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INVITED REVIEW

Prostate Cancer

Neuroendocrine cells of prostate cancer: biologic functions and molecular mechanisms

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Prostate cancer (PCa) is a major health risk for older men worldwide. Existing systemic therapies mostly target androgen receptor (AR). Although treatments are initially effective, the disease always recurs. A potential mechanism for the treatment failure is that PCa contains, in addition to the AR-positive luminal type tumor cells, a small component of neuroendocrine (NE) cells. The function of NE cells in PCa remains poorly understood, and one important characteristic of these cells is their lack of expression of AR and resistance to hormonal therapy. In addition, many patients develop the more aggressive small-cell neuroendocrine carcinoma (SCNC) after hormonal therapy. Although this clinical phenomenon of disease transformation from adenocarcinoma to SCNC is well established, the cell of origin for SCNC remains unclear. Recently, loss of function of Rb and TP53 and amplification and overexpression of MYCN and Aurora A kinase have been identified as important biomarkers and potential disease drivers. In this article, we systematically review the histology of normal prostate and prostate cancer including the main histologic types: adenocarcinoma and SCNC. We also review the findings from many studies using cellular and animal models as well as human specimens that attempt to understand the molecular mechanisms of treatment failure, disease progression, and tumor transformation from adenocarcinoma to SCNC.

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INTRODUCTION

Prostate cancer (PCa) is the most common noncutaneous malignancy in men in Western countries and a leading cause of cancer-related deaths. Although it had been considered a relatively uncommon disease in Asian countries including China,[1] its incidence has been rising rapidly in recent years, which makes PCa an important health risk for older men worldwide.

The management of PCa has evolved over the years as our understanding of the disease increases. It is now widely accepted that many localized, low-grade cancers do not need to be treated.[2] For patients who choose radical treatment, many can be cured by local therapies such as surgery or radiation. Unfortunately, a significant number of patients experience biochemical recurrence followed by metastasis requiring systemic treatment. Since 1940s, systemic treatment has mostly targeted androgen receptor (AR) signaling by inhibiting androgen production (surgical or medical castration) and/or blocking AR function with competitive inhibitors.[3] Because AR signaling is essential for the survival and proliferation of PCa, vast majority of the patients treated will experience tumor regression. However, the therapeutic efficacy is temporary and the patients will inevitably experience disease recurrence and develop castration-resistant prostate cancer (CRPC). It has been demonstrated that AR signaling continues to be critical for CRPC. Based on this notion, newer agents have been developed to inhibit intratumoral androgen synthesis (*i.e.*, abiraterone) or more effectively inhibit AR signaling (*i.e.*, enzalutamide). They achieve therapeutic efficacy in many CRPC patients, but the disease will eventually progress despite maximal AR inhibition. Therefore, novel therapies targeting AR-independent mechanisms are urgently needed to revolutionize the treatment for patients with advanced PCa.

Developing novel therapies requires better understanding of the disease. It has been hypothesized that cellular heterogeneity may contribute to the eventual failure of AR-targeted agents.[4] Histologically, PCa cells are heterogeneous, with the majority of tumor cells possessing luminal (secretory) phenotype and a minor cell population demonstrating neuroendocrine (NE) features. A significant portion of advanced and recurrent tumors has pure NE phenotype known as small-cell neuroendocrine carcinoma (SCNC). These NE tumor cells do not express AR and are resistant to hormonal therapy which has attracted major attention from clinicians and researchers.

