Human Reproduction, Vol.34, No.1 pp. 69–78, 2019
Advanced Access publication on November 14, 2018 doi:10.1093/humrep/dey332

human reproduction

ORIGINAL ARTICLE ***Gynaecology***

Iatrogenic endometriosis harbors somatic cancer-driver mutations

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Submitted on April 25, 2018; resubmitted on September 5, 2018; accepted on November 1, 2018

STUDY QUESTION: Does incisional endometriosis (IE) harbor somatic cancer-driver mutations?

SUMMARY ANSWER: We found that approximately one-quarter of IE cases harbor somatic-cancer mutations, which commonly affect components of the MAPK/RAS or PI3K-Akt-mTor signaling pathways.

WHAT IS KNOWN ALREADY: Despite the classification of endometriosis as a benign gynecological disease, it shares key features with cancers such as resistance to apoptosis and stimulation of angiogenesis and is well-established as the precursor of clear cell and endometrioid ovarian carcinomas. Our group has recently shown that deep infiltrating endometriosis (DE), a form of endometriosis that rarely undergoes malignant transformation, harbors recurrent somatic mutations.

STUDY DESIGN, SIZE, DURATION: In a retrospective study comparing iatrogenically induced and endogenously occurring forms of endometriosis unlikely to progress to cancer, we examined endometriosis specimens from 40 women with IE and 36 women with DE. Specimens were collected between 2004 and 2017 from five hospital sites in either Canada, Germany or the Netherlands. IE and DE cohorts were age-matched and all women presented with histologically typical endometriosis without known history of malignancy.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Archival tissue specimens containing endometriotic lesions were macrodissected and/or laser-capture microdissected to enrich endometriotic stroma and epithelium and a hypersensitive cancer hotspot sequencing panel was used to assess for presence of somatic mutations. Mutations were subsequently validated using droplet digital PCR. PTEN and ARID1A immunohistochemistry (IHC) were performed as surrogates for somatic events resulting in functional loss of respective proteins.

MAIN RESULTS AND THE ROLE OF CHANCE: Overall, we detected somatic cancer-driver events in 11 of 40 (27.5%) IE cases and 13 of 36 (36.1%) DE cases, including hotspot mutations in *KRAS*, *ERBB2*, *PIK3CA* and *CTNNB1*. Heterogeneous PTEN loss occurred at similar rates in IE and DE (7/40 vs 5/36, respectively), whereas ARID1A loss only occurred in a single case of DE. While rates of detectable somatic cancer-driver events between IE and DE are not statistically significant ($P > 0.05$), *KRAS* activating mutations were more prevalent in DE.

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LIMITATIONS, REASONS FOR CAUTION: Detection of somatic cancer-driver events were limited to hotspots analyzed in our panel-based sequencing assay and loss of protein expression by IHC from archival tissue. Whole genome or exome sequencing, or epigenetic analysis may uncover additional somatic alterations. Moreover, because of the descriptive nature of this study, the functional roles of identified mutations within the context of endometriosis remain unclear and causality cannot be established.

WIDER IMPLICATIONS OF THE FINDINGS: The alterations we report may be important in driving the growth and survival of endometriosis in ectopic regions of the body. Given the frequency of mutation in surgically displaced endometrium (IE), examination of similar somatic events in eutopic endometrium, as well as clinically annotated cases of other forms of endometriosis, in particular endometriomas that are most commonly linked to malignancy, is warranted.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by a Canadian Cancer Society Impact Grant [701603, PI Huntsman], Canadian Institutes of Health Research Transitional Open Operating Grant [MOP-142273, PI Yong], the Canadian Institutes of Health Research Foundation Grant [FDN-154290, PI Huntsman], the Canadian Institutes of Health Research Project Grant [PJT-156084, PIs Yong and Anglesio], and the Janet D. Cottrelle Foundation through the BC Cancer Foundation [PI Huntsman]. D.G. Huntsman is a co-founder and shareholder of Contextual Genomics Inc., a for profit company that provides clinical reporting to assist in cancer patient treatment. R. Aguirre-Hernandez, J. Khattra and L.M. Prentice have a patent MOLECULAR QUALITY ASSURANCE METHODS FOR USE IN SEQUENCING pending and are current (or former) employees of Contextual Genomics Inc. The remaining authors have no competing interests to declare.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: endometriosis / somatic mutations / targeted sequencing / iatrogenic disease / cesarean section / incisional scar

Introduction

Endometriosis is a chronic, estrogen-dependent, inflammatory gynecological disease characterized by the growth and persistence of endometrial-like glands and stroma outside of the uterus (Giudice, 2010; Vercellini *et al.*, 2014). Roughly 10% of reproductive-aged women and 50% of women experiencing chronic pelvic pain or infertility may be affected by endometriosis (Giudice, 2010). Clinical symptoms associated with endometriosis include chronic and/or cyclical pelvic pain, dyspareunia, dysmenorrhea and subfertility (Giudice, 2010; Vercellini *et al.*, 2014). Not only does endometriosis greatly impair the health-related quality of life and work productivity of affected women (Nnoaham *et al.*, 2011), but it poses a substantial economic burden on the healthcare system. The estimated cost of endometriosis (including both direct and indirect costs) to the USA in 2009 was roughly $69.4 billion USD (Simoens *et al.*, 2012), thereby highlighting the importance of studying this disease beyond associated risk of certain cancers.

