HoxB13 expression in ductal type adenocarcinoma of prostate: clinicopathologic characteristics and its utility as potential diagnostic marker

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The histologic criteria and selective biomarkers of prostate ductal type adenocarcinoma (DAC) are relatively unknown compared to that known about acinar type adenocarcinoma (AAC). It is known that genetic alteration in \(Hox13\) gene is associated with carcinogenesis of prostate cancer. In this study, we investigated clinicopathologic characteristics of HoxB13 expression in prostate cancer and compared clinicopathologic profiles of DAC and AAC of prostate. After slide review, some morphological variants of DAC, equivalent to Gleason pattern 3 and 5 of AAC were identified. High level of HoxB13 expression was identified in 46.5% (46 out of 99 cases) and 39.2% (31 out of 79 cases) of cases that belong to the training set and test set, respectively. In the training set, high level of HoxB13 expression was significantly correlated with DAC \((P < 0.001)\), higher Gleason score \((P < 0.001)\), advanced pathologic T stage \((P = 0.010)\), and occurrence of biochemical recurrence (BCR; \(P < 0.001)\). The test set confirmed that high level of HoxB13 expression was associated with DAC \((P < 0.001)\), higher Gleason score \((P = 0.001)\), advanced pathologic T stage \((P < 0.001)\), and occurrence of BCR \((P < 0.001)\). Our findings suggest that HoxB13 may be a useful diagnostic marker for detection of DAC and a prognostic marker for prediction of BCR.

Prostate cancer is one of the most common cancers in males, especially in developed countries\(^1\). The majority of prostate cancer is acinar type adenocarcinoma (AAC); however, there are several variants of prostate cancer causing diagnostic difficulties due to the overlapping features with AAC\(^2\). Thus, variant forms are often misdiagnosed as AAC when using histology samples, causing difficulties in the histologic evaluation of prostate cancer\(^3\).

Ductal type adenocarcinoma (DAC) is another common subtype of prostate adenocarcinoma, and its incidence has been gradually increasing\(^4,5\). When compared with AAC, patients with DAC are more often diagnosed with an advanced T stage and exhibit greater mortality\(^6,9\). Generally, DAC shows papillary architecture lined by pseudostratified columnar epithelium with voluminous cytoplasm\(^6,7\); however, due to its broad spectrum of morphological presentations, DAC cases are often assigned one of several differential diagnoses: metastatic adenocarcinoma from the colorectal area, urothelial carcinoma, high-grade prostatic intraepithelial neoplasia (HGPIN), and intraductal prostate cancer (IDC-P)\(^7,8\).

A study about interobserver variabilities in the diagnosis of DAC was conducted, and among several diagnostic parameters, papillary architecture was found to be the most useful feature for diagnosis of DAC. Interobserver discrepancies, however, still remain a major obstacle in its diagnosis\(^7\). To address this problem, several studies have been performed to identify diagnostic markers of DAC, although, distinguishing DAC from AAC remains difficult\(^8-11\).

\(Hox\) genes, composed of four paralogous clusters, are located on four different chromosomes\(^12\). Among these genes, posterior \(Hox\) genes, particularly \(HoxA13, HoxB13,\) and \(HoxD13\), are important for the development of...
the separate lobes of the prostate gland, seminal vesicles, and epididymis. In addition, each Hox13 gene is associated with a lobe-specific prostatic identity and cellular differentiation\(^{13,14}\). Genetic alteration of HoxA13 and HoxB13 genes is associated with the development of prostate cancer\(^ {14–17}\). Specifically, a germline G84E mutation in HoxB13 was associated with hereditary prostate cancer\(^ {16}\). Furthermore, dysregulation of HoxB13 has been reported in colon, breast, and lung cancers as well as in cutaneous melanoma\(^ {18–20}\). Despite these findings, differential HoxB13 expression according to histologic subtype and the clinical implications of Hox expression in prostate cancer have not been fully investigated. Thus, in this study, we evaluated the expression of HoxA13 and HoxB13 in DAC versus AAC to identify their roles as diagnostic markers for DAC.

**Results**

### Histopathologic reassessment of 178 prostate cancer cases.

After the slide review, 25 cases previously diagnosed as DAC, including 19 equivocal cases, were reclassified as AAC, and 18 cases previously diagnosed as AAC were reclassified as DAC. Therefore, 68 cases of DAC and 110 cases of AAC were used for comparison of the clinicopathologic characteristics based on the histologic subtypes.

