

KI67 and DLX2 predict increased risk of metastasis formation in prostate cancer - a targeted molecular approach

William JF Green BSc MRCS¹, Graham Ball PhD², Geoffrey Hulman MRCPATH³, Catherine Johnson BSc², Gerry Van Schalwyk MRCPATH⁴, Hari L Ratan MD FRCS¹, Daniel Soria PhD⁵, Jonathan M Garibaldi PhD⁵, Richard Parkinson PhD FRCS¹, Joshua Hulman³, Robert Rees PhD², Desmond G Powe PhD^{2,3*}

¹Department of Urology, Queen's Medical Centre, Nottingham University Hospitals NHS Trust, Nottingham, NG7 2UH, UK

²The John van Geest Cancer Research Centre, Nottingham Trent University, Nottingham, NG11 8NS, UK

³Department of Cellular Pathology, Nottingham University Hospitals NHS Trust, Nottingham, NG7 2UH, UK

⁴Pathology Department, Division of Planned Care, Royal Derby Hospital, Derby, DE22 3NE, UK

⁵Advanced Data Analysis Centre, Intelligent Modelling and Analysis Research Group, School of Computer Science, University of Nottingham, NG8 1BB, UK

***Corresponding author:**

Dr DG Powe

Department of Cellular Pathology

Queen's Medical Centre

Nottingham University Hospitals NHS Trust

Nottingham, NG7 2UH UK

Tel: +44 (0) 115 9249924 ex 63484

E-Mail: des.powe@nottingham.ac.uk

Word count: 2942

Abstract

Background:

There remains a need to identify and validate biomarkers for predicting prostate cancer (CaP) outcomes using robust and routinely available pathology techniques to identify men at most risk of premature death due to prostate cancer. Previous immunohistochemical studies suggest the proliferation marker Ki67 might be a predictor of survival, independently of PSA and Gleason score. We performed a validation study of Ki67 as a marker of survival and disease progression and compared its performance against another candidate biomarker, DLX2, selected using artificial neural network (ANN) analysis.

Methods: A tissue microarray (TMA) was constructed from transurethral resected prostatectomy (TURP) histology samples (n=192). ANN analysis was used to identify candidate markers conferring increased risk of death and metastasis in a public cDNA array. Immunohistochemical analysis of the TMA was carried out and univariate and multivariate tests performed to explore the association of tumour protein levels of Ki67 and DLX2 with time to death and metastasis.

Results: Univariate analysis demonstrated Ki67 as predictive of CaP-specific survival (DSS; p=0.022), and both Ki67 (p=0.025) and DLX2 (p=0.001) as predictive of future metastases. Multivariate analysis demonstrated Ki67 as independent of PSA, Gleason score and D'Amico risk category for DSS (HR=2.436, p=0.029) and both Ki67 (HR=3.296, p=0.023) and DLX2 (HR=3.051, p=0.003) as independent for future metastases.

Conclusion: High Ki67 expression is only present in 6.8% of CaP patients and is predictive of reduced survival and increased risk of metastasis, independent of PSA, Gleason score and D'Amico risk category. DLX2 is a novel marker of increased metastasis risk found in 73% patients and 8.2% showed co-expression with a high Ki67 score. Two cancer cell proliferation markers, Ki67 and DLX2, may be able to inform clinical decision making when identifying patients for active surveillance.

Introduction

The last decade has seen the development and refinement of predictive risk stratification models in the management of prostate cancer (CaP) (Parekh et al., 2006). These tools are validated for clinical decision-making and facilitating informed patient consent to treatment.

These tools aim to apply an objective, evidence-based algorithm to a set of disease parameters, thus moving away from subjective judgments based on clinical experience. Unsurprisingly given the genetic and molecular heterogeneity between individuals, these tools are far from perfect. They cannot always determine an individual's likelihood of clinically significant disease, particularly in classically low to intermediate risk groups, and in those patients undergoing active surveillance (AS) (Loeb et al., 2014; Wang et al., 2013). Increased understanding of the molecular biology of CaP has identified some biomarkers that are predictive of disease outcome and therapeutic response to treatment (Ben-Porath et al., 2008), for complementary use with the serum PSA test and histological Gleason score assessment.

The TransAtlantic Prostate Cancer Group developed a 31 gene cell cycle progression (CCP) signature for predicting outcome in prostate cancer (Cuzick et al., 2012, 2011) using conservatively treated transurethral resected prostate (TURP) samples, and validated CCP in radical prostatectomy and initial needle core biopsy (Cuzick et al., 2012). Another predictive test is the commercially available multigene RT-PCR Oncotype DX Prostate Cancer Assay. A 17-gene panel assesses markers of 4 distinct biological targets: the androgen pathway (AZGP1, KLK2, SRD5A2, and FAM13C), cellular organisation (FLNC, GSN, TPM2, and GSTM2), proliferation (TPX2) and

stromal response (BGN, COL1A1, and SFRP4), to produce a 'genomic prostate score' (GPS) that predicts the likelihood of high grade/stage disease at diagnosis (Klein et al., 2014; Knezevic et al., 2013).

Cancer cell proliferation is a surrogate of tumour growth and is associated with worsened prognosis in prostate cancer and other cancer types (Ramsay et al., 2011) (Nagalla et al., 2013). Unsurprisingly, measuring the proportion of cancer cells undergoing cell proliferation using the immunohistochemical proliferation marker Ki67 has shown promise as a biomarker for predicting biochemical recurrence in patients with localised prostate cancer (Berney et al., 2009). In addition, Ki67 has been reported to predict distant metastasis formation in intermediate prostate cancer, following radiation therapy (Verhoven et al., 2013) and in conjunction with other markers post docetaxel chemotherapy (Antonarakis et al., 2012). It has also demonstrated predictive value in assessing overall survival (OS) and disease-specific survival (DSS) (Fisher et al., 2013). However there is a lack of consensus when assigning thresholds for dichotomously categorising patients, which have varied from 2.4-26% for biochemical recurrence and from 3-10.3% for DSS.

There remains an unmet need to identify markers capable of reliably assessing an individuals' risk of developing metastasis and castrate resistance. These are important end-points for clinical assessment because they are associated with worsening prognosis. In this study we attempt to validate recent work carried out by Fisher et al in which they used a single biomarker, Ki67, to predict outcome in a conservatively managed group of patients diagnosed on needle core biopsy (Fisher et al., 2013). They demonstrated that Ki67 has significant ability to predict prostate cancer specific

death on both univariate and multivariate analysis (compared with PSA and Gleason Score). Ki67 has previously been demonstrated to predict survival and recurrence in patients undergoing radical treatment (Khor et al., 2009; Pollack et al., 2004; Zellweger et al., 2009). However Fishers work was the first study to demonstrate its prognostic utility from diagnostic biopsy in conservatively managed patients. This is important as this prognostic information could be used as an adjunct in clinical decision making to reduce the number of patients requiring radical treatment, with all its attendant risks.

