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Original Article

Increased proliferation in atypical hyperplasia/endometrioid intraepithelial neoplasia of the endometrium with concurrent inactivation of ARID1A and PTEN tumour suppressors

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Abstract

Uterine endometrioid carcinoma is the most common neoplastic disease in the female genital tract and develops from a common precursor lesion, atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN). Although the genomic landscape of endometrioid carcinoma has been recently revealed, the molecular alterations that contribute to tumour progression from AH/EIN to carcinoma remain to be elucidated. In this study, we used immunohistochemistry to determine if loss of expression of two of the most commonly mutated tumour suppressors in endometrioid carcinoma, *PTEN* and *ARID1A*, was associated with increased proliferation in AH/EIN. We found that 80 (70%) of 114 cases exhibited decreased or undetectable PTEN and 17 (15%) of 114 cases had focal loss of ARID1A staining. ARID1A loss was focal, while PTEN loss was diffuse, and all specimens with ARID1A loss had concurrent PTEN loss ($p = 0.0003$). Mapping the distribution of PTEN and ARID1A staining in the same specimens demonstrated that all AH/EIN areas with ARID1A loss were geographically nested within the areas of PTEN loss. A significant increase in the proliferative activity was observed in areas of AH/EIN with concurrent loss of PTEN and ARID1A compared to immediately adjacent AH/EIN areas showing only PTEN loss. In a cell culture system, co-silencing of ARID1A and PTEN in human endometrial epithelial cells increased cellular proliferation to a greater degree than silencing either ARID1A or PTEN alone. These results suggest an essential gatekeeper role for ARID1A that prevents PTEN inactivation from promoting cellular proliferation in the transition of pre-cancerous lesions to uterine endometrioid carcinoma.

Keywords: atypical hyperplasia; endometrioid intraepithelial neoplasia; PTEN; ARID1A; tumour suppressor; proliferation; immunohistochemistry; co-silencing; in vitro cell culture model

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The authors declare no conflict of interest.

Introduction

Uterine endometrioid carcinoma is the most common malignant neoplasm of the female genital tract and is the fourth most common malignancy among women in the United States. Although several genome-wide studies including The Cancer Genome Atlas (TCGA) have revealed the molecular landscape of uterine endometrioid carcinoma and substantially improved our understanding of endometrial cancers [1,2], its pathogenesis, especially during the transition from a precursor lesion to cancer, remains to be further elucidated. Delineating the molecular genetic events involved in tumour progression has implications for detection, diagnosis and treatment of uterine endometrioid carcinoma.

It is thought that atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN) is the precursor lesion of many endometrioid carcinomas of the uterus. AH/EIN is not synonymous with carcinoma but it is a lesion that may regress, persist or progress to invasive carcinoma. Approximately 37% of women diagnosed with AH/EIN have a concurrent endometrioid carcinoma, and its presence is associated with an increased risk of carcinoma beyond AH/EIN [3]. Morphologically, AH/EIN is defined as cytological atypia superimposed on endometrial hyperplasia [4], and the lesion shares several molecular genetic alterations with uterine endometrioid carcinoma such as microsatellite instability and somatic mutations of *PTEN* [5].

The tumour suppressor, *PTEN*, encodes a phosphatase and acts through an Akt-dependent pathway to suppress cell division and promote apoptosis [6]. Mutations and deletions of *PTEN* represent the most common genetic change found in uterine endometrioid carcinoma; genetic inactivation is found in approximately 80% of cases [1,7]. On the other hand, *ARID1A* has emerged as a tumour suppressor that is mutated in a wide variety of human neoplasms, most frequently in endometriosis-related ovarian cancers including clear cell and endometrioid carcinomas as well as uterine endometrioid carcinomas [8–12]. *ARID1A* encodes a subunit of the switch-sucrose nonfermentable chromatin remodelling family, which plays an essential role in regulating DNA synthesis, transcription, DNA methylation and DNA damage repair. In human cancer, ARID1A acts as a gatekeeper regulating cellular proliferation and as a caretaker preventing genomic instability [13–15]. In uterine endometrioid carcinoma, inactivating *ARID1A* mutations occur in approximately 40% of cases [1,11].

