Ductal and acinar components of mixed prostatic adenocarcinoma frequently have a common clonal origin

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Abstract
Background: Ductal adenocarcinoma (DA) is an aggressive subtype of prostate cancer. It is most commonly seen in mixed tumors together with conventional acinar adenocarcinoma (AA). The genetic profile of DA and its clonal origin is not fully characterized.

Objective: To investigate whether DA represents a distinct genetic subtype and to investigate the somatic relationship between the ductal and acinar components of mixed cancers.

Design, Setting, and Participants: In 17 radical prostatectomy specimens ductal and acinar tumor components from the same tumor foci were dissected. DNA was extracted and genomic sequencing performed. After exclusion of two cases with low cell yield, 15 paired samples remained for analysis.

Results: In 12 of 15 cases a common somatic denominator was identified, while three cases had clonally separate components. In DA, TMPRSS2-ERG gene fusions were detected in 47% (7/15), clonal FOXA1 alterations in 33% (5/15) and SPOP alterations in 27% (4/15) of cases. In one case KIAA1549-BRAF fusion was identified. Genome doubling events, resulting in an increased ploidy, were identified in the DA in 53% (8/15) of cases, but not seen in any AA. PTEN and CTNNB1 alterations were enriched in DA (6/15) but not seen in any AA. No cancers showed microsatellite instability or high tumor mutation burden.

Conclusions: Ductal and acinar prostate adenocarcinoma components of mixed tumors most often share the same origin and are clonally related. DA components in mixed tumor often exhibit genome doubling events resulting in aneuploidy, consistent with the aggressive nature of high grade prostate cancer.

KEYWORDS
clonality, ductal adenocarcinoma, genetics, prostate cancer
INTRODUCTION

Ductal adenocarcinoma (DA) of the prostate was first described in 1967 by Melicow and Pachter as endometrioid carcinoma of the prostatic utricle. Since then it has been debated whether DA is a true subtype of prostate cancer or a morphological variant of common acinar adenocarcinoma (AA). Bock and Bostwick proposed that no histologic features are unique for DA, other than site of growth in large periurethral ducts or in the verumontanum. The term DA has been broadened to include tumors with this morphological phenotype arising in the peripheral parts of the prostate, often intermixed with AA.

Currently, the diagnosis of DA of the prostate is based on morphology, being characterized by tall, columnar, pseudostratified epithelium, with elongate nuclei and a papillary, cribriform, or glandular architecture. In the World Health Organization Classification of Tumors, DA is ranked as the second most frequent cancer type in the prostate accounting for 3.2% of all prostatic carcinomas. DA occurs most commonly in association with AA and is seen in isolation in only 0.2%–0.4% of all prostate cancers.

In the absence of necrosis, DA without an AA component is, by definition, assigned a Gleason score of 4 + 4 = 8 (ISUP Grade 4), as its clinical behavior has been shown to be similar to that of AAs of this grade. DA is reported to have a less favorable prognosis than AA following radical prostatectomy or radiotherapy.

The genetic profile of DA is not fully characterized. Earlier studies have reported a similar rate of copy number alterations as in metastatic prostate cancer, as well as an increased number of mutations in DNA damage repair genes and fewer ERG fusions than are seen in AA. It has also been suggested that the DA and AA components in mixed prostate cancer may share a clonal origin. This conclusion is based on the observation that ERG expression and loss of phosphatase and tensin homolog (PTEN) is less common in both DA and AA components of mixed tumors when compared to pure AAs that are matched according to stage and grade. In a recent study, the integrative genomic and transcriptomic analyzes of coincident foci of DA and AA components of prostate cancers, with both ductal and acinar features, indicated that DA and AA components had diverged from a common progenitor.

In this study, we have undertaken genomic profiling of a series of mixed prostate cancers with DA and AA components to investigate the clonal relationship between morphologically distinct tumor components within the same cancer focus.

MATERIALS AND METHODS

2.1 Study participants and tissues

The records of Aquesta Uropathology, Brisbane, Australia, were searched for DA in radical prostatectomy specimens accessioned in the years 2012–2019. A total of 51 radical prostatectomy specimens with a DA component were identified and the diagnosis was confirmed independently by two pathologists (Hemamali Samaratunga and Lars Egevad).

Radical prostatectomy specimens had been handled according to a routine protocol. Following formalin-fixation, the gland was sliced horizontally, with slices cut into standard-size blocks. The prostate gland was totally embedded. For this study, further sections were cut, stained with hematoxylin and eosin and reviewed by two of the authors (Claes Lindh and Lars Egevad) for the presence of sufficient volumes of DA and AA for genetic analysis. Cases were included only if spatially separate and morphologically distinct areas of DA and AA, without overlap, were found within the same tumor focus (Figure 1).