Despite the prevalence of endometriosis, its pathogenesis and mechanism of dissemination are poorly understood. Endometriosis primarily manifests itself in the pelvic region in three distinct forms: superficial peritoneal endometriosis, deep infiltrating endometriosis (DE) and ovarian endometriomas (cystic masses affecting the ovary) (Vercellini *et al.*, 2014). It is important to note that endometriosis is not restricted to the pelvis (Giudice, 2010; Matalliotakis *et al.*, 2017); past studies have reported on rare cases of extra-pelvic endometriosis in the lungs, liver, pericardium, surgical scars and even the central nervous system (Gunes *et al.*, 2005; Ceccaroni *et al.*, 2010; Bourgioti *et al.*, 2017; Matalliotakis *et al.*, 2017). Moreover, the specific origin of endometriosis is contentious and several theories on its etiology have been proposed including retrograde menstruation (the reflux of endometrial fragments through the fallopian tubes during menstruation), coelomic metaplasia, Müllerian remnants and lymphatic/vascular dissemination (Vercellini *et al.*, 2014). While each theory is supported by at least circumstantial evidence, none appears sufficient to fully explain every incident case of endometriosis—it may be plausible that different mechanisms may give rise to distinct types of endometriosis.

Although endometriosis is estimated to progress to cancer in only 1% of cases (Vercellini *et al.*, 2014), studies have established endometriosis as the precursor to clear cell ovarian carcinomas (CCOCs) and endometrioid ovarian carcinomas (ENOCs) (Anglesio *et al.*, 2015; Anglesio and Yong, 2017). Women suffering from endometriosis have a 2–4-fold greater risk of developing these cancers (Pearce *et al.*, 2012), moreover, mutational studies demonstrate a clonal relationship between endometriosis-associated ovarian carcinomas and endometriotic lesions. For instance, CCOCs or ENOCs and concurrent endometriotic lesions from the same cases harbored identical somatic mutations in *ARID1A* or *PIK3CA* (Wiegand *et al.*, 2010; Anglesio *et al.*, 2015)—consequently, these alterations contribute to the mutational burden in ovarian endometriosis and have widely been considered early events in the malignant transformation of such lesions.

Our group has recently shown that DE harbors recurrent somatic cancer-driver mutations (Anglesio *et al.*, 2017). Malignant transformation of this particular form of endometriosis is exceedingly rare. Nonetheless, we identified somatic cancer-driver hotspot mutations in *PIK3CA*, *KRAS* and *PPP2R1A*, and loss of function mutations in *ARID1A*, all together affecting 7/27 (25.9%) cases of DE subjected to broad genomic analysis. *KRAS* activating mutations were confirmed in 3/12 additional cases with focused analysis (Anglesio *et al.*, 2017). The function of these mutations in endometriosis is unclear despite the presence of these mutations being clearly non-random. While endometriosis is not considered a malignancy, it shares many notable pathophysiological features with cancers, such as resistance to apoptosis and stimulation of angiogenesis (Taylor *et al.*, 2002; Bulun, 2009). Endometriosis is also capable of invading local tissue, such as in DE (Kavallaris *et al.*, 2003). Therefore, such somatic cancer-driver mutations may be advantageous for the overall survival and growth of endometrial tissue outside of a

native uterine microenvironment. Considering the relative rarity of malignant transformation of endometriosis overall, and particularly DE, it is unlikely that these mutations act solely as early events in malignant transformation. Nevertheless, to understand the importance of these cancer-driver mutations in a non-malignant etiology, it is crucial to determine their prevalence across various forms of the disease.

In the current study, we sought to investigate the prevalence of somatic cancer-driver mutations in endometriosis by comparing DE to another tissue-infiltrating form of endometriosis that is unlikely to undergo malignant transformation. Specifically, we examined incisional endometriosis (IE), an iatrogenic form of endometriosis that occurs in the resulting surgical scars of obstetric or gynecological procedures (Leng *et al.*, 2006). Unlike other forms of endometriosis, the uterine origin of cells is well accepted for IE: endometrial cells, both stroma and epithelium, are mechanically transferred to the abdominal fascia or subcutaneous tissue around sites of incision following procedures such as cesarean sections, hysterectomies, myomectomies appendectomies, tubal ligations and episiotomies (Kaloo *et al.*, 2002; Gunes *et al.*, 2005; Nominato *et al.*, 2010). We compared somatic driver mutation profiles between DE and IE to determine whether there were differences in mutation profile between these two types of endometriosis with unique differences in their etiologies (endogenously occurring vs iatrogenically induced, respectively).

Materials and Methods

Patient identification and tissue collection

We obtained formalin-fixed and paraffin-embedded (FFPE) tissue specimens from four independent cohorts of women with IE. The Vancouver General Hospital in Vancouver, BC, Canada contributed endometriotic tissue samples from 12 IE patients. The Referral Centre for Gynecopathology in Mannheim, Germany contributed tissue samples from 10 IE patients. The University Hospital Tuebingen in Tuebingen, Germany contributed tissue samples from 15 IE patients. Lastly, the VU University Medical Center (VUMC) in Amsterdam, The Netherlands contributed tissue samples from three IE patients. Inclusion criteria for the IE cohort were diagnosis with incisional, umbilical or post C-sectional endometriosis lesions containing both epithelial and stromal components by extensive pathology review, the absence of cancer or dysplasia, and a lesion size sufficient for tissue coring, macrodissection, and/or laser-capture microdissection (LCM). Details of prior surgery and the time interval between suspected inciting surgery and subsequent diagnosis with IE were available for most, but not all, patients (Supplementary Table SI). Note that a few cases included in our IE cohort lacked details of surgical history, or only had a history of surgical abortion, and therefore may represent spontaneous cases of abdominal wall or subcutaneous endometriosis (rather than iatrogenic disease). Adjacent tissue blocks of endometriosis (same anatomical site) were available for sampling for some IE patients (Supplementary Table SI).

