Metastatic Prostate Cancer Diagnosed by Fine Needle Aspiration: Contemporary Cytopathologic and Biomarker Assessment with Clinical Correlates

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ABSTRACT

Introduction - The diagnosis of metastatic prostatic cancer (MPC) by fine needle aspiration (FNA) can usually be rendered by typical cytomorphologic and immunohistochemical (IHC) features. However, MPC diagnosis may be complicated by transformation to atypical phenotypes such as small cell carcinoma, typically under pressure from androgen deprivation therapy (ADT). Predictive and prognostic biomarkers can also be assessed by IHC. This study illustrates how careful assessment of cytologic and biomarker features may provide therapeutic and prognostic information in MPC.

Design - We reviewed our anatomic pathology archives for MPCs diagnosed by FNA from 1/2014 - 6/2021. Clinical histories, cytology slides, and cell blocks were reviewed. Extensive IHC biomarker workup was performed, including markers of prostate lineage, cell cycle dysfunction, Ki-67, neuroendocrine markers, PDL1, and androgen receptor splice variant 7. Cases were reclassified into three categories: Conventional type, Intermediary type, and High Grade Neuroendocrine Carcinoma (HGNC).

Results - 18 patients were identified. 12 had conventional MPC, including 6/6 ADT-naive patients. 6/12 (50%) with prior ADT were reclassified as intermediary or HGNC. 4 intermediary cases included squamous differentiation (2) and pro-proliferative features (2). 2 HGNC cases had typical small cell carcinoma cytomorphology. PDL1 (2) and ARv7 (3) expression was identified. 5/5 intermediary and HGNC patients died of disease versus 6/11 conventional type.

Conclusions - Aggressive cytomorphologic variants were commonly identified in patients with prior ADT. Identification of non-conventional cytomorphology and increased proliferation can provide important prognostic information. Clinically actionable biomarkers such as PDL1 and ARv7 can be assessed by IHC.

INTRODUCTION

Prostate cancer is the third most diagnosed malignancy worldwide, with approximately 270,000 new cases and 34,500 deaths annually in the US.1 Initial therapy for newly diagnosed primary prostatic adenocarcinoma or prostate cancer is based primarily on clinical stage, pathologic grade, prostate specific antigen (PSA) levels, and life expectancy. Most patients have localized disease at the time of initial diagnosis, but more advanced disease is present in 15-20% of patients.1-2 Patients with carcinoma confined to the prostate are typically treated with either radical prostatectomy or definitive radiotherapy. Active surveillance is also an option for those afflicted with low-risk disease or reduced life expectancy.3 For patients with distant metastatic disease, ADT continues to be a mainstay of therapy.4-6Metastasic prostate cancer can be readily diagnosed by fine needle aspiration (FNA).7

The diagnosis of metastatic prostate cancer (MPC) is relatively straight-forward for conventional or acinar MPC. Morphologically, tumor cells are present in crowded, three-dimensional groups of cells with indistinct cell borders. Nuclei are usually round and uniform, with easily identified single central prominent nucleoli. Acinar arrangements of tumor cells can be appreciated, both within larger groups and as individual follicle-like structures.8-9 The unique immunohistochemical profile of PC aids the FNA diagnosis when needed. Conventional forms of PC usually express prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), NK3 Homeobox 1 (NKX3.1), and androgen receptor (AR). ERG rearrangements are common early in prostate cancer development and can be detected with high sensitivity and specificity by immunohistochemistry (IHC).8-9

While conventional forms of PC are relatively easy to diagnose on FNA, metastatic disease may display variant morphology which can complicate the diagnosis. Such variant morphology may exhibit uncommon histologic variants of PC (e.g. ductal, squamous cell) or morphologic

phenotypes (pro-proliferative, neuroendocrine) reflective of lineage plasticity developing as a manifestation of therapeutic pressure and/or treatment resistance.11-12 Pro-proliferative and other variant forms of MPC may lack the acinar morphology of conventional PC, and exhibit necrosis and high mitotic activity. Transformation to non-acinar forms, including small cell carcinoma and squamous cell carcinoma, can be seen.10-14

AR plays a critical role in the development and ultimate metastatic progression of PC.15-17 AR is a hormone ligand-dependent transcription factor which regulates cell cycle progression in prostate origin tissue and induces transcription of prostate specific genes, including PSA and NKX3.1. ADT is a mainstay of treatment for PC patients presenting with metastatic disease, who develop metastatic disease, or may have other high-risk factors.6 Conventional ADT include luteinizing hormone-releasing hormone (LHRH) receptor agonists (e.g. leuprorelin) and -non-steroidal anti-androgens (NSAA) (e.g. bicalutamide).5,18More recently, novel androgen receptor signaling inhibitor (ARSI) therapies have shown improvement in disease-free survival in PC. Examples include abiraterone, which directly blocks androgen production from all sources, and enzalumatide, which directly sequesters AR and blocks nuclear translocation.

Despite improved disease-free survival, most patients receiving ADT eventually develop castrate-resistant prostate cancer (CRPC).19-20 Early castrate-resistance often develops through reactivation of AR signaling, such as the constitutive activation that occurs through the AR splice variant ARv7.17 Eventually PC can acquire additional alterations that lead to “androgen indifference,” such as PI3K/AKT pathway mutations.21 Androgen indifference, in turn, may be associated with lineage plasticity and pro-proliferative features. Androgen indifferent PCs include those with mixed conventional acinar morphology and neuroendocrine features. CRPCs can also differentiate to non-acinar, non-neuroendocrine phenotypes, such as squamous cell carcinoma. Ultimately, PC can become hormone refractory. Such cancers may exhibit complete loss of acinar morphology, loss of PLM expression, and Ki-67 elevation. Many of these cases exhibit neuroendocrine marker expression and typical cytologic features of HGNC.12 Androgen-refractory PCs, lacking neuroendocrine differentiation (“double-negative” PC) may also develop

through alternate pathways, such as fibroblastic growth factory pathway.12 After an androgen deprivation refractory state develops in CRPC, non-hormonal chemotherapeutics are preferred and may still be effective for HGNC and "double negative" MPC. Taxane chemotherapies (docetaxel and cabazitaxel) are mainstays for conventional prostate cancer, and they are used in combination with platinum-based chemotherapy (e.g. cisplatin) for HGNC.6

