Optimised ARID1A immunohistochemistry is an accurate predictor of ARID1A mutational status in gynaecological cancers

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Abstract

ARID1A is a tumour suppressor gene that is frequently mutated in clear cell and endometrioid carcinomas of the ovary and endometrium and is an important clinical biomarker for novel treatment approaches for patients with ARID1A defects. However, the accuracy of ARID1A immunohistochemistry (IHC) as a surrogate for mutation status has not fully been established for patient stratification in clinical trials. Here we tested whether ARID1A IHC could reliably predict ARID1A mutations identified by next-generation sequencing. Three commercially available antibodies – EPR13501 (Abcam), D2A8U (Cell Signaling), and HPA005456 (Sigma) – were optimised for IHC using cell line models and human tissue, and screened across a cohort of 45 gynaecological tumours. IHC was scored independently by three pathologists using an immunoreactive score. ARID1A mutation status was assessed using two independent sequencing platforms and the concordance between ARID1A mutation and protein expression was evaluated using Receiver Operating Characteristic statistics. Overall, 21 ARID1A mutations were identified in 14/43 assessable tumours (33%), the majority of which were predicted to be deleterious. Mutations were identified in 6/17 (35%) ovarian clear cell carcinomas, 5/8 (63%) ovarian endometrioid carcinomas, 2/5 (40%) endometrial carcinomas, and 1/7 (14%) carcinosarcomas. ROC analysis identified greater than 95% concordance between mutation status and IHC using a modified immunoreactive score for all three antibodies allowing a definitive cut-point for ARID1A mutant status to be calculated. Comprehensive assessment of concordance of ARID1A IHC and mutation status identified EPR13501 as an optimal antibody, with 100% concordance between ARID1A mutation status and protein expression, across different gynaecological histological subtypes. It delivered the best inter-rater agreement between all pathologists, as well as a clear cost-benefit advantage. This could allow patients to be accurately stratified based on their ARID1A IHC status into early phase clinical trials.

Keywords: ARID1A immunohistochemistry; clear cell ovarian cancer; next generation sequencing; biomarker

Introduction

Recent studies have highlighted that somatic loss of function mutations in the tumour suppressor gene ARID1A underpin 57% of ovarian clear cell carcinomas (OCCC) [1,2], 40% of uterine endometrioid carcinomas [3] and between 20 and 36% of uterine carcinosarcomas [4–6]; however, they are rare in high-grade serous ovarian carcinoma (HGSOC) [1,2]. HGSOCs are underpinned by TP53 mutations, where p53 immunohistochemistry...
ARID1A is routinely used in clinical practice to aid diagnosis [7]. However, TP53 mutations are uncommon in OCCC; these differences in driver mutational profiles highlight the distinct aetiology of the two diseases. Moreover, the presence of ARID1A missense mutations may not alter ARID1A protein expression, unlike TP53 missense mutations. Loss or significant reduction of ARID1A protein expression is associated with heterozygous ARID1A mutations [1,8,9], suggesting a dominant-negative tumour suppressor role (see [10] for a comprehensive review). ARID1A forms a key DNA binding subunit in the ATP-dependent Switch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodelling complex that modulates the winding of DNA around histone cores allowing access to the DNA to enable transcription, DNA repair, and replication [11,12]; loss of function of this protein leads to aberrant cell cycle and loss of proliferation control [13]. Two genetically engineered mouse models (GEMM) have been created with ARID1A alterations that led to tumour formation with loss of ARID1A protein expression observed by IHC. The first was an ovarian endometrioid tumour with co-existent PTEN loss [14] and, more recently, a GEMM harbouring both ARID1A loss and a PIK3CA (H1047R) mutation promoted ovarian clear-cell tumourigenesis [15]. ARID1A mutations are also detected at high frequencies in other solid tumours including 23% of pancreatic ductal adenocarcinoma, 23% of advanced urothelial carcinomas, 18% hepatocellular carcinomas, and 6% of metastatic castrate-resistant prostate cancers [16–20].