We recently demonstrated in *ARID1A*/*PTEN* conditional knockout mice that co-deletion of both genes, but not deletion of the individual genes, in ovarian surface epithelium resulted in ovarian endometrioid carcinoma [12]. It is, thus, likely that loss of both tumour suppressors plays a critical role in tumour progression of uterine endometrioid carcinoma. To test this possibility, we performed immunohistochemistry to determine the effects of co-loss of PTEN and ARID1A on cellular proliferation in AH/EIN tissues. A conventional approach to examining the involvement of specific molecular genetic changes in tumour progression would be to compare the frequency of a given molecular alteration between the lesion and its precursor using mutational analysis in the whole lesions and precursors. However, it would be a challenge to apply this strategy to recapitulate a clonal evolution process. As an alternative, we performed geographical mapping of ARID1A and PTEN immunoreactivity, and carefully correlated the staining patterns with histopathological features and cellular proliferation, which allowed us to define whether inactivation of both ARID1A and PTEN pathways created advantageous clones with a higher proliferative ability. In addition, we applied a new *in vitro* cell culture model to determine if silencing PTEN and ARID1A enhanced cellular proliferation. The results from this study provide new insight into tumour progression of uterine endometrioid carcinoma, suggesting that ARID1A renders an essential gatekeeper role preventing PTEN inactivation from promoting proliferation essential for tumour progression from AH/EIN to uterine endometrioid carcinoma.

Materials and methods

Case selection

AH/EIN cases from endometrial curettage and biopsy were retrieved by diagnostic review of pathology files from the Department of Pathology, Seirei Mikatahara Hospital, Hamamatsu, Japan and the Department of Pathology, the National Taiwan University Hospital, Taipei, Taiwan. Haematoxylin and eosin stained sections from the study cases were reviewed by pathologists (AA, TM, HO and IS) to confirm the diagnosis, based on criteria described in the 4th edition of the WHO Classification of Tumours of Female Reproductive Organs [4]. A total of 114 patients were identified. One or two paraffin blocks from the qualified cases were retrieved and sequential unstained sections prepared to ensure tissue continuity in successive slides. We did not include non-atypical hyperplasia cases herein because our previous study did not report any ARID1A loss in those specimens [16]. The study was approved by the institutional review boards (14–46; 2014/12/15).

Immunohistochemistry

Immunohistochemical analysis of whole tissue sections was performed either on a Leica bond-max immunostainer or manually. For manual staining, antigen retrieval was performed by placing sections in citrate buffer (pH 6.0) and autoclaved at 120°C for 10 min. The following antibodies were used: a polyclonal rabbit anti-ARID1A antibody (Sigma-Aldrich, HPA005456, 1:1000 dilution), a mouse monoclonal anti-PTEN antibody (6h2.1, Dako, M362729-2, 1:100 dilution) and a mouse monoclonal anti-Ki-67

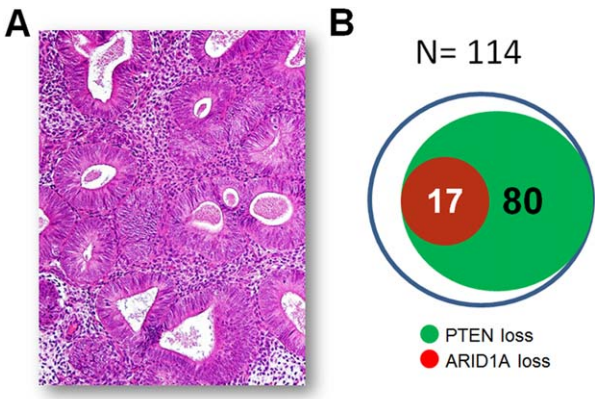

Figure 1. The distribution of AH/EIN cases harbouring loss of PTEN and/or ARID1A expression. (A) Morphological features from a representative AH/EIN case (haematoxylin-and-eosin stain). (B) Among 114 AH/EIN cases, 17 exhibited focal ARID1A loss and 80 exhibited diffuse PTEN loss. All 17 cases of ARID1A loss harboured concurrent PTEN loss and were geographically nested within the area of PTEN loss.