Cytomorphology and immunohistochemical features of MPC diagnosed by FNA can provide important prognostic and predictive information. The presence of non-acinar phenotypes, especially SCC, are associated with poor prognosis.22 Identifying HGNC transformation in MPC is important, as alternate therapies (e.g. cisplatin) are considered. Immunohistochemical stains for RB, PTEN, and ARv7 may provide further prognostic implication, helping to identify high risk cases.23-24 Ki-67 labeling index is typically <10% in conventional MPC.25 However, Ki-67 index may be significantly elevated in prostate cancer with pro-proliferative, neuroendocrine transformation, or small cell phenotype. Expression of Programmed Death Ligand 1 (PDL1), an important predictive marker involved in immune evasion by carcinomas, has also been identified in a significant subset of MPC and can be targeted with the monoclonal antibody therapy pembrolizumab via compassionate use. PDL1 immunohistochemistry is considered positive when any specific (>1%) expression is seen.26-27 Finally, ERG expression is highly specific for MPC and hence of diagnostic utility, and may also serve as a potential predictive marker for docetaxel response.28

In this study, we retrospectively reviewed cases of MPC diagnosed by FNA and correlated the findings with clinicopathologic features and contemporary prostate cancer therapies including CRPC. Cases were specifically evaluated for variant morphologies or other pro-proliferative or variant features and the impact of prior therapy on pathologic features and clinical outcome.

Finally, we investigated the feasibility of several novel prognostic and predictive biomarkers in FNA material of MPC.

METHODS AND MATERIALS

Patient Selection

This study was performed under institutional review board-approved protocols (with a waiver of informed consent). We searched our anatomic pathology archives for a period of 7.5 years (1/1/2014 - 6/30/2021) for FNA cases flagged as malignant with “prostate” or “prostatic” in the diagnostic line or comment section. We identified a total of 19 FNA cytology cases from 18 patients. FNAs were performed under endobronchial ultrasound-guidance (7), ultrasound-guidance (6), and computer tomographic-guidance (6). In all cases, cell block and hematoxylin and eosin-stained slides were prepared. Cytologic material was prepared as either Diff-Quik and Pap stained smear slides (15) or one liquid-based cytology (ThinPrep) slide (4). All slides, including cytology, cell block, and immunohistochemical stains were retrieved from the study cases identified. All cytologic material and patient clinical histories were reviewed by two study pathologists with expertise in cytopathology and genitourinary pathology (RC, RM) to confirm the diagnosis of MPC as well as assess the cytomorphologic features of the cases in this cohort. Prostate cancer histories were reviewed, including prior prostate surgical pathology material and prior metastatic disease. When available, prior surgical pathology and cytopathology material was re-reviewed. Therapeutic records were obtained, including history of definitive or salvage radiation, chemotherapy, and androgen deprivation therapies, including second generation agents such as abiraterone and enzalutamide.

Immunohistochemistry

To further categorize study cases and assess for prognostic and predictive markers, immunohistochemical stains were performed on archived formalin-fixed, paraffin-embedded cell block material. Stains were performed using antibodies against NKX3.1 (BioCare CP42213), prostate specific membrane antigen (PSMA, Dako M3620), prostate specific antigen (PSA,

Ventana 760-2506), androgen receptor (AR, Ventana 760-4605), synaptophysin (Ventana 790-4407), chromogranin (Ventana 760-2519), Ki-67 (Ventana 790-4286), phosphatase and tensin homolog (PTEN, Cell Signaling 9188), erythroblast transformation-specific-related gene protein (ERG, Ventana 790-4576), programed death-ligand 1 (PDL1, Spring Biosciences M4420), retinoblastoma protein (RB, Cell Signaling 9309), cyclin D1 (Cell Marque 241R-18), and androgen receptor variant 7 (ARv7 RevMAb Biosciences 31-1109-00). IHC was performed on the Ventana Discovery XT automated slide staining system using ultraView or OptiView DAB detection kit (Roche-Ventana, ZA). All cases were re-reviewed and assessed for cytologic features, including the presence or absence of typical PC acinar morphology, solid tumor cell sheets, non-acinar morphology (e.g. small cell carcinoma, squamous cell carcinoma), mitoses, and necrosis.

Tumor Categorization

Based on our previous experience as well as published data reflecting prostate cancer tumor biology (Figure 1), cases of MPC were classified into three categories based on cytomorphologic and immunohistochemical features: conventional, intermediary, or HGNC.11-13 Conventional cases were categorized by the presence of acinar morphology, retained expression of prostatic-specific markers, and absent neuroendocrine expression. Intermediary cases were categorized by the presence of either pro-proliferative features (i.e. Ki67>=20% +/- neuroendocrine marker expression) or non-conventional prostatic morphology (e.g. squamous cell, ductal, or sarcomatoid differentiation). HGNC cases were categorized by morphologic and immunohistochemical features typical of small cell carcinoma, and/or tumors exhibiting strong immunohistochemical expression of NE markers along with high Ki-67 labeling index, or loss of pathway markers Rb and Cyclin D1.

RESULTS

Clinical and Radiographic Features

We identified a total of 19 FNA cytology cases from 18 patients (Table 1). Patients ranged in age from 53-88 years. In 14 patients there was a known prior history of PC, initially diagnosed from 1 to 21 years prior to FNA. In 6 patients there was prior MPC diagnosed before the current FNA (5 bone, 1 iliac lymph node). 4 patients were de novo presentations of MPC.