Novel ways of treating patients with ARID1A mutations have focused largely on using synthetic-lethal approaches. Bitler et al highlighted the potential of targeting the antagonistic activity between SWI/SNF and EZH2 methyltransferase with the EZH2 small molecule inhibitor GSK126, which triggered apoptosis in ARID1A mutated cells [21]. This was mediated via upregulation of PIK3IP1, a direct target of EZH2, and selectivity was further enhanced upon inhibition of PI3K-AKT signalling [21]. Subsequent work by Bitler et al has shown that ARID1A-mutated ovarian cancers are selectively dependent on HDAC6 activity, due to HDAC6 upregulation in ARID1A mutant cells that mechanistically inactivates the apoptosis-promoting function of TP53 due to deacetylation of histone lysine 120 [22]. The work showed that treating ARID1A-mutated tumours with the small molecule HDAC6 inhibitor, ACY1215, showed a survival benefit in vivo. Additional approaches identified reliance on the ARID1A parologue ARID1B in mutant cells [23]. Our group has found ARID1A defective OCCC tumours can be targeted with the multikinase inhibitor dasatinib, mediated through addiction to the dasatinib target YES1 [9] and more recently a profound sensitivity to inhibition of the DNA repair kinase ATR, leading to premature mitotic entry, genmic instability, and apoptosis [24]. Shen et al have additionally shown that loss of ARID1A leads to impaired G2-M DNA damage checkpoint activation and repair of DNA double strand breaks (DSB), causing sensitivity to DSB inducing treatments such as the Poly (ADP-ribose) polymerase (PARP) inhibitor talazaparib, both in vitro and in vivo [25]. Together, these observations open up the possibility of assessing these therapeutic approaches in clinical trials, in particular for tumour types with high frequencies of ARID1A mutations, such as OCCC.

Currently there are no clinical trials recruiting patients that prospectively assess ARID1A mutational status. However, there are three early phase trials investigating ARID1A mutational status and response to therapy, the first of which allocates treatment to patients whose biopsies are sequenced as part of ongoing clinical sequencing programmes outside of the remit of the clinical trial [26]. Patients with PIK3CA, AKT, or ARID1A mutations will receive olaparib with the AKT inhibitor AZD5363 [26]. A second is a randomised phase II study of nintedanib (an oral tyrosine kinase inhibitor targeting VEGF receptors 1–3, FGFR 1–3, and PDGFR α and β) compared to chemotherapy in patients with clear cell carcinoma of the ovary or endometrium, which will assess ARID1A mutational status retrospectively and correlate with outcome [27]. A third trial, which is ongoing but not currently recruiting, will assess dasatinib in patients with recurrent or persistent ovarian, Fallopian tube, endometrial, or peritoneal carcinoma and will retrospectively compare ARID1A mutational and IHC status [28]. These trials highlight that prospectively assessing ARID1A mutational status is potentially cost-prohibitive and the turn-around time can make it difficult for trial recruitment. Therefore, a surrogate biomarker of mutational status such as IHC is needed.

Despite the clinical importance of ARID1A mutations, rapid sequencing is not widely available. Hence, IHC would be the commonest method to infer mutational status, highlighted by the fact that a number of open clinical trials are performing retrospective ARID1A mutational assessment. Studies that have so far assessed the correlation between ARID1A mutational status and IHC have demonstrated a good concordance [1,3,29]. However, the accuracy of IHC as a predictor of ARID1A mutation in ovarian carcinoma has not been precisely defined as there is no uniform scoring system or specific antibody that is recommended for clinical IHC use. In the largest concordance study to date, Wiegand et al assessed ARID1A in 182 gynaecological tumours, defining positive staining as definitive nuclear staining and...
negative as no immunoreactivity [1]. There was a statistically significant correlation between the loss of ARID1A protein expression and ARID1A mutational status in both OCCC and endometrioid carcinomas [1]. In total, 73% of OCCC (27/37 cases) with a known ARID1A mutation showed loss of ARID1A expression. However, 11% of OCCC (4/36 cases) with no ARID1A mutation also showed loss of ARID1A protein expression [1]. Of note this study used the mouse clone 3H2 (Abgent, CA), which targets a region of 111 amino acids (aa 1216 to 1326) but is no longer commercially available. Patients with recurrent or metastatic disease who will be entering the growing number of clinical trials where a robust assessment of ARID1A protein expression in the tumour could be informative will require a test that is accurate and reproducible with a fast turn-around time.

Here our aims were to develop ARID1A IHC as a surrogate predictive biomarker for diagnostic assessment of ARID1A mutational status in gynaecological tumours. In particular, we sought to compare the concordance of a number of commercially available antibodies using a standardised scoring system and identify the most optimal assay for clinical assessment of mutation status.