DACs that fulfilled all diagnostic criteria for ductal component were all assigned as Gleason pattern 4. The majority of cases arose from the inner zone of periurethral primary ducts with expansile invasive pattern (Fig. 1A,B). They showed complex papillary patterns with fibrovascular cores, and the papillary architecture was composed of tall columnar tumor cells, mimicking colon or endometrioid adenocarcinoma. Coagulative necrosis was rarely identified, unlike in colon or endometrioid type adenocarcinoma. In addition, less pleomorphism and stratification were identified compared to those of urothelial carcinoma.

In addition to the Gleason pattern 4 DACs, which fulfilled all diagnostic criteria, 10 cases that had been originally classified as DAC showed some morphological variants in the juxtaposed: 6 cases with tubular structures, 2 cases with growth patterns equivalent to Gleason pattern 5 of AAC and 2 cases with both variants. Simple tubular structures were composed of tall columnar tumor cells with elongated nuclei and were intermingled with conventional DACs; however, no evidence of papillary cores was observed (Fig. 1C,D). These tubular structures showed different morphological features than those of AAC, raising the possibility of DACs with Gleason pattern 3.

Two growth patterns equivalent to Gleason pattern 5 of AAC were observed adjacent to conventional DACs (Fig. 2A). Two cases showed central comedo-type necrosis (Fig. 2B). Having a comparatively larger size than AAC was a prerequisite for determination of DAC. Infiltrative cord-like patterns that mimicked invasive lobular carcinoma of the breast was observed in two cases (Fig. 2C).

### Clinicopathologic features of patients.

Clinicopathologic analysis was performed on the training set and test set, respectively. In the training set, DAC was significantly associated with a higher Gleason score (\(P < 0.001\)), tumor volume more than 5cc (\(P = 0.004\)), presence of extraprostatic extension (EPE; \(P = 0.004\)), advanced pathologic T stage (\(P = 0.016\)), intact PTEN expression (\(P < 0.001\)) and occurrence of biochemical recurrence (BCR; \(P = 0.004\)). The results of clinicopathologic analysis on the test set was similar to that of training set. DAC was significantly correlated with a higher Gleason score (\(P < 0.001\)), presence of EPE (\(P < 0.001\)), advanced pathologic
T stage ($P < 0.001$), ERG positivity ($P = 0.012$) and occurrence of BCR ($P = 0.004$). The results of chi-square analysis of the clinicopathologic factors are summarized in Table 1.

Clinicopathologic characteristics according to HoxA13 and HoxB13 expression status. In the almost all of cases, HoxA13 and HoxB13 were expressed in the nucleus of tumor cells, with concomitant non-specific cytoplasmic staining. HoxA13 is highly expressed in tumor cells of Gleason pattern 4 DACs, especially those surrounding papillary cores and comprising large ducts (Fig. 3A). For HoxB13, the expression patterns within each tumor subtype and Gleason pattern differed. The expression of HoxB13 was similar to that of HoxA13 in Gleason pattern 4 DACs (Fig. 3B). For HoxB13, the expression patterns within each tumor subtype and Gleason pattern differed. The expression of HoxB13 was similar to that of HoxA13 in Gleason pattern 4 DACs (Fig. 3C). Morphological variants of DAC, which is located adjacent to Gleason pattern 4 DACs, also showed expression of HoxB13 in columnar cells of Gleason pattern 3 DACs (Fig. 3D) and singly scattered tumor cells or those comprising large nests with central comedo-type necrosis of Gleason pattern 5 DACs (Fig. 3E). On the contrary, HoxB13 expression was lower in AAC cases compared with DACs (Fig. 3F). All of 19 equivocal cases showed high HoxB13 expression. The validation results of HoxA13 and HoxB13 antibody are presented in Supplementary Fig. 1.

High HoxB13 expression was identified in 46.5% (46 out of 99 cases) and 39.2% (31 out of 79 cases) of cases that belong to the training set and test set, respectively. In the training set, high level of HoxB13 expression was significantly associated with DAC ($P < 0.001$), a higher Gleason score ($P < 0.001$), tumor volume more than 5cc ($P = 0.042$), presence of EPE ($P = 0.001$), advanced pathologic T stage ($P = 0.010$), intact PTEN expression ($P < 0.001$) and occurrence of BCR ($P < 0.001$). The test set showed the results similar to those of the training set. High level of HoxB13 expression was significantly correlated with DAC ($P < 0.001$), a higher Gleason score ($P = 0.001$), presence of EPE ($P < 0.001$), presence of lymphovascular invasion (LVI; $P = 0.001$), involvement of seminal vesicle ($P = 0.043$), advanced pathologic T stage ($P < 0.001$), lower ERG expression ($P = 0.016$), and occurrence of BCR ($P < 0.001$). In addition, high level of HoxB13 expression showed a tendency toward tumor volume more than 5cc ($P = 0.056$), and frequent intact PTEN expression ($P = 0.065$). The results of chi-square analysis are summarized in Table 2.