All 14 patients with a prior diagnosis of PC had a documented history of prior therapy in the form of either surgical, radiotherapy, hormonal or chemotherapy (Table 2). Of these, while 9 (64%) patients had undergone prior prostatectomy, 10 (71%) patients had prior radiation therapy, including 5 (36%) with definitive radiation therapy and 5 (36%) with salvage radiation therapy. 12/14 (86%) patients received ADT, including 4 (29%) with combination of leuprorelin and bicalutamide, 4 (29%) leuprorelin alone, 2 (14%) with combination of leuprorelin and abiraterone, 1 (7%) with combination of leuprorelin, abiraterone, and enzalutamide; and 1 (7%) with combination of abiraterone and enzalutamide. 2 (14%) patients received prior docetaxel therapy exclusively. None received cisplatin.

11/18 (61%) patients had radiologic evidence of bone metastatic disease at the time of FNA, including 4 (22%) with axial and appendicular metastases, 5 (28%) with axial metastases, and 2 (11%) with isolated rib metastases. 16/18 (89%) patients had lymph node involvement by radiographic imaging, including 7 (39%) mediastinal lymphadenopathy, 2 (11%) iliac lymphadenopathy, 2 (11%) retroperitoneal lymphadenopathy, 1 (6%) with cervical lymphadenopathy, and 4 (22%) with multisystem lymphadenopathy. 2/18 (11%) patients had soft tissue metastatic disease on radiography, including 1 (6%) abdominal soft tissue and 1 (6%) with lung metastases and pelvic soft tissue metastases. 9/18 (50%) patients had both bone and lymph node metastatic disease, including 4/4 (100%) patients without prior PC treatment and 5/14 (36%) with prior PC diagnosis and treatment. Amongst the 4 patients with a de novo diagnosis of MPC and no past history, 4 (100%) had axial bone involvement, while 2 (50%) had local and 2 (50%) had distal lymph node metastases.

Cytologic and Diagnostic Immunohistochemical Features

Among the 18 patients, 19 FNA sites were sampled: 14 lymph node (7 mediastinal, 5 left cervical or supraclavicular, 2 retroperitoneal), 4 bone (2 sacrum, 1 femur, 1 rib), and 1 abdominal soft tissue mass. One patient had sampling of different mediastinal lymph node sites in separate procedures. All other patients had only 1 FNA site sampled.

FNA cytology and cell block features were examined in all 19 cases (Table 3). Typical PC acinar morphology was observed in 16 (84%) cases, and prominent nucleoli in 18 (95%) cases. Solid tumor sheets were seen in 10 (53%) cases. Mitotic activity was identified in 5 (26%) cases, and tumor cell necrosis in 7 (37%). Non-acinar morphologic variants were seen in 5 (26%) cases. Neuroendocrine differentiation reflective of small cell carcinoma features, including high nuclear to cytoplasmic ratios, fine chromatin, absence of prominent nucleoli, nuclear molding, and crush artifact, were seen extensively in 2 (11%) cases and focally in 1 (5%) case. Squamous differentiation with keratinization was seen extensively in 1 (5%) case and focally in 1 (5%) case.

In 17/19 (89%) cases, one or more PLM (NKX3.1, PSMA, and PSA) were strongly positive (>50% staining). 2 (11%) cases had weak focal positivity for PSMA and PSA but were negative for NKX3.1 and AR. 17 (89%) cases strongly expressed AR. 2 (11%) cases expressed neuroendocrine (NE) markers (synaptophysin, chromogranin). Ki-67 labeling index ranged from 1-70%. 12 (63%) had a labeling index of <=5%. 5 (26%) had labeling index of ≥ 20% (Table 4).

Metastatic Prostatic Cancer Subcategorization

Based on cytomorphology and immunohistochemical features, cases of MPC were further subclassified into one of three categories: Conventional Type, Intermediary Type, or High-Grade Neuroendocrine Carcinoma (Table 3).

There were 13 samples from 12 patients that were classified as conventional type (Figures 1-2). All cases had focal to diffuse acinar formation, and no variant morphologic forms of carcinoma were seen. PSM and AR expression was seen in all cases by immunohistochemistry. No NE

expression by immunohistochemistry was identified. In 12 (92%) cases, Ki-67 was estimated at <=5%. 1 (8%) case had Ki-67 labeling index of 15%.

There were 4 (22%) cases that were classified as intermediary type. All intermediary cases showed at least focal expression of PLMs, including strong expression in 3 cases. Solid sheets of tumor cells and necrosis were identified in all cases. 2/4 (50%) cases showed solid growth with squamous cell carcinoma features, including keratinization (Figures 3-4). 1 case showed diffuse squamous cell cytomorphology. 1 showed patchy squamous differentiation, solid growth, and Ki-67 of 20%. 2/4 (50%) exhibited pro-proliferative and/or neuroendocrine features. 1 case showed mixed acinar and neuroendocrine cytomorphology (Figure 5), with synaptophysin expression and Ki-67 index of 20%. 1 showed solid growth with central necrosis, mitotic activity, and Ki-67 index of 60% (Figure 6).

There were 2 (11%) cases classified as HGNC type (Figures 7-8). Both cases showed cytomorphology typical of NE carcinoma, including high nuclear to cytoplasmic ratios, fine chromatin, nuclear molding, crush artifact, necrosis, and frequent mitoses. Absent in both cases was acinar PC morphology. Ki-67 labeling index was highly elevated in both cases (60-70%). 1 had retained expression of PLMs in addition to NE biomarker expression. 1 lacked PLM and NE biomarker expression by immunohistochemical stain, despite typical NE carcinoma cytologic features and lost expression of Rb and Cyclin D1.. The latter patient had a recent rise in PSA and radiographic findings consistent with multifocal metastatic disease involving the pelvis, lung, and multiple lymph node sites, all clinical features known to be consistent with a small cell disease phenotype.