However Fisher's group did not report whether Ki67 is predictive of metastasis. We therefore decided to examine the relationship between Ki67 expression and subsequent metastases and also applied a bioinformatic learning tool, Artificial Neural Network (ANN) analysis, to identify further biomarkers of metastasis development and DSS in a contemporary gene array library of an unselected population of prostate cancer patients.

Materials and Methods

Patient selection and clinical data collection

365 Patients diagnosed with CaP between 1999 and 2001 were identified. These were consecutive non-selected patients and all underwent ‘best-practice’ treatment at Nottingham City Hospital, UK. Initial histological cancer diagnosis was made using tissue obtained by prostate needle core biopsy or TURP specimens. A total of 192 patients were selected for inclusion in the study; 155 patients were excluded to improve cohort homogeneity. Exclusion criteria included patients not undergoing TURP (29), patients lost to follow up or key parameters not recorded (114). Gleason scoring was modified in 2005 by the International Society of Urological Pathology (ISUP) consensus (Epstein et al., 2005) and for this reason all cases were histologically reviewed and Gleason scored using contemporary ISUP guidelines. Multiple clinicopathological variables were recorded for each patient including PSA and histological Gleason score at diagnosis, time taken to metastasis formation and time taken to prostate cancer specific death post-diagnosis. Patient management was based on PSA levels, histology Gleason score, and clinical staging. Patients were clinically followed-up at 3-6 monthly intervals and the majority of patients received ‘watchful waiting’. Androgen deprivation therapy (ADT) was given for disease progression, indicated by a rapid rise in PSA level, symptoms or metastasis development. Palliative radiotherapy or chemotherapy was administered for symptom control (bone pain or prostate bleeding) in patients with late stage cancer.

Use of the tissue samples for this study was approved by the North West 7 Research Ethics Committee – Greater Manchester Central REC number 10/H1008/72.

Tissue microarray (TMA) construction

A TMA was constructed using archival wax-embedded TURP tissue samples sourced via the Nottingham Health Science BioBank. Histology sections were reviewed by a pathologist (GH) and 0.6mm diameter donor cores were sampled from at least two different tumour regions per patient using an automated TMA Grand Master instrument (3DHistech Ltd, Hungary).

Biomarker selection

ANN techniques were applied to a publically available prostate cancer cDNA gene expression array (Wang et al., 2010) to identify biomarkers for predicting tumour metastasis (Powe et al., 2014) so that their performance could be compared to the proliferation marker Ki67. We have previously reported on the use of an Artificial Neural Network technique to identify predictors of metastasis (Powe et al., 2014). Here the ANN model was reiterated 50 times with random sampling and the average mean square error of a test subset for each input variable was considered to determine the predictive capability for metastasis class.

The top 10 genes ranked for association with metastasis development are included in Table 1. Four of these had commercially available antibodies (AMACR (Racemase), DLX2, PAICS and MYO6). DLX2 was selected for validation due to its novelty as a candidate marker in prostate cancer, its putative oncogenic function (Cantile et al., 2005; Morini et al., 2010), and its high ANN ranking. For comparison, a curated literature search was performed to identify evidence for the application of Ki67 as a marker of outcome in prostate cancer

(Antonarakis et al., 2012; Berney et al., 2009; Fisher et al., 2013; Verhoven et al., 2013).

Immunohistochemistry (IHC)

Optimal antibody dilutions and antigen retrieval conditions were performed using positive and negative control tissues suggested by the antibody suppliers. After microwave antigen retrieval in 10mM sodium citrate slides were sequentially incubated in the primary antibody, detection reagents (Novolink, Leica), and haematoxylin.

Immunostained TMA sections were assessed to determine the appropriate scoring technique for quantifying protein expression levels. Sections were independently scored (WG, DP) without knowledge of pathology grade. Ki67 and DLX2 nuclear staining was microscopically assessed at x20 magnification in tumour cells present in the TMA core, using a Histochemical score technique (H-score (McCarty et al., 1985)). The H-score is achieved by summing the product of percentage cells showing each level of staining intensity where 0=absence of staining, 1=weak staining, 2=moderate staining, and 3=strong staining intensity. Staining thresholds used for dichotomous categorisation were chosen using the software program X-tile (Camp et al., 2004), or by those given in previously published studies. Patients were dichotomously categorised according to the nuclear H-score: positive expression was defined as a score equal to or greater than 110 for Ki67 and 10 for DLX2.

We used REMARK guidelines (McShane et al., 2006) for reporting on prognostic biomarkers in the whole patient series. The proportion of patients with scorable tissue

sections was less than the total number of patients originally incorporated in the TMA due to detachment of cores during processing and because not every section taken from every core contained cancer tissue (independently reviewed by GH). Missing data was assessed for randomness using a Little's test (Little, 1986) and Wilcoxon-Mann-Whitney test, both at 95% confidence level. We failed to reject the null hypothesis of data being missing completely at random ($p > 0.05$). The proportion of patients with tissue sections suitable for scoring is shown in Table 2.

Statistical analysis

Statistical analysis was performed using SPSS (Version 21; IBM, US) applied to verified cancer samples. Pearson Chi-square tests were performed to assess biomarker associations with clinicopathological variables including initial PSA and Gleason score. Kaplan-Meier plots with log-rank tests were used to model biomarker associations with disease-specific survival (DSS; months) and time (months) to metastasis development from diagnosis. Biomarkers also underwent multivariate Cox proportional hazards regression modelling to assess the additional prognostic value to the initial PSA at diagnosis, Gleason score, and initial D'Amico risk category. The significance level used was $P < 0.05$. If during biomarker analysis a particular clinicopathological variable was missing (for example if it had never been recorded in the notes) then that patient would be excluded from the statistical calculation.

Results

Patient characteristics are shown in Table 3. Examples of nuclear Ki67 and DLX2 staining are shown in Figure 1.

Ki67

A total 161/192 (83.9%) patients samples had scorable tissue with 11 (6.8%) and 150 (93.2%) showing positive and negative staining, respectively. Increased nuclear Ki67 expression showed a negative association with OS ($\chi^2=9.493$, $p=0.002$) and DSS ($\chi^2=5.222$, $p=0.022$; Figure 2a) and a positive association with metastatic disease ($\chi^2=5.058$, $p=0.025$; Figure 2b). Subsequent multivariate Cox regression analysis demonstrated that Ki67 contributed additional predictive ability to PSA concentration, Gleason score and initial D'Amico risk for DSS (HR=2.436, $p=0.029$, 95%CI=1.096-5.416) and metastasis risk (HR=3.296, $p=0.023$, 95%CI=1.1814-9.196). All Ki67 positive patients had high grade cancer (Gleason score 8-10, Groups 4-5) and in addition, 8/11 (72.7%) Ki67 positive patients showed co-expression with DLX2.