High HoxA13 expression was identified in 60.6% (60 out of 99 cases) and 58.2% (46 out of 79 cases) of cases that belong to the training set and test set, respectively. In the training set, high level of HoxA13 expression was significantly associated with presence of EPE ($P = 0.008$), and advanced pathologic T stage ($P = 0.015$). However, no significant correlation between high HoxA13 expression and various clinicopathologic factors was identified in the test set. The results of chi-square analysis are summarized in Supplementary Table S1, and representative immunoprofiles are presented in Supplementary Fig. 2.

Interobserver agreement. Prior to estimating the interobserver agreement, we established two diagnostic criteria for the reproducible assessment of DAC. Morphologic criteria were described in the Materials and methods section as a diagnostic criteria for the ductal component. Immunophenotypic criteria were based on the
Table 1. Clinicopathological characteristics of 178 prostate cancers according to the histologic subtype in training set and test set. Abbreviations: DAC, ductal type adenocarcinoma; AAC, acinar type adenocarcinoma; PSA, prostate-specific antigen; EPE, extraprostatic extension; PNI, perineural invasion; LVI, lymphovascular invasion; RM, resection margin; SV, seminal vesicle; IHC, immunohistochemistry; BCR, biochemical recurrence. *Evaluated in 113 prostatectomy specimens.

| Category         | Variables | Training set | Test set |
|------------------|-----------|--------------|----------|
|                  | No. of cases (n = 99) | DAC (%) (n=36) | AAC (%) (n=63) | P-value | No. of cases (n = 79) | DAC (%) (n=32) | AAC (%) (n=47) | P-value |
| Age (y)          | 67.7 ± 7.90 | 66.0 ± 7.59 | 0.272 | 66.3 ± 5.90 | 65.2 ± 7.79 | 0.513 |
| Pre-operative PSA (ng/mL) | 12.8 ± 9.76 | 11.4 ± 14.5 | 0.612 | 11.7 ± 7.46 | 9.54 ± 6.99 | 0.179 |
| Gleason score    | 8 17 (47.2) | 52 (82.5) | <0.001 | 55 15 (46.9) | 40 (85.1) | <0.001 |
|                  | 9–10 30 19 (52.8) | 11 (17.5) | 24 | 17 (53.1) | 7 (14.9) | 0.650 |
| Location         | Unilateral 23 8 (22.2) | 15 (23.8) | 0.857 | 14 5 (15.6) | 6 (12.2) | 0.687 |
|                  | Bilateral 76 28 (77.8) | 48 (76.2) | | 65 27 (84.4) | 38 (80.9) | |
| Tumor volume     | ≤5 cc 74 21 (53.8) | 53 (84.1) | 0.004 | 60 21 (65.6) | 39 (83.0) | 0.076 |
|                  | >5 cc 25 15 (60.0) | 10 (15.9) | | 19 11 (34.4) | 8 (17.0) | |
| EPE              | Absent 52 12 (38.9) | 40 (63.1) | 0.004 | 47 10 (31.3) | 37 (78.7) | <0.001 |
|                  | Present 47 24 (61.1) | 23 (36.9) | | 32 22 (68.7) | 10 (21.3) | |
| PNI              | Absent 7 3 (38.9) | 4 (6.3) | 0.703 | 5 3 (9.4) | 2 (4.3) | 0.390 |
|                  | Present 92 33 (41.7) | 59 (89.7) | | 74 29 (90.6) | 45 (95.7) | |
| LVI              | Absent 88 31 (61.1) | 57 (90.5) | 0.522 | 67 25 (78.1) | 42 (89.4) | 0.210 |
|                  | Present 11 5 (13.9) | 6 (9.5) | | 12 7 (21.9) | 5 (10.6) | |
| RM extension     | Absent 34 14 (41.7) | 20 (41.7) | 0.472 | 42 18 (56.3) | 24 (51.1) | 0.650 |
|                  | Present 56 22 (58.6) | 34 (58.3) | | 37 14 (43.7) | 23 (48.9) | |
| SV involvement   | Absent 82 27 (75.0) | 55 (87.3) | 0.118 | 69 25 (78.1) | 44 (93.6) | 0.081 |
|                  | Present 17 5 (29.4) | 12 (21.9) | | 10 7 (21.9) | 3 (6.4) | |
| Pathologic T stage| T2 46 11 (30.6) | 35 (55.6) | 0.016 | 47 10 (31.3) | 37 (78.7) | <0.001 |
|                  | T3 and T4 53 25 (69.4) | 28 (44.4) | | 32 22 (68.7) | 10 (21.3) | |
| Pathologic N stage* | N0 68 32 (48.9) | 36 (100.0) | 0.115 | 40 26 (66.0) | 14 (100.0) | >0.999 |
|                  | N1 4 4 (11.1) | | | 1 1 (3.7) | | |
| PTEN IHC         | Intact 55 30 (54.5) | 25 (39.7) | <0.001 | 33 10 (31.3) | 23 (48.9) | 0.118 |
|                  | Loss 44 6 (16.7) | 38 (60.3) | | 46 22 (68.7) | 24 (51.1) | |
| ERG IHC          | Negative 83 32 (38.9) | 51 (80.1) | 0.302 | 60 29 (90.6) | 31 (60.0) | 0.012 |
|                  | Positive 16 4 (11.1) | 12 (19.0) | | 19 3 (9.4) | 16 (34.0) | |
| BCR              | Absent 57 14 (25.0) | 43 (68.3) | 0.004 | 61 19 (59.4) | 42 (89.4) | 0.004 |
|                  | Present 42 22 (61.1) | 20 (31.7) | | 18 13 (40.6) | 5 (10.6) | |