NE CELLS IN BENIGN PROSTATE AND PROSTATIC ADENOCARCINOMA

Prostate is an epithelial organ. Epithelial cells form glands and produce secretions contributing to semen. Two major epithelial cell types can be easily identified by light microscopy: luminal cells (or secretory cells) and basal cells. Luminal cells are located on the luminal side of the glands and possess secretory functions including the synthesis and secretion of prostate specific antigen (PSA). The luminal cells are

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terminally differentiated and express differentiation markers AR and PSA. Basal cells, in contrast, are proliferating cells that do not express AR and PSA. It has been reported that basal cell is a cell of origin for human prostate cancer,[5] although, under artificial conditions, certain luminal cells may give rise to PCa in mice.[6] Basal and luminal cells make up the vast majority of the epithelium in human prostate, but there is a third, minor component of epithelial cells known as NE cells.[7] The NE cells comprise no more than 1% of the total epithelial population and are scattered among the more abundant basal and luminal cells. NE cells cannot be easily identified under light microscope, but can be discovered by electron microscopy due to their elongated cell bodies and intracytoplasmic dense-core secretory granules. A much more practical method to highlight NE cells in human prostate tissue is immunohistochemistry (IHC) using antibodies against markers of NE differentiation such as chromogranin A (CgA), synaptophysin (SYN), and neuron-specific enolase (NSE).[8,9] The function of NE cells in benign prostate remains largely unknown.

Prostate carcinogenesis undergoes distinct steps, and the detailed mechanisms of tumor development are discussed in an review article.[10] It is commonly accepted that the first step is *in situ* malignant transformation of luminal cells termed "high-grade prostate intraepithelial neoplasia (HGPIN)."[11] This lesion presents with both architectural and cytologic abnormalities. In addition to malignant features present in the luminal cell compartment, an important criterion for the histologic diagnosis of HGPIN is the presence of basal cells, although their numbers may be significantly reduced and the basal layer can be incomplete. The HGPIN lesions can eventually progress to invasive adenocarcinoma, which is characterized by cancerous glands invading prostate stroma. The cancerous glands are composed of luminal type tumor cells without basal cells. Importantly, every case of adenocarcinoma also contains rare NE cells[12] (**Figure 1**). The number of NE cells varies from case to case, but they generally comprise no more than 1% of the entire tumor cell population. Similarly, IHC using antibodies against NE cell markers is the most commonly used method for the detection of NE cells in prostate adenocarcinoma.

The function of NE cells in adenocarcinoma remains largely unclear. Studies have shown that NE tumor cells possess secretory function and secrete many biologically active molecules including biogenic amines, neuropeptides, and cytokines.[13] The luminal type tumor cells, on the other hand, express receptors for many of the secreted NE cell products.[13] It is therefore possible that paracrine interactions exist between NE cells and luminal type tumor cells in PCa, which may be critical for the survival of the latter. In contrast to luminal type tumor cells which demonstrate uncontrolled proliferative activity, NE cells are quiescent. Our lab has discovered that NE cells secrete interleukin-8 (IL-8) and express IL-8 receptor CXCR2.[14] The autocrine activation of CXCR2 by IL-8 activates P53 pathway inside the NE tumor cell and leads to a quiescent state of the NE cells.[15]

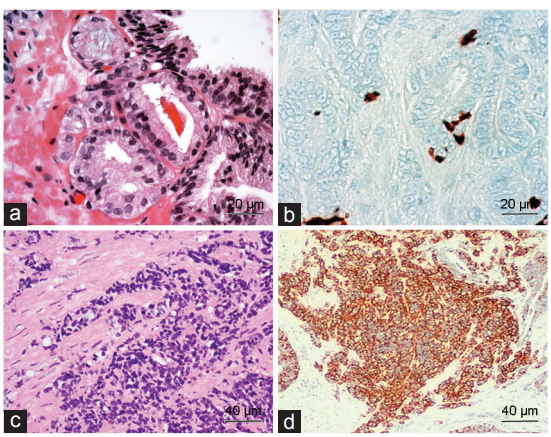

Figure 1: (**a**) A histologic picture of prostatic adenocarcinoma. (**b**) Immunohistochemical stain of prostatic adenocarcinoma showing rare NE cells positive for NE marker chromogranin A. (**c**) A histologic picture of prostatic small-cell carcinoma. (**d**) Immunohistochemical stain of prostatic adenocarcinoma showing positive staining for NE marker chromogranin A. NE: neuroendocrine.