DLX2

A total 185/192 patients samples had scorable tissue with 135 (73%) and 50 (27%) showing positive and negative staining, respectively. DLX2 expression was not associated with Gleason score or PSA levels. DLX2 alone was not found to be predictive of DSS ($\chi^2=2.282$, $p=0.131$) (Figure 2c) but increased nuclear DLX2 expression showed a positive association with metastasis development ($\chi^2=10.207$, $p=0.001$; Figure 2d), independent of PSA concentration, Gleason score and initial D'Amico risk using multivariate Cox regression analysis (HR=2.754, $p=0.003$,

95%CI=1.423-5.332).. Coexpression with high Ki67 staining was seen in 8.2% DLX2 positive tumours.

Discussion

This study compares the utility of two biomarkers to predict prostate cancer survival and risk of metastasis in an unselected cohort of prostate cancer patients with at least ten years of clinical follow up. Biomarker selection was based on recent studies highlighting Ki67 as a marker of cell proliferation and outcome in prostate cancer (Antonarakis et al., 2012; Berney et al., 2009; Khor et al., 2009; Pollack et al., 2004; Verhoven et al., 2013; Zellweger et al., 2009) and a bioinformatic ANN approach applied to a gene expression array derived from prostate cancer patients, interrogated for markers of metastases. Development of metastasis was associated with increased expression of the tumour markers Ki67 and DLX2. To the best of our knowledge this is the first study of DLX2 being used as a marker of disease progression in prostate cancer. Interestingly co-expression of Ki67 and DLX2 occurred in 6.8%(11/161) scorable patients and was characterised by high cancer grade (Gleason 8-10) and high risk of metastasis.

Ki67 is functionally associated with cellular proliferation and is a surrogate for the growth fraction of tumours. It has been reported to be predictive of CaP specific mortality (Antonarakis et al., 2012; Verhoven et al., 2013; Zellweger et al., 2009) and we confirm that Ki67 provides prognostic information on disease-specific and overall survival in prostate cancer. Furthermore, we demonstrated that Ki67 provides additional prognostic utility (HR:2.19) to the PSA and Gleason score, validating the study by Fisher et al. (Fisher et al., 2013) who recently reported that Ki67 from biopsy tissue independently predicts survival in prostate cancer (HR:2.78).

In addition to validating Fishers work we also demonstrated that Ki67 expression is predictive of future metastases in prostate cancer, adding to the small but growing cohort of potential markers of metastasis in this disease. For example, recent work by Columbel et al has examined the expression of three putative stem cell markers (integrin alpha 2 and 6 and CMET) in men with high risk CaP. They concluded that the proportion of stem cell-like cancer cells is predictive of bone metastases.

Interestingly only 6.8% (11 patients) of our cancer cohort had a significantly raised Ki67 and we propose that such patients could be counselled regarding an increased risk of death and metastasis, particularly if they are also positive for DLX2, and should be considered for an active surveillance programme. Both markers are known to be functionally important in cancer cell proliferation and, coupled with their association with high Gleason grade reported here, these findings fit with a hypothesis that tumour proliferation rates are a surrogate for tumour aggression and poor prognosis.

Using an Artificial Neural Networking approach DLX2 was identified as a marker for metastasis prediction. The distal-less homeobox (DLX) gene family are involved in embryonic development, tissue homeostasis, cell cycle and apoptosis (Tang et al., 2013). A growing number of homeobox genes have been shown to be deregulated in a variety of human tumors, and their deregulation is known to enhance cell survival and proliferation and prevent differentiation (Lee et al., 2011). Aberration is reported in breast, lung, ovarian and colon tumours (Morini et al., 2010) and early work has shown that these genes may be involved in neuroendocrine differentiation seen in

advanced prostate cancers (Cantile et al., 2005) but no association with clinical outcomes has ever been reported.

DLX2 has been reported to be involved in shifting TGF β from a tumour suppressor to a tumour promoting function by repressing TGF β RII and the cell cycle inhibitor p21CIP1, and simultaneously increasing the mitogenic transcription factor c-Myc and epidermal growth factor (EGF) (Yilmaz et al., 2011). In addition it has been suggested that DLX2 activity may suppress TGFbeta-mediated cell adhesion and migration inhibition (Massagué, 2008). Our novel DLX2 findings validate the ANN bioinformatics approach in revealing it to be a strong predictor of increased metastasis risk (HR:3.311), more so than either the PSA or Gleason score. DLX2 therefore warrants further investigation because of its ability to assess cancer cell survival potential.

In summary, we demonstrate that two cell proliferation markers, Ki67 and DLX2, appear to predict CaP specific survival and metastasis. Independent validation of these findings is needed to establish if Ki67 and DLX2 (specially co-expression) should be considered for prospective clinical trials.

Acknowledgements

The authors thank Christopher Nolan for his assistance and the Nottingham Hospitals Charity (Special Trustees) for their financial assistance.

Conflicts of interest

None declared

Ethics

Use of the tissue samples for this study was approved by the North West 7 Research Ethics Committee – Greater Manchester Central REC number 10/H1008/72

References

- Antonarakis, E.S., Keizman, D., Zhang, Z., Gurel, B., Lotan, T.L., Hicks, J.L., Fedor, H.L., Carducci, M.A., De Marzo, A.M., Eisenberger, M.A., 2012. An immunohistochemical signature comprising PTEN, MYC, and Ki67 predicts progression in prostate cancer patients receiving adjuvant docetaxel after prostatectomy. *Cancer* 118, 6063–6071. doi:10.1002/cncr.27689
- Ben-Porath, I., Thomson, M.W., Carey, V.J., Ge, R., Bell, G.W., Regev, A., Weinberg, R.A., 2008. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat. Genet.* 40, 499–507. doi:10.1038/ng.127
- Berney, D.M., Gopalan, A., Kudahetti, S., Fisher, G., Ambroisine, L., Foster, C.S., Reuter, V., Eastham, J., Moller, H., Kattan, M.W., Gerald, W., Cooper, C., Scardino, P., Cuzick, J., 2009. Ki-67 and outcome in clinically localised prostate cancer: analysis of conservatively treated prostate cancer patients from the Trans-Atlantic Prostate Group study. *Br. J. Cancer* 100, 888–893. doi:10.1038/sj.bjc.6604951
- Camp, R.L., Dolled-Filhart, M., Rimm, D.L., 2004. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 10, 7252–7259. doi:10.1158/1078-0432.CCR-04-0713
- Cantile, M., Kisslinger, A., Cindolo, L., Schiavo, G., D'Antò, V., Franco, R., Altieri, V., Gallo, A., Villacci, A., Tramontano, D., Cillo, C., 2005. cAMP induced modifications of HOX D gene expression in prostate cells allow the identification of a chromosomal area involved in vivo with neuroendocrine differentiation of human advanced prostate cancers. *J. Cell. Physiol.* 205, 202–210. doi:10.1002/jcp.20384
- Cuzick, J., Berney, D.M., Fisher, G., Mesher, D., Møller, H., Reid, J.E., Perry, M., Park, J., Younus, A., Gutin, A., Foster, C.S., Scardino, P., Lanchbury, J.S., Stone, S., Transatlantic Prostate Group, 2012. Prognostic value of a cell cycle progression signature for prostate cancer death in a conservatively managed needle biopsy cohort. *Br. J. Cancer* 106, 1095–1099. doi:10.1038/bjc.2012.39
- Cuzick, J., Swanson, G.P., Fisher, G., Brothman, A.R., Berney, D.M., Reid, J.E., Mesher, D., Speights, V.O., Stankiewicz, E., Foster, C.S., Møller, H., Scardino, P., Warren, J.D., Park, J., Younus, A., Flake, D.D., Wagner, S., Gutin, A., Lanchbury, J.S., Stone, S., 2011. Prognostic value of an RNA expression signature derived from cell cycle proliferation genes for recurrence and death from prostate cancer: A retrospective study in two cohorts. *Lancet Oncol.* 12, 245–255. doi:10.1016/S1470-2045(10)70295-3
- Epstein, J.I., Allsbrook, W.C., Amin, M.B., Egevad, L.L., ISUP Grading Committee, 2005. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. *Am. J. Surg. Pathol.* 29, 1228–1242.
- Fisher, G., Yang, Z.H., Kudahetti, S., Møller, H., Scardino, P., Cuzick, J., Berney, D.M., Transatlantic Prostate Group, 2013. Prognostic value of Ki-67 for prostate