antibody (Dako, clone MIB-1, M7240, 1:100 dilution). The sections were incubated with the appropriate secondary antibodies. A positive reaction was detected using the EnVision+System (Dako, Carpinteria, CA). Tumour stromal cells served as positive internal controls for ARID1A and PTEN antibodies. The specificity of the ARID1A antibody was confirmed in a previous study [11]. Because loss of nuclear expression of ARID1A was almost always complete, we scored ARID1A staining either as (complete) loss or retention. Similarly, we compared PTEN immunoreactivity between glands and adjacent stromal cells and determined if there was loss of PTEN expression, which was scored as either undetectable or reduced staining due to deletion or inactivation in one allele. The Ki-67 labelling index was recorded both in AH/EIN areas of concomitant ARID1A and PTEN loss and in ARID1A positive/PTEN loss AH/EIN areas by manual counting on photomicrographs.

Normal human endometrial epithelial cell culture

Anonymous endometrial tissue was obtained from pre-menopausal women who underwent hysterectomy for a benign reason. Acquisition of tissue specimens was approved by the Institutional Review Board at the Johns Hopkins Hospital, Baltimore, Maryland. Fresh samples were directly processed after arrival in the surgical pathology unit. Endometrial tissue was rinsed with phosphate buffered saline (Life Technologies) and minced thoroughly in a petri dish. The specimen was resuspended in 5 ml Hank's Balanced Salt Solution (HBSS, Life Technologies) containing collagenase IV (Life Technologies) (1 mg/ml) and incubated at 37°C with gentle agitation for 25 min. The cells were further dissociated using 5 ml of 0.05% trypsin (Life Technologies), and trypsin activity was later neutralised using 10 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) containing 10% Fetal bovine serum (FBS, Sigma). The cell suspension was then filtered through a 40-μm cell-strainer and spun down at 1000 $\times$ g for 5 min. After washing in fresh HBSS, the cell pellet was resuspended in RPMI1640 medium supplemented with 15% FBS, 1% penicillin/streptomycin (Life Technologies) and 1% non-essential amino acids (Life Technologies) and seeded on 0.1% gelatin-coated dishes at 37°C and 5% CO_2. Epithelial cells were immortalised using SV40 adenovirus and were designated as hEM2 cells.

Lentivirus production and cell growth assay

ARID1A short hairpin RNAs (shRNAs; TRCN00000 59090 and TRCN0000059091, 1:1 mixture) and PTEN shRNA (TRCN0000002746) were obtained from the RNAi Consortium. Lentiviral vectors expressing ARID1A shRNAs and PTEN shRNAs were produced using HEK293FT cells transfected with pLKO.1-puro lentiviral plasmids (the RNAi consortium) and second generation packaging systems, pSPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259). Lentivirus targeting green fluorescence protein (GFP) was used as a negative control. hEM2 cells were transduced with GFP virus only, ARID1A virus, PTEN virus, or with a combination of ARID1A and PTEN virus. Two days after transduction, cells were seeded into 96-well gelatin--coated plates at 3000 cells per well in RPMI1640 medium supplemented with 2% FBS. Cell growth was assessed using CellTiter-Blue® Cell Viability Assay (Promega) at different time points.

RNA extraction and qPCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). First strand cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription-PCR was performed using Platinum Taq DNA polymerase (Life Technologies) and SYBR Green I (Life Technologies). The primers used were: ARID1A (forward)-CAGTACCTGCCT CGCACATA, ARID1A (reverse)-GCCAGGAGACC AGACTTGAG, PTEN (forward)-TGAAGGCGTATA CAGGAACAAT, PTEN (reverse)-CGGTGTCATAA TGTCTTTCAGC, β-actin (forward)-GTTGTCGAC GACGAGCG and β-actin (reverse)-GCACAGAGCC TCGCCTT.

