images of ALDH7A1 come from patient sample C1 (left) and I1 (right). All exposures were 3 seconds except for ALDH7A1 which was 1 second.

4 Discussion

Prostate cancer is one of the leading causes of cancer deaths for U.S. men. While early stages of the disease can be effectively treated, the late stage androgen-independent PrCa remains untreatable. The causes and the mechanisms underlying androgen-independent PrCa remain unknown. Previously, the Haas group found that cells grown from prostatectomy samples gave rise to a group of androgen-independent PrCa stem cells, and it is hypothesized that these cancer stem cells could be the cause of the lethal androgen-independent PrCa.

To explore the timing of the detection of PrCa stem cells, patient needle biopsy samples confirmed by pathologists as containing PrCa were stained with antibodies against six stem cell markers: CD133, CD44, ALDH7A1, LGR5, Nanog and Oct4. The immunofluorescent stains of these stem cell markers show their expression in the exact structures clinical pathologists use as a diagnosis of prostate cancer in H&E stained tissue sections. The six stem cell markers, except for CD44, were expressed in all of the available needle biopsy samples. CD44 is not expressed in sample K1, and CD44 only has a low expression in the other samples. The minimal expression of CD44 in prostate biopsies and radical prostatectomy samples has been documented previously by Korski *et al* [33]. The reactivity of six stem cell specific antibodies in the same regions that were diagnosed as adenocarcinoma by histopathological diagnosis suggests cancer stem cells comprise the pathological structures of early prostate cancer. The presence of cancer

stem cells in the earliest diagnostic stage of prostate cancer further provides evidence to the hypothesis of prostate cancer being a cancer stem cell derived disease.

In addition to PrCa, areas containing benign prostatic hyperplasia (BPH) were also seen in the needle biopsy samples. BPH is a disease of the epithelial cells and the stromal cells that surround the prostatic glands, and it can often lead to bladder obstruction and urinary problems [34]. BPH and PrCa are two distinct diseases in terms of their pathology and morphology. Another difference between the two diseases are observed from the results in the experiment.

Stem cell markers CD133, Oct4 and ALDH7A1 show different expression pattern between cancer and hyperplasia samples as seen in table 1 and figure 5. CD133, a membrane glyocoprotein usually found in the endoplasmic reticulum and plasma membrane of stem cells [35, 36], is expressed in the cytoplasm of cancer glands but not expressed in the cytoplasm of hyperplastic areas. Oct4, a transcription factor closely associated with stem cell pluripotency [37], is expressed in the nucleus of cancer glands but not expressed in the nucleus of hyperplastic areas. ALDH7A1, a detoxifying enzyme that also plays a role in cell cycle progression in the nucleus [38, 39], is weakly expressed in the nucleus of adenocarcinoma but not expressed in the nucleus of hyperplasia. The absence of these three stem cell markers in their location of expression in the hyperplastic glands suggest that hyperplasia may consist of more differentiated stem cells than adenocarcinoma does. This interpretation is in agreement with the inability to culture hyperplastic prostate cells, which the Haas group has attempted on hundreds of hyperplasia samples, while prostate adenocarcinoma samples derived from prostatectomy tissue could be routinely established in culture [14].

Cancer stem cells are defined as a class of stem cell-like cells capable of tumor initiation and metastasis [40]. Finones *et al*. showed that PrCa stem cells are androgen-independent [14], and others report that PrCa stem cells are more resistant to PrCa

treatments than differentiated tumor cells [41, 42]. The androgen independence and treatment resistance suggest PrCa stem cells may be the cause to androgen-independent PrCa. The expression of stem cell markers found in this study may further support this. One of the stem cell markers, CD133, was found in PrCa cells that did not express markers for androgen receptor [15]. When these tumor $CD133^{+}$ cells derived from prostatectomy samples were treated with differentiating conditions, CD133 expression was reduced and high expression of androgen receptor was found. Another stem cell marker, LGR5, was also found to be androgen-independent in the prostate. After castrating mice and allowing their prostates to regress, $LGR5^{+}$ cells were still found in the regressed prostates and LGR5 mRNA level did not decrease [23]. Most interestingly, Nanog might be specifically related to the development of androgen-independent PrCa. PrCa cells induced with Nanog overexpression lead to development of tumors in androgen-deficient mice [43]. The information on these stem cell markers further show an association between PrCa stem cells and androgen-independence.

If cancer stem cells indeed are the initiating cells of androgen-independent PrCa, then this lethal class of prostate cancer cells could potentially exist at the early stage of locally invasive adenocarcinoma. Since cancer stem cells have the ability to differentiate into non-tumorigenic tumor cells [43, 44] and only exist in a small amount [45, 46], as the disease progress the androgen-independent cancer stem cells may self-renew and differentiate into androgen-dependent cells that make up the bulk of the tumor. When androgen deprivation therapy eliminates the majority of the cancer cells that are androgen-dependent, PrCa may regress in patients. However, the androgen-independent cancer stem cells still exist, and can further proliferate into tumors that are unresponsive to androgen, and cause some patients to relapse into the lethal androgen-independent PrCa.

To further investigate the idea mentioned above, data on patient outcomes from the patients that provided the needle biopsy samples would be helpful. Knowing whether

those patients have developed the lethal type of PrCa can help to further correlate the relationship between cancer stem cells and androgen-independent PrCa. However, those data were not available to be included in this study. In addition to the lack of patient outcomes, not all eight patient samples have been stained with all six stem cell markers due to the limited number of sections that can be cut from the needle biopsy paraffin blocks. Only patient samples C1 and K1 were stained with all six markers. Nevertheless, consistent staining results have been obtained with the available patient needle biopsy samples to support the presence of cancer stem cells in early prostate cancer.

The existence of cancer stem cells at an early stage of PrCa suggests the possibility to specifically target these cells to prevent further progression of the disease. A novel treatment in addition to the current burn (radiotherapy), cut (radical prostatectomy) and poison (chemotherapy) methods could be developed, e.g. differentiation therapy. Differentiating the PrCa stem cells to mature cells where they can no longer self-renew and proliferate may stop the progression of prostate cancer.

Differentiation treatments have been used for over thirty years in another progenitor stem cell disease, acute promyelocytic leukemia (APL). APL, a cancer in which the promyelocytes are unable to differentiate and accumulate in the bone marrow, is being routinely and effectively treated with a differentiating agent, all trans-retinoic acid (ATRA). The use of ATRA leads to the maturation of promyelocytes into terminally differentiated granuloctyes and about 90% of patients were able to attain complete remission without significant side effects [47, 48]. ATRA has also been shown to inhibit proliferation of breast cancer cell lines while having a lesser effect on normal breast cell lines. Tumor growth in mice that were injected with breast cancer cells was inhibited by ATRA as well [49].

The results in this experiment showed the expression of six stem cell markers in the same structures used by pathologists as diagnosis of prostate cancer in H&E staining

procedures. This strongly suggests that the adenocarcinoma structures in prostate cancer are composed of stem cell-like cells. The stem cell nature of cancer cells in early prostate cancer provides insight to a new treatment, differentiation therapy, to target the disease. Using differentiation agents that do not produce significant side effects such as ATRA may be an effective treatment for early prostate cancer.

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