- cancer death in a conservatively managed cohort. *Br. J. Cancer* 108, 271–277. doi:10.1038/bjc.2012.598
- Khor, L.-Y., Bae, K., Paulus, R., Al-Saleem, T., Hammond, M.E., Grignon, D.J., Che, M., Venkatesan, V., Byhardt, R.W., Rotman, M., Hanks, G.E., Sandler, H.M., Pollack, A., 2009. MDM2 and Ki-67 predict for distant metastasis and mortality in men treated with radiotherapy and androgen deprivation for prostate cancer: RTOG 92-02. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 27, 3177–3184. doi:10.1200/JCO.2008.19.8267
- Klein, E.A., Cooperberg, M.R., Magi-Galluzzi, C., Simko, J.P., Falzarano, S.M., Maddala, T., Chan, J.M., Li, J., Cowan, J.E., Tsiatis, A.C., Cherbavaz, D.B., Pelham, R.J., Tenggara-Hunter, I., Baehner, F.L., Knezevic, D., Febbo, P.G., Shak, S., Kattan, M.W., Lee, M., Carroll, P.R., 2014. A 17-gene Assay to Predict Prostate Cancer Aggressiveness in the Context of Gleason Grade Heterogeneity, Tumor Multifocality, and Biopsy Undersampling. *Eur. Urol.* doi:10.1016/j.eururo.2014.05.004
- Knezevic, D., Goddard, A.D., Natraj, N., Cherbavaz, D.B., Clark-Langone, K.M., Snable, J., Watson, D., Falzarano, S.M., Magi-Galluzzi, C., Klein, E.A., Quale, C., 2013. Analytical validation of the Oncotype DX prostate cancer assay - a clinical RT-PCR assay optimized for prostate needle biopsies. *BMC Genomics* 14, 690. doi:10.1186/1471-2164-14-690
- Lee, S.Y., Jeon, H.M., Kim, C.H., Ju, M.K., Bae, H.S., Park, H.G., Lim, S.-C., Han, S.I., Kang, H.S., 2011. Homeobox gene *Dlx-2* is implicated in metabolic stress-induced necrosis. *Mol. Cancer* 10, 113. doi:10.1186/1476-4598-10-113
- Little, R., 1986. A test of missing completely at random for multivariate data with missing values. *J. Am. Stat. Assoc.* 83, 1198–1202.
- Loeb, S., Bruinsma, S.M., Nicholson, J., Briganti, A., Pickles, T., Kakehi, Y., Carlsson, S.V., Roobol, M.J., 2014. Active Surveillance for Prostate Cancer: A Systematic Review of Clinicopathologic Variables and Biomarkers for Risk Stratification. *Eur. Urol.* doi:10.1016/j.eururo.2014.10.010
- Massagué, J., 2008. TGF β in Cancer. *Cell* 134, 215–230. doi:10.1016/j.cell.2008.07.001
- McCarty, K.S., Miller, L.S., Cox, E.B., Konrath, J., McCarty, K.S., 1985. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch. Pathol. Lab. Med.* 109, 716–721.
- McShane, L.M., Altman, D.G., Sauerbrei, W., Taube, S.E., Gion, M., Clark, G.M., Statistics Subcommittee of NCI-EORTC Working Group on Cancer Diagnostics, 2006. REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res. Treat.* 100, 229–235. doi:10.1007/s10549-006-9242-8
- Morini, M., Astigiano, S., Gitton, Y., Emionite, L., Mirisola, V., Levi, G., Barbieri, O., 2010. Mutually exclusive expression of *DLX2* and *DLX5/6* is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231. *BMC Cancer* 10, 649. doi:10.1186/1471-2407-10-649
- Nagalla, S., Chou, J.W., Willingham, M.C., Ruiz, J., Vaughn, J.P., Dubey, P., Lash, T.L., Hamilton-Dutoit, S.J., Bergh, J., Sotiriou, C., Black, M.A., Miller, L.D., 2013. Interactions between immunity, proliferation and molecular subtype in breast cancer prognosis. *Genome Biol.* 14, R34. doi:10.1186/gb-2013-14-4-r34