DA was defined morphologically as a tumor with tall, columnar, pseudostratified epithelium arranged in a papillary and/or cribriform architecture. The nuclei were large and elongate, and contained macronucleoli. Tumor purity was assessed through semiquantitative assessment of the cancer cell fraction in the DA and AA components in tiers of 10%. Only samples with >20% cancer cell fraction were accepted for microdissection.

Following the morphological selection process, 17 cases remained for genetic analysis. Two of these cases were excluded because of low DNA yield. From the remaining 15 cases, sections of 10 μm thickness were cut from the tissue blocks and the morphologically distinct tumor components were microdissected using light microscopy. Tissues were then collected into tubes for DNA extraction. Morphologically normal tissue from paraffin blocks of the same patient was similarly dissected for analysis of germline DNA.

2.2 Sample processing and sequencing

Nucleic acids were extracted using the AllPrep FFPE kit (Qiagen). Kapa DNA hyper (Roche) was applied to construct libraries for sequencing using UMI xGen CS Adapters (IDT). On average 299 ng of DNA (range 10–402) was used from tumor FFPE tissue and 178 ng of DNA (range 31–400) from noncancerous FFPE tissue. Subsequently, targeted sequencing was performed using in-solution hybridization based capture. Baits (120 bp oligos) were obtained from Twist
Bioscience (San Francisco, USA). The baits were designed to target unique regions in the human genome commonly altered in prostate cancer to allow for detection of point mutations (78 genes), structural variants (11 genes), copy-number alterations (genome wide), microsatellite instability (MSI) and hypermutation (Table S1). The design targeted approximately 3000 common SNPs which enables genome-wide copy-number alteration profiling and ploidy assessment. Illumina paired-end sequencing (2 x 150 bp) was performed on the NovaSeq system (Illumina). For tumor and germline DNA, 20 x 10^6 and 15 x 10^6, respectively, read pairs were ordered (Table S2).

The AutoSeq pipeline was applied for bioinformatic processing and manual curation of data. Various inhouse and publicly available bioinformatic tools are integrated into the AutoSeq pipeline. Purity and ploidy analysis was performed using PureCN with manual verification by requiring distinct copy-number states in samples with a ploidy increase (Figure S1). Visualization of variants was undertaken in the integrated genomics viewer and statistical analysis was performed in R.

For two cases without any available germline DNA, healthy donor DNA was applied as a germline DNA reference. To enable identification of somatic variants, all small variants with >0.01 population frequency in the Genome Aggregation Database (gnomAD) were assumed to be germline variants. The remaining variants were manually investigated and only clonal variants were retained for further analysis. Each variant type (mutations, copy-number alterations, and structural variants) from each pair was inspected to remove artifacts, to catalog cancer drivers, to estimate the cancer DNA fraction and to determine ploidy and the somatic relationship between the DA and AA components. To ensure that somatic alterations detected in either the DA or AA component were not overlooked due to low cancer DNA fraction, all variant positions were manually inspected in all tissues from the same study participant.

Spatially separated components of DA and AA of the prostate were harvested from the same tumor foci of 17 men. Targeted sequencing by in-solution hybridization based capture was performed using a design specifically optimized for prostate cancer (Table S1). The panel design has a genomic footprint of 1.39 Mb and allows for identification of somatic and germline small variants, genomic structural rearrangements, genome-wide copy-number alterations, assessment of ploidy and cancer cell purity, microsatellite instability and tumor mutational burden (TMB). The panel enables comprehensive analysis of DNA repair and homologous recombination repair (HRR) genes commonly altered in prostate cancer. Genes reported to be inactivated (either by mutations or copy-number alterations) in ≥1% of localized and advanced prostate cancer were included in our assay and analyzed accordingly. Genes in the BRCA complex were included despite being relevant in a very small fraction of cases without any available germline DNA, healthy donor DNA was applied as a germline DNA reference. To enable identification of somatic alterations, all small variants with >0.01 population frequency in the Genome Aggregation Database (gnomAD) were assumed to be germline variants. The remaining variants were manually investigated and only clonal variants were retained for further analysis. Each variant type (mutations, copy-number alterations, and structural variants) from each pair was inspected to remove artifacts, to catalog cancer drivers, to estimate the cancer DNA fraction and to determine ploidy and the somatic relationship between the DA and AA components. To ensure that somatic alterations detected in either the DA or AA component were not overlooked due to low cancer DNA fraction, all variant positions were manually inspected in all tissues from the same study participant.