**morphologic criteria and HoxB13 expression.** Equivocal cases that exhibited high level of HoxB13 expression were classified as DAC.

In the training set, interobserver agreement was 66.7% (63 out of 99 cases), and kappa value was 0.214 [95% confidence interval (CI), 0.017–0.418] without specific diagnostic criteria. However, in the second round, following application of the morphologic criteria, the interobserver agreement increased to 69.7% (69 out of 99 cases), and the kappa value was 0.353 (95% CI, 0.154–0.539). With the additional application of the immunophenotypic criteria, the interobserver agreement further increased to 83.8% (83 out of 99 cases), and the kappa value was 0.670 (95% CI, 0.515–0.805). In the test set, the interobserver agreement after the application of the morphologic criteria was 75.9% (60 out of 79 cases) and kappa value was 0.498 (95% CI, 0.286–0.677). With application of the immunophenotypic criteria, the interobserver agreement further increased to 83.5% (66 out of 79 cases), and the kappa value was 0.657 (95% CI, 0.476–0.821).

**Impact of histologic subtype and HoxB13 expression on BCR-free survival and prognosis.** In the training set, no significant differences in BCR-free survival between AAC and DAC were observed (P = 0.141; Fig. 4A) before the application of our diagnostic criteria. However, after the application of our diagnostic criteria, significant difference was identified between the subgroups. When applying morphologic criteria, DAC and AAC were found to have significantly different BCR-free survival (P < 0.001; Fig. 4B). When based on immunophenotypic criteria, DAC cases had significantly shorter BCR-free survival than AAC cases with low level of HoxB13 expression, and 13 equivocal cases with high level of HoxB13 expression showed similar BCR-free survival as DAC cases (P < 0.001 and P = 0.001, respectively; Fig. 4C).

Survival analysis on the test set validated our diagnostic criteria for DAC. When applying morphologic criteria in the test set, DAC and AAC showed significantly different BCR-free survival (P < 0.001; Fig. 4D). When applying immunophenotypic criteria, DAC cases had significantly shorter BCR-free survival than AAC cases with low
level of HoxB13 expression, and 6 equivocal cases with high level of HoxB13 expression showed similar BCR-free survival as DAC cases ($P < 0.001$ and $P = 0.003$, respectively; Fig. 4E).

Univariate analysis identified the following characteristics that were associated with shorter BCR-free survival: DAC ($P < 0.001$), higher Gleason score ($P < 0.001$), bilateral location ($P = 0.032$), tumor volume more than 5 cc ($P < 0.001$), presence of EPE ($P < 0.001$), presence of LVI ($P = 0.001$), extension to resection margin ($P < 0.001$), involvement of seminal vesicles and/or lymph node metastasis ($P < 0.001$), and high level of HoxB13 expression ($P < 0.001$). In contrast, loss of PTEN expression ($P < 0.001$) was associated with longer BCR-free survival. Following multivariate analysis, DAC ($P = 0.045$), extension to the resection margin ($P = 0.001$) and high level of HoxB13 expression ($P < 0.001$) were associated with shorter BCR-free survival. In contrast, loss of PTEN expression ($P = 0.049$) was associated with longer BCR-free survival. The results of univariate and multivariate analyses are summarized in Table 3.