- Parekh, D.J., Ankerst, D.P., Higgins, B.A., Hernandez, J., Canby-Hagino, E., Brand, T., Troyer, D.A., Leach, R.J., Thompson, I.M., 2006. External validation of the Prostate Cancer Prevention Trial risk calculator in a screened population. *Urology* 68, 1152–1155. doi:10.1016/j.urology.2006.10.022
- Pollack, A., DeSilvio, M., Khor, L.-Y., Li, R., Al-Saleem, T.I., Hammond, M.E., Venkatesan, V., Lawton, C.A., Roach, M., Shipley, W.U., Hanks, G.E., Sandler, H.M., 2004. Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 22, 2133–2140. doi:10.1200/JCO.2004.09.150
- Powe, D.G., Dhondalay, G.K.R., Lemetre, C., Allen, T., Habashy, H.O., Ellis, I.O., Rees, R., Ball, G.R., 2014. DACH1: its role as a classifier of long term good prognosis in luminal breast cancer. *PloS One* 9, e84428. doi:10.1371/journal.pone.0084428
- Ramsay, A.K., McCracken, S.R.C., Soofi, M., Fleming, J., Yu, A.X., Ahmad, I., Morland, R., Machesky, L., Nixon, C., Edwards, D.R., Nuttall, R.K., Seywright, M., Marquez, R., Keller, E., Leung, H.Y., 2011. ERK5 signalling in prostate cancer promotes an invasive phenotype. *Br. J. Cancer* 104, 664–672. doi:10.1038/sj.bjc.6606062
- Tang, P., Huang, H., Chang, J., Zhao, G.-F., Lu, M.-L., Wang, Y., 2013. Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma. *World J. Gastroenterol. WJG* 19, 2697–2703. doi:10.3748/wjg.v19.i17.2697
- Verhoven, B., Yan, Y., Ritter, M., Khor, L.-Y., Hammond, E., Jones, C., Amin, M., Bahary, J.-P., Zeitzer, K., Pollack, A., 2013. Ki-67 is an independent predictor of metastasis and cause-specific mortality for prostate cancer patients treated on Radiation Therapy Oncology Group (RTOG) 94-08. *Int. J. Radiat. Oncol. Biol. Phys.* 86, 317–323. doi:10.1016/j.ijrobp.2013.01.016
- Wang, S.-Y., Cowan, J.E., Cary, K.C., Chan, J.M., Carroll, P.R., Cooperberg, M.R., 2013. Limited ability of existing nomograms to predict outcomes in men undergoing active surveillance for prostate cancer. *BJU Int.* doi:10.1111/bju.12554
- Wang, Y., Xia, X.-Q., Jia, Z., Sawyers, A., Yao, H., Wang-Rodriguez, J., Mercola, D., McClelland, M., 2010. In silico estimates of tissue components in surgical samples based on expression profiling data. *Cancer Res.* 70, 6448–6455. doi:10.1158/0008-5472.CAN-10-0021
- Yilmaz, M., Maass, D., Tiwari, N., Waldmeier, L., Schmidt, P., Lehembre, F., Christofori, G., 2011. Transcription factor Dlx2 protects from TGFβ-induced cell-cycle arrest and apoptosis. *EMBO J.* 30, 4489–4499. doi:10.1038/emboj.2011.319
- Zellweger, T., Günther, S., Zlobec, I., Savic, S., Sauter, G., Moch, H., Mattarelli, G., Eichenberger, T., Curschellas, E., Rüfenacht, H., Bachmann, A., Gasser, T.C., Mihatsch, M.J., Bubendorf, L., 2009. Tumour growth fraction measured by immunohistochemical staining of Ki67 is an independent prognostic factor in preoperative prostate biopsies with small-volume or low-grade prostate cancer. *Int. J. Cancer J. Int. Cancer* 124, 2116–2123. doi:10.1002/ijc.24174

Titles and Legends to Figures

Figure 1: Examples of positive Ki67 (a) and DLX2 (b) nuclear prostate cancer staining compared to tumours that did not express Ki67 (c) or DLX2 (d)

