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ANTIBODY VALIDATION ARTICLE

REVISED Application of ARID1A to murine formalin-fixed paraffin embedded tissue using immunohistochemistry [version 3; peer review: 2 approved]

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Abstract

ARID1A is a known suppressor of tumour formation and the Human Protein Atlas antibody HPA005456 has been demonstrated in previous literature to stain tumour tissue by immunohistochemistry (IHC) in formalin-fixed paraffin embedded human tissue and human cell lines. This article details the validation of this antibody for immunohistochemistry of formalin-fixed paraffin embedded murine tissue using a Leica BondMax immunostainer. Using Western blot and IHC on murine wild-type and knockout tissue we have demonstrated that this antibody to ARID1A correctly stains murine tissue by immunohistochemistry.

Keywords

immunohistochemistry , antibody , ARID1A , tissue

This article is included in the **Antibody** **Validations** gateway.

Open Peer Review

Approval Status **✓✓**

	1	2
version 3 (revision) 04 Jun 2015		
version 2 (revision) 06 Jan 2015	✓ view	✓ view
version 1 15 Oct 2014	? view	? view

1. **Andrew D. Chalmers**, University of Bath,
Bath, UK

2. **Stephen McQuaid**, Queen's University
Belfast, Belfast, UK

Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 2

The article type has been changed from 'Research Note' to 'Antibody Validation Article' to better reflect the type of study presented.

See referee reports

Introduction

ARID1A (AT-rich interactive domain 1a) is a member of the SWI/SNF family and its loss has been implicated as a factor in multiple premalignant and malignant conditions, including Barrett's oesophagus and oesophageal carcinoma as well as endometrial and clear cell ovarian carcinomas and their precursor endometriotic lesions¹⁻⁴. The ARID1A antibody from Human Protein Atlas is a rabbit antibody generated against a PrEST (Protein Epitope Signature Tag) fragment of the ARID1A gene and affinity purified against the same fragment⁵. It is thus designated as being "mono-specific" in that the affinity purification removes any non-specific or low affinity binders to the peptide. Through the Human Protein Atlas, the antibody has been tested on a wide variety of human tissue types and human malignancies, as well as for expression in immunofluorescence on U-2 OS, A-431 and U-251 MG cell lines.

This demonstrates a nuclear expression in all cell lines and in the majority of tissue types⁶. However, the Western blot data were not supportive and did not produce staining corresponding to the expected size, although the data from a protein array did confirm a peak at the expected size. The antibody has been used to stain human colorectal cancers⁷, clear cell carcinomas⁸ and on a variety of clear cell cancer cell lines⁹ by immunohistochemistry.

To our knowledge, whilst the sequence homology between mouse and human ARID1A is 95%, this antibody has not been qualified using knockout tissue and has not been tested or published on murine tissue and this work represents the first data to do so.

Materials and methods

Reagent details

Details of all reagents with reference to the immunohistochemical staining procedure can be found in [Table 1](#).

Antibody details

Anti-ARID1A is a monospecific rabbit polyclonal generated to a PrEST sequence – PGLGNVAMGPRQHYYPYGGPYDRVRTEPG IGPEGNMSTGAPQPNLMPSPNDSGMYSPSRYPQQQQQQQ QQRHDSYGNQFSTQGTSPSGSPFSPQQTMTMYQQQQQNYK ([Table 2](#)). The homology of the PrEST sequence used as immunogen

Table 1. Details of ancillary reagents for immunohistochemistry.

Process	Reagent	Manufacturer	Catalogue Number	Concentration
Fixation	Neutral Buffered Formaldehyde	Sigma	HT501128	10%
Pretreatment	ER1 (Sodium Citrate, pH6)	Leica Biosystems	AR9961	Proprietary
	ER2 (Tris/EDTA, pH9)	Leica Biosystems	AR9640	Proprietary
	Enzyme 1 (Proteinase K)	Leica Biosystems	AR9551	100 µg/ml
Staining	Peroxide Block	Leica Biosystems	DS9263	Proprietary
	Streptavidin – HRP	Leica Biosystems	DS9263	Proprietary
	Diaminobenzidine (DAB)	Leica Biosystems	DS9263	Proprietary
	Haematoxylin	Leica Biosystems	DS9263	Proprietary
	DAB Enhancer	Leica Biosystems	AR9452	Proprietary
Washes/Blocks	Bond Wash (Tris Buffer)	Leica Biosystems	AR9590	Proprietary
	Antibody Diluent	Leica Biosystems	AR9352	Proprietary
	Avidin/Biotin Block	Vector Laboratories	SP-2001	Proprietary

Table 2. Details of primary and secondary antibodies.