HORMONAL THERAPY FOR PROSTATE CANCER AND THERAPY-INDUCED SMALL-CELL NEUROENDOCRINE CARCINOMA

An important feature of NE cells is that they do not express AR[16] and are resistant to hormonal therapy that targets AR signaling. It is therefore proposed that hormonal therapy, while inhibiting luminal type tumor cells to achieve symptomatic relief, will spare NE cells, select for their survival, and further enrichment. The increased NE cells may support the survival of the adjacent luminal type tumor cells in the androgen-deprived condition, leading to therapeutic resistance. Histologically, the majority of the recurrent tumors after hormonal therapy are classified as adenocarcinoma (CRPC-adeno) with both luminal and NE cells, and NE cells usually comprise a larger proportion of the tumor cells in CRPC compared with untreated tumors.[17]

While the vast majority of human PCa is classified as adenocarcinoma in both untreated and castration resistant stages, there is a variant form of PCa known as SCNC which is composed of pure NE tumor cells.[18] *De novo* SCNC is very rare and comprises no more than 1% of all clinically diagnosed cases of PCa. However, this variant form becomes rather common in recurrent tumors after hormonal therapy, including conventional hormonal therapeutic agents or the newer agents abiraterone and enzalutamide.[19] In comparison to adenocarcinoma, SCNC does not form glandular structures but grows as solid sheets, cords, and single cells (**Figure 1**). The tumor cells are smaller with scant cytoplasm, high N/C ratio, and frequent mitotic and apoptotic figures. The nuclei of SCNC cells are darkly stained with homogeneous chromatin pattern and no nucleoli. The diagnosis of SCNC may be confirmed by diffuse positivity of tumor cells for the expression of NE markers CgA and SYN by IHC.[20,21] However, a significant number of cases are negative for these markers and negative staining should not preclude the diagnosis of SCNC. We have recently identified forkhead box A2 (FOXA2) as a sensitive and specific marker for SCNC which may help pathologists in the diagnosis of challenging cases.[22] Interestingly, a related transcription factor, FOXA1, inhibits NE differentiation.[23] Other IHC markers that are potentially useful include positive staining for CD56,[20] P53,[15] thyroid transcription factor-1 (TTF-1),[20] and CD44[24] and negative staining for Rb[25] and cyclin D1.[26] It is important to note that the IHC profiles vary from case to case and there is some overlap between SCNC and adenocarcinoma. Histologic morphology remains the gold standard of pathologic diagnosis of SCNC.

Although pure SCNC exists, it is more often intermixed with adenocarcinoma with the two histologic types present in various proportions in a given tumor.[19] Previous clinical observations indicated that SCNC and adenocarcinoma may have different patterns of metastasis, with the former commonly homing to lymph nodes and bone and the latter frequently homing to visceral organs particularly the liver. However, the results of a recent large clinical study found no preferential metastatic patterns for the two histologic types.[19] Clinically,

SCNC is highly aggressive, and the survival of patients with SCNC is significantly shorter than that of patients with adenocarcinoma after treatment failure.[19] Because tumor cells of SCNC do not express PSA, patients often show low serum PSA levels relative to their tumor burdens. Previous studies, mostly using *de novo* cases, have largely demonstrated that SCNC generally does not express AR.[20] However, our recent study of metastatic SCNC in the setting of treatment failure (therapy-induced SCNC or t-SCNC) showed that AR is generally positive in tumor cells.[19] Interestingly, AR's transcriptional activity appears low despite protein expression in the nucleus, suggesting that epigenetic mechanisms may inhibit AR function.[19] Since SCNC cells are independent to AR function and do not respond to AR-targeted therapy, clinicians often treat patients with chemotherapy, but the benefits are usually limited.[27] As transformation into SCNC appears to be a common pathway of disease progression, particularly at the end stage of the disease, this disease has attracted major attention by clinicians and researchers.