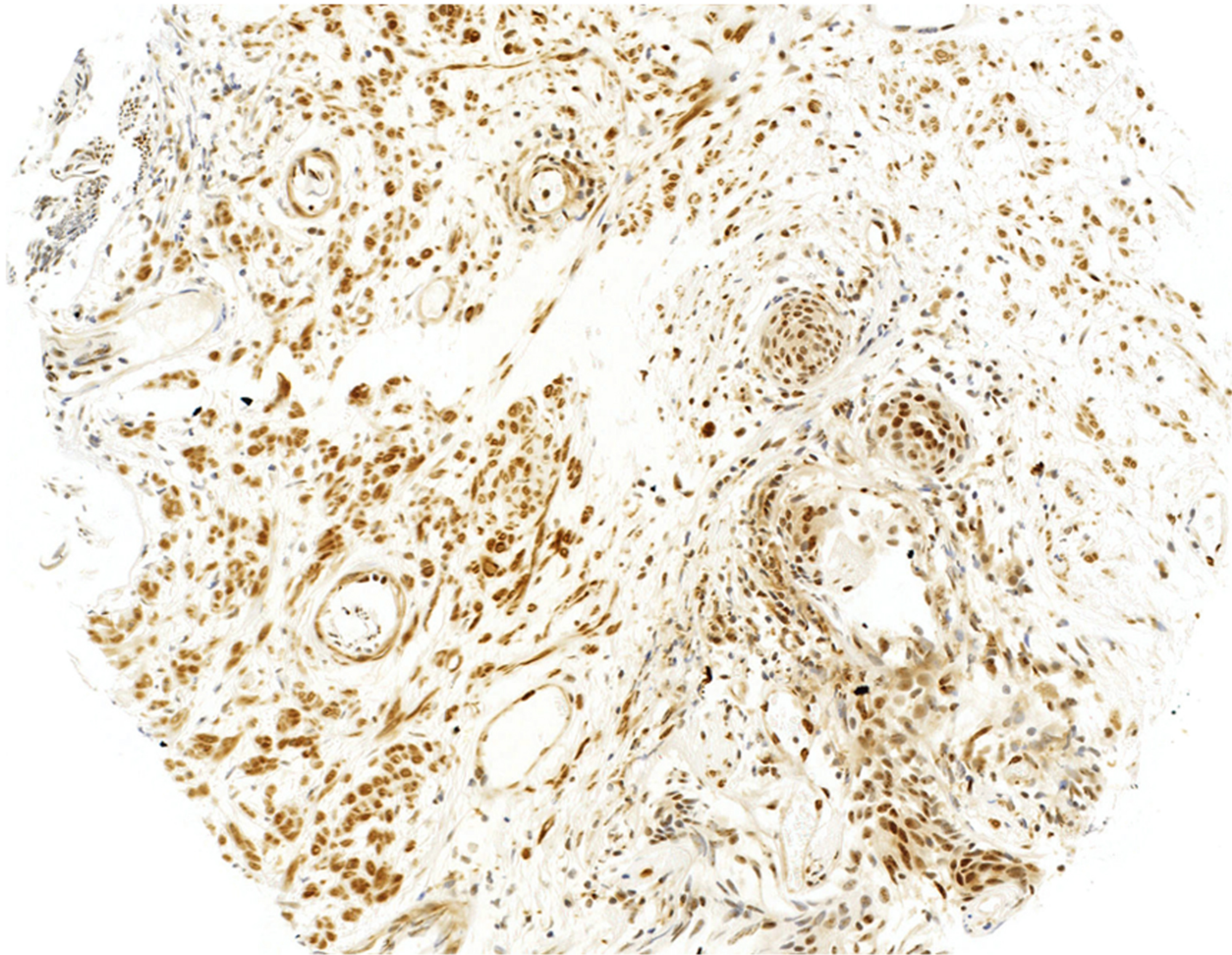
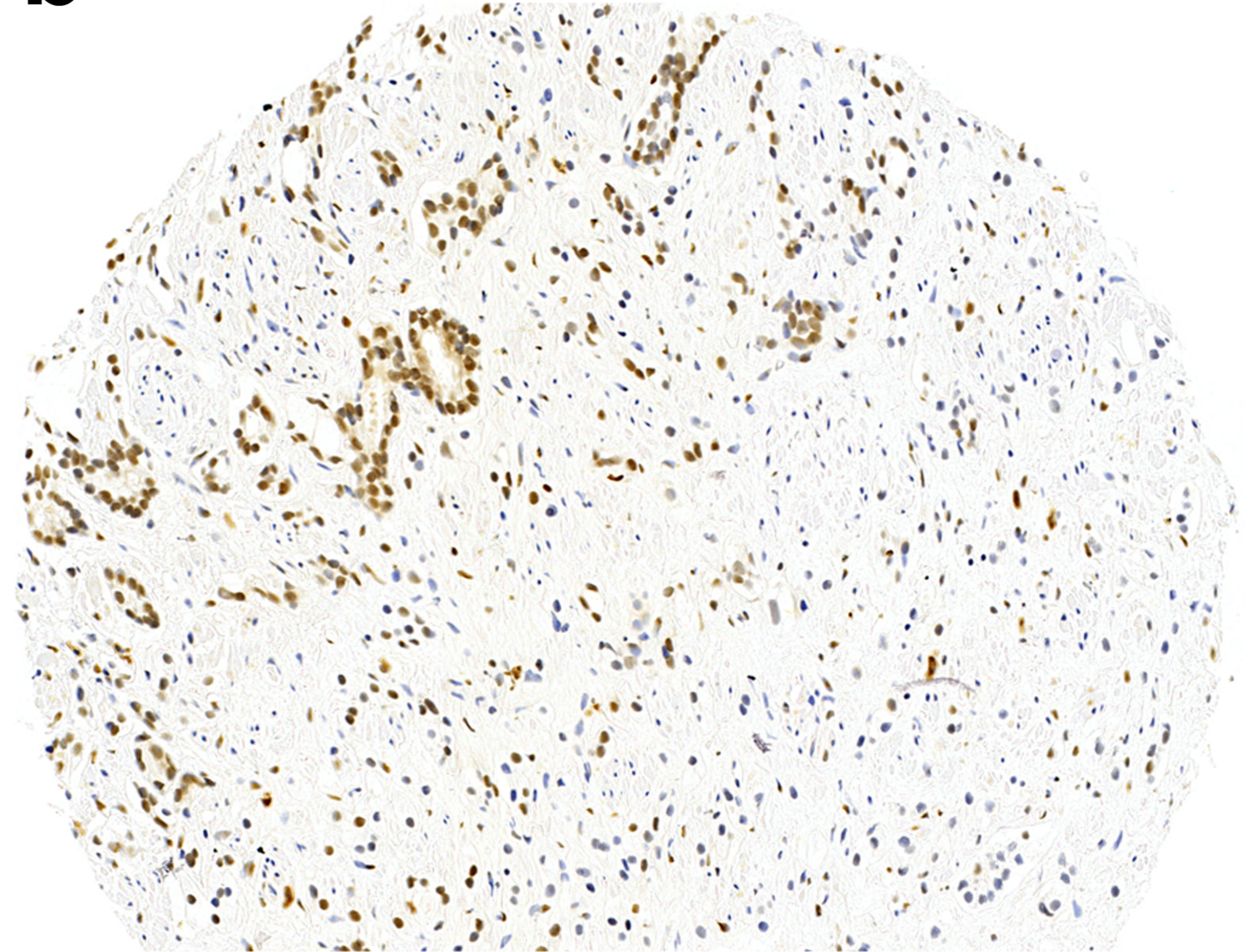
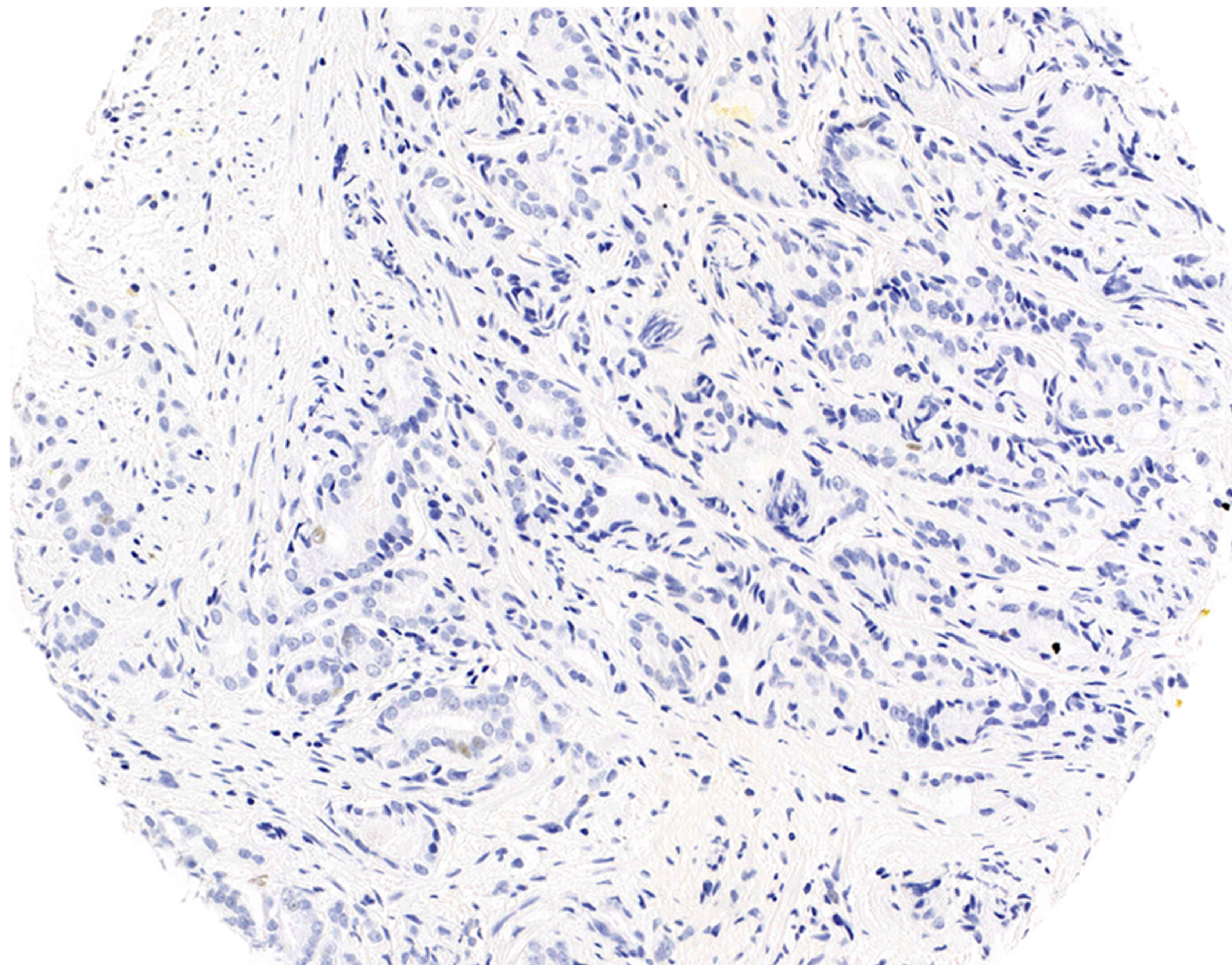
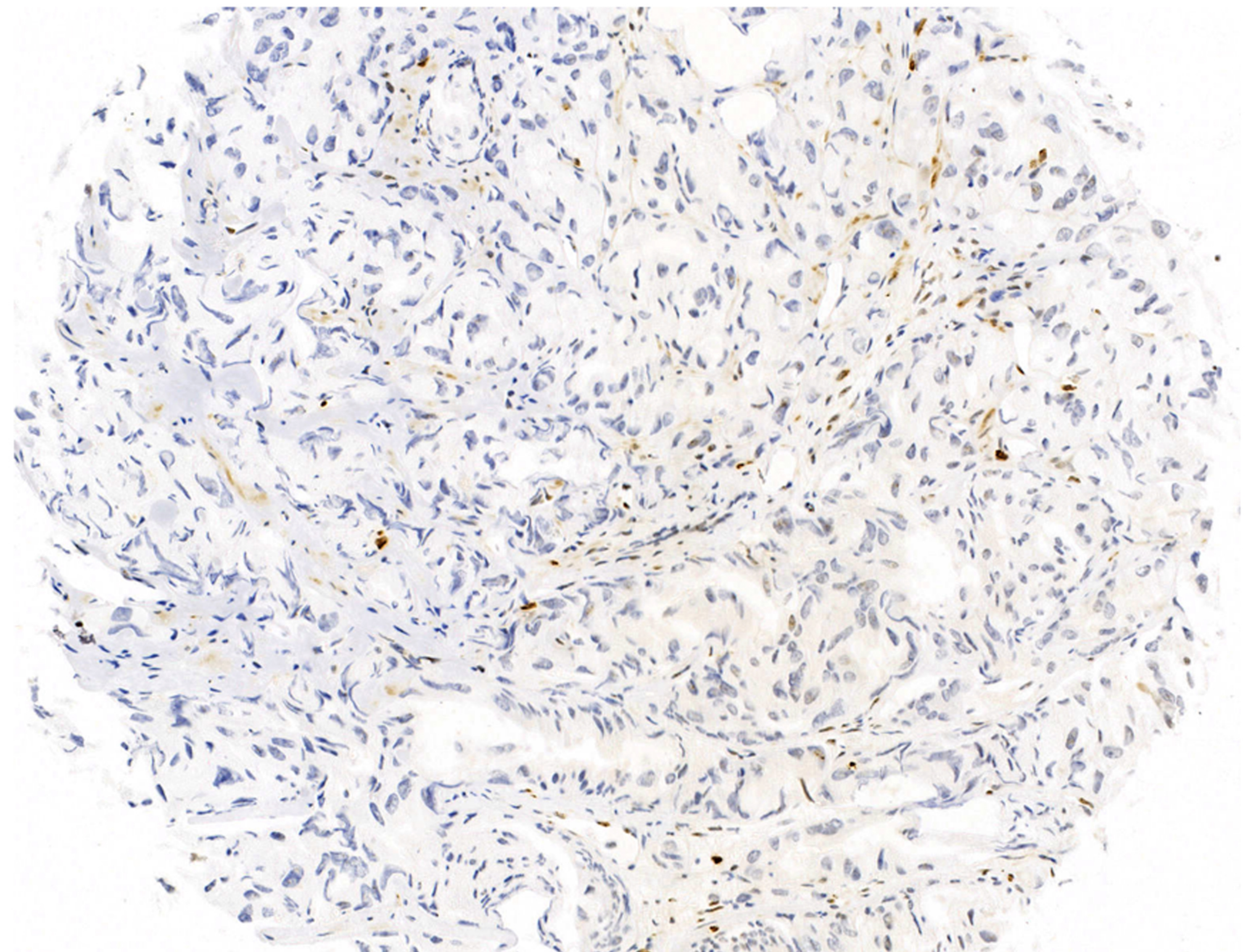
Figure 2: Kaplan–Meier plots demonstrating a) Prostate specific cancer death over time according to Ki-67 score; b) Metastasis development over time according to Ki-67 score; c) Prostate specific cancer death over time according to DLX2 score d) Metastasis development over time according to DLX2 score.