Antibody	Manufacturer	Catalogue Number	RRID
ARID1A	Atlas Antibodies	HPA005456	AB_1078205
GAPDH (Clone 14C10)	Cell Signaling	2118	AB_561053
Donkey anti-Rabbit Biotin	Jackson ImmunoResearch	711-065-152	AB_2333077
Goat anti-Rabbit IRDye 680LT	Li-Cor Biosciences	926-68021	AB_10706309
Goat anti-Rabbit IRDye 800CW	Li-Cor Biosciences	926-32213	AB_621848

is 95%, when verified with BLAST against the mouse sequence. The lot number used for all validations was A40072 and for subsequent staining was D81856. A concentration of 1 µg/ml was used for initial validations and 0.5 µg/ml for final runs.

Donkey anti-rabbit biotin (Jackson ImmunoResearch, Table 2) is specific for Rabbit IgG (Heavy and Light chains) and was affinity purified to remove cross-reactions to Bovine, Chicken, Goat, Guinea Pig, Syrian Hamster, Horse, Human, Mouse, Rat and Sheep. All slides were stained with a concentration of 4.8 µg/ml.

Anti-GAPDH was used as a loading control for Western blots and was a rabbit monoclonal (Cell Signaling, Table 2). Detection antibody for the Western blot for ARID1a was Goat anti-rabbit IR Dye 680LT (Li-Cor Biosciences, Table 2) used at a concentration of 0.1 µg/ml and detection of GAPDH was Goat anti-rabbit IR Dye 800CW (Li-Cor Biosciences, Table 2).

Tissue details

All tissues and cell pellets used during the validation were fixed in Neutral Buffered Formaldehyde as specified (Table 3) before being transferred directly to 70% ethanol for no longer than 3 days. Tissue processing was conducted on a Leica ASP300 through graded ethanols before clearing in xylene and impregnation in molten paraffin wax (Fisher). All tissue sections were cut on a Leica rotary microtome at 3 µm.

Arid1a^{-/-} mice were created by crossing Floxed *Arid1a* mice with *ROSA26 Cre-ERT2* mice and resultant genotyping. Loss of Arid1a expression is expected following intraperitoneal injection of Tamoxifen.

Floxed *Arid1a* mice were a gift from Dr. Peri Tate, Sanger Institute, Hinxton UK; *ROSA26 Cre-ERT2* mice were a gift from Prof Chambon, IGBMC, France. ES-2 cells were purchased from ATCC and RMG-II were a gift from Prof Huntsman, British Columbia Cancer Agency, Vancouver, Canada.

Experiment details

Western blot. Protein was extracted from the two clear cell carcinoma cell lines using a Tris-EDTA lysis buffer and run on a non-denaturing 3–8% Tris-acetate gel (Life Technologies). Following

electrophoresis, the transfer membrane was probed with 0.2 µg/ml of anti-rabbit ARID1A (HPA005456) at 4°C overnight and 0.1 µg/ml anti-GAPDH (14C10) for the same length of time. Detection of the anti-rabbit ARID1A was with Goat anti-rabbit IRDye 680LT (Li-Cor Biosciences) and the GAPDH was with Goat anti-rabbit IRDye 800CW (Li-Cor Biosciences) both at 0.1 µg/ml.

Immunohistochemistry. Slides were deparaffinised and rehydrated on a Leica ST5020 using Xylene (Sigma) for 2 × 10 mins and ethanol (Fisher), 2 × 100% ethanol followed by 1 × 70% ethanol for 5 mins each. Following staining, all slides were dehydrated, cleared and mounted and coverslipped in DPX (Fisher).

The antibody was validated on a Leica BondMax instrument using a Leica Intense R kit to a standardised in-house protocol. All reagents were from Leica as part of the Intense R kit and were conducted at room temperature, unless otherwise specified. All staining steps included individual washes in Leica Bond Wash after each step, as part of the protocol (Table 4). A full protocol for the validated conditions can be found in the [supplementary material](#). In this protocol, the step named “primary” refers to the anti-ARID1a primary antibody.