Materials and methods

Cell lines

Cell lines ES2 and TOV21G were obtained from the American Type Tissue Collection (ATCC). HCT116 isogenic ARID1A (Q456*/Q456*) and parental lines were purchased from Horizon Discovery (Cambridge, UK). These were developed by knock-in of a premature stop codon (Q456*). Cell lines were cultured in a humidified 37 °C incubator with 5% CO₂. Cell lines were tested to confirm no mycoplasma infection using Mycoalert™ Mycoplasma Detection Kit as per manufacturer’s instructions (Lonza, Slough, UK). Cell line identity was confirmed with short tandem repeat typing using the Promega GenePrint® system (Promega, Southampton, UK). Cell pellets were formalin fixed and paraffin-wax embedded (FFPE) for antibody optimisation.

Clinical samples

All patients gave written consent for the use of material for research purposes and tissue samples were obtained with appropriate ethical approval under the Royal Marsden Hospital (RMH) NHS Foundation Trust study: CCR3705 “Analysis of tumour specimens for biomarkers in gynaecological cancers” (Table 1 and supplementary material, Table S1). All patient samples were reviewed at RMH and appropriate FFPE tissue blocks were selected from their histology reports. Haematoxylin and eosin (H&E) sections were reviewed by a pathologist (DK) to confirm appropriate tumour tissue and content. Five thick (8 μm) sections were cut for DNA extraction, with an additional H&E slide and three unstained sections for ARID1A IHC. Whole serial sections were cut from the same diagnostic block to minimise heterogeneity between analysis for the ARID1A IHC and next-generation sequencing (NGS). If no germline blood sample was available, then non-malignant FFPE blocks were obtained and sections cut for DNA extraction.

DNA extraction and library preparation

DNA extraction and NGS took place in Good Clinical Laboratory Practice (GCLP)-accredited laboratories at
The Centre for Molecular Pathology, The Royal Marsden NHS Foundation Trust, Sutton. Genomic DNA from FFPE tissue sections was extracted using QIAamp FFPE Tissue Kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions for both tumour and non-malignant content. Genomic DNA from blood was extracted using the QIAamp Blood mini kit (manual) or QIAasymphecy DNA Midi Kit (automated) (Qiagen) according to the manufacturer’s instructions. DNA quality was assessed on the Agilent 2200 Tapestation (Agilent, Stockport, UK) and the Qubit Fluorometer (Fisher Scientific, Loughborough, UK).

Targeted ARID1A sequencing

ARID1A mutations were identified using a targeted capture panel ( Nimblegen, Roche, Welwyn Garden City, UK), designed to target 59 genes for the FORMAL clinical trial (Feasibility of Molecular Characterization Approach to Treatment, CCR3994, Royal Marsden NHS Hospital, Foundation Trust). The panel typically covers ARID1A at 99% >250X (supplementary material, Table S2). Individual sample library preparation was performed using the KAPA Biosystem HyperPlus kit using 50–200 ng DNA. Pooling and capture steps were performed using the Nimblegen SeqCap Capture Protocol (Roche). KAPA Library Quant Kit Universal qPCR Mix (Illumina), was used to quantify the libraries before combining in equimolar concentrations prior to sequencing on the Illumina MiSeq. A high confidence call covered >95% of a region; medium confidence >85% and a failure if the coverage was <85% coverage. MiSeq Reporter software (v2.5.1) was used to align sequences to version hg19 of the human genome using aligner: BWA v0.61 and Somatic Caller v3.5.2.1 to call variants alongside in-house web server 1.0 and 2.0 software. Variants were called at a frequency of 5% and present in at least 5 reads, with a minimum read depth of 10 reads. Mutations were manually visualized in the Integrated Genomics Viewer. All mutations were validated using a custom AmpliSeq panel (Thermo Fisher, Wilmington, USA) and run on the Ion Proton panel (Thermo Fisher) with 10 ng input DNA. Sequencing data were analysed using the Ion Torrent software suite (version 5.2.2). Sequences were aligned to version hg19 of the human genome and mutations were called using the variantCaller (version v5.2.0.34). For pairs of tumour-normal samples, mutation calls were intersected using vcf-icsec (VCFtools version 0.1.10) and filtered to retain somatic mutations. Oncotator and SnpEff (version 3.3h) were used to annotate somatic variants reporting the most deleterious effect. Further annotations were added to each mutation using ANNOVAR and the following databases: ClinVar, COSMIC, dbnsfp33a, clinvar_20170130, ljb23_ma, exac03, and exac03nontcga. Only those mutations that were confirmed with both sequencing platforms were included in the final concordance analysis. Raw targeted sequencing data have been deposited into the NCBI Sequence Read Archive under the accession PRJNA432413 and PRJNA432343.