Proposed diagnostic algorithm for DAC. Morphologic criteria and immunophenotypic criteria showed strong correlation in the training set (Spearman’s correlation coefficient = 0.685, $P < 0.001$) and the test set (Spearman’s correlation coefficient = 0.657, $P < 0.001$), respectively. In addition, the immunophenotypic criteria showed superior interobserver agreement compared to morphologic criteria. Thus, HoxB13 immunohistochemistry (IHC) can be used as a diagnostic marker for DAC in cases with uncertain morphologic features. Based on these findings, we propose a new diagnostic algorithm for DAC (Fig. 5).

Discussion
Histologic subtypes other than AAC represent less than 10% of all prostate cancer cases. DAC is usually combined with AAC to yield mixed type adenocarcinomas. According to several studies, the proportion of cases classified as this mixed type adenocarcinoma varies from 0.13% to 12.7%6,21–23. Although less frequent, the biologic behavior of DAC is aggressive, exhibiting frequent EPE, involvement of seminal vesicles, extension to the surgical resection margins, presence of LVI, and BCR4,24. Moreover, DAC also metastasizes to unusual sites, such as the lung, liver, and brain.

Despite the high mortality rate of DAC, it is difficult to detect this tumor type because of the frequent subnormal prostate-specific antigen (PSA) levels26. A study about the interobserver variability in the diagnosis of DAC was conducted and papillary architecture was proven to be the most important factor for the diagnosis of DAC3. However, despite several studies to elucidate the immunoprofile of DAC, definitive diagnostic markers for DAC have not yet been identified.

In this study, we evaluated the morphological patterns of DAC and the differences in expression of HoxA13 and HoxB13 between DAC and AAC. HoxB13 was strongly expressed in Gleason pattern 4 DACs. In addition, HoxB13 was expressed in tumor cells that exhibited tubular structures or growth patterns equivalent to Gleason pattern 5 of AAC. These findings raise the possibility of variable Gleason patterns, including 3 and 5, for DACs. DACs with Gleason pattern 3 and 5 were observed in the vicinity of DAC nodules. DACs with tubular features (Gleason pattern 3) were more frequently identified than DACs with Gleason pattern 5; however, DACs
Table 2. Clinicopathological characteristics of 178 prostate cancers HoxB13 expression status in training set and test set. Abbreviations: DAC, ductal type adenocarcinoma; AAC, acinar type adenocarcinoma; PSA, prostate-specific antigen; EPE, extraprostatic extension; PNI, perineural invasion; LVI, lymphovascular invasion; RM, resection margin; SV, seminal vesicle; IHC, immunohistochemistry; BCR, biochemical recurrence *Evaluated in 113 prostatectomy specimens.