In addition, we obtained FFPE or molecular-fixed (Sakura Finetek, USA) and paraffin-embedded (MFPE) tissue specimens from two independent cohorts of women with DE. Endometriotic tissue samples from 23 DE patients were retrieved from local pathology archives and the prospective tissue bank at the BC Women's Centre for Pelvic Pain and Endometriosis in Vancouver, BC, Canada. Ten cases (Patients 41–50) overlap with our previous study (Supplementary Table SII) wherein they were analyzed by droplet digital PCR for *KRAS* mutations alone (Anglesio *et al.*, 2017). Here we include them with a broader genomic analysis as noted below. The VUMC contributed tissue samples from an additional 13 DE patients. Inclusion criteria for the DE cohort were local invasion >5 mm, pathologist-confirmed endometriosis, the absence of cancer or dysplasia, and a lesion size sufficient for tissue coring, macrodissection and/or LCM. Blocks of tissue representing DE at distant/anatomically distinct sites were available for several cases (Supplementary Table SII).

Ethics approval

Institutional review boards at each respective hospital approved tissue collection and collection of clinical data. See the Supplemental methods for further details.

Sample processing and DNA extraction

Except as noted below, specimens were sectioned at 8 µm onto glass slides, deparaffinized with xylene and stained with 10% diluted hematoxylin and eosin (H&E; Supplementary Fig. S1). Using a standard H&E slide as a guide, we manually macrodissected the stained specimens under a stereo microscope using the tip of a 20-guage needle. All tissues for patients 38–40 and 64–76 were enriched by laser-captured microdissection (LCM) by sectioning at 5 µm onto PEN membrane slides (Leica Microsystems Inc., Switzerland), staining with Toluidine blue, and dissecting stromal and epithelial components of endometriosis together using the Leica Laser MicroDissection (LMD) 7 system (Leica Microsystems Inc., Switzerland).

DNA from all enriched specimen was extracted using the ARCTURUS® PicoPure® DNA Extraction Kit (ThermoFisher Scientific, USA) and quantitated using the Qubit 2.0 Fluorometer (ThermoFisher Scientific, USA).

Additionally, a subset of samples initially macrodissected and where somatic mutations were observed, were subject to LCM of distinct stromal and epithelial compartments of endometriosis so as to ascertain which cell populations were affected. DNA was extracted as noted above for other LCM samples.

Targeted sequencing

A proprietary hypersensitive cancer hotspot assay, FIND IT™ version 3.4 (Contextual Genomics, Canada), was used to sequence macrodissected or laser captured endometriotic specimens. Hotspot regions from 33 genes were analyzed (Supplementary Table SI). Libraries were constructed using 45–75 ng of total DNA input. Quality assurance methods based on DNA sequence barcodes were incorporated into the assay and bioinformatics pipeline to increase sensitivity of called mutations. Candidate variants for orthogonal validation were selected as those with probability scores ≥0.8 and variant allele frequency (VAF) ≥0.8% for macrodissected samples or VAF ≥ 5.0% for laser-captured samples, as well as having been previous reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) (Forbes *et al.*, 2017).

Droplet digital PCR assays

We used droplet digital polymerase-chain-reaction (ddPCR) assays to orthogonally validate hotspot mutations identified by targeted sequencing. Moreover, independent of the targeted sequencing results, one sample from each patient was also analyzed for all common *KRAS* activating G12 mutations (G12A, G12C, G12D, G12V, G12R, G12S, a subset of samples also included G13D) by ddPCR assays to rule out false positive and false negative next-generation sequencing-based errors related to *KRAS* (see Supplemental methods for primer/probes sequence). DNA from macrodissected specimens was pre-amplified for 10 cycles before subsequent droplet generation using the QX200 Droplet Generator (Bio-Rad Laboratories, USA). After thermal cycling, the QX200 Droplet Reader (Bio-Rad Laboratories, USA) was used to quantify droplets.

ddPCR was also used to detect select mutations from distinct LCM-enriched stromal and epithelial compartments from a subset of endometriosis lesions (see text and Supplemental methods).

ARID1A and PTEN immunohistochemistry

Loss of nuclear ARID1A immunoreactivity was used as a surrogate for *ARID1A* loss-of-function mutations (Khalique *et al.*, 2018; Kobel *et al.*, 2018). Specimens were stained either on the Dako Omnis automated immunostainer (Agilent Technologies, USA) using a 1:150 dilution of an ARID1A rabbit polyclonal antibody, HPA005456 (Sigma-Aldrich), or on the BenchMark Ultra autostainer (Ventana Medical Systems, USA) using a 1:100 dilution of the same ARID1A rabbit polyclonal antibody (HPA005456). Similarly, loss of PTEN immunoreactivity was used as a surrogate for *PTEN* loss-of-function mutations. Specimens were stained either on the Ventana Discovery Ultra (Ventana Medical Systems, USA) immunostainer using a 1:25 dilution of rabbit monoclonal antibody, 138G6 (Cell Signaling, USA), or on the BenchMark Ultra autostainer (Ventana Medical Systems, USA) using a 1:100 dilution of rabbit monoclonal antibody, SP218 (Spring Bioscience, USA). T.M.N., B.T.C. or H.M.H. scored all ARID1A and PTEN immunostained slides. Specific detail on assays used for individual specimens can be found in the Supplemental methods.

Statistical analyses

The Student's *t*-test was used to compare mean age of IE and DE patients included in this study. Since somatic cancer-driver events are not mutually exclusive of one another, we conducted the Fisher's exact test on each individual pairwise comparison of driver event rates in IE and DE to assess whether they were significantly different. All tests were two-sided and a *P*-value <0.05 was considered to be statistically significant.