ARID1A immunohistochemistry

IHC was performed on 3–4 μm thick whole tissue sections. The slides were incubated with antibodies to: Anti-ARID1A, rabbit monoclonal 1:1000, EPR13501 (Abcam, Cambridge, UK), ARID1A/BAF250A, rabbit monoclonal 1:250, D2A8U (Cell Signaling Technology Europe, Leiden, The Netherlands), and anti-ARID1A, rabbit polyclonal 1:400, HP A005456 (Sigma-Aldrich, Gillingham, Dorset, UK), using the Dako-Autostainer Link 48 with the EnVision FLEX kit as per manufacturer’s instructions (Agilent Technologies, Cheddle, Cheshire, UK). Human breast, prostate, and kidney tissues were used as positive controls and xenograft models were obtained as previously described [24].

Cases were independently scored using an immunoreactive scoring system [30] by three pathologists, DK (Pathologist 1) KN (Pathologist 2), and AA (Pathologist 3), who were blinded to the sequencing results. Sections were evaluated for both intensity (0 = negative, 1 = weak staining; 2 = moderate; 3 = strong) and proportion of positively stained cells expressed as a percentage (0 = 0%; 1+ ≤10%; 2+ = 11–50%; 3+ = 51–80%; 4+ >80%). The intensity and proportion of stained cells were multiplied to produce the final score between 0 and 12 [30]. Stromal cells were used as an internal positive control. The pathologists’ scores were averaged to give a combined score used in further analyses. An IHC score “cut-off” for loss of expression was defined in conjunction with the genomic deleterious mutation results for each antibody using receiver operating characteristic (ROC) statistical analysis. Fleiss kappa statistics were used to assess interrater variability [31].

Results

ARID1A IHC optimisation shows nuclear immunoreactivity in ARID1A wild-type cases and absence in ARID1A mutant cases