| Category                  | Variables | Training set | Test set |
|---------------------------|-----------|--------------|----------|
|                           |           | No. of cases (n = 99) | High (%) (n = 46) | Low (%) (n = 53) | P-value | No. of cases (n = 79) | High (%) (n = 31) | Low (%) (n = 48) | P-value |
| Age (y)                   |           | 68.2 ± 7.80 | 65.3 ± 7.45 | 0.062 | 65.9 ± 6.66 | 65.6 ± 7.38 | 0.871 |
| Pre-operative PSA (ng/mL) |           | 11.8 ± 9.05 | 12.0 ± 15.7 | 0.934 | 11.1 ± 7.81 | 10.0 ± 8.67 | 0.516 |
| Histologic subtype        |           | 83 13 (28.3) | 50 (94.3) | <0.001 | 47 6 (19.4) | 41 (85.4) | <0.001 |
| Gleason score             |           | 69 23 (50.0) | 46 (86.8) | <0.001 | 55 15 (48.4) | 40 (83.3) | 0.001 |
| Location                  |           | 73 (34.8) | 9 (17.0) | 0.010 | 47 (22.6) | 7 (14.6) | 0.363 |
| Tumor volume              |           | 74 30 (65.2) | 44 (83.0) | 0.042 | 60 20 (64.5) | 40 (83.3) | 0.056 |
| EPE                       |           | 52 16 (34.8) | 36 (67.9) | 0.001 | 47 10 (32.3) | 37 (77.1) | <0.001 |
| PNI                       |           | 92 42 (91.3) | 50 (94.3) | 0.701 | 32 21 (67.7) | 11 (22.9) |
| LVI                       |           | 88 40 (87.0) | 48 (90.6) | 0.569 | 67 21 (67.7) | 46 (95.8) | 0.001 |
| RM extension              |           | 34 16 (34.8) | 18 (34.0) | 0.932 | 42 17 (54.8) | 25 (52.1) | 0.811 |
| SV involvement            |           | 82 35 (76.1) | 47 (88.7) | 0.098 | 69 24 (77.4) | 45 (93.8) | 0.043 |
| Pathologic T stage        |           | 79 48 (61.8) | 35 (71.7) | 0.015 | 46 22 (71.0) | 24 (50.0) | 0.065 |
| Pathologic N stage*       |           | 68 40 (90.9) | 28 (100.0) | 0.152 | 40 23 (100.0) | 17 (43.7) | 0.439 |
| PTEN IHC                  | Intact    | 55 36 (67.3) | 19 (35.8) | <0.001 | 46 22 (71.0) | 24 (50.0) | 0.065 |
|                           | Loss      | 64 10 (21.7) | 34 (64.2) | 0.33 | 33 9 (29.0) | 24 (50.0) |
| ERG IHC                   | Negative  | 83 40 (87.0) | 43 (81.2) | 0.432 | 60 28 (90.3) | 32 (66.7) | 0.016 |
|                           | Positive  | 16 6 (13.0) | 10 (18.9) | 19 3 (9.7) | 16 (33.3) |
| BCR                       | Absent    | 57 15 (32.6) | 42 (79.2) | <0.001 | 61 15 (48.4) | 46 (95.8) | <0.001 |
|                           | Present   | 42 31 (67.4) | 11 (20.8) | 18 16 (51.2) | 2 (4.2) |

with Gleason pattern 3 formed larger tubular glands than those of AAC and were composed of tall columnar cells with pseudostratification. DACs having Gleason pattern 5 manifested as central comedo-type necrosis or infiltrative cord-like patterns mimicking invasive lobular carcinoma of the breast. These Gleason pattern 5 DACs were comparatively larger in size than AACs and intermingled with Gleason pattern 4 DACs. However, we did not identify any other variants with a Gleason pattern 5, such as a solid sheet-like growth corresponding to that of AAC. Further studies are necessary to investigate the various variants with Gleason pattern 3 and 5 in DACs.

No significant correlation was identified between HoxA13 expression and histologic subtype by morphologic criteria; however, the majority of DAC cases showed high level of HoxB13 expression and were associated with intact PTEN protein expression and ERG negativity, consistent with previous study results19. In a previous study, Morais et al. suggested the possibility of a clonal relationship between ductal and acinar components of mixed type adenocarcinomas10. In addition, the HoxB13 gene regulates luminal differentiation of prostatic epithelium in animal models13. Thus, it is plausible to assume that the expression level of the HoxB13 gene is associated with the development of DAC. Further studies are required to elucidate the relationship between HoxB13 expression and the development of DAC.

We identified a strong correlation between subgroups based on the morphologic and immunophenotypic criteria in the training set and the test set. In addition, interobserver agreement based on the immunophenotypic criteria was better than that based on the morphologic criteria in both cohorts. In addition to diagnostic reproducibility, the changes in diagnostic criteria affected prognostic classification of prostate cancer patients. Before the application of morphologic criteria, no significant differences in BCR-free survival were identified between AAC and DAC; however, after the application of morphologic criteria, DACs were found to have a shorter BCR-free...
survival than AACs. In addition, after application of the immunophenotypic criteria, the equivocal cases with high level of HoxB13 expression exhibited BCR-free survival similar to that of DACs. Thus, our findings suggest that immunophenotypic criteria could be useful to determine the histologic subtypes of equivocal cases. Upon univariate and multivariate analysis, the high level of HoxB13 expression was identified as a significant factor for the prediction of BCR, which is similar findings to those of the previous study. Based on our IHC and survival analyses results, we conclude that HoxB13 can be used as a diagnostic marker for DAC. In addition, HoxB13 expression can also be used as a prognostic marker, regardless of histologic subtype. Despite these promising findings, this study has a limitation because it is performed on the single cohort from the single institute. Therefore, additional studies using larger and independent cohorts are necessary to validate these conclusions. In summary, we investigated the morphological features of DAC and the expression of HoxB13 in 178 radical prostatectomy (RP) specimens. DACs showed various morphological features that lead to diagnostic difficulties; however, using HoxB13 expression analysis for immunophenotypic criteria combined with morphologic characteristics resulted in improved interobserver agreement and prognostic significance. Therefore, we suggest that when a final diagnosis remains equivocal, HoxB13 IHC can be an excellent ancillary measure to diagnose DAC.