A slide using the same conditions and retrieval but omitting the primary antibody was used to control for any background staining due to the retrieval and detection steps.

Imaging. All slides were digitised using a Leica Scanscope AT2 at 0.5 µm/pixel resolution. Datasets can be viewed by downloading the Leica Imagescope free viewer at <http://www.leicabiosystems.com/pathology-imaging/aperio-epathology/integrate/imagescope/>.

Table 4. Staining protocol for ARID1A immunohistochemistry.

Protocol steps	Reagent	Time (mins)
Antigen Retrieval	ER1	20
	Or ER2	20
	Or Enz1	10
Staining	Peroxide Block	5
	Avidin	10
	Biotin	10
	ARID1A	15
	Donkey anti-rabbit Biotin	8
	SA-HRP	8
	DAB	5
	DAB Enhancer	10
Counterstaining	Haematoxylin	5

Table 3. Details of tissue and cell pellet used during the validation.

Species	Tissue Type	Strain/Cell line	Details	Fixation Time
Murine	Uterus	C57Bl6	Female	16 hrs
Murine	Uterus	C57Bl6 KO <i>Arid1a</i> ^{fl/fl}	Female	24 hrs
Human	Cell Pellet	ES-2		20 hrs
Human	Cell Pellet	RMG-II		20 hrs

Results

To determine the correct cell line to utilise and to confirm the equivocal Western blot data from Human Protein Atlas, the antibody was used to stain a Western blot of two cell lines; ES-2 and RMG-II, both of which are cell lines derived from clear cell carcinoma and have been previously demonstrated as ARID1a wild-type and mutated, respectively⁹. It could be demonstrated that the HPA ARID1A antibody showed positive expression in ES-2 cell lines at the expected size of 270 kDa and no staining for RMG-II. The loading control of GAPDH showed that there were no loading issues (Figure 1). Thus, these cell lines were chosen to be grown, formalin fixed and processed into paraffin wax for immunohistochemical validation.

For immunohistochemical validation, ES-2 and RMG-II cell lines were stained using three antigen retrieval conditions; ER1 (Sodium Citrate, pH6), ER2 (Tris/EDTA, pH9) and Enzyme 1 (Proteinase K, 100 µg/ml) at a fixed antibody concentration of 1 µg/ml. The enzyme retrieval demonstrated no nuclear signal for either ES2 or RMG-II cell pellets and was discarded for future work (Figure 2; Dataset a). The ER2 condition did demonstrate significant nuclear staining in the ES2 cell pellet with minimal background staining in the RMG-II cell pellet (Figure 2; Dataset b).

Figure 1. Western blot of ES2 and RMG-II clear cell carcinoma cells lines. ARID1A (red band) can be seen to be present at approximately 270kD in ES2 cell line only. GAPDH at 37kD (green band) represents loading control.

Figure 2. ES2 and RMG-II cell lines stained by immunohistochemistry with anti-ARID1A using three antigen retrieval conditions, ER1, ER2 and Enzyme 1. NPA denotes No Primary antibody control and represents the ER2 condition. Bar = 200 µm.

However, the staining in the ER1 condition was determined to give the best signal:noise ratio with no background cytoplasmic staining and crisp nuclear staining for the cell pellet (Figure 2; Dataset c). Control slides, omitting the primary antibody, were negative except for the ER2 condition in the RMG-II cell pellet where a weak cytoplasmic background could be seen (Figure 2; Dataset d). Thus there was minimal background inherent in the staining procedure. It was therefore determined that the antibody showed specificity for formalin-fixed paraffin embedded tissues and could be run on murine tissue.

Murine uterine tissue was used as positive control tissue samples, given the literature data on cell lines and endometrial tissue. The ER1 condition at 1 µg/ml demonstrated clean nuclear staining in the uterine epithelial compartment as well as nuclear staining of stromal cells. However, the nuclear staining in the stroma was not universal and distinct negative nuclei could be seen (Figure 3A; Dataset e). There was no cytoplasmic or extracellular stromal background staining present and the antibody titrated successfully losing the intensity of staining, as expected (Dataset e). Following this, a concentration of 0.5 µg/ml was used for future preparations which provided clear and consistent staining in repeated batches using a different antibody lot (Dataset f). A No Primary antibody control (NPA) showed no staining in the epithelial or nuclear compartment (Figure 3B; Dataset e).