Prognostic and Predictive Immunohistochemistry

Table 5 summarizes previously described prognostic and predictive immunohistochemistry results (Figure 10). ARv7 expression was identified by an immunohistochemical stain in 4 FNAs from 3 (18%) patients, 1 each from the HGNC, intermediary, and conventional sub-categories

(Figure 9). The patient with two mediastinal lymph node samples had expression of ARv7 in both sites. PTEN expression was identified in 1 case, that of a SCC. No PTEN expression was seen in conventional subtype cases. ERG expression was seen in 6 (33%) cases, including strong (>50%) expression in 2 of 4 intermediary cases and moderate (<50%) expression in 4 of 13 conventional cases. RB protein and cyclin D1 expression were both lost in 2 (11%) cases, 1 each of HGNC and conventional types. There were 2 additional cases (11%) that showed loss of RB protein with retained cyclin D1 expression. PDL1 expression was identified in 2 cases (11%) (Figure 9). All other cases showed complete absence of PDL1 expression (0%).

Prior Therapy and Metastatic Prostate Cancer Subcategory

Review of clinical history revealed a history of PC treatment for 6/6 (100%) patients with HGNC or intermediary types versus 8/12 (67%) of patients with conventional type MPC. Overall, 6/12 (50%) with a history of ADT were categorized as intermediary or HGNC, compared to 0/6 (0%) without prior ADT. Both HGNC cases occurred after prostatectomy and treatment with multiple anti-androgenic medications; one receiving leuprorelin, abiraterone, and enzalutamide and one leuprorelin and bicalutamide. Neither had prior radiation therapy. Among the intermediary type, 4/4 patients received both radiation and anti-androgenic therapy. 75% (3/4) of intermediary patients received initial definitive radiation therapy for PC, compared to 7% (1/14) of HGNC and conventional type cases. Among conventional type cases, 8 patients had a prior history of PC therapy, including 6 with antiandrogen therapy, 1 with definitive radiation, and 1 with prostatectomy with subsequent salvage radiation. Prior salvage radiation was common overall in this cohort (5/8). 4/4 cases with no prior therapy were of conventional type.

Clinical Outcomes

Clinical follow-up was available for 16 patients (Table 6). There were 11 (69%) patients that died of PC, with survival times from 5 to 35 months. There were 5 (31%) patients alive at the time of this study, including 3 without radiographic evidence of disease. Three surviving patients (60%)

had 2 years or less of ongoing follow-up. Two patients (40%) were without evidence of disease after longer periods of ongoing follow-up (5 and 7 years, respectively). The 3 patients alive without evidence of disease all had conventional MPC at FNA and no prior ADT history.

6/11 (55%) of patients with conventional type metastasis on FNA died of disease, compared to 5/5 (100%) with HGNC or intermediary type who died of disease. 2 patients with HGNC died at 6 and 8 months after FNA diagnosis. 3 patients with intermediary type died at 5 months, 14 months, and 18 months. 1 patient with intermediary disease was lost to follow-up.

DISCUSSION

The diagnosis of MPC on FNA is often uncomplicated. Conventional PC shows characteristic cytomorphology (acinar formations, prominent nucleoli) and immunohistochemical staining (NXK3.1 and PSA expression).8-10 Therapy-naive MPC is generally of conventional type. However, prior treatment history can have a significant impact on the features of MPC. Clinical and radiographic features, such as widespread axial metastases and rising serum PSA, can assist the diagnosis. However, while conventional PC is easy to diagnose, transdifferentiation to atypical forms can occur. Transformation to castration-resistant and hormone-refractory phenotypes occurs first through reactivation of the AR pathway and then through acquisition of additional mutations which may allow for independence from the AR pathway as a driver of proliferation.16,20 Cytopathologists must be aware of these variant morphologies, such as HGNC including small cell carcinoma, to avoid misclassification of metastatic carcinoma. Serum PSA can be a helpful ancillary study even in patients with non-conventional disease, as significant PSA elevations were seen in patients with not only conventional subtypes (e.g. patients 3-5) but also HGNC (patient 18) and intermediary (patients 15-16) subtypes (Table 1). The recognition of variant morphologies is also important for prognostic and predictive purposes. Identification of HGNC is particularly consequential, as such cancers are typically treated with platinum-based chemotherapeutics. Notably, neither case of HGNC was identified as such by report in our study.

A number of other genetic alterations can affect PC progression and contribute to the development of advanced stage PC and CRPC.17,21,29 Rearrangements of the *ERG* oncogene, that encodes the transcription factor ERG protein, leading to fusion with *TMPRSS2*, a prostate-specific cell-surface serine protease gene, are identified in over half of PC, and may represent an early step in the development of many cases.29 Alterations of the tumor suppressor gene *PTEN*, especially deletions, are uncommon in localized PC but are present in up to 60% of metastatic cases.30 *Rb* and *p53* gene mutations are also more commonly identified in metastatic disease and in PC with neuroendocrine phenotype.22,31

In this retrospective study, we found that cytomorphologic and immunohistochemical features can allow for identification of PC subtypes of interest. Conventional MPC was the most common subtype, including among patients with prior treatment for PC. Such cases were characterized by the presence of typical acinar PC morphology, diffuse PLM expression, and low Ki-67 labeling index. HGNC was distinguished by typical small cell carcinoma morphology with or without associated NE biomarker expression, with Ki-67 index of >=50%. In between were intermediary cases, which included cases with transformation to non-acinar (squamous) morphology and pro-proliferative cases with features intermediate between conventional acinar MPC and HGNC. In our study, 6 cases (33%) displayed atypical PC features on FNA, including 2 that were HGNC (11%). There were 4 cases (22%) that showed intermediary variant morphologies, including 2 (11%) with squamous cell carcinoma and 2 (11%) with pro-proliferative phenotype. These 4 intermediary cases maintained at least partial expression of PLMs, with strong PLM staining in 3 (75%).

