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ORIGINAL PAPER

Subtype-specific mutation of *PPP2R1A* in endometrial and ovarian carcinomas

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Abstract

PPP2R1A **mutations have recently been described in 3/42 (7%) of clear cell carcinomas of the ovary.** ***PPP2R1A*** **encodes the α-isoform of the scaffolding subunit of the serine/threonine protein phosphatase 2A (PP2A) holoenzyme. This putative tumour suppressor complex is involved in growth and survival pathways. Through targeted sequencing of** ***PPP2R1A***, **we identified somatic missense mutations in 40.8% (20/49) of high-grade serous endometrial tumours, and 5.0% (3/60) of endometrial endometrioid carcinomas. Mutations were also identified in ovarian tumours at lower frequencies: 12.2% (5/41) of endometrioid and 4.1% (2/49) of clear cell carcinomas. No mutations were found in 50 high-grade and 12 low-grade serous carcinomas. Amino acid residues affected by these mutations are highly conserved across species and are involved in direct interactions with regulatory B-subunits of the PP2A holoenzyme.** ***PPP2R1A*** **mutations in endometrial high-grade serous carcinomas are a frequent and potentially targetable feature of this disease. The finding of frequent** ***PPP2R1A*** **mutations in high-grade serous carcinoma of the endometrium but not in high-grade serous carcinoma of the ovary provides clear genetic evidence that these are distinct diseases.**

Keywords: endometrial carcinoma; ovarian carcinoma; PPP2R1A; PP2A; high-grade serous; mutations; uterine carcinoma

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Introduction

Endometrial, or uterine, carcinoma is the most common gynaecological cancer. Ovarian cancer, while rarer, is a more frequent cause of mortality, being the fifth most deadly cancer overall affecting women in Canada and the USA [1,2]. Endometrial carcinomas are histopathologically subclassified based on tumour cell type, and endometrioid carcinomas are most common. High-grade serous carcinomas account for only 10% of endometrial carcinomas, however are responsible for approximately 50% of deaths, reflecting its aggressive behaviour [3,4]. Ovarian carcinomas are similarly classified based on tumour cell type and it is widely accepted that within both endometrial and ovarian carcinoma there are specific molecular alterations that characterize each subtype [5–7], and compelling evidence that ovarian carcinoma subtypes are distinct diseases [8]. Although there are striking clinical and pathological similarities between high-grade serous carcinoma of endometrium and ovary, and between endometrioid carcinoma of endometrium and ovary, it is less clear whether tumours of comparable cell type arising in different organs are also similar with respect to underlying molecular abnormalities. This is an important consideration if treatments are based on tumour cell type. Rather than organ of origin. In the case of endometrial carcinomas a handful

of studies have suggested that endometrioid carcinomas possess recurrent mutations in *PTEN, PIK3CA, KRAS* and *CTNNB1* and are frequently microsatellite-unstable [9–11], whereas high-grade serous carcinomas have frequent abnormalities of *p53*, *ERBB2*, *p16* and *E-cadherin* [11]. There has been a call in recent literature to identify new markers to define patient groups that would benefit from alternative or aggressive treatments for both ovarian and endometrial cancers [8,12]. To accomplish this, molecular and mutational characterization is needed to better understand the carcinoma subtypes and their relationship within a spectrum of gynaecological malignancies.

Protein phosphatase 2A (PP2A) is one of the four major serine/threonine phosphatases [13]. It is involved in a wide array of cellular pathways, including cell growth and survival, and has been implicated to have a tumour suppressor role [14,15]. The core enzyme is a heterodimer made up of a 65 kDa scaffolding A subunit (α- or β-isoform) and a 36 kDa catalytic C subunit (α- or β-isoform), which associates with a regulatory B subunit (multiple potential family members and isoforms) to make up the heterotrimeric holoenzyme [16,17]. PPP2R1A (PR65a) is the α-isoform of the scaffolding A subunit that interacts with both the B and C subunits of the protein. This α-isoform is ubiquitously expressed and is typically 10–100 times more abundant than the β-isoforms (PPP2R1B) [18].The structure of PPP2R1A is composed of a curved, hook-like helical structure formed by 15 tandemly repeated Huntington-Elongation-A subunit-TOR (HEAT) motifs [19]. The helical conformation of PPP2R1A contains an intra-repeat region, which is highly conserved, and an inter-repeat region formed by the turns and loops of the helices [20,21]. The intra-repeat HEAT motifs 11–15 form strong hydrogen bonds and hydrophobic interactions with the catalytic C subunits of PP2A, while the HEAT motifs 2–7, located on the apical side of the A subunit, loosely interacts with the isoforms of the regulatory B subunit and has no interaction with the catalytic C subunit [13,22].