Results

Sample description

We examined somatic mutations in common cancer hotspots in 40 women with IE (total of 59 specimens studied), and in 36 women with DE (total of 43 specimens studied). The mean age of women with IE (Patients 1–40) was 36.5 years (28–49 years) (Supplementary Table SII and Supplementary Fig. S2). Between one and four tissue blocks from each patient were collected and analyzed. In four patients (Patients 5, 10, 12 and 28) eutopic endometrium samples were available for sequencing—we did not detect somatic cancer-driver mutations in the available eutopic endometrium specimens. Of IE cases with obtainable surgical history, the original surgical procedure performed was most often cesarean section and the interval between the most recent gynecological or obstetric surgery and subsequent diagnosis with IE ranged from 1 month to 11 years. The mean age of women with DE (Patients 41–76) was 33.9 years (22–50 years) (Supplementary Table SIII and Supplementary Fig. S2). Although most women were affected with DE at a single anatomical site, several women had multiple DE lesions at distinct anatomical sites, these additional lesions were included in analysis when available. The mean age of women in the IE and DE cohorts were not significantly different ($P = 0.0765$, Student's *t*-test) (Supplementary Fig. S2).

Sequencing findings

Of 40 patients with IE, four patients (10.0%) harbored somatic COSMIC hotspot mutations in either *KRAS* (2), *PIK3CA* (1) or *ERBB2* (1) (Table I). Of 36 patients with DE, eight patients (22.2%) harbored somatic cancer-driver mutations in either *KRAS* (7) or *CTNNB1* (1) (Table I and Supplementary Table SIV). No association between detection of mutations and institutional cohort or processing was observed.

Analyzing laser-captured microdissected epithelial and stromal cell fractions using ddPCR, we previously determined *KRAS* G12 mutations to be restricted to the glandular epithelial component of endometriotic lesions (Anglesio *et al.*, 2017). In this study, using the same method, we were able to confirm that similar to *KRAS* observations: *CTNNB1*, *PIK3CA* and *ERBB2* hotspot mutations were also enriched only in the glandular-epithelial compartment of endometriotic lesions (Table I and Fig. 1; Supplementary Fig. S3). Hotspot *KRAS* mutations remained the most common somatic cancer-driver mutations detected in both IE and DE: there were *KRAS* mutations in 2 of 40 patients with IE (5%) compared to 7 of 36 patients with DE (19.4%), however, given our limited sample sizes, this difference is not significant ($P = 0.076$, Fisher's exact test).

Immunohistochemistry

Immunohistochemical (IHC) staining revealed loss of ARID1A protein to be a rare event with only a single case of ARID1A-loss in a DE case (1/36; 3%) and no detectable loss in IE cases (Fig. 2A and B; Supplementary Tables SV and SVI). Conversely, 7 of 40 patients with IE (18%), and 5 of 36 patients with DE, (14%) exhibited loss of PTEN. Whole slide sections revealed a heterogeneous pattern of PTEN loss in endometriotic lesions, wherein only some glands demonstrate loss of PTEN immunoreactivity (Fig. 2C and D; Supplementary Fig. S4; Supplementary Tables SV and SVI). While in some cases PTEN-null glands tended to be clustered (Supplementary Fig. S4), in others the number of endometriosis glands were limited and thus difficult to infer spatial distribution. Consistent with laser-capture analysis of cancer-driver mutations, we observed ARID1A-loss and PTEN-loss only in the epithelial compartment of endometriotic lesions.

Total mutation rates

Accounting for both ddPCR-validated somatic COSMIC hotspot mutations and the IHC findings, the overall rate of somatic cancer-driver events in IE and DE was 27.5% (proportion) and 36.1% (proportion), respectively. The pattern of somatic mutations compared between the IE and DE cases is illustrated in Fig. 3 (see also Supplemental methods and Supplementary Table SVII).

Discussion

Beyond the association of endometriosis and ovarian cancer, endometriosis is an understudied disease as its origin remains contentious and pathophysiology poorly understood. An estimated 176 million women are affected by endometriosis worldwide (Adamson *et al.*, 2010) and thus the disease has a profound impact on the healthcare costs and wellbeing of women in many different countries. Current standard treatment for endometriosis consists of adjunctive medical therapy or surgical resection of endometriotic lesions (Giudice, 2010), however, rates of endometriosis recurrence may be as high as 43–68% (Tandoi *et al.*, 2011). Furthermore, surgical staging of endometriosis, largely based on anatomical presentation, does not appear to correlate well with pain symptoms (Vercellini *et al.*, 2007) and lacks prognostic value for clinical endpoints such as recurrence or risk of malignant transformation (Johnson *et al.*, 2017). Expanding recent finding of somatic molecular alterations across endometriosis types stands to benefit endometriosis classification and may lead to a novel and

Table I Somatic cancer-driver mutations detected in endometriosis (EMS) specimens from women with incisional endometriosis (IE) and deep infiltrating endometriosis (DE). The variant allele frequency (VAF) of macrodissected or laser-capture microdissected (LCM) specimens as determined by means of targeted panel sequencing and corresponding droplet digital PCR (ddPCR) assays are presented below. 'Adjacent' refers to tissue specimens obtained from a different archival tissue block yet the same anatomical site as the index block. 'Separate' refers to specimens obtained from an anatomically distinct site from the index block.