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3 RESULTS

The pathological characteristics of the 15 cases finally included in the study are summarized in Table 1. The cancer DNA fraction as assessed by sequencing data (median 0.39, range 0.051–0.84) was lower than expected based on semiquantitative pathology assessment of the cancer cell fraction (median 0.80, range 0.45–0.90). The mean target coverage was 547 (range 234–1810, Table S2).

Sequencing findings are summarized in Table S3 and also displayed in Figure 2. In 12 cases the genomic data indicated that the DA and AA components had originated from a common denominator, while they were clonally independent in three cases (Cases 1, 5, and 8). TMPRSS2-ERG gene fusions were detected in 53% (8/15), FOXA1 alterations in 33% (5/15) and SPOP in 27% (4/15) of cases. In the eight cases with TMPRSS2-ERG gene fusions, alterations were seen in both DA and AA components in 6 cases, only in the AA component in one case and only in the DA component in one case. In the five cases with clonal FOXA1 alterations, alterations were seen in both DA and AA components in three cases and only in the DA component in two cases. In the four cases with SPOP mutations, mutations were seen in both DA and AA components in three cases and only in the DA component in one case. One study participant carried a rare KIAA1549–BRAF fusion event.

Tissue heterogeneity was pronounced and for this reason each pair of DA or AA components were analyzed separately and the genetic alterations compared (Figure 2). In three cases no common somatic denominator could be identified, despite detected variants in one component that should have been identified with certainty if present in the other, based on coverage and tumor purity. Case 8 harbored TMPRSS2-ERG gene fusions in both the DA and AA component; however, these were of different types and structural variant start/stop coordinates. TMPRSS2-ERG gene fusion was detected in the AA component of case 13, whereas a clonal high-impact FOXA1 variant was detected in the DA component. The only common somatic alteration was a large deletion event on chromosome 13, which is, therefore, likely to have occurred in a cancer precursor. Six of the cases had mutually exclusive CTNNB1 hotspot mutations or PTEN alterations in the DA component (three cases with CTNNB1 hotspot mutations and three cases with PTEN alterations). No CTNNB1 hotspot mutations or PTEN alterations were identified in the acinar foci.

In 53% (8/15) of cases the DA components showed genome doubling events resulting in a increased ploidy. This ploidy increase was only present in DA in six cases, where the ploidy of both components could be assessed and it was not observed in any of the AAs within the series. Four of the remaining DA tumors harbored SPOP hotspot mutations. MSH6 alterations were noted in both the DA and AA components in one case and in the AA component in one case. No other DNA Mismatch Repair (MMR) gene alterations were found. No samples showed signs of MSI. The TMB was less than 15 mutations per megabase of the genome coding area of DNA in all DA and AA samples. Thus, no cases showed signs of high TMB. We did not detect any HRR gene alterations in this set of mixed acinar and DAs.
The architectural and cellular features of the DA and AA components showed no obvious morphological differences between cases, with and without a clonal relationship of the tumors (Figure 3).

4 | DISCUSSION

Prostate cancer is inherently heterogeneous with areas of different tumor grade and morphological differentiation.\textsuperscript{20–22} Numerous clonally separate tumor foci often co-exist in radical prostatectomy specimens,\textsuperscript{23} and it is apparent that independent tumors sometimes merge with each other to form collision tumors. Thus, there are two possible scenarios for the origin of DA of the prostate. DA may arise de novo as a pure DA and mixed tumors would thus be collision tumors between two independent tumor clones. Alternatively, the DA component in mixed tumors may represent differentiation or dedifferentiation of AA.

A pronounced genetic heterogeneity, characteristic for localized prostate cancer, was found.\textsuperscript{20,21,23,24} Three cases in our study showed no common somatic denominator between the DA and AA components. These cases could thus be true collision tumors, where two clonally independent tumors have arisen independently and grown into each other. In the remaining 12 cases the genomic data indicated that the DA and AA components had originated from a common denominator. The identified somatic alteration spectrum concurred with previous reports on prostate cancer,\textsuperscript{22} but with skewed alteration frequencies of individual genes as previously reported for DA (Figure 2).\textsuperscript{8,11} Variants characterizing the prostate cancer molecular subtypes were detected in at least one tissue in all study participants.\textsuperscript{22}

The genetic profile of DA is not fully characterized. Our group has earlier demonstrated that copy number alterations occur as frequently in DA as in Gleason score 8–9 AA, but less frequently than in metastatic prostate cancer.\textsuperscript{8} DA harbors somatic changes seen in advanced and/or metastatic castration-resistant AA, which may account for its aggressive biological behavior. In a study of 51 specimens, targeted next-generation sequencing showed that 25 cases (49%) had at least one DNA damage repair gene alteration.\textsuperscript{9} It has been reported that MSI is common in DA,\textsuperscript{25} but in a study from our group deficient MMR proteins were identified in only one out of 33 DAs and three out of 40 grade-matched AAs.\textsuperscript{26} In this present study MSH6 alterations were noted in both the DA and AA components in one case and in the AA component in another case. This is in line with the findings of our earlier study, where mutations in MMR genes were uncommon in DA.\textsuperscript{26} It is noteworthy that in our sequencing analysis no cases showed MSI. HRR gene mutations have previously been shown to be enriched in DA,\textsuperscript{9} but no HRR gene alterations were detected in our series.