**Materials and Methods**

**Patient selection and clinical information.** All 1460 consecutive RP specimens from 2008 to 2014 were selected from the archive of the Severance Hospital. Cases with neoadjuvant androgen deprivation therapy were excluded. To rule out the possibility of other conditions, such as HGPIN or IDC-P that mimic DAC, dual IHC for high molecular weight cytokeratin and α-methylacyl-CoA racemase was performed. After evaluation of hemotoxylin and eosin (H&E)-stained slides and dual IHC, 75 cases, including 15 mixed-type adenocarcinoma cases, were eventually selected as the DAC group. As a control group, 103 consecutive RP specimens diagnosed as AAC from 2008 to 2014 were included and matched with a corresponding Gleason score of ≥ 8. The entire cases were randomly assigned to 99 cases of training set and 79 cases of test set. Several clinical factors, including age at the time of operation, follow-up level of PSA, and other follow-up data were obtained via medical record review. Cases with serum PSA > 0.2 ng/mL at least 6 weeks after surgery and a second confirmatory increase thereafter were considered to have BCR. BCR-free time was estimated from the
date of the first curative surgery to the date of BCR or death without any type of relapse. This study was approved by the Institutional Review Board of the Severance Hospital (4-2018-0641) and informed consent were obtained from all patients. This study was performed in accordance with the Declaration of Helsinki.

**Histopathological evaluation.** All cases were reviewed by three independent pathologists via evaluation of H&E-stained whole-section slides. Pathologic factors, including Gleason score (based on the 2014 International Society of Urological Pathology consensus)28, EPE, LVI, perineural invasion, and pathologic stage based on the 8th American Joint Committee on Cancer criteria29 were acquired. Tumor volume was calculated by visual inspection method as previously described30.

For the diagnosis of DAC, we newly defined the following as diagnostic criteria for the ductal component: (1) topographical origin of central primary ducts close to urethral lumen, (2) true papillary and/or cribriform architecture more than three times larger than typical acini, (3) tall columnar epithelium that was at least three times longer than the height of the nuclei and stratified or elongated nuclei with prominent nucleoli. Cases that satisfied at least two of diagnostic criteria were classified as DAC. Equivocal cases that satisfied only one criterion were considered to be AAC.

**IHC and interpretation.** The antibodies used for IHC on formalin-fixed paraffin-embedded tissue whole sections are shown in Supplementary Table S2. IHC was conducted with the Ventana Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA) according the manufacturer’s protocol. Cell Conditioning 1 buffer (EDTA, pH 8.0, Ventana Medical Systems) was used for antigen retrieval.

Interpretation of IHC results was performed by a urologic pathologist. Cytoplasmic staining of HoxA13 and HoxB13 was considered non-specific, and only nuclear staining was evaluated. The results of HoxA13 and