While the vast majority of newly diagnosed PC show conventional features, therapy can induce changes leading to atypical phenotypes. All patients with aggressive subtypes of MPC had prior therapy, including 6 (100%) with prior ADT. Notably, both patients with HGNC subtype had received at least 2 forms of androgen-targeting therapy. The 4 patients with intermediary subtype had all received at least 1 ADT and radiotherapy, with 3 (75%) of patients having been treated with both definitive radiotherapy and ADT. Among patients with conventional subtype of MPC, prior therapy (8/12, 67%) and prior ADT (6/12, 50%) was less common. Like ADT,

radiation therapy may be associated with progression of disease.32-33 In this study, an initial prostate cancer treatment of definitive radiation was present in 3/6 (50%) of non-conventional forms of MPC compared to 1/12 (8%) of acinar MPC (Table 6).

Overall outcomes in our study correlated with MPC subtype. The presence of HGNC or intermediary morphology was an ominous finding, as 5/5 (100%) of patients died within 14 months. HGNC and intermediary cases had a median survival of just 6 months; interestingly, intermediary cases exhibited aggressive disease similar to fully developed HGNC phenotype. Among patients with conventional MPC, 6/11 (55%) died of disease after a median survival of 12 months. Of the 5 surviving patients, 3 had no clinicoradiological evidence of disease at 2, 5, and 7 years. Notably, all 3 patients were naive to ADT at the time of FNA. Overall survival was 60% (3/5) in ADT-naive patients compared to 18% (2/11) for patients previously treated with at least 1 ADT agent.

From a diagnostic perspective, immunohistochemistry for PLMs, such as AR, NKX3.1, PSMA, PSA, and ERG, helped confirm a MPC diagnosis (Table 7). In particular, PSMA showed at least focal staining in all cases. It is important to remember that AR pathway can frequently be dysfunctional in metastatic, treated PC, especially during the castrate resistant phase. Such dysfunctionality also affects and frequently leads to under-expression of androgen signaling-dependent biomarkers like PSA and ERG.34 Hence, while positive ERG immunohistochemical expression is highly specific for a cancer of primary prostatic origin, negative ERG expression could result from dysfunctional androgen signaling; in such cases, genomic *ERG* rearrangement can be detected by utilizing an *ERG* break-apart fluorescent *in situ* hybridization assay.35 ERG expression may be a negative predictor for docetaxel response, and was identified in 6/18 (33%) of cases. Complete loss of AR expression was identified in only 2 cases, both of which exhibited variant morphology: 1 AR-null HGNC and 1 non-neuroendocrine AR-null (squamous cell) case.

While immunohistochemistry for PLMs is generally sufficient for diagnosing and characterizing therapy-naïve MPC, our findings suggest that an expanded diagnostic panel may be considered

in ADT-treated patients, especially in those with non-acinar morphologic phenotypes. A Ki-67 labeling index of 20% or greater was seen to be associated with aggressive features in our study (5/6 cases). Previous studies of primary prostate cancer have set Ki-67 thresholds of 5% or 10% for high-risk disease.36-38 15/18 (83%) cases in our study had a Ki-67 labeling index of at least 5%. One case of therapy-naïve, conventional subtype MPC (patient 1) had a Ki-67 labeling index of 15%. All other cases of conventional MPC showed Ki-67 labeling indices of <10%. 3 intermediary cases had Ki-67 labeling index of more than 20%, while one intermediary case with squamous phenotype had 10% labeling. As expected, the two HGNC cases demonstrated Ki-67 labeling of 50% or greater. In addition to Ki-67, synaptophysin and chromogranin immunohistochemistry should be considered in any post-therapy cases with non-acinar morphology to help identify neuroendocrine transdifferentiation.

Immunohistochemical stains can highlight important prognostic and predictive features of MPC. Loss of PTEN expression, typically through deletion of the gene locus, is a common early event in the development of MPC, and in fact all cases of conventional PC showed lost PTEN expression in our study. PTEN expression was only identified in 1 HGNC. Loss of RB is also associated with aggressive phenotypes, including androgen-refractory forms with or without HGNC transformation. 1/2 HGNC cases displayed loss of RB by immunohistochemistry, along with 1/4 intermediary and 2/12 conventional subtypes. 3/3 patients with RB loss and clinical follow-up died within 12 months of FNA.

Constitutive activity of AR pathway through the ARv7 splice variant can allow for evasion of AR suppression by PTEN. ARv7 is associated with aggressive disease, and 2/2 patients with ARv7 positivity and clinical follow-up died within 14 months of FNA. Predictive immunohistochemistry for PDL1 and ERG may also provide useful clinical information. PDL1 immunohistochemistry is predictive for response to pembrolizumab in other cancers, and PDL1 expression of 1% of greater was identified in 2/18 (11%) of cases.31 ARv7 and PDL1 expression were only identified in ADT-treated cases.

To our knowledge, this is the first study that details the cytopathologic features in patients with MPC relative to their post multi-modality therapy, including second generation androgen receptor signaling inhibitors. The strengths of this study include a contemporary assessment utilizing current and novel biomarkers to effectively subcategorize MPC cases. Limitations of this study include the small patient numbers and lack of corresponding biopsy material for definitive biopsy-cytology based correlations.