We first evaluated three ARID1A antibodies based upon their current availability and recent use in the
| Patient identifier | Diagnosis | IHC ARID1A antibody EPR13501 average score (Pathologists 1, 2, and 3) | IHC ARID1A antibody D2A8U average score (Pathologists 1, 2, and 3) | IHC ARID1A antibody HPA005456 average score (Pathologists 1, 2, and 3) | IHC: ARID1A protein loss | Sequencing: ARID1A mutation and protein change |
|-------------------|-----------|-------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------|--------------------------|-------------------------------------------------|
| 3705-0442         | CS        | 12                                                                 | 12                                                                 | 10                                                                 | No                       | No                                                             |
| 3705-0449         | CS        | 12                                                                 | 11                                                                 | 10                                                                 | No                       | No                                                             |
| 3705-0456         | CS        | 10                                                                 | 11                                                                 | 9                                                                  | No                       | No                                                             |
| 3705-0470         | CS        | 12                                                                 | 8                                                                  | 10                                                                 | No                       | No                                                             |
| 3705-0484         | CS        | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0500         | CS        | 5                                                                  | 5                                                                  | 4                                                                  | Yes                      | p.Tyr1377Ter, p.Glu1542Ter                                    |
| 3705-0510         | CS        | 12                                                                 | 12                                                                 | 8                                                                  | No                       | No                                                             |
| 666179            | ECC       | 12                                                                 | 11                                                                 | 8                                                                  | No                       | No                                                             |
| 3705-0142         | EA0       | 7                                                                  | 1                                                                  | 4                                                                  | Yes                      | p.Pro7fs                                                       |
| 3705-0341         | EA0       | 12                                                                 | 11                                                                 | 9                                                                  | No                       | No                                                             |
| 3705-0481         | EA0       | 0                                                                  | 0                                                                  | 2                                                                  | Yes                      | p.Pro1135fs                                                    |
| 3705-0541         | EA0       | NA                                                                 | NA                                                                 | NA                                                                 | NA                       | p.Gln428Ter                                                    |
| 3705-0323         | EA0       | 3                                                                  | 3                                                                  | 6                                                                  | Yes                      | p.Gln512Ter, p.Arg1899Ter                                     |
| 3705-0460         | EA0       | 1                                                                  | 1                                                                  | 3                                                                  | Yes                      | p.Arg1505Ter                                                   |
| 3705-0482         | EA0       | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0529         | EA0       | 12                                                                 | 12                                                                 | 9                                                                  | No                       | No                                                             |
| 3705-0199         | EAE       | 3                                                                  | 2                                                                  | 1                                                                  | Yes                      | p.Ile1975fs, p.Gln799fs                                       |
| 3705-0438         | EAE       | 1                                                                  | 1                                                                  | 1                                                                  | Yes                      | p.Arg1504Ter                                                   |
| 3705-0548         | EAE       | 12                                                                 | 11                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0553         | DCE       | 9                                                                  | 6                                                                  | 7                                                                  | No                       | No                                                             |
| 3705-0475         | HGSOC     | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0487         | LGSOC     | 11                                                                 | 12                                                                 | 11                                                                 | No                       | No                                                             |
| 3705-0493         | LGSOC     | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0525         | LGSOC     | 11                                                                 | 11                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0501         | MAOE      | 12                                                                 | 11                                                                 | 12                                                                 | No                       | NA                                                             |
| 3705-0308         | MAO       | 12                                                                 | 12                                                                 | 7                                                                  | No                       | No                                                             |
| 3705-0145         | OCCC      | 0                                                                  | 3                                                                  | 2                                                                  | Yes                      | p.Ser614Ter                                                    |
| 3705-0207         | OCCC      | 12                                                                 | 10                                                                 | 8                                                                  | No                       | No                                                             |
| 3705-0346         | OCCC      | 2                                                                  | 1                                                                  | 3                                                                  | Yes                      | p.Ala42fs, p.Gln723Ter                                       |
| 3705-0379         | OCCC      | 12                                                                 | 11                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0383         | OCCC      | 12                                                                 | 11                                                                 | 11                                                                 | No                       | No                                                             |
| 3705-0416         | OCCC      | 0                                                                  | 1                                                                  | 2                                                                  | Yes                      | p.Gly191fs, p.Tyr485Ter                                       |
| 3705-0435         | OCCC      | 11                                                                 | 12                                                                 | 8                                                                  | No                       | No                                                             |
| 3705-0453         | OCCC      | 12                                                                 | 12                                                                 | 9                                                                  | No                       | No                                                             |
| 3705-0464         | OCCC      | 1                                                                  | 1                                                                  | 1                                                                  | Yes                      | p.Gly1848fs, p.Ile2275Ser, p.Gln2070Ter                       |
| 3705-0468         | OCCC      | 12                                                                 | 11                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0497         | OCCC      | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0514         | OCCC      | 12                                                                 | 11                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0540         | OCCC      | 1                                                                  | 0                                                                  | 1                                                                  | Yes                      | p.Gln932fs                                                    |
| 3705-0544         | OCCC      | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0545         | OCCC      | 0                                                                  | 0                                                                  | 3                                                                  | Yes                      | p.Gly319fs, p.Leu1100fs                                       |
| 3705-0558         | OCCC      | 11                                                                 | 11                                                                 | 11                                                                 | No                       | No                                                             |
| 3705-0435*        | OCCC      | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0466         | SCCOHT    | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |
| 3705-0551         | SCCOHT    | 12                                                                 | 12                                                                 | 12                                                                 | No                       | No                                                             |

CS, carcinosarcoma of the ovary; DCE, de-differentiated carcinoma of the endometrium; ECC, endometrial clear cell; EAE, endometrioid adenocarcinoma of the endometrium; EA0, endometrioid adenocarcinoma of the ovary (encompassed within ENOC, endometrioid ovarian carcinoma); LGSOC, low grade serous ovarian carcinoma; HGSOC, high grade serous ovarian carcinoma; SCCOHT, small cell carcinoma of the ovary, hypercalcaemic type; MAOE, mesonephric adenocarcinoma of the ovary and endometrium; MAO, mesonephric adenocarcinoma of the ovary; NA, not available; OCCC, ovarian clear cell carcinoma.