The most striking characteristic of the DA components were genome doubling events resulting in an increased ploidy, which was seen in 53% (8/15) of cancers. This ploidy increase was only present in DA in the six cases where the ploidy of both components could be

| Case | Age | PSA | Case GS | PT category | Surgical margins | LNs sampled | LNs involved | % Ductal | Anatomic zone | GS in sampled AA |
|------|-----|-----|---------|-------------|------------------|-------------|--------------|----------|---------------|-----------------|
| 1    | 70  | N/A | 4 + 3 = 7 | T3a         | Negative        | 21          | 0            | 25       | PZ            | 3 + 4           |
| 2    | 64  | 12  | 5 + 4 = 9 | T3b         | Positive        | 6           | 0            | 10       | PZ            | 5 + 4           |
| 3    | 61  | N/A | 4 + 3 = 7 | T3a         | Negative        | -           | -            | 30       | PZ + TZ       | 3 + 4           |
| 4    | 54  | N/A | 5 + 4 = 9 | T3a         | Negative        | -           | -            | 10       | PZ            | 3 + 4           |
| 5    | 70  | 5.5 | 4 + 3 = 7 | T2          | Negative        | -           | -            | 50       | PZ            | 3 + 3           |
| 6    | 60  | 24  | 4 + 3 = 7 | T3a         | Focally positive| 44          | 0            | 25       | PZ + TZ       | 3 + 4           |
| 7    | 61  | 5.9 | 4 + 3 = 7 | T3a         | Negative        | -           | -            | 25       | PZ            | 3 + 4           |
| 8    | 69  | 6.5 | 4 + 4 = 8 | T3a         | Negative        | -           | -            | 70       | PZ            | 3 + 3           |
| 9    | 62  | 7.2 | 4 + 5 = 9 | T3a         | Negative        | -           | -            | 25       | PZ            | 3 + 3           |
| 10   | 72  | 3   | 4 + 5 = 9 | T3b         | Negative        | -           | -            | 35       | PZ + TZ       | 4 + 3           |
| 11   | 66  | 13  | 4 + 5 = 9 | T3a         | Positive        | -           | -            | 30       | PZ + TZ       | 4 + 4           |
| 12   | 61  | 17  | 4 + 3 = 7 | T3a         | Focally positive| -           | -            | 70       | PZ            | 3 + 3           |
| 13   | 67  | 3.5 | 4 + 5 = 9 | T3a         | Negative        | -           | -            | 80       | PZ + TZ       | 3 + 4           |
| 14   | 72  | 6.9 | 4 + 5 = 9 | T3b         | Focally positive| -           | -            | 35       | PZ + TZ       | 4 + 5           |
| 15   | 71  | 2.1 | 4 + 3 = 7 | T3a         | Positive        | -           | -            | 30       | PZ            | 3 + 3           |

Abbreviations: GS, Gleason score; LN, lymph node; PSA, prostate-specific antigen.
FIGURE 2  Broad genomic profiling of paired samples of tissues from ductal and acinar adenocarcinoma. The top heatmap displays the somatic alterations detected from tumor tissue profiling. The type of alteration is coded according to the top legend. Subclonal mutations and structural variants are defined as having an allele frequency <1/4 of the cancer DNA fraction. Synonymous point mutations are not displayed here. Variants of unknown significance are non-synonymous single nucleotide variants outside hotspots and not annotated as pathogenic. The single-row middle heatmaps provide information on clonal origin, shared variants by variant type, tumor mutation burden and ploidy. The bottom panel displays the estimated fraction of cancer DNA in each sequenced tissue sample. The dashed lines at 0.01, 0.10, and 0.20 denote the cutoffs for reliable detection of point mutations, loss of heterozygosity, and homozygous deletions, respectively [Color figure can be viewed at wileyonlinelibrary.com]
assessed and it was not observed in any of the AAs of the series. It has previously been reported that such events are associated with advanced prostate cancer. Of the remaining DA tumors, four harbored SPOP hotspot mutations, which are not associated with advanced disease. Both tissue compartments of one case carried a rare KIAA1549–BRAF fusion event, which has been previously reported in prostate cancer.