In summary, MPC can show a spectrum of cytomorphologic and immunohistochemical features on FNA. Most cases are of conventional acinar type, but transformation to variant forms can occur as a result of prior therapy with ADT with or without radiation. Prior treatment with multiple modalities was associated with an enrichment of cases exhibiting transformation to intermediary (non-conventional) forms and HGNC in our study. Careful cytomorphologic assessment of FNA material is necessary for patients with previously anti-androgen-treated MPC to rule out transformation to atypical phenotypes. A panel of biomarkers (Table 7) which can further help annotate and correctly categorize such MPC cases and hence carry clinical import include PLMs (AR, NKX3.1, PSMA, PSA, ERG), Ki-67, and neuroendocrine markers such as synaptophysin and chromogranin, along with signal pathway markers like RB and Cyclin D1. An elevated Ki-67 labeling index in the correct morphologic and clinical context may help confirm such transformed MPC. NE marker expression and Ki-67 >50% can help confirm HGNC diagnosis, which is particularly important as HGNC is typically non-responsive to AR-targeted therapies but may be responsive to platinum-based therapy (e.g. cisplatin). Intermediary transformed cases include MPCs that show either partial transformation to neuroendocrine carcinoma or transformation to other non-acinar epithelial subtypes, such as squamous cell carcinoma. They show aggressive features, including non-acinar morphology, necrosis, and high Ki-67 labeling index (pro-proliferative phenotype), while maintaining at least partial PLM expression. Ancillary studies identified biological aberrations such as ARv7 expression and any PDL1 expression in patients treated with anti-androgen therapy. Ancillary immunohistochemical studies can accordingly provide important prognostic and predictive information for MPC in FNA material.

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FIGURE LEGENDS

Figure 1: Schematic of prostate cancer progression. Conventional prostate cancers exhibit acinar architecture with canonical androgen receptor signaling and prostate lineage marker expression. However, prostate cancer can undergo progression to pro-proliferative or trans-differentiated (neuroendocrine and/or squamous) states under therapeutic pressure and then exhibit reduced prostate lineage marker expression, reduced dependence on androgen signaling, and increased proliferation. Prostate cancer can ultimately develop full progression to high grade neuroendocrine carcinoma, with elevated proliferation and neuroendocrine markers, a dysfunctional AR pathway, and loss of RB and Cyclin D1.

Figure 2 Conventional acinar prostatic adenocarcinoma metastatic to the sacrum in a therapy naive patient (Patient #2). Adenocarcinoma is present in cohesive acinar arrangements (a-b, smear slides, DiffQuik-stained 100x and Papanicolaou-stained, 200x). NKX3.1 (c) and PSMA (d) are diffusely expressed by immunohistochemical stain.

Figure 3. Conventional acinar prostatic adenocarcinoma metastatic to mediastinal lymph nodes in a patient who had received prior anti-androgen therapy (patient 12). The adenocarcinoma retains the conventional acinar morphology and prominent nucleoli typical of prostatic adenocarcinoma (a-b, smear slides, DiffQuik-stained 100x and Papanicolaou-stained, 200x). As in untreated cases, tumor cells retained expression of NKX3.1 (c) and PSMA (d).

Figure 4 Squamous cell carcinoma differentiation in metastatic prostatic cancer to the femur (patient 13). Carcinoma is present as solid sheets with keratinization (a, cell block, hematoxylin and eosin, 200x) and as clusters with markedly pleomorphic forms (b, ThinPrep, Papanicolaou-

stained, 400x). Patchy retained expression of PSMA (c) was present, while NKX3.1 (d) was negative.

Figure 5 Squamous cell carcinoma differentiation in metastatic prostatic cancer to the mediastinal lymph nodes (patient 14). Carcinoma is present as solid sheets of large tumor cells with necrosis (a, smear slide, Papanicolaou-stained, 200x), including prominent keratinization (cell block, hematoxylin and eosin, 400x). Prostate lineage marker expression, such as NXK3.1 (c) was retained. Ki-67 labeling index was elevated (d).

Figure 6. Pro-proliferative features in metastatic prostatic cancer to the subcarina (patient 15). Tumor is present in solid sheets and poorly-formed acinar arrangements (a-b, smear slide, Papanicolaou stained, 400x and cell block, hematoxylin and eosin, 200x). NKX3.1 expression is retained (c), but Ki-67 labeling index is above 50% (d).

Figure 7. Neuroendocrine transdifferentiation in metastatic prostatic cancer with retained acinar features in a mediastinal lymph node (patient 16). Smear slides show clear acinar formations (a, smear slide, DiffQuik-stained, 200x), but in other areas tumor cells are present as single cells and in loose sheets, with fine chromatin lacking nucleoli (b, smear slide, Papanicolaou stained, 400x). NKX3.1 expression was retained (c), but moderate expression of synaptophysin (d) was also noted.

Figure 8. Small cell carcinoma (high grade neuroendocrine) transformation in metastatic prostatic cancer to a left cervical lymph node (patient 17). Tumor is present exclusively in loose sheets of cells exhibiting typical small cell features, including high nuclear to cytoplasmic ratios, fine chromatin, nuclear molding, and absent nucleoli (a-b, smear slides, DiffQuik stained, 200x and 400x). Acinar morphology is absent. Ki-67 labeling index is elevated over 50% (c), while Rb and cyclinD1 (d) expression are lost.

Figure 9. Small cell carcinoma (high grade neuroendocrine) transformation in metastatic prostatic cancer to a left cervical lymph node (patient 18). Like patient 17, typical features of small cell carcinoma are noted, such as loss of cohesion, fine chromatin, and nuclear molding. (a-b, smear slides, Papanicolaou-stained, 200x and DiffQuik stained, 400x). NKX3.1 (c) expression was retained, but diffuse synaptophysin (d) expression was also present.

Figure 10. Biomarker immunohistochemistry in metastatic prostate cancer. PDL1 staining was identified in 2 cases, including patient 7 (a). ARv7 expression was present in 4 FNAs from 3 patients, including patient 5 (b).