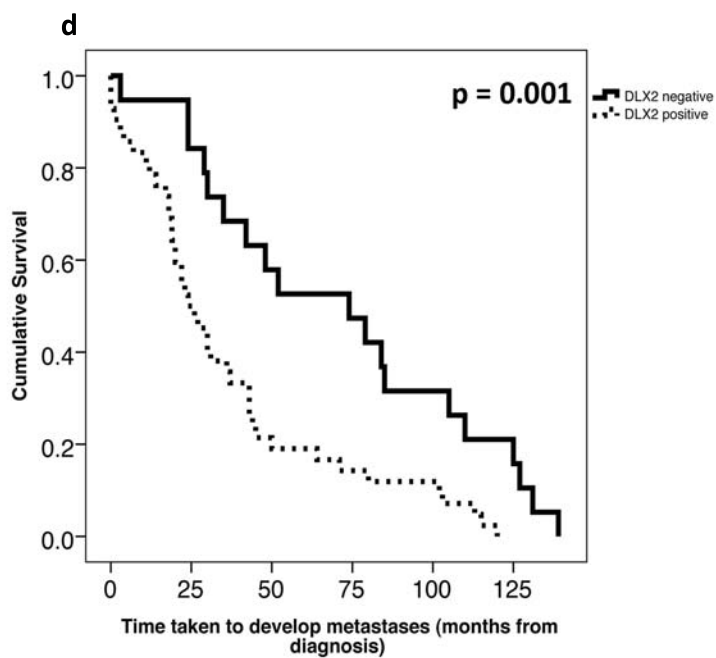
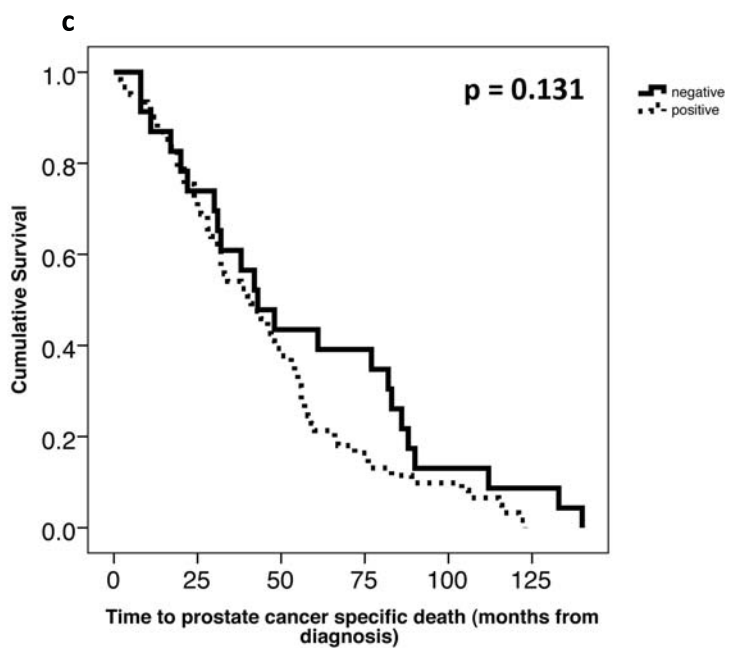
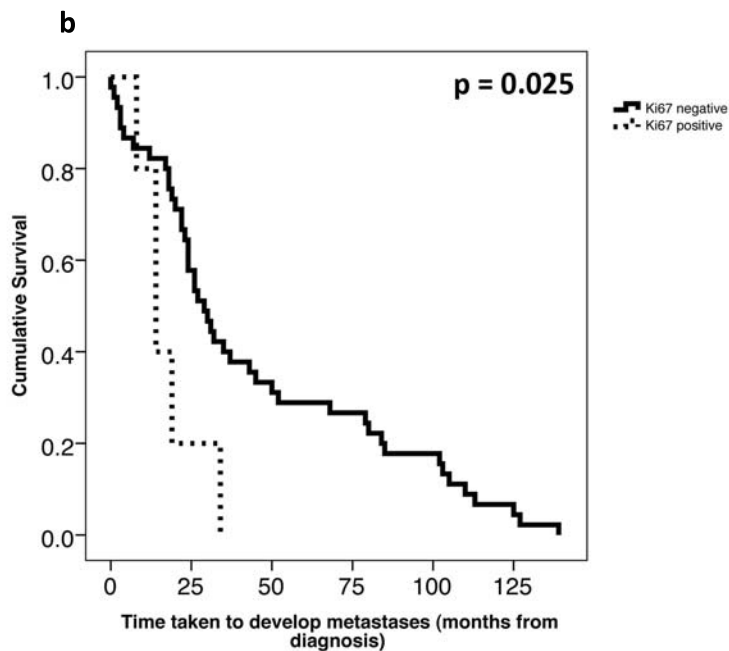
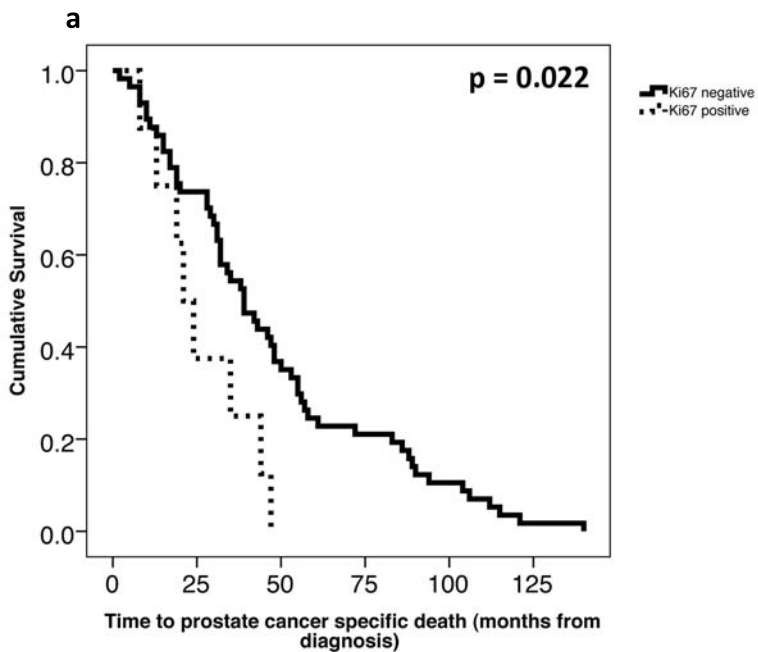
Table 1: ANN ranked gene list showing association with prostate cancer metastasis