MOLECULAR MECHANISMS OF SCNC

The very first animal model, transgenic adenocarcinoma of mouse prostate (TRAMP), was developed by expressing SV40 virus early genes in the mouse under the control of the probasin promoter.[28] The resulting mouse tumor has a phenotype characteristic of SCNC.[28] Another study later demonstrated that both Rb and P53 need to be knocked out for the SCNC phenotype to occur.[29] The findings in the mouse models are consistent with clinical findings in human SCNC specimens, in which mutations of the *Rb* and *P53* genes are frequent.[15,25,30] Mechanistically, our lab has demonstrated that autocrine activation of CXCR2 activates the P53 pathway in the NE cells of adenocarcinoma, and *P53* mutation inactivates the IL-8-CXCR2-P53 signaling cascade. This removes a major growth inhibitory signal, leading to hyperproliferation and aggressive behavior of the NE cells and the emergence of SCNC.[15] Mouse models show that Rb1 loss facilitates lineage plasticity and metastasis of prostate adenocarcinoma initiated by *Pten* mutation. Additional loss of Trp53 causes resistance to antiandrogen therapy. SCNC tissue from both human and mouse demonstrates increased expression of epigenetic reprogramming factors such as EZH2 and SOX2.[31,32]

Beltran and colleagues observed frequent amplification and overexpression of MYCN and Aurora A kinase in human SCNC cases.[30] A subsequent study by Lee *et al*.[33] demonstrated that forced overexpression of MYCN, in combination with activation of the PI3K-AKT-mTOR pathway, was sufficient to induce SCNC in fresh benign human prostatic epithelial cells, confirming that MYCN is a driver of SCNC. Rickman's group showed that N-Myc induces an EZH2-mediated transcriptional program, which likely mediates the development of SCNC.[34]

In modeling the transition from conventional prostatic adenocarcinoma to SCNC using a patient-derived xenograft, Akamatsu and colleagues found that the placental gene *PEG10* is de-repressed during the adaptive response to AR interference and becomes highly upregulated in clinical SCNC. The AR and the E2F/RB pathway dynamically regulate distinct posttranscriptional and posttranslational isoforms of PEG10 at distinct stages of SCNC development. *In vitro*, PEG10 promoted cell-cycle progression from G0/G1 in the context of TP53 loss and regulated Snail expression via transforming growth factor β (TGF-β) signaling to promote invasion, which may underline part of the mechanism of RB1 and TP53 loss in SCNC.[35]

Lapuk and colleagues observed reduced expression of REST, a transcription factor and master repressor of neuronal differentiation, in a case of SCNC. REST binds to target sites within genes important for a neuronal phenotype and prevents transcription, leading to NE differentiation.[36] Subsequently, Li *et al*.[37] observed alternative splicing involving REST as a mechanism driving the NE phenotype. The authors developed a novel bioinformatic tool to analyze alternative RNA splicing in RNA-sequencing data from publicly available databases. They discovered that most of the splice events are regulated by the RNA splicing factor SRRM4, a master regulator required for transdifferentiation of embryonic stem cells to neural cells. Experimental studies confirmed that SRRM4 modulates the splicing of REST, resulting in lower levels of REST transcripts and higher levels of the truncated variant transcript REST4. In human SCNC tumor samples, elevated SRRM4 expression is negatively associated with the REST/REST4 expression ratio. While SRRM4 targets alternative splicing of REST, blockage of AR signaling inhibits posttranscriptional REST protein expression, and the two pathways have additive effects on NE differentiation. Importantly, loss of Rb1 and TP53 function enhances SRRM4-induced NE transdifferentiation.[37] A recent histologic study of paraffin-embedded human tumor samples suggested that the expression of SRRM4 is associated with SCNC.[38]