Patient	Age at FNA	History of prostate cancer?	Years since prostate cancer diagnosis	Prior metastatic disease	Serum PSA at FNA (ng/mL)	Bone metastases*	Soft tissue metastases*	Site of FNA
1	65	No	0	-	Not performed	Axial skeleton	Widespread lymph nodes	Lymph node - left cervical
2	88	No	0	-	Not performed	Axial skeleton	Mediastinal lymph nodes	Bone - left sarcum
3	64	No	0	-	15.8	Isolated rib metastasis	Iliac lymphadenopathy	Bone - rib
4	67	No	0	-	36.5	Axial skeleton	Iliac lymphadenopathy	Bone - right sacrum
5	74	Yes	9	Yes (bone)	115.4	Axial skeleton	Mediastinal lymph nodes	Lymph nodes - mediastinal
6	67	Yes	11	No	4.1	None	Retroperitoneal lymph nodes	Lymph node - retroperitoneal
7	66	Yes	16	No	6.2	None	Retroperitoneal lymph nodes	Lymph node - retroperitoneal
8	67	Yes	12	Yes (bone)	Not performed	Axial skeleton	Cervical and supraclavicular lymph nodes	Lymph node - left cervical
9	67	Yes	5	No	0.6	None	Abdominal soft tissue	Soft tissue - abdominal
10	73	Yes	14	Yes (iliac lymph nodes)	4.1	None	Mediastinal lymph nodes	Lymph node - mediastinal
11	75	Yes	10	Yes (bone)	2.7	Widespread bone metastases	Widespread lymph nodes	Lymph node - left supraclavicular
12	68	Yes	7	No	1.2	None	Mediastinal lymph nodes	Lymph node - mediastinal
13	58	Yes	1	Yes (bone)	0.9	Widespread bone metastases	None	Bone - left femur
14	71	Yes	9	No	2.0	Isolated rib metastasis	Mediastinal lymph nodes	Lymph node - mediastinal
15	85	Yes	21	No	115.6	Axial skeleton and humerus	Mediastinal lymph nodes	Lymph node - mediastinal
16	80	Yes	11	No	24.5	None	Mediastinal lymph nodes	Lymph node - subcarinal
17	53	Yes	6	No	Not performed	None	Widespread lymph nodes, lung, pelvic mass	Lymph node - left cervical
18	65	Yes	10	Yes (bone)	463.4	Widespread bone metastases	Widespread lymph nodes	Lymph node - left cervical

TABLE 1: Clinical Histories and Radiographic Findings in Metastatic Prostate Cancer Patients

Abbreviations: PSA, prostate specific antigen; FNA, fine needle aspiration

* Based on radiographic evidence of disease at time of FNA

Patient	Category	Prostatectomy	Radiation Therapy (RT)	Androgen Deprivation Therapy	Abiraterone	Enzalutamide	Docetaxel	Cisplatin
5	Conventional	Yes	Yes, prostate-bed RT	No	Yes	Yes	Yes	No
6	Conventional	No	Yes, definitive RT	No	No	No	No	No
7	Conventional	Yes	Yes, prostate-bed RT	Leuprorelin	No	No	No	No
8	Conventional	Yes	Yes, prostate-bed RT	Leuprorelin and Bicalutamide	No	No	No	No
9	Conventional	Yes	Yes, prostate-bed RT	No	No	No	No	No
10	Conventional	Yes	No	Leuprorelin	No	No	No	No
11	Conventional	Yes	Yes, prostate-bed RT	Leuprorelin	Yes	No	No	No
12	Conventional	Yes	No	Leuprorelin	Yes	No	No	No
13	Intermediary	No	Yes, bone metastasis RT	Leuprorelin	No	No	Yes	No
14	Intermediary	No	Yes, definitive RT	Leuprorelin and Bicalutamide	No	No	No	No
15	Intermediary	No	Yes, definitive RT	Leuprorelin	No	No	No	No
16	Intermediary	No	Yes, definitive RT	Leuprorelin and Bicalutamide	No	No	No	No
17	gh-grade neuroendocrine carcinor	Yes	No	Leuprorelin and Bicalutamide	No	No	No	No
18	gh-grade neuroendocrine carcinor	Yes	No	Leuprorelin	Yes	Yes	No	No

TABLE 2: Prostate Cancer Therapy History in Metastatic Prostate Cancer Patients
Note: Patients 1-4 were therapy-naive (no prior history of prostate cancer) with conventional metastatic prostate cancer

Patient	Acinar morphology	Non-acinar morphology	Necrosis	Mitotic Activity	Category
1	Present	Absent	Present	Absent	Conventional
2	Present	Absent	Absent	Absent	Conventional
3	Present	Absent	Absent	Absent	Conventional
4	Present	Absent	Absent	Absent	Conventional
5A	Present	Absent	Absent	Absent	Conventional
5B	Present	Absent	Absent	Absent	Conventional
6	Present	Absent	Absent	Absent	Conventional
7	Present	Absent	Absent	Absent	Conventional
8	Present	Absent	Absent	Absent	Conventional
9	Present	Absent	Absent	Absent	Conventional
10	Present	Absent	Absent	Absent	Conventional
11	Present	Absent	Absent	Present	Conventional
12	Present	Absent	Absent	Absent	Conventional
13	Absent	Squamous cell carcinoma with keratinization	Present	Present	Intermediary
14	Present	Squamous cell carcinoma with keratinization	Present	Absent	Intermediary
15	Present	Solid growth with comedo necrosis	Present	Present	Intermediary
16	Present	olid growth with neuroendocrine differentiatio	Present	Absent	Intermediary
17	Absent	Small cell carcinoma	Present	Present	High-grade neuroendocrine carcinoma
18	Absent	Small cell carcinoma	Present	Present	High-grade neuroendocrine carcinoma