*Matched patient lung metastasis.
literature [24,32,33]; Abcam EPR13501 monoclonal antibody, Cell Signaling D2A8U monoclonal antibody, and Sigma HPA005456 polyclonal antibody, hereafter abbreviated to clone details (Figure 1A). Antibodies were optimised on human tissue, HCT116 ARID1A isogenic (mutant and wild-type) cell lines, Figure 1.
ES2 (ARID1A wild-type), TOV21G (ARID1A mutant) ovarian clear cell carcinoma cell lines, and in HCT116 ARID1A isogenic xenograft models (Figure 1B-D). These cell lines are known to either express or not express ARID1A as previously evaluated by western blot (supplementary material, Figure S1 and [24]). Antibodies were diluted accordingly to ensure a contrast between mutant and wild-type cell lines. ARID1A immunoreactivity was detected in the nucleus, in both malignant epithelial tumour and stromal cells, which were used as a positive internal control in all samples. Of all three antibodies tested, D2A8U showed the strongest immunoreactivity in the cell line models (Figure 1C), with background staining most visible with HPA005456.

We next evaluated ARID1A protein expression in a cohort of 45 gynaecological cancers with all three antibodies (Figure 2 and Table 1). All three antibodies demonstrated ARID1A immunoreactivity and performed well on archival tissue, with the oldest block evaluated from 2005 (3705-0481) and the most recent from 2016 (666179) (Table 2 and supplementary material, Table S3). One case (3705-0541) was not fixed appropriately at the time of resection and we were unable to process it for IHC, although we were able to extract good quality DNA. Twenty-four cases scored a maximum immunoreactive score of 12 with EPR13501, compared to 16 with D2A8U and 13 with HPA005456. Four cases scored 0 with EPR13501, three cases scored 0 with D2A8U and none scored 0 with HPA005456 (Table 2). The scoring concordance of the antibodies between the pathologists varied, with EPR13501 showing the best inter-rater agreement of 0.78, followed by D2A8U (0.67) and HPA005456 (0.57), Fleiss’ kappa statistics [31] (supplementary material, Table S4).

Endometriosis-related carcinomas show enrichment of ARID1A mutations

ARID1A mutations were next evaluated using a 59-gene targeted DNA capture panel, followed by NGS with a median depth of 699X for ARID1A, (mean depth range of ARID1A 1117X-2711X) (Figure 2 and supplementary material, Table S2). We were able to

Figure 1. ARID1A antibody optimisation in tumour cell lines and tumour xenograft models. (A) Schematic of the ARID1A protein illustrating epitope regions of all three antibodies studied. The lollipop plot shows mutation loci of two cell lines used in antibody optimisation: HCT116 ARID1A isogenic mutant cell line Q456*/Q456*, nonsense mutation (depicted in orange); and TOV21G, a compound heterozygous cell line with mutation loci TOV21G p.548fs and p.756fs, frameshift mutations (depicted in mauve). (B) Immunoreactivity of ARID1A detected by all three antibodies in HCT116 ARID1A isogenic cell lines embedded in FFPE blocks. HCT116 –/– (ARID1A wild-type) cell line. EPR13501, rabbit monoclonal, antigen retrieval using microwave and dilution 1:1000. D2A8U, rabbit monoclonal, antigen retrieval with pretreatment module and dilution 1:250. HPA005456, rabbit polyclonal, antigen retrieval with pretreatment module and dilution 1:400. Scale bar is equal to 100 μm. (C) ARID1A immunoreactivity in ovarian clear cell carcinoma cell lines. TOV21G (ARID1A mutant) shows loss of ARID1A immunoreactivity whereas nuclear immunoreactivity was preserved in the ES2 (ARID1A wild-type) cell line. Scale bar is equal to 100 μm. (D) ARID1A immunoreactivity was detected with all three antibodies in xenograft models of HCT116 –/+ with loss of ARID1A expression in HCT116 –/– xenograft models. The background staining seen in cell lines was reduced in xenografts. Scale bar is equal to 100 μm.

Figure 2. Study workflow. Modified CONSORT diagram showing the processing of the 45 gynaecological cancer cases identified. After histopathology review, a representative block was chosen. The same FFPE block was used for extracting DNA for next-generation sequencing (NGS) and IHC. One case failed NGS quality control and one case was not suitable for IHC analysis. Three histopathologists independently reviewed and scored cases. These were then averaged and compared to mutational status to establish concordance (n = 43).
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Table 3. ROC curve analysis to define mutant immunoreactive score and concordance

| Antibody | All gynaecological cases: ARID1A mutant score | Concordance (%) | OCCC cases: ARID1A mutant score | Concordance (%) |
|----------|---------------------------------------------|-----------------|---------------------------------|-----------------|
| EPR13501 | <8 100% sensitivity and 100% specificity     | 100             | <6.5 100% sensitivity and 100% specificity | 100             |
| D2A8U    | <5.5 100% sensitivity and 100% specificity  | 100             | <6.5 100% sensitivity and 100% specificity | 100             |
| HPA005456| <6.5 100% sensitivity and 96% specificity    | 97              | <5.5 100% sensitivity and 100% specificity | 100             |