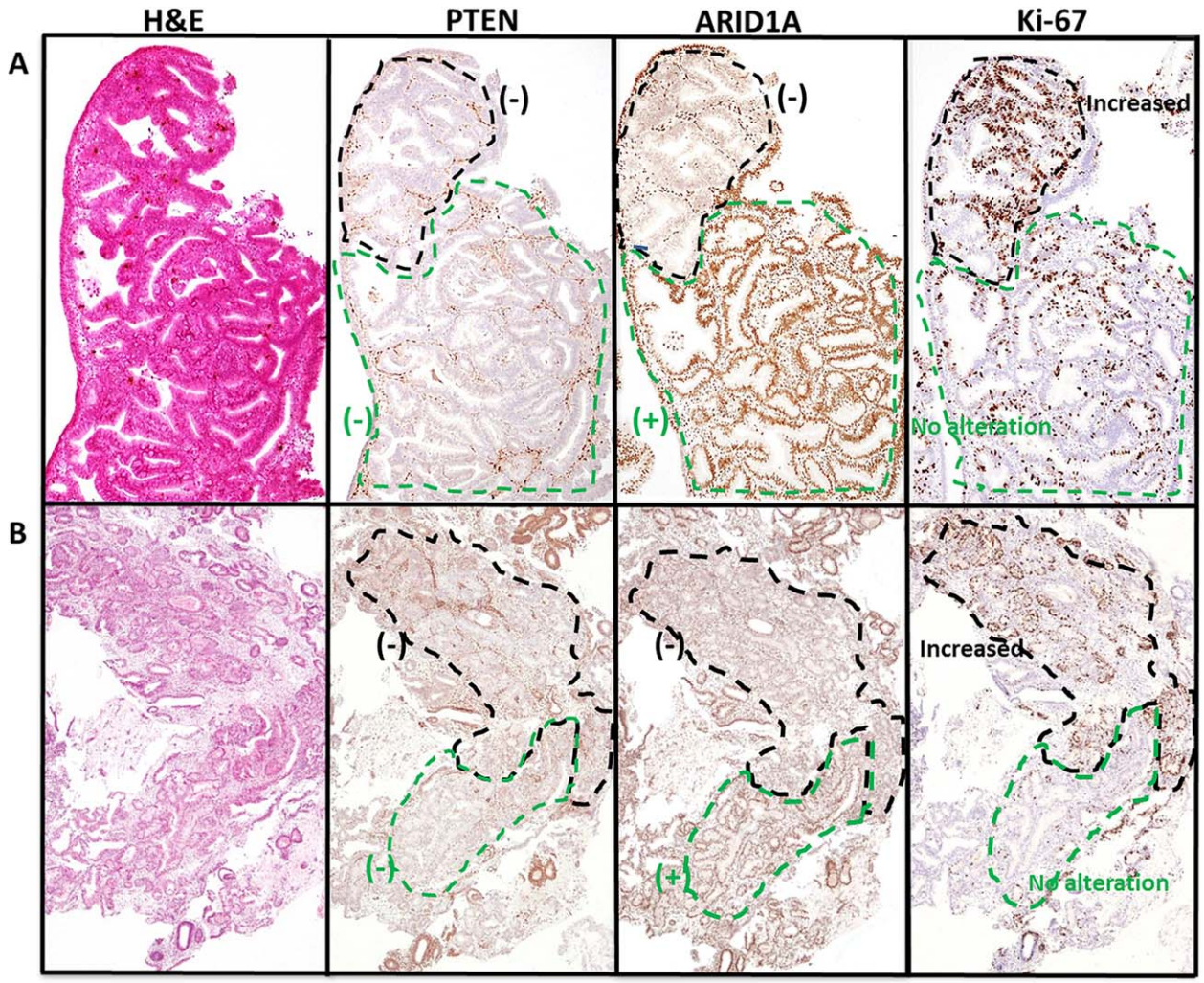

Figure 2. Patterns of ARID1A, PTEN and Ki-67 immunostaining in two representative AH/EIN cases (A, B). Areas of ARID1A and PTEN coloss are circled with black dashed lines, and areas with only PTEN loss are circled with green dashed lines.