TABLE 3: Cytomorphologic Findings in Metastatic Prostate Cancer
Note: Patient 5 underwent sampling of mediastinal sites twice in separate procedures

Patient	Category	NKX3.1	PSMA	Androgen Recepto	Synaptophysin	Chromogranin	Ki-67 labeling index
1	Conventional	+++	+++	+++	-	-	15%
2	Conventional	+++	+++	+++	-	-	5%
3	Conventional	+++	++	+++	-	-	5%
4	Conventional	+++	+	+++	-	-	2%
5A	Conventional	+++	+++	+++	-	-	5%
5B	Conventional	+++	+++	+++	-	-	5%
6	Conventional	++	+	+++	-	-	1%
7	Conventional	+++	+++	+++	-	-	2%
8	Conventional	+++	+	+++	-	-	5%
9	Conventional	+++	+++	+++	-	-	5%
10	Conventional	+++	++	+++	-	-	5%
11	Conventional	+++	+++	+++	-	-	5%
12	Conventional	+++	+++	+++	-	-	5%
13	Intermediary	-	+	-	-	-	10%
14	Intermediary	+++	+++	+++	-	-	20%
15	Intermediary	+++	+++	+++	-	-	60%
16	Intermediary	+++	+++	+++	++	+	20%
17	High-grade neuroendocrine carcinoma	-	+	-	-	-	60%
18	High-grade neuroendocrine carcinoma	+++	++	+++	+++	++	70%

Immunohistochemical stain grading: +++, >50% expression; ++, 25-50% expression; +, <25% expression

TABLE 4: Diagnostic Immunohistochemistry in Metastatic Prostate Cancer

Patient	Category	ARv7	PTEN	ERG	PDL1	Rb	CyclinD1
1	Conventional	Negative	Negative	Negative	Negative	Retained	Retained
2	Conventional	Negative	Negative	Positive, diffuse	Negative	Retained	Retained
3	Conventional	Negative	Negative	Negative	Negative	Retained	Retained
4	Conventional	Negative	Negative	Negative	Negative	Lost	Lost
5A	Conventional	Positive	Negative	Negative	Negative	Retained	Retained
5B	Conventional	Positive	Negative	Negative	Negative	Retained	Retained
6	Conventional	Negative	Negative	Negative	Negative	Retained	Retained
7	Conventional	Negative	Negative	Negative	Positive	Retained	Retained
8	Conventional	Negative	Negative	Negative	Negative	Retained	Retained
9	Conventional	Negative	Negative	Negative	Negative	Retained	Retained
10	Conventional	Negative	Negative	Positive, focal	Negative	Retained	Retained
11	Conventional	Negative	Negative	Positive, focal	Negative	Lost	Retained
12	Conventional	Negative	Negative	Positive, focal	Negative	Retained	--- (No residual tissue)
13	Intermediary	Negative	Negative	Negative	Negative	Lost	Retained
14	Intermediary	Negative	Negative	Positive, diffuse	Negative	Retained	Retained
15	Intermediary	Positive	Negative	Positive, diffuse	Negative	Retained	Retained
16	Intermediary	Positive	Negative	Negative	Positive	Retained	Retained
17	gh-grade neuroendocrine carcinon	Negative	Negative	Negative	Negative	Lost	Lost
18	gh-grade neuroendocrine carcinon	Negative	Positive	Negative	Negative	Retained	Retained

TABLE 5: Biomarker Immunohistochemistry in Metastatic Prostate Cancer

Patient	Tumor classification	History of 1st generation ADT	History of SGAAT	History of definitive radiation	Clinical outcome
1	Conventional	No	No	No	DOD - 8 months
2	Conventional	No	No	No	DOD - 30 months
3	Conventional	No	No	No	Alive - NED at 7 years
4	Conventional	No	No	No	Lost to follow-up
5	Conventional	No	Yes	No	DOD - 12 months
6	Conventional	No	No	Yes	Alive - NED at 5 years
7	Conventional	Yes	No	No	DOD - 35 months
8	Conventional	Yes	No	No	DOD - 10 months
9	Conventional	No	No	No	Alive - NED at 2 years
10	Conventional	Yes	No	No	Alive with disease at 2 years
11	Conventional	Yes	Yes	No	DOD - 12 months
12	Conventional	Yes	Yes	No	Alive with disease at 1 year
13	Intermediary	Yes	No	No	DOD - 7 months
14	Intermediary	Yes	No	Yes	DOD - 5 months
15	Intermediary	Yes	No	Yes	DOD - 14 months
16	Intermediary	Yes	No	Yes	Lost to follow-up
17	gh-grade neuroendocrine carcinor	Yes	No	No	DOD - 6 months
18	gh-grade neuroendocrine carcinor	Yes	Yes	No	DOD - 8 months

Abbreviations: ADT, androgen-deprivation therapy; SGAAT, second generation anti-androgen therapy; DOD, died of disease; NED, no evidence of disease

TABLE 6: Clinical Trajectories for Metastatic Prostate Cancer Patients

Marker Category	Immunohistochemical Stains
Prostate Lineage	AR NKX3.1 PSMA PSA ERG
Proliferation	Ki-67
Neuroendocrine	Synaptophysin Chromogranin
Prognostic and Predictive	Rb Cyclin D1 ARv7 PTEN PDL1

Abbreviations: AR, androgen receptor; PSMA, prostate specific membrane antigen; PSA, prostate specific antig

Table 7 - Proposed Biomarkers for Metastatic Prostate Cancer and Transformed States

ıen; ERG, *ETS* family transcription factor ERG; Rb, retinoblastoma protein; ARv7, androgen receptor variant 7; PTI

ΞN, phosphatase and tensin homolog; PDL1, programmed death-ligand 1