CELL OF ORIGIN OF SCNC

The transformation of adenocarcinoma into SCNC after AR-targeted therapy is a very intriguing phenomenon. Because adenocarcinoma contains both luminal type tumor cells and NE tumor cells, it would be very interesting and highly significant to determine if SCNC develops from the former (transdifferentiation) or the latter (clonal expansion). Buttyan's group reported that withdrawal of androgen from the culture media changes LNCaP cells from a luminal phenotype (expression of AR and PSA) to a NE phenotype with changes in cell morphology and expression of NE markers.[39] Adding androgen back to the media will cause the opposite changes.[40] Subsequent studies demonstrate that the NE phenotype may also be induced by many other stimuli.[41–56] The different stimuli include cytokines (*e.g.*, IL-1, IL-1beta, IL-2, interferon [IFN]-gamma) and growth factors (*e.g.*, fibroblast growth factor receptor 2 [FGFR2IIIb]). Multiple intracellular signaling molecules and pathways may participate in the process (*e.g.*, protein tyrosine kinase and MAP kinase pathway, alter cyclooxygenase-2 [COX-2] expression and its enzymatic product prostaglandin E2, G protein-coupled receptor signaling, activated 3',5'-cyclic AMP-dependent protein kinase, protein kinase A). Because a large variety of stimuli and signaling pathways can induce a similar NE phenotype, it is possible that this may be a default state for the cells under stressful conditions. In addition, these studies provide experimental evidence for "transdifferentiation" and suggest that NE tumor cells may be derived from luminal type tumor cells upon hormonal therapy. However, it is important to point out that the LNCaP model only produces NE cells in a quiescent, nonproliferative state, similar to the NE cells in adenocarcinoma but different from the NE cells in SCNC which are highly proliferative and extremely aggressive. The mouse SCNC models resulting from loss of function of Rb and P53[28,29] and the NMYC study by Lee *et al*.[33] do not address the issue of cell of origin. Studies in human patients have not obtained conclusive evidence about cell of origin of SCNC. The results from some studies appear to support the transdifferentiation model. In a patient-derived xenograft (PDX) model, Wang's group observed transformation from adenocarcinoma to SCNC after castration of the mouse. Longitudinal observations demonstrated a gradual loss of luminal signature and a corresponding increase in the NE signature.[57] The evolutionary relationships gleaned from sequencing studies in Beltran *et al*.[19] suggest that SCNC cancers have same genomic changes as surrounding adenocarcinomas. Aggarwal *et al*. study[19] also found

that similar to adenocarcinoma, treatment-induced SCNCs express high levels of AR but with much lower AR transcriptional activity. However, our work demonstrating loss of P53 in NE cells as a basis for the development of SCNC would be more consistent with the clonal expansion model.[15] Overall, the cell of origin of SCNC remains an unresolved issue and further investigation is warranted.

NOMENCLATURE

Researchers, pathologists, and clinicians have not used standardized terminology to describe the complex and variable phenomena observed in pathologic specimens of PCa patients, which may lead to misunderstanding. For example, some may not realize that NE tumor cells are present in essentially every case of adenocarcinoma and their numbers can vary significantly from case to case. As a result, some pathologists would use the imprecise term "prostatic adenocarcinoma with NE differentiation" if they happen to stain a case of adenocarcinoma and see more than the "normal" number of NE tumor cells. Such a diagnosis should be avoided because, if the histologic diagnosis is adenocarcinoma, the presence of more abundant NE cells carries unknown significance. A potential pitfall is that such a diagnosis may be misinterpreted as SCNC and the patient may be mismanaged as a result. Another term that we also discourage is neuroendocrine prostate cancer (NEPC) because it can potentially include the very rare carcinoid tumor and large cell neuroendocrine carcinoma in addition to the more common SCNC.[18] Worse yet, a high-grade adenocarcinoma with abundant NE tumor cells, particularly when highlighted by IHC, may also be misclassified as such. We favor the term small-cell carcinoma as proposed by an expert panel or small-cell neuroendocrine carcinoma because it is a well-recognized pathologic entity and experienced pathologists can apply the same, standardized histologic criteria which will avoid unnecessary inconsistencies.

AUTHOR CONTRIBUTIONS

YHH, YQZ and JTH discussed the contents and wrote the review.

COMPETING INTERESTS

All authors declared no competing interests.

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