EMS type	Patient and block	Descriptor	Driver mutation identified	Collection method and component	VAF (%)—targeted sequencing	VAF (%)— ddPCR
IE	8A	Index	KRAS G12V	Macrodissection: mixed	3.04	2.53
			KRAS G12V	LCM: mixed		28.7
	8B	Adjacent	KRAS G12V	Macrodissection: mixed	Not detected	3.29
IE	16A	Index	ERBB2 S310F	Macrodissection: mixed	3.336	3.97
			ERBB2 S310F	LCM: mixed		18.2
			ERBB2 S310F	LCM: epithelium		21.4
			ERBB2 S310F	LCM: stroma		1.36
IE	19A	Index	KRAS G12C	Macrodissection: mixed	4.833	3.32
			KRAS G12C	LCM: mixed		29.5
IE	25A	Index	PIK3CA H1047R	Macrodissection: mixed	5.359	5.79
			PIK3CA H1047R	LCM: epithelium		24.9
			PIK3CA H1047R	LCM: stroma		0.567
DE	41A	Index	CTNNB1 G34V	Macrodissection: mixed	3.933	3.88
			CTNNB1 G34V	LCM: epithelium		19.5
			CTNNB1 G34V	LCM: stroma		0.664
DE	42A	Index	KRAS G12D	Macrodissection: mixed	2.807	2.14
			KRAS G12D	LCM: epithelium		38.125
			KRAS G12D	LCM: stroma		0.002
DE	45A	Index	KRAS G12D	Macrodissection: mixed	0.932	n/a
			KRAS G12D	LCM: mixed		2.065
DE	50A	Index	KRAS G12V	Macrodissection: mixed	Not detected	0.941
			KRAS G12V	LCM: mixed		3.589
DE	51A	Index	KRAS G12D	Macrodissection: mixed	1.108	1.03
DE	54A	Index	KRAS G12C	Macrodissection: mixed	1.05	1.19
DE	61B	Separate	KRAS G12V	Macrodissection: mixed	2.627	2.81
DE	72A	Index	KRAS G12A	LCM: mixed	10.749	10.41

more biologically informative system of classification. Widespread knowledge on the prevalence of mutations may highlight common pathway dysfunction. Even with difficulties in targeting RAS-pathway (Samatar and Poulikakos, 2014) and potential toxicities related to PI3K-Akt pathway inhibitors (Engelman, 2009), molecular characterization may justify the use of targeted therapies in select circumstances and will undoubtedly drive innovation for novel intervention strategies.

Our previous study revealed the presence of recurrent somatic cancer-driver mutations (particularly *KRAS*) in DE (Anglesio *et al.*, 2017). In the current study, we analyzed the prevalence of somatic cancer-driver events in IE, another form of endometriosis with little malignant potential, using a hypersensitive cancer hotspot assay combined with orthogonal validation by ddPCR or IHC staining. We found that the overall rates of somatic cancer-driver events to be similar for IE and DE, moreover, the spectrum of affected pathways was similar. The similarity in the rates of mutation and mutational profile of IE and DE is consistent with endometriotic cells in both forms of endometriosis originating from a similar etiology. Because IE is accepted to originate from endometrial cells in the uterus via iatrogenic transplantation, this may further the uterine origin of DE as well (e.g. secondary to retrograde menstruation).

Although our sample sizes were insufficiently large to conclude differences in either overall rates of somatic events or enrichment of particular alterations when comparing IE and DE, it is apparent that alterations resulting in upregulation of the MAPK/RAS or PI3K-Akt-mTOR signaling pathways are present in a substantial fraction of endometriosis cases. In endometriosis, somatic alterations in these pathways may confer a survival advantage to cells. For example via production of high levels of VEGF and BCL-2 or BCL-xL and therefore the stimulation of angiogenesis and resistance to apoptosis respectively (Taylor *et al.*, 2002; Beliard *et al.*, 2004; Braun *et al.*, 2007; Pylayeva-Gupta *et al.*, 2011). In fact, studies have demonstrated the upregulation of VEGF and other pro-angiogenic factors in oncogenic *KRAS*-transformed epithelial cells (Matsuo *et al.*, 2009; Wang *et al.*, 2015), though not yet in the context