Western blot analysis

Cells were rinsed with Phosphate Buffered Saline (PBS) and lysed in cell lysis buffer (Cell Signalling) supplemented with the complete protease inhibitor tablet (Roche). Proteins were separated using 4–15% Mini-PROTEAN® TGX™ Gel (Biorad) and transferred to PVDF membrane (Biorad). Western blotting was performed using the following antibodies: ARID1A (HPA005456, Sigma-Aldrich), cytokeratin8 (TROMA-I, DSHB) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, G9545, Sigma-Aldrich).

Results

We identified 114 patients whose endometrial biopsy or curettage specimens contained AH/EIN, characterised by crowded tubular or branching glands with nuclear atypia and the area of glands exceeding that of stroma [4]. The histological features of one representative case are shown in Figure 1(A). The ages of patients whose biopsies were used in this study ranged from 20 to 78 with an average of 45.1 ± 8.9. We performed immunohistochemistry using ARID1A and PTEN antibodies on all specimens and found that 80 (70%) of 114 cases had decreased or undetectable levels of PTEN immunoreactivity (ie, PTEN loss) and 17 (15%) of 114 cases had loss of ARID1A staining [Figure 1(B)]. The occurrence of co-loss of ARID1A and PTEN was highly significant and non-random ($p = 0.0003$, Fisher's exact test, two-tailed). In contrast to diffuse loss of PTEN staining, loss of ARID1A immunoreactivity was (multi-)focal in which negative ARID1A immunoreactivity was present in small aggregates of individual glands. Similar

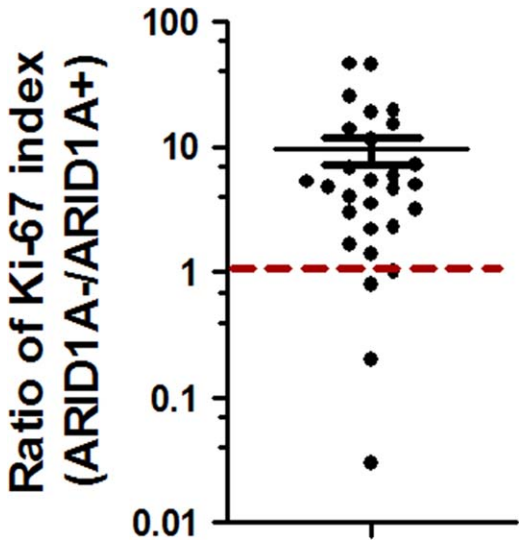

Figure 3. Normalised Ki-67 indices in foci of PTEN loss with or without loss of ARID1A expression. Data are presented as Ki-$67^{ARID1A-}$/Ki-$67^{ARID1A+}$.

to our previous report [16], we did not identify any AH/EIN specimens showing diffuse loss of ARID1A involving all hyperplastic glands. All 17 cases showing ARID1A loss had concurrent loss of PTEN staining [Figure 1(B)]. As eight specimens harboured more than one focus of ARID1A loss, we recorded 28 small regions showing ARID1A loss among these 17 cases. Careful mapping of these 28 AH/EIN foci with ARID1A and PTEN loss demonstrated that areas with PTEN loss encompassed larger areas than the regions of ARID1A loss. All 28 AH/EIN foci with ARID1A loss were geographically nested within the areas exhibiting PTEN loss (Figure 2). Based on haematoxylin and eosin staining, there were no noticeable differences in morphology between AH/EIN foci with ARID1A and PTEN co-loss and those adjacent foci with only PTEN loss (Figure 2).