We now provide evidence supporting a recent publication [23] finding low-frequency mutations of *PPP2R1A* in 7% (3/42) of ovarian clear cell carcinomas, in which these mutations impacted neighbouring codons in exon 5 of the gene. Furthermore, we present the novel finding that this gene is also mutated in endometrioid-type endometrial and ovarian carcinomas and in a significantly higher percentage of high-grade serous endometrial carcinomas, but not high-grade serous carcinomas of the ovary.

Methods

Patient samples

The tumour specimens analysed in this study for DNA and RNA sequencing were collected via several tumour banks and tissue repositories, at the BC Cancer Agency and Vancouver General Hospital (via OvCaRe), the Australian Ovarian Cancer Study (AOCS) and the Montreal tumour bank (Banque de Tissus et de Données, of the Réseau de Recherche sur le Cancer of the Fonds de la Recherche en Santé du Québec, affiliated with the Canadian Tumour Repository Network) (see also Supporting information, Table S2). Patients were approached for written informed consent before undergoing surgery, to donate tissue surplus to diagnostic requirements plus a blood sample and for use in a research ethics board (REB)-approved research protocol. All patients were informed at the time of consent about the potential risks of loss of confidentiality arising from use of their samples in research, and that none of the research study data would ever form part of the clinical record or be reported back to the care physicians (see also Wiegand *et al* [24]).

Whole-transcriptome sequencing analysis

Whole-transcriptome data from Wiegand *et al* (European Genome–Phenome Archive, Accession No., EGAS00000000075) was analysed as previously described [24,44].

DNA extraction

DNA and RNA were extracted using standard methodologies, as previously described [24,44].

PCR and Sanger sequencing

PCR primer sets were designed to amplify exons 5 and 6 of *PPP2R1A*. Priming sites for −12 M13 forward and −27 M13 reverse were added to the 5′ ends to allow direct Sanger sequencing of amplicons: *PPP2R1A* exons 5 and 6, forward primer 5′-ACAGAGAGGGGGTCATCACTT-3′, reverse 5′-GCCTAATGGAAACCTCAGCTC-3′; FFPE samples were amplified using exon 5, forward 5′-AAAACCTG GACCCACACAAC-3′, reverse 5′-TTGGAGAACATG GGGATGAT-3′; and exon 6 using forward 5′-CTCTC CTCTCCCTAGGACTCG-3′, reverse 5′-GTGTCAGT GTCCCCACCAGT-3′. For PCR reactions, Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) reagents were used. After denaturation at 94 °C for 3 min, DNA was amplified for 35–45 cycles (94 °C 45 s, 64 °C 30 s, 72 °C 30 s), final extension was at 72 °C for 5 min using a MJ Research Tetrad (Ramsey, MN, USA). PCR products were purified using ExoSAP-IT® (USB® Products, Affymetrix, Cleveland, OH, USA) and amplified in both forward and reverse directions, using M13 oligos (M13, forward 5′-TGTAAAACGACGGCCAGT-3′, reverse 5′-CAGGAAACAGCTATGAC-3′) and the ABI BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Amplified products were then sequenced using an ABI Prism 3130×1 Genetic Analyser (Applied Biosystems). All capillary traces were visually inspected to confirm their presence in tumour and absence from germline traces, using Finch

TV (Geospiza, Seattle, WA, USA) and Mutation Surveyor (SoftGenetics LLC). All mutations were confirmed by duplicate PCR reactions.