Discussion

In this study, we assessed the concordance of three commercially available antibodies for the assessment of ARID1A protein expression as a surrogate biomarker of mutation status. Overall, we found that IHC is an excellent surrogate biomarker of loss of function mutational status and were able to establish thresholds with each antibody to reliably identify mutant cases to be used in prospective patient assessment that improves upon published concordance rates.

Concordance rates for all cases were 100% (EPR13501), 100% D2A8U, and 97% (HPA005456). The histograms for all antibodies show that overall there is a clear bimodal distribution in immunoreactive scores between the mutant and wild-type cases. This is best exemplified with EPR13501 and D2A8U, with HPA005456 showing the greatest variation, perhaps due to the polyclonal nature of the antibody, whereas the D2A8U and EPR13501 are monoclonal. For instance, comparing OCCC cases alone, our concordance was 100% with all antibodies,
Figure 3. Distribution of ARID1A mutations identified by targeted sequencing in gynaecological cancers. (A) A lollipop plot showing the distribution and classes of validated loss of function mutations in ARID1A detected by both sequencing platforms in 44 cases. Twenty-one ARID1A mutations were identified in 14 patients. The majority were frameshift mutations (9, in mauve), with 11 nonsense mutations (orange) and 1 missense mutation (green). (B) Bar chart showing the frequency of ARID1A mutations according to histology. An enrichment of ARID1A mutations was seen in endometriosis-related tumours and carcinosarcomas, with 63% of endometroid ovarian carcinomas (light blue), and 35% of OCCCs (dark blue) having an ARID1A mutation. Endometrial carcinoma includes ECC, EAE and DCE. (C) A VAF plot shows each case with the type of validated mutation (colour of circle) and coverage (size of circle) with corresponding protein expression on IHC (boxes below). Six cases had more than one mutation in ARID1A. Case 3705-0541 was not suitable for IHC processing.
compared to 73% (27/37 samples) as reported by Wiegand et al. [1]. Given that the number of OCCC mutant cases, we assessed is small due to the rarity of cases and the fact that trials combine gynaecological histologies, we propose utilising the cut-offs derived from all gynaecological cases. As all three antibodies had excellent specificity and sensitivity, other factors come into consideration when deciding which antibody to recommend taking forward for potential clinical use. The interpathologist scores were most consistent with EPR13501, and the dilution factor of 1:1000 for EPR13501 compared to D2A8U’s 1:250 means that this would be more cost-effective with less batch-to-batch variation. D2A8U’s staining was most intense in cell line models, but weaker in human tissue, whereas EPR13501 had the strongest intensity in human tissue out of all three antibodies. We investigated HPA005456 as it had been used in a number of recent papers [3,33,35]; however, as it is a rabbit polyclonal antibody, it will not be available for investigators once the stock runs out and is therefore not a long term viable option.

One of the limitations of this study is that even though we identified 45 patient samples initially, we were only able to assess 43 cases. However, the rarity of these tumours in general renders larger series challenging. In order to correct for this, we were stringent with our analysis of the sequencing data, including only cases where mutations were identified by both sequencing platforms. Thus, we could be confident with the mutation calls that we made. Overall, we have shown that IHC is generally concordant with mutational status; we did observe some variation in the carcinosarcoma cases, which may, in