Finally, when applied to a genetically engineered, tamoxifen-induced, Arid1a knockout mouse model, the staining in the uterine epithelium could be almost completely abrogated (Arrow, Figure 4b) when compared to the same area in a wild-type animal (Arrow, Figure 4a) with a small focal area of epithelial staining still present. There was no effect of the KO on the staining in the stromal compartment.

Figure 3. A) Murine uterine tissue stained by immunohistochemistry with anti-ARID1A using the ER1 condition and a concentration of 1 µg/ml demonstrating clear nuclear staining of the epithelial compartment (E) and negative nuclei in the stromal compartment (S). B) No primary antibody control of a similar area of epithelium/stroma. Bar = 100 µm.

Figure 4. Murine uterine tissue stained by immunohistochemistry with anti-ARID1A demonstrating nuclear staining in wild-type mice (A) but loss of epithelial staining after ARID1A knock-out in *Arid1a*^{fl/fl} mice (B). Bar = 100 µm.

Dataset 1. Whole slide images from antibody validation of HPA005456 for immunohistochemistry - Version 2

<http://dx.doi.org/10.5256/f1000research.5514.d41579>

Detailed legends for each dataset (Datasets a–f) can be found in the text file provided. For Version 2 an additional image was added to Dataset e.

Conclusions

It is clear from the use of ES2 and RMG-II cell lines that the Atlas Antibodies ARID1A antibody is specific for ARID1A in both Western blots and formalin-fixed paraffin embedded preparations of human origin and, coupled with the literature evidence, that it is validated in human tissue.

The staining pattern when applied to murine uterus showing a clear nuclear pattern, where there is a high level of sequence homology between the two species, is again consistent with the literature on this protein. When stained on an ARID1A knockout mouse model, the staining could be almost completely abrogated in the epithelial compartment but not in the stroma. Knockout mice generated in this manner are almost never 100% complete as in some cells recombination will not be induced due to issues such as low ligand (Tamoxifen) penetration or failure of the ligand to induce recombination, thus explaining the small focal area of epithelial staining. The difference in staining in the two compartments is also likely related to the same effect, as all other controls, such as omission of primary antibody remained negative. Thus, given the overwhelming data from other sources, it is likely the stromal staining reflects continuing Arid1a expression in this specific model system.

Therefore, in conclusion when taken in combination, it is clear that the anti-human ARID1A antibody is cross-reactive with murine tissue and can be used for this purpose.

Data availability

F1000Research: Dataset 1. Whole slide images from antibody validation of HPA005456 for immunohistochemistry - Version 2, [10.5256/f1000research.5514.d41579](http://dx.doi.org/10.5256/f1000research.5514.d41579)¹⁰

Author contributions

WH wrote and conceived the idea behind the article, IG performed the Western blots and mouse experiments and requested validation of ARID1A in murine tissue, JM performed all immunohistochemical staining.

Competing interests

No competing interests were disclosed.

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Supplementary material

Full name: B AB DABe Rabbit**ID:** 10008**Version:** 0(current)**Type:** IHC staining**Created by:** BondPowerUser**Creation time:** 17/12/2013 11:28**Facility:** <not defined>**Staining status:** Single

Protocol: BABDABR b

Bond-max and Bond-x

Step Reagent		<i>Supplier: Leica Microsystems</i>	
1 *Peroxide Block			
Step type: Reagent	Incubation time: 5min0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Leica Microsystems</i>	
2 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Leica Microsystems</i>	
3 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Open
Step Reagent		<i>Supplier: Leica Microsystems</i>	
4 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Vector Laboratories</i>	
5 Avidin Block			
Step type: Reagent	Incubation time: 10min0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Leica Microsystems</i>	
6 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Leica Microsystems</i>	
7 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.
Step Reagent		<i>Supplier: Leica Microsystems</i>	
8 *Bond Wash Solution			
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.