Next, we determined proliferative activity using the Ki-67 labelling index in these 28 AH/EIN foci showing concurrent decreases in ARID1A and PTEN immunoreactivity and compared the index with the adjacent glands which are ARID1A positive and PTEN negative. We found that Ki-67 positive epithelial cells were more abundant in hyperplastic glands showing concomitant loss of ARID1A and PTEN than in adjacent glands showing only PTEN loss (Figure 2). Because the Ki-67 labelling index varied among cases, we normalised the Ki-67 index of the ARID1A negative area to the corresponding ARID1A positive area in the same case. The normalised Ki-67 index were significantly higher in ARID1A loss/PTEN loss glands than in the matched ARID1A positive/PTEN loss glands from the same case ($p = 0.001$, paired t-test, two-tailed) (Figure 3). Specifically, among the 28 pairs of ARID1A loss/PTEN loss and adjacent ARID1A positive/PTEN loss glands that were compared, all except four pairs demonstrated an increase in the Ki-67 index ratio in ARID1A loss/PTEN loss as compared to the adjacent ARID1A positive/PTEN loss glands. When we separated the PTEN undetectable foci from the PTEN decreased foci, we failed to observe any significant difference in Ki-67 labelling index.

To determine whether co-loss of ARID1A and PTEN elevates proliferative activity in endometrial epithelial cells, we isolated and cultured normal endometrial epithelial cells from a woman who underwent total hysterectomy due to leiomyomata. The established epithelial cell culture (named hEM2) grew with a cobble stone appearance [Figure 4(A)] and expressed ARID1A and cytokeratin 8 [Figure 4(B)]. To mimic the downregulation of ARID1A and PTEN in AH/EIN, we silenced *ARID1A* or *PTEN* or both in these cells using RNA interference. Lentiviruses carrying shRNAs targeting either *ARID1A* or *PTEN* were used to transduce hEM2 cells. Knockdown efficiency was robust as evidenced by a significant decrease in mRNA levels of *ARID1A* and *PTEN* after transduction [Figure 4(C)]. We then measured the proliferation indexes based on the growth rates of hEM2 cells from the four transduced groups: control *GFP* shRNA, *ARID1A* shRNA, *PTEN* shRNA and combined *ARID1A* and *PTEN* shRNAs. Cell numbers were normalised to the number of cells initially seeded. As shown in Figure 4(D), hEM2 cells transduced with the combination of *ARID1A* and *PTEN* shRNAs exhibited the highest proliferative activity compared to other groups of cells, a result supporting the observations derived using the immunohistochemical approach.

Discussion

Genetic or epigenetic inactivation of *PTEN* resulting in loss of its expression is prevalent in uterine endometrioid carcinoma. As sporadic PTEN-negative glands due to inactivating mutations or deletions are present in 43% of normal-appearing premenopausal endometria [17], alteration in PTEN is thought to be one of the earliest molecular changes in the development of endometrioid carcinoma. Despite the frequent PTEN loss in normal-appearing glands, only a very small fraction of them will progress to