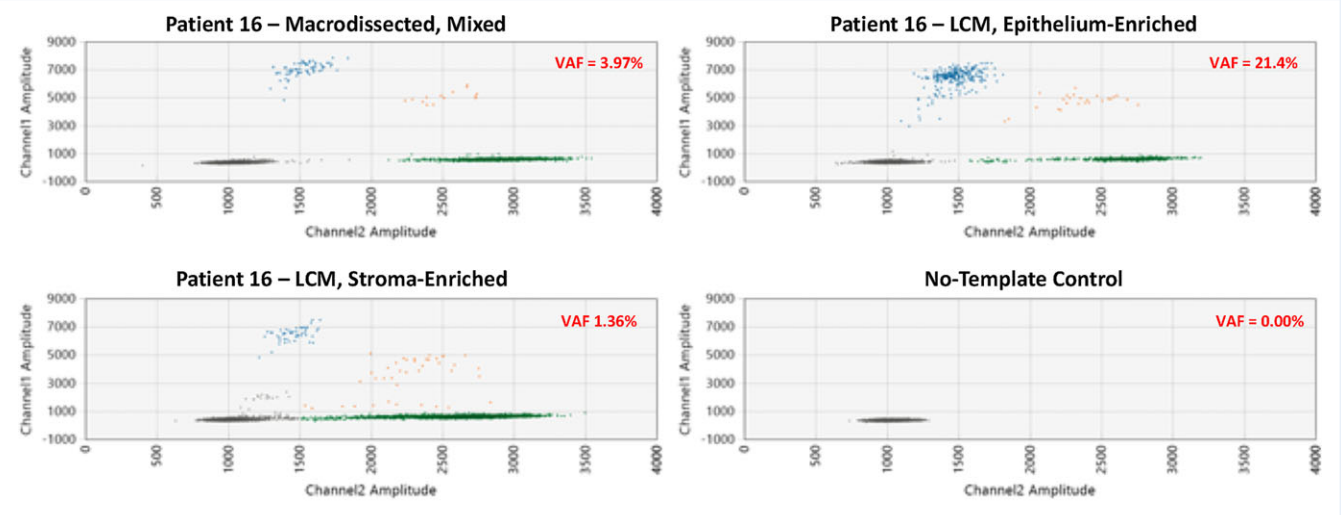

Figure 1 Droplet digital PCR (ddPCR) validation of *ERBB2* c.929C>T (p.S310F) mutation in the epithelial component of endometriosis in Patient 16. Patient 16 harbors an *ERRB2* S310F mutation as detected by ddPCR in a macrodissected endometriosis sample. The variant allelic frequency (VAF) in the epithelium-enriched, laser-captured microdissected (LCM) sample (from the same tissue block) is notably higher than in the stroma-enriched LCM sample (21.4 vs 1.4%, respectively), consistent with this somatic alteration being epithelial-restricted.

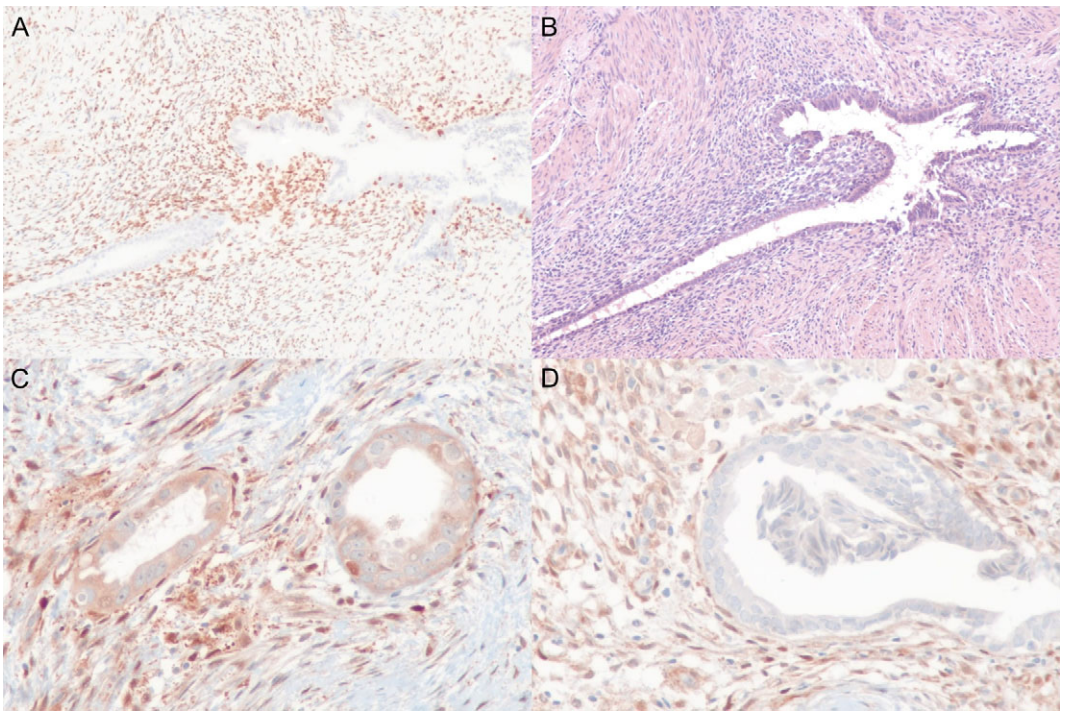

Figure 2 Immunohistochemistry of deep and incisional endometriosis samples showing (A**) loss of ARID1A in epithelial endometriosis cells and (**B**) matching haematoxylin and eosin staining from deep endometriosis patient 47.** Similarly, we observed both deep and incisional endometriosis with heterogeneous expression of PTEN. For example, incisional endometriosis patient 17 (**C**) shows intermediate PTEN expression in some regions of glandular epithelium, while (**D**) loss of PTEN expression was apparent in other regions. In both the context of ARID1A and PTEN immunohistochemistry, the endometriosis stromal compartment (and surrounding normal tissues) provides a clear, block-specific, positive control with strong expression visible for both proteins, respectively.