In a study of 38 DAs with 38 matched pure AAs, fluorescence in situ hybridization showed that TMPRSS2-ERG gene fusion was less common in DA than in AA (4/38 vs. 17/38). In 21 of the DAs there was also an adjacent AA component and in these mixed tumors, TMPRSS2-ERG gene fusion was only present in one AA (1/21). Thus, TMPRSS2-ERG gene fusion was much less common in the AA component of mixed cancers than in pure AA controls (5% vs. 45%). In a follow-up study by Morais et al., immunohistochemistry showed that increased ERG expression and loss of PTEN were less common in both DA and AA components of mixed tumors than in matched pure AAs. These findings led the authors to hypothesize that mixed tumors, with both DA and AA, may be clonally related in some cases and show important molecular differences from pure AA. Furthermore, a study utilizing next generation sequencing technique on 51 cases of DA has reported that ETS fusions are significantly less common in DA compared with patients with both localized and castration resistant prostate cancer.

In this study, TMPRSS2-ERG gene fusions were detected in both DA and AA components in 33% of the tumors. Also, in one case TMPRSS2-ERG gene fusions were noted in both DA and AA, but of different types and structural variant start/stop coordinates. In one case a TMPRSS2-ERG gene fusion was detected in the AA component, but not in the DA component, while in another case TMPRSS2-ERG gene fusion was detected in the DA component, but not in the AA component. In contrast to earlier findings we found that TMPRSS2-ERG gene fusion is a common event in both the DA and AA component of mixed tumors. The discrepancy may be explained by the different analytical methods used in the studies. We used targeted genetic sequencing, while earlier studies have used immunohistochemical or fluorescence in situ hybridization techniques. It is more difficult to explain why our study showed a similar frequency of TMPRSS2-ERG gene fusions in both DA and AA components, while the study of Schweizer et al, utilizing next generation sequencing, has reported these event to be uncommon in DA.

Recently, Gillard et al. performed laser microdissection of DA and AA components of ten cases of coincident foci of prostate cancer with ductal and acinar features. DNA and RNA were extracted and used for integrative genomic and transcriptomic analyzes. The results indicated that coincident DA and AA diverged from a common progenitor, yet they harbored distinct alterations unique for each case. AR expression and activity were similar in both tumor components. Nine of ten cases had mutually exclusive CTNNB1 hotspot mutations or PTEN alterations in the DA component, which were absent in the acinar foci. The authors concluded that both tumor components in mixed tumors are clonally related and had thus arisen from the same cell of origin. Genome duplication events were not analyzed, but in...
our study gene duplication events were a characteristic of the DA component, being present in 8/15 cases. Whole genome doubling/ploidy increase is arguably the genomic feature most strongly associated with advanced prostate cancer.27 These findings are in accordance with earlier clinical descriptions of DA as an aggressive neoplasm.7

In this study, we found mutually exclusive mutations in PTEN and CTNNB1 in 6 of 15 cases, while Gillard et al. identified such mutations in 9 of 10 cases. These two studies are rather small and we cannot exclude the possibility that the differences in the incidence of PTEN and CTNNB1 alterations are random. A major difference between the studies is that we analyzed truly mixed prostate cancers with morphologically distinct DA and AA components, while Gillard et al. included coincident prostate cancer foci with ductal and acinar features. However, our study confirms their findings that PTEN and CTNNB1 alterations are enriched in DA and absent in AA. Whether these were separate foci or mixed tumors, containing both DA and AA, components is unclear. Two cases were excluded from the study because of low DNA yield and the cancer DNA fraction as assessed by sequencing data was lower than expected based on semiquantitative pathology assessment of the cancer cell fraction, in line with earlier reports.31

This is, to our knowledge, the first genomic study of DA and AA within the same tumor focus. We show that the majority of DA and AA in mixed prostate cancers share a common somatic denominator. Less commonly they represent collision tumors, where two clonally independent tumors have grown into each other, forming a mixed tumor. Also, we describe that genomic doubling events, resulting in a ploidy increase, is characteristic for DA. This is well in accordance with the characterization of DA as a high-grade cancer.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

ETHICAL CONSIDERATIONS
The study was approved by the Regional Ethic Review Board, Stockholm (2010/710-31/2, 2013/1451-32, 2018/827-32) and the Auesta Ethics Committee, Brisbane (AQ421010).

DATA AVAILABILITY STATEMENT
The authors confirm that data supporting these findings of this study are available within this article and its supplementary material.

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