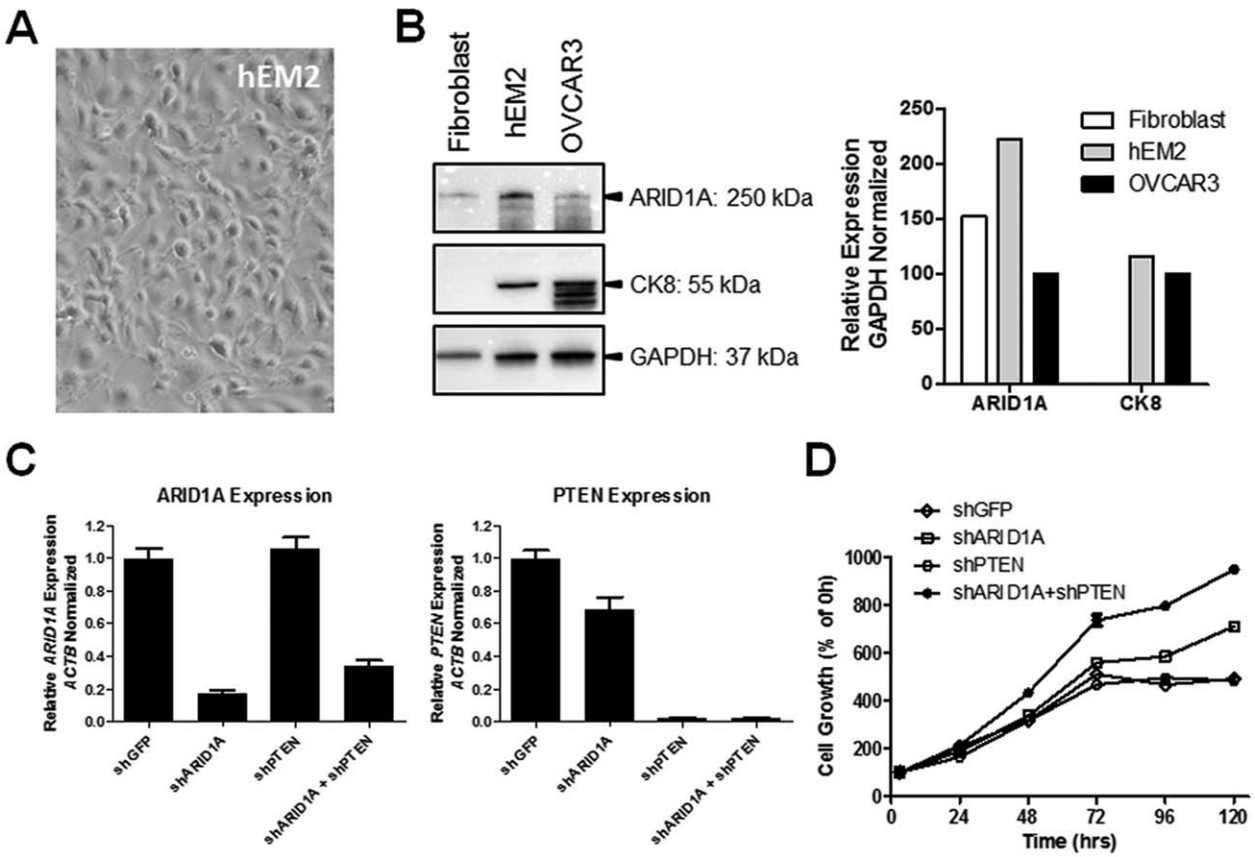

Figure 4. The effect of silencing expression of ARID1A, PTEN or both in an *in vitro* endometrial epithelial cell culture. (A) The human endometrial epithelial cell culture, hEM2, shows a cobble stone appearance under phase contrast microscopy. (B) (left) Western blot analysis demonstrates that hEM2 cells, like an ovarian cancer cell line (OVCAR3), express ARID1A and cytokeratin 8 (CK8). A fibroblast line was used as a control. GAPDH was used as the loading control. (right) Densitometric quantification of the western blot. (C) Knockdown efficiency in hEM2 cells transduced with lentiviruses expressing ARID1A shRNA and PTEN shRNA. Lentivirus expressing GFP shRNA (shGFP) was used as a negative control. mRNA levels were determined by quantitative real-time PCR. (D) Growth curves of hEM2 cells treated with different shRNAs. Data were expressed as mean ± SEM.