Protocol: BABDABR b

Bond-max and Bond-x

Full name: B AB DABe Rabbit

ID: 10008

Version: 0(current)

Type: IHC staining

Created by: BondPowerUser

Creation time: 17/12/2013 11:28

Facility: <not defined>

Staining status: Single

Step Reagent				<i>Supplier: Vector Laboratories</i>
9 Biotin Block				
Step type: Reagent	Incubation time: 10min0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
10 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
11 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
12 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Not applicable</i>
13 Primary				
Step type: Reagent	Incubation time: 15min0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
14 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
15 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				<i>Supplier: Leica Microsystems</i>
16 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.

Protocol: BABDABR b

Bond-max and Bond-x

Full name: B AB DABe Rabbit

ID: 10008

Version: 0(current)

Type: IHC staining

Created by: BondPowerUser

Creation time: 17/12/2013 11:28

Facility: <not defined>

Staining status: Single

Step Reagent				Supplier: Jackson ImmunoResearch
17 Biotinylated Anti-Rabbit				
Step type: Reagent	Incubation time: 8min0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
18 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
19 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
20 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
21 *Streptavidin-HRP				
Step type: Reagent	Incubation time: 8min0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
22 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier: Leica Microsystems
23 *Bond Wash Solution				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.	
Step Reagent				Supplier:
24 *Deionized Water				
Step type: Wash	Incubation time: 0s	Temperature: Ambient	Dispense type: Selected vol.

Protocol: BABDABR b

Bond-max and Bond-x

Full name: B AB DABe Rabbit

ID: 10008

Version: 0(current)

Type: IHC staining

Created by: BondPowerUser

Creation time: 17/12/2013 11:28

Facility: <not defined>

Staining status: Single

Step Reagent

Supplier: Leica Microsystems

25 *Mixed DAB Intense

Step type: Reagent

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

26 *Mixed DAB Intense

Step type: Reagent

Incubation time: 5min0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier:

27 *Deionized Water

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier:

28 *Deionized Water

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier:

29 *Deionized Water

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

30 *Bond DAB Enhancer

Step type: Reagent

Incubation time: 10min0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

31 *Bond Wash Solution

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

32 *Bond Wash Solution

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Protocol: BABDABR b

Bond-max and Bond-x

Full name: B AB DABe Rabbit

ID: 10008

Version: 0(current)

Type: IHC staining

Created by: BondPowerUser

Creation time: 17/12/2013 11:28

Facility: <not defined>

Staining status: Single

Step Reagent

Supplier: Leica Microsystems

33 *Bond Wash Solution

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

34 *Hematoxylin

Step type: Reagent

Incubation time: 5min0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier:

35 *Deionized Water

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier: Leica Microsystems

36 *Bond Wash Solution

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Step Reagent

Supplier:

37 *Deionized Water

Step type: Wash

Incubation time: 0s

Temperature: Ambient

Dispense type: Selected vol.

Open Peer Review

Current Peer Review Status:

Version 2

Reviewer Report 12 January 2015

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Stephen McQuaid

Center for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, UK

All concerns have been addressed.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 January 2015

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Andrew D. Chalmers

Department of Biology and Biochemistry, University of Bath, Bath, UK

The authors have addressed my comments/concerns satisfactorily and I am happy to approve the manuscript.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 02 December 2014

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Stephen McQuaid

Center for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, UK

This article, by Howatt *et al.* validates an antibody to ARID1A in murine FFPE samples, is a good example of the correct validations procedures that must be met when validating an antibody for use in tissue sections. Issues are relatively minor:

1. What was the rationale for a starting concentration of 1µg/ml for initial validations?
2. There is a focal region of the KO epithelium which clearly appears to be positive. The authors would need to explain this.
3. Can the authors please add more discussion of the nature of the staining of the stromal cells. Do they consider this to be non-specific? Is such staining of stroma present in human tissue sections?
4. Control slides, omitting primary antibody, were carried out during the cell line phase of the experiments. Were such slides run on the murine tissue sections and can the results be included in the data. I note that the stromal staining was still present at selected concentration of 0.5µg/ml (dataset f)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 31 Dec 2014

Will Howat, University of Cambridge, Li Ka Shing Centre, Cambridge, UK

Dear Dr McQuaid,

Thank you for your comments which we agree with and have taken on board. Specifically:

- All of the validations conducted at the Histopathology/ISH facility start with a dilution of 1:100 from the manufacturers provided stock concentration and we perform 3 retrieval methods; Sodium Citrate pH6, Tris EDTA pH9 & Proteinase K. We titrate as appropriate following establishment of the optimal retrieval method. The

manufacturers original stock concentration was 100ug/ml, hence the 1ug/ml starting concentration. They have subsequently changed their stock concentration to 200ug/ml.