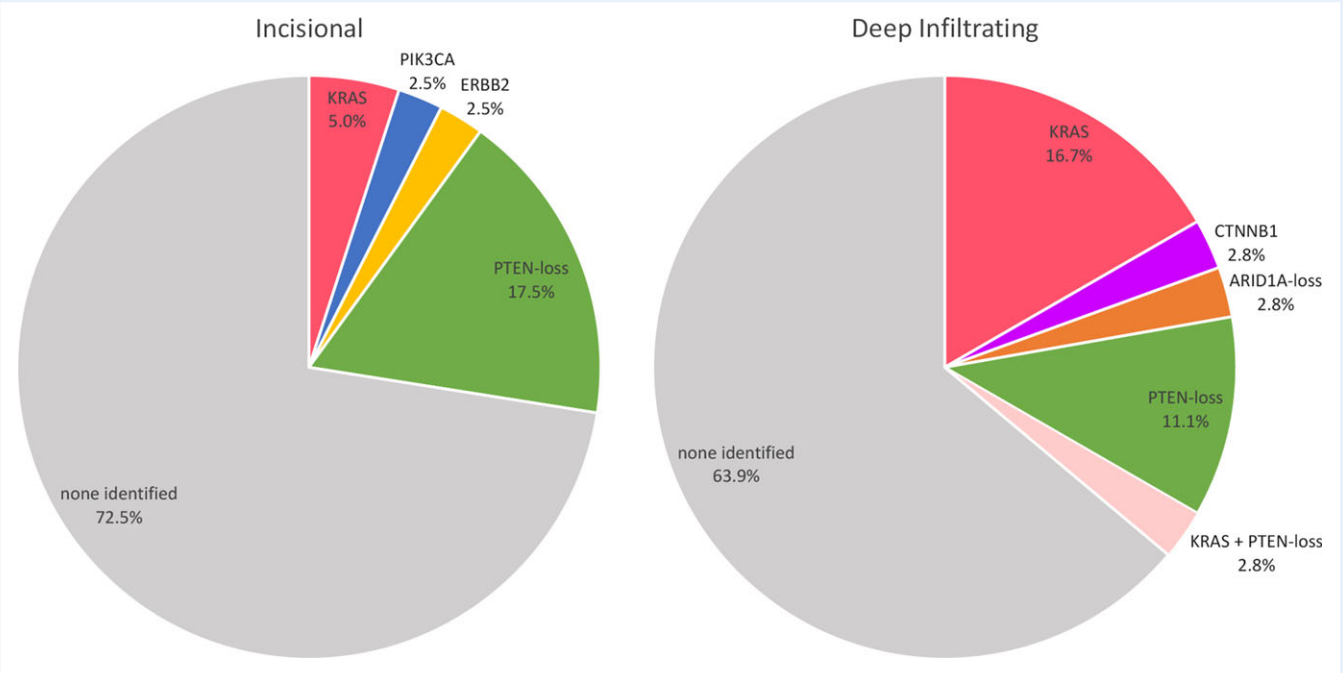

Figure 3 Overview of somatic cancer-driver events in incisional endometriosis (left) and deep infiltrating endometriosis (right). The reported proportion of cases is based on an observed event from any specimen(s) from each case, this is important in the context of deep endometriosis especially as multiple blocks and anatomical sites were assayed. Our results suggest that somatic alterations were sub-clonal in all informative cases, therefore, it is possible that additional re-sampling may increase the proportion of patients in both deep or incisional categories with one or more observable somatic alteration in one or more lesion(s).

of endometriosis. Additionally, the expression of oncogenic Ras results in the upregulation of BCL-xL in colon cancer cells and the upregulation of both BCL-2 and BCL-xL in hematopoietic cells *in vitro* (Kinoshita *et al.*, 1995; Okamoto *et al.*, 2015). In support of our hypothesis, Cheng *et al.* (2011) were able to develop a mouse model of endometriosis by transplanting endometrium from $KRAS^{G12V/+}$ donor mice into subcutaneous, abdominal pockets of immunocompetent recipient mice. In this model, oncogenic *KRAS* promoted the formation of endometriosis and enabled the prolonged survival of endometriotic lesions but does not result in malignant transformation (Cheng *et al.*, 2011). In a more recent study, *KRAS* activation in (mouse) eutopic endometrial tissues was suggested to regulate progesterone receptor transcriptional function via SIRT1 and lead to progesterone resistance (Yoo *et al.*, 2017). SIRT1 has also been shown to be required in maintaining stemness/enabling transformation in a Ras-driven model of glial tumors and increases activation of p44/42 (ERK) seen in combination with reduced cellular senescence in lung fibroblasts (Huang *et al.*, 2008; Lee *et al.*, 2015; O'Callaghan and Vassilopoulos, 2017). However, it should be noted that neither of these models are estrogen/progesterone dependent. While SIRT1 activity linked to *KRAS* activation may indeed be important in endometriosis pathogenesis, any link to progesterone receptor activity remains tenuous, in particular since the Yoo *et al.* (2007) model may be confounded due to its use of the Pgr-CRE promotor to induce $KRAS^{G12D}$ expression. Nonetheless, before accepting any potential mechanism related to our observed cancer-driver mutations, model systems incorporating somatic mutations into non-malignant endometriosis must be improved and interrogated.

Our data also broadly question the defacto-labeling of 'cancer driver' genes as single mutational hits do not appear adequate to trigger malignant progression of endometriosis. To date, the generation of (mouse) models resembling the subtypes of ovarian cancer associated with endometriosis (CCOCs or ENOCs) require at minimum two somatic alterations (Dinulescu *et al.*, 2005; Wu *et al.*, 2007; Chandler *et al.*, 2015), and none develop a validated physiological analogue of endometriosis. Furthermore, mutations in oncogenes such as *KRAS* and tumor suppressor genes such as *PTEN* are well described to trigger cellular senescence in benign lesions after an initial period of proliferation (Courtois-Cox *et al.*, 2008). It remains to be seen if such a paradigm of negative feedback could apply to endometriosis carrying driver gene alterations, e.g. could such alterations provide sufficient proliferative advantage for lesion establishment yet ultimately abrogate progression? However, endometriotic lesions grow, or persist, in 78% of cases and rarely subside spontaneously (Abbott *et al.*, 2004) and endometriosis is reported to exhibit aberrant response to anti-proliferative signals.

Similar to our previous report, ddPCR assays and IHC staining revealed that all somatic cancer-driver events observed (hotspot mutations in *KRAS, CTNNB1 and PIK3CA*, loss of PTEN, or loss of ARID1A) affected only the epithelial compartment of endometriotic lesions. Moreover, visualization of lesions with PTEN-loss or ARID1A-loss revealed that only some, generally clustered, glands were affected by these somatic events whereas other surrounding glands had normal expression—consistent with clonal expansion of an affected epithelial cell. While our methods do not allow us to make similar observation

for the hotspot mutations, we would expect this to be the case. Curiously, we observed PTEN-loss in several women with IE or DE yet, based on our analysis, it is unclear by which mechanism loss of PTEN occurs in endometriosis. Targeted panel sequencing did not reveal loss-of-function hotspot point mutations in *PTEN*, despite partial coverage over this gene. However, it is possible that PTEN is lost through large-scale deletion or epigenetic mechanisms/methylation.