carcinoma. A recent longitudinal study that analysed clone-specific *PTEN* mutations in the endometrium over time in women who have increased or minimal risk for developing endometrial neoplasia failed to show that early PTEN loss in endometrial glands contributed to tumour progression [18]. Collectively, these studies suggest that PTEN loss alone is insufficient to drive tumour development; therefore, additional molecular alterations are required to collaborate with PTEN loss to drive tumourigenesis. In this study, we provide new evidence that ARID1A loss (presumably due to somatic inactivating mutation) is a critical molecular factor that collaborates with down-regulation of PTEN to promote cellular proliferation. We demonstrate that proliferative activity increases in AH/EIN glands showing co-loss of ARID1A and PTEN compared to the immediately adjacent glands with only PTEN loss. Thus, ARID1A seems to be a determining factor for controlling rate of cell division, a key factor in tumour evolution. Second, we carried out an *in vitro* functional study in endometrial epithelial cell culture to simulate the loss of ARID1A and/or PTEN in patient samples. Compared to knockdown of either ARID1A or PTEN alone, the growth rate of endometrial epithelial cells was significantly elevated when both *ARID1A* and *PTEN* genes were silenced simultaneously. This result is consistent with findings from a recent study demonstrating molecular collaboration between ARID1A and PTEN using inducible knockout mouse models. In that study, we reported that co-deletion of *ARID1A* and *PTEN,* but not the individual genes, in ovarian surface epithelium results in the genesis of ovarian endometrioid carcinoma in mice [12]. Analysis of TCGA endometrial cancer dataset demonstrated that 66% of well-differentiated endometrioid carcinomas

contained *PTEN* inactivating mutations and 28% had concurrent *ARID1A* and *PTEN* mutations.

From a technical perspective, this report underscores the usefulness of applying immunostaining to the study of clonal evolution based on the expression patterns of ARID1A and PTEN. In comparison to analysis of somatic mutations, loss of ARID1A and PTEN immunoreactivity appears to provide a more sensitive marker for inactivation of the two tumour suppressor pathways, in part because loss of expression can be a result of deletion or epigenetic silencing in addition to sequence mutations [11,19–22]. Furthermore, immunohistochemical analysis enables construction of a detailed map of ARID1A and PTEN loss in AH/EIN, a task that cannot be easily accomplished by mutational analysis, as AH/EIN areas with ARID1A loss tend to be small and morphologically indistinguishable from AH/EIN areas with retained ARID1A.

Based on this study and previously published data, we suggest a tumour progression model for uterine endometrioid carcinoma. Loss of PTEN occurs in sporadic glands in the normal endometrium, but this loss is insufficient to drive tumour initiation unless these PTEN negative glands acquire additional molecular 'hits' which contribute to the formation of AH/EIN [5,23]. Tumour progression from AH/EIN to carcinoma may depend on whether the hyperplastic glands acquire new molecular genetic changes including *ARID1A*. In a background of *PTEN* inactivation, an acquired mutation in *ARID1A* leads to an increased rate of cell proliferation. These *ARID1A*-mutated clones gradually outgrow the surrounding *ARID1A*-wildtype hyperplastic glands which have less proliferative activity. Consequently, *ARID1A*-negative clones progress to endometrioid carcinoma. It needs to be noted that not all endometrioid carcinomas harbour *ARID1A* mutations or have loss of ARID1A expression; therefore, molecular alterations that demonstrate similar functional consequences to *ARID1A* mutation may contribute to tumourigenesis in these cases without ARID1A loss.

In summary, we performed a detailed analysis of ARID1A and PTEN immunostaining and assessed the association between the pattern of their expression and proliferative activity in AH/EIN. We observed intralesional heterogeneity of ARID1A expression in AH/EIN and demonstrated that ARID1A loss in a PTEN loss background correlates with increased proliferation in hyperplastic glands. The above result was validated in our *in vitro* cell culture model. These findings, together with our previous studies [16], suggest a gatekeeper role for ARID1A in preventing tumour progression from AH/EIN to endometrioid carcinoma despite the presence of *PTEN* mutation. It will be important in the future to determine if co-loss of ARID1A and PTEN expression provides a useful tissue biomarker to predict the risk of developing uterine endometrioid carcinoma.

Author contributions

AA and T-LM contributed equally. AA, T-LM, YSR, HO, FZ, R-CW, T-LW, I-MS conceived and AA, T-LM, YSR, HO, FZ, R-CW carried out experiments. All authors were involved in analyzing and interpreting the data, writing the paper and had final approval of the submitted version. AA and I-MS are corresponding authors.

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