- We believe that the knockout model recombining a floxed Arid1a with CRE-ERT2 is not 100% complete as in some cells recombination will not be induced due to issues such as low ligand (tamoxifen) penetration or failure of the ligand to induce recombination. Thus explaining the area of focal staining in the epithelium. Similarly, although we have not investigated this further, we believe that the same problem underlies the stromal staining and is likely due to penetrance of the tamoxifen after being delivered intraperitoneally. Thus, given the wealth of data and the no primary antibody controls, now included in Fig 3, we feel that the stromal staining is not non-specific. Stromal staining does indeed occur in human tissue.

All of the above have now been included in the conclusion section of the article.

Kind Regards,

Will Howat

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 November 2014

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Andrew D. Chalmers

Department of Biology and Biochemistry, University of Bath, Bath, UK

The manuscript by Will Howat and colleagues validates an anti-ARID1A antibody and provides a good example of an antibody validation paper. In particular the use of negative cell lines and knockout mouse tissue demonstrate the extent of specificity of the antibody. The data is mostly well presented and clearly explained in the text.

The manuscript would be suitable for indexing if some, mostly minor, issues can be addressed.

1. I think the title should mention "antibody" to make clear it's an antibody that is being validated.
2. The authors say there is 95% sequence homology between human and mouse, it would be interesting to see how conserved the sequence the antibody was raised against is.
3. The Materials and Methods give a good overview of the reagents and methods used for the

IHC, but there is little information about the western blotting. The reagents and methods used should be added.

4. It would be good to cite the source of the cells used and the mouse tissue.
5. I was not clear if the lack of staining in RMG-II cells was expected or an unexpected but useful result? This should be explained.
6. Figure 1 legend. Should read "represents *the* loading control"
7. In figure 4B the KO epithelium is clearly negative, except one region in the top left which looks positive, I wondered what the authors felt about this?
8. It is interesting that the stromal staining appears to be non-specific, I wonder if the authors ever did a no primary control? Does the stroma still stain? Also it would be worth mentioning this staining in the conclusions.
9. Author contributions. Should it read "conceived the idea behind the article" or similar wording ?

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 31 Dec 2014

Will Howat, University of Cambridge, Li Ka Shing Centre, Cambridge, UK

Dear Dr Chalmers,

Many thanks for your comments which we agree with and have taken on board. Specifically:

- We have modified the title to read "Application of anti-ARID1a antibody..."
- We have BLAST searched the protein sequence and it confirms 95% homology for the sequence used for immunisations. This has been added to the methods.
- We accept that the methods detailing the Western blotting are more limited than the IHC methods, as is the case of the methods detailing the production of the knockout mouse model. However, we feel that the article was designed as an example of antibody validation for immunohistochemistry and that while western blotting provides important additional data, it is not the focus of the article. We thus feel that there is sufficient information in the details behind the western blotting to repeat the experiment, without clouding the article with the full methods.
- The sources of the cells and mouse tissue have now been cited.

- The lack of staining of RMG-II cells was consistent with the literature (Anglesio *et al.*) and this information has been added to the results section.
- The figure legend has been modified.
- We believe that the focal staining is a result of incomplete recombination floxed Arid1a allele and Cre/ERT2. The resulting knockout is almost never 100% complete as in some cells recombination will not be induced due to issues such as low ligand (tamoxifen) penetration or failure of the ligand to induce recombination. Similarly, we believe that the staining in the stromal compartment is specific but represents a failure of delivery of Tamoxifen or of recombination. The data from no primary antibody (NPA) control which are all completely clean, now included in Fig 3, helps to demonstrate this. We have included these points in the conclusion.
- The author contributions have been modified.

Kind Regards,

Will Howat

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 2

Reader Comment 12 Jan 2015

Jan Voskuil, Everest Biotech Ltd, Oxfordshire, UK

Well done!

Competing Interests: No competing interests were disclosed.

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