Table 2: The number of patients within the prostate cancer cohort that were dichotomously categorised for each biomarker

Table 3: Characteristics of prostate cancer patients incorporated in the TMA

Table 4: Multivariate analysis was performed to assess the predictiveness of Ki67 and DLX2 compared to PSA concentration, Gleason groups and D'Amico risk.

a**b****c****d**



Tables 1-4

Table 1

Rank	Gene accession number	Gene name	Gene Product (protein)
1	AK022765.1	AMACR	Alpha-methylacyl-CoA racemase
2	AI796120	AMACR	Alpha-methylacyl-CoA racemase
3	AF047020.1	AMACR	Alpha-methylacyl-CoA racemase
4	NM_004405.2	DLX2	Distal-less homeo box 2
5		PCA3	Prostate cancer antigen 3
6	NM_012485.1	HMMR	Hyaluronan-mediated motility receptor (RHAMM or CD168)
7	U90236.2	MYO6	Myosin VI
8	NM_017636.1	FLJ20041	Hypothetical protein FLJ20041 Alias: TRPM4B
9	BF511718	RHO7	GTP-binding protein Rho7 Alias: RND2
10	NM_006452.1	ADE2H1	Multifunctional polypeptide similar to SAICAR synthetase and AIR carboxylase Alias: PAICS
11	NM_002570.1	PACE4	Paired basic amino acid cleaving system
12	NM_004503.1	HOXC6	Homeo box C6

Table 2

Biomarker (score)	Number of cancer patients scored	Number positive (%)	Number negative (%)
Ki67 (>110)	161	11 (6.8)	150 (93.2)
DLX2 (>10)	185	135 (73)	50 (27)

Table 3

Clinical variable		Number of patients (%)	Chemotherapy	Androgen depletion therapy including orchidectomy (% all patients)	Radiotherapy (% all patients)
PSA (ng/ml) at diagnosis	≤4	18 (9.4)			
	>4	168 (87.5)			
	Not recorded	6 (3.125)			
Gleason Score	Group 1: ≤6	7 (3.6)	1 (0.52)	6 (3.1)	0
	Group 2: 3+4	22 (11.5)	5 (2.6)	4 (2)	0
	Group 3: 4+3	31 (16.1)	5 (2.6)	6 (3.1)	2 (1)
	Group 4: 8	42 (21.9)	9 (4.7)	8 (4.1)	0
	Group 5: 9-10	90 (46.9)	43 (22.4)	30 (15.6)	3 (1.5)
Metastasis	At diagnosis	32 (16.6)			
	Subsequent metastasis	73 (38)			
	Never developed metastasis	87 (45.3)			
Death due to prostate cancer	Yes	105 (54.7)			
	No	55 (28.6)			
	Still alive	20 (10.4)			
	Not recorded	12 (6.2)			

Table 4

Variable	Significance	Hazard ratio	95% Confidence interval	
			Lower	Upper
Ki67 Survival prediction				
Ki67	0.029	2.436	1.096	5.416
PSA concentration	0.290	1.00	1.000	1.000
Gleason categories	0.936	1.018	0.659	1.572
D'Amico risk	0.964	0.919	0.347	2.433
Ki67 Metastasis prediction				
Ki67	0.023	3.296	1.181	9.196
PSA concentration	0.131	1.001	1.000	1.002
Gleason categories	0.146	1.469	0.874	2.469
D'Amico risk	0.325	0.600	0.217	1.660
DLX2 Metastasis prediction				
DLX2	0.003	3.051	1.451	6.418
PSA concentration	0.202	1.002	0.999	1.004
Gleason categories	0.768	1.081	0.643	1.819
D'Amico risk	0.827	0.891	0.316	2.511