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| Category        | Variables       | BCR-free survival |          |          |          |          |
|-----------------|-----------------|-------------------|----------|----------|----------|----------|
|                 |                 |                   | Univariate | Multivariate |       |       |
|                 |                 |                   | HR (95% CI) | P-value | HR (95% CI) | P-value |
| Age (y)*        | ≤67             | 1                 | —        | —        | —        | —        |
|                 | >67             | 1.132 (0.703–1.823) | 0.610  | —        | —        | —        |
| Histologic subtype | AAC            | 1                 | —        | —        | —        | —        |
|                 | DAC             | 4.127 (2.422–7.032) | <0.001 | 1.907 (1.015–3.585) | 0.045 |
| Gleason score   | 8               | 1                 | —        | —        | —        | —        |
|                 | 9–10            | 2.777 (1.721–4.482) | <0.001 | —        | —        | —        |
| Location        | Unilateral      | 1                 | —        | —        | —        | —        |
|                 | Bilateral       | 2.169 (1.070–4.396) | 0.032  | —        | —        | —        |
| Tumor volume    | ≤5 cc           | 1                 | —        | —        | —        | —        |
|                 | >5 cc           | 3.997 (2.447–6.529) | <0.001 | —        | —        | —        |
| EPE             | Absent          | 1                 | —        | —        | —        | —        |
|                 | Present         | 2.986 (1.814–4.914) | <0.001 | 0.975 (0.506–1.880) | 0.940 |
| Perineural invasion | Absent       | 1                 | —        | —        | —        | —        |
|                 | Present         | 0.729 (0.315–1.687) | 0.460  | —        | —        | —        |
| LVI             | Absent          | 1                 | —        | —        | —        | —        |
|                 | Present         | 2.707 (1.521–4.817) | 0.001  | 0.882 (0.413–1.882) | 0.745 |
| RM extension    | Absent          | 1                 | —        | —        | —        | —        |
|                 | Present         | 2.289 (1.608–4.836) | <0.001 | 2.957 (1.546–5.655) | 0.001 |
| SVI and/or LNM  | Absent          | 1                 | —        | —        | —        | —        |
|                 | Present         | 3.308 (2.008–5.450) | <0.001 | 1.862 (0.977–3.547) | 0.059 |
| Pathologic T stage | T2            | 1                 | —        | —        | —        | —        |
|                 | T3 and T4       | 3.098 (1.853–5.177) | <0.001 | —        | —        | —        |
| HoxA13 IHC      | Low             | 1                 | —        | —        | —        | —        |
|                 | High            | 0.939 (0.562–1.569) | 0.810  | —        | —        | —        |
| HoxB13 IHC      | Low             | 1                 | —        | —        | —        | —        |
|                 | High            | 6.742 (3.721–12.217) | <0.001 | 4.293 (2.013–9.152) | <0.001 |
| PTEN IHC        | Loss            | 1                 | —        | —        | —        | —        |
|                 | Intact          | 0.331 (0.182–0.603) | <0.001 | 0.531 (0.282–0.999) | 0.049 |
| ERG IHC         | Negative        | 1                 | —        | —        | —        | —        |
|                 | Positive        | 0.616 (0.303–1.251) | 0.180  | —        | —        | —        |

**Table 3.** Univariate and multivariate analysis of BCR-free survival in 178 prostate cancers. Abbreviations: DAC, ductal type adenocarcinoma; AAC, acinar type adenocarcinoma; EPE, extraprostatic extension; LVI, lymphovascular invasion; RM, resection margin; SVI, seminal vesicle involvement; LNM, lymph node metastasis; IHC, immunohistochemistry *Median age of 178 patients was 67.0 y.
HoxB13 IHC were evaluated using a classification system based on the proportion and intensity of staining, as previously described\(^3\). The proportion category was assigned as follows: 1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79%, and 6 = 80–100%. Intensity category was assigned as follows: 0 = no staining, 1 = weak, 2 = intermediate, and 3 = strong. Quickscore was defined as the product of the proportion and intensity scores\(^3\). Quickscores \(\geq 3\) were considered to be high expression, and those \(< 3\) were regarded as low expression.

PTEN expression was evaluated by comparing staining between malignant glands and adjacent benign glands or stroma. As previously described, cases with markedly decreased or completely negative staining across entire tumor glands compared with the adjacent benign glands or stroma were considered to have loss of PTEN expression\(^1,3\). The other cases were considered to have intact PTEN expression. Cases with any tumor cells showing nuclear ERG expression were considered positive for expression.

**Evaluation of correlation and interobserver agreement.** Samples were examined in a double-blind manner by two independent experienced pathologists (YA Cho [observer A] and JW Joo [observer B]). Both pathologists were also blinded to the results of the histologic evaluation, which was performed by other pathologists. Before training, interobserver agreement was evaluated on the training set. After that, two independent pathologists were trained via morphologic criteria for DAC and IHC slides of HoxB13 that were matched to the H&E-stained slides. The results of HoxB13 IHC performed by the urologic pathologist were blinded to the observers. And then, interobserver agreement was evaluated on the training set and the test set after each training.

**Statistical analysis.** Data were analyzed using SPSS for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Student’s \(t\)-test and chi-square test were used for continuous and categorical variables, respectively. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate regression analysis was performed using a Cox proportional hazards model. Statistical significance was assumed when \(P < 0.05\).

Spearman’s correlation coefficient was used to evaluate correlation between two diagnostic criteria, and interobserver agreement was evaluated by calculating percentage agreement. Cohen’s kappa was used to compare the observed agreement and that expected by chance. Kappa values were categorized as previously described\(^3\).

**Data availability**
All data of this study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare no competing interests.

Additional information

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