Figure 4. ARID1A IHC shows good concordance with mutational analysis for all three antibodies in a variety of gynaecological cancers. (A) ARID1A immunoreactivity in grade 2 endometrioid adenocarcinoma of the ovary. Case 3705-0460, with one mutation (p.Arg1505Ter), shows a lack of tumour cell staining with positive stromal staining (immunoreactive score with EPR13501: 1, D2A8U: 1 and HPA005456: 3). Case 3705-0529, ARID1A wild-type, shows positive tumour cell nuclear staining with an immunoreactive score with EPR13501: 12, D2A8U: 12 and HPA005456: 9. Scale bar is equal to 100 μm. (B) Histograms showing the distribution of all immunoreactive scores (n = 43) with all three antibodies, annotated with validated mutational status. The majority of ARID1A mutant cases (red) show low immunoreactivity scores and ARID1A wild-type cases (blue) show high immunoreactivity scores. There is greatest variation in the HPA005456 scores.
itself, reflect the heterogeneity of these tumours and the limitations of using an immunoreactive score. For example, in case 3705-0416, with two mutations (p.Gly191fs, p.Tyr485Ter), shows a lack of tumour cell staining with positive stromal staining (imunoreactive score with EPR13501: 0, D2ABU: 1 and HPA005456: 2). Case 3705-0514, ARID1A wild-type, shows positive tumour cell nuclear staining with an immunoreactive score with EPR13501: 12, D2ABU: 11, and HPA005456: 12. Scale bar is equal to 100 μm. (B) Histogram showing immunoreactivity scores for OCCC cases shows a bimodal distribution between mutant cases (loss of immunoreactivity) and ARID1A wild-type cases (retention of immunoreactivity).

Figure 5. ARID1A IHC shows good concordance with mutational analysis for all three antibodies in OCCC. ARID1A immunoreactivity in OCCC. Case 3705-0416, with two mutations (p.Gly191fs, p.Tyr485Ter), shows a lack of tumour cell staining with positive stromal staining (imunoreactive score with EPR13501: 0, D2ABU: 1 and HPA005456: 2). Case 3705-0514, ARID1A wild-type, shows positive tumour cell nuclear staining with an immunoreactive score with EPR13501: 12, D2ABU: 11, and HPA005456: 12. Scale bar is equal to 100 μm. (B) Histogram showing immunoreactivity scores for OCCC cases shows a bimodal distribution between mutant cases (loss of immunoreactivity) and ARID1A wild-type cases (retention of immunoreactivity).
be extended to other tumour types to ascertain its general applicability.

A recent sequencing study identified cancer-associated inactivating ARID1A mutations in deep infiltrating endometriosis, with loss of ARID1A immunoreactivity serving as a surrogate for ARID1A inactivating mutations using the HPA005456 antibody [35]. Using the monoclonal EPR13501 antibody, which had the best inter-rater agreement in our study, would allow comprehensive analysis of endometriosis to determine the clinical significance of ARID1A loss and be of potential diagnostic use.

In conclusion, we have systematically assessed a number of commercially available antibodies and identified EPR13501 as a robust biomarker of ARID1A status with a cut-off of <8 using our optimised scoring system. This will be useful for recruiting patients for clinical trials based on ARID1A mutational status. An international academic trial of ATR inhibition in combination with a PARP inhibitor in ARID1A-stratified gynaecological cancers that utilises our findings is planned to open in 2018 using this approach, allowing validation and evaluation of the IHC scoring system in the context of a prospective clinical trial.

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Author contributions statement

RN, CJL, and SB conceived and designed the study. SK, KN, AA, DK, FD, AL, TJ, MH, KF, and NM carried out the experiments and undertook IHC scoring. JC, TJ, MH, AGR, and RN performed the bioinformatics analysis. SK, KN, AA, DK, FD, CJL, and SB discussed and interpreted the results. SK, CJL, SB, and RN wrote the first draft. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. ARID1A status of OCCC cell lines. Western blot of ARID1A protein expression in ES2 (OCCC ARID1A wild-type cell line) and TOV21G (OCCC ARID1A mutant cell line) using the EPR13501, D2A8U, and HPA005456 antibodies. ARID1A (250 kDa fragment), loading control β-Actin (42 kDa)

Figure S2. Subclonal expression in a Grade 2 endometrioid adenocarcinoma of the ovary (case 3705-0482). Images of an area of subclonal expression with IHC scores and sequencing results. Top Row: Low power magnification, arrow shows area of absent protein expression surrounded by area of positive expression with all three antibodies (scale bar 1 mm). Bottom Row: High power magnification, arrow shows area of absent protein expression surrounded by area of positive expression with all three antibodies (scale bar 100 μm)

Table S1. Extended table of clinicopathological features of the patient cohort

Table S2. Summary of ARID1A sequencing metrics from the targeted capture panel

Table S3. Summary of individual and combined scores for ARID1A IHC

Table S4. Interpathologist concordance metrics

Table S5. Summary of ARID1A mutations identified and their validation