Finally, it is unclear whether mutations independently arise in implanted endometrial cells or whether they are already present in the endometrium prior to implantation/seeding. In other words, are such cancer-driver events naturally present at low levels in the eutopic endometrium? It is conceivable that accumulation of somatic alterations, including driver mutations in selective/permissive microenvironments, reflects the aging of tissues (Risques and Kennedy, 2018). A recent study analyzing uterine lavage fluid reported cancer-associated mutations, including mutations in *KRAS* and *PIK3CA*, in roughly half of women analyzed (51 of 95) that lacked histopathological evidence of (endometrial) cancer (Nair *et al.*, 2016). Likewise, peritoneal washing revealed *TP53* mutations in 19 of 20 control women (women unaffected by cancer or reported benign pathology), albeit at ultra-low allelic frequencies (<0.1%), with an apparent increase in mutational burden correlating with age (Krimmel *et al.*, 2016). As the mutations we have identified in women with IE and DE are common in CCOCs and ENOCs (Committee on the State of the Science in Ovarian Cancer Research, 2016; Wang *et al.*, 2017), establishing the prevalence of these mutation across types of endometriosis is warranted, in particular for endometriomas where relative risk of malignant transformations is considerably higher (Saavalainen *et al.*, 2018).

By taking into account the diffuse cellular make-up of endometriosis specimens and challenges of ultra-low input sequencing from FFPE tissue, we have confirmed the presence of somatic cancer-driver events in women with IE as well as DE. These two forms of endometriosis are associated with very low malignant potential, however, nearly one-third (31.6%) of all endometriosis cases analyzed harbored cancer-driver events, most commonly activating mutations of *KRAS* and loss of PTEN expression. Our screen for mutations is not exhaustive, and it is possible that whole genome/exome sequencing, or epigenetic analysis, may uncover additional somatic 'driver' events. Nevertheless, it is evident that these somatic events, particularly those involving the RAS/MAPK pathway or PI3K-Akt-mTOR pathway, are inherent features of endometriosis outside of the context of cancer and represent potential mechanisms that contribute to endometriosis pathology. Further exploration on the prevalence and function of these alterations is greatly needed to enhance our understanding and management of this vastly understudied disease.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We thank the women who have allowed us to use their tissue samples in our research study. We would also like to thank the staff at the Genetic Pathology Evaluation Centre (GPEC) and the Department of Anatomical Pathology at the Vancouver General Hospital in Vancouver for their assistance with specimen collection, immunohistochemical staining and optimization, and tissue sectioning. Lastly, we would like to acknowledge the NKI-AVL Core Facility Molecular Pathology & Biobanking (CFMPB) for supplying NKI-AVL Biobank material and /or lab support.

Author's Roles

V.L., H.M.H., P.J.Y., D.G.H. and M.S.A. designed the study. V.L., L.V., T.M.N., B.T.C., T.P., N.L.O., H.N., A.L., J.K., L.M.P., D.C., M.K., V.M., A.F.L., J.P., M.C.B., B.K., S.Y.B., F.K. and S.K. collected specimens and data. T.M.N., B.T.C. and H.M.H. scored immunostained slides. V.L. and R.A.H. performed data analysis. V.L. and L.V. drafted the article. All authors revised the manuscript and approved submission of the final version.

Funding

Canadian Cancer Society Impact Grant [701603, PI Huntsman], Canadian Institutes of Health Research Transitional Open Operating Grant [MOP-142273, PI Yong], the Canadian Institutes of Health Research Foundation Grant [FDN-154290, PI Huntsman], the Canadian Institutes of Health Research Project Grant [PJT-156084, PIs Yong and Anglesio], and the Janet D. Cottrelle Foundation through the BC Cancer Foundation [PI Huntsman]. The BC Women's Hospital and Health Centre Foundation and Women's Health Research Institute (Nelly Auersperg Grant) provided support to the BC Women's Centre for Pelvic Pain and Endometriosis. The BC Cancer Foundation and the VGH and UBC Hospital Foundation provided funding to OVCARE: BC's Ovarian Cancer Research Team (including V.L., T.M.N., B.C.C., T.P., A.L., D.C., P.J.Y., D.G.H. and M.S.A.). A Frederick Banting and Charles Best Canada Graduate Scholarship from the Canadian Institutes of Health Research provided support to V.L. The Dutch Cancer Society translational research fellowship [KWF 2013-5869] provided support to H.M.H. The Michael Smith Foundation for Health Research Health Professional Investigator Award provided support to P.J. Yong. The Janet D. Cottrelle Foundation Scholars fund provided support to M.S. Anglesio. The Dr Chew Wei Memorial Professorship in Gynecologic Oncology and the Canada Research Chairs program (Research Chair in Molecular and Genomic Pathology) provided support to D.G. Huntsman.

Conflict of interest

D.G.H. is a co-founder and shareholder of Contextual Genomics Inc., a for profit company that provides clinical reporting to assist in cancer patient treatment. R.A.-H., J.K. and L.M.P. have a patent MOLECULAR QUALITY ASSURANCE METHODS FOR USE IN SEQUENCING pending and are current (or former) employees of Contextual Genomics Inc. The remaining authors have no competing interests to declare.

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