Immunohistochemistry staining

Sections (4 μm) were processed using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol with proprietary reagents. After baking at 60 °C for 1 h, they were deparaffinized on the automated system using EZ Prep solution (Ventana). A heat-induced antigen retrieval method was used in cell conditioning solution (CC1–Tris-based EDTA buffer, pH 8.0, Ventana). The rabbit monoclonal ER, clone SP1 (cat. no. RM-9101) antibody was obtained from Thermo Fisher Scientific (Ottawa, Ontario, Canada); the mouse monoclonal p53, clone DO-7 (cat. no. M7001) antibody, and mouse monoclonal WT-1, clone 6F-H2 (cat. no. M3561) antibody were obtained from Dako (Burlington, Ontario, Canada). All primary incubations were performed for 60 min with heat, at 1 : 25 dilution for ER, 1 : 400 dilution for p53 and 1 : 50 dilution for WT-1 in Ventana antibody diluents. The Ventana Universal Secondary Antibody was used for 32 min at 37 °C. The detection system used was the Ventana DABMap kit, and slides were then counterstained with haematoxylin and treated with a proprietary blueing agent (p53 and WT-1 used Ventana; ER used Tissue Tek Prisma). All washes were conducted with the Ventana reaction buffer, and the dehydration steps and coverslip procedure were completed according to the manufacturer's recommendations.

Results

From our whole-transcriptome sequencing data [24] we identified and validated one mutation within exon 5 of *PPP2R1A*, corresponding to amino acid residue R183, in an ovarian clear cell carcinoma (OCCC1). To determine whether such mutations are present in other ovarian or endometrial carcinomas, we used Sanger sequencing to analyse exons 5 and 6 of *PPP2R1A* in DNA extracted from 271 tumours (see Methods and Table 1). Although mutations of exon 6 have not been previously described, it was included due to known interactions of amino acids P179, R183, R182 (coded within exon 5) and W257 (coded within exon 6) with the PP2A regulatory B subunits [20–22,25]. We identified both novel and recurrent somatic mutations in both ovarian and endometrial carcinomas. Mutations appeared to be subtype-enriched (Fisher's exact test, $p < 0.0001$): 40.8% (20/49 cases) of high-grade serous endometrial carcinomas compared to only 5.0% (3/60 cases) of endometrial endometrioid carcinomas and 12.2% (5/41 cases) within ovarian endometrioid carcinomas (Figure 1A, Table 1). Mutations were also observed at a lower frequency of 4.1% (2/49 cases) in ovarian clear cell carcinomas (Table 1; see also Supporting information, Table S1), including two distinct missense mutations observed in neighbouring nucleotides, resulting in two apparent amino acid changes in a single case of ovarian clear cell carcinoma (Table 1; see also Supporting information, Table S1). No other cases had more than a single mutation, and none of the mutations appeared to be homozygous. In 28/31 cases, which tested positive for mutations in *PPP2R1A*, we were able to obtain germline DNA to test somatic status. In all 28 cases the normal germline DNA tested negative for *PPP2R1A* mutations, demonstrating that all mutations are somatic (see Supporting information, Tables S1, S2). Also, to further assess the sensitivity of mutation detection by sequencing, we determined the tumour cellularity for the majority of endometrial and ovarian tumours (see Supporting information, Table S2). We confirmed that there are no significant cellularity differences between endometrial high-grade serous carcinomas with and without mutations ($p > 0.3$, range 20–90%), giving a good indication that it is possible to detect *PPP2R1A* mutations in tumours with low cellularity.

With the exception of mutations observed at R182 and R183, none of the other *PPP2R1A* mutations described in this study have been found in other human cancers [26]. All mutations appear to be confined to intra-repeat regions of the PPP2R1A HEAT motifs, with mutations in exon 5 and 6 corresponding to HEAT 5 and 7 motifs, respectively (Figure 1B). These regions are known to interact loosely with the various isoforms of the regulatory B subunit of the PP2A holoenzyme [13,22]. There is no correlation between the intra-repeat loop affected, carcinoma type or subtype. All except one of the observed mutations resulted in a predicted radical amino acid change (see Supporting information, Table S1), and none of the mutations are predicted to result in premature termination or frameshifts.

To give some insight into possible morphological differences within endometrial high-grade serous carcinomas with and without mutations, we assessed expression of p53, WT-1 (Wilms' tumour 1) and ER (oestrogen receptor) by immunohistochemistry (IHC) (see Supporting information, Table S2). The frequency of IHC staining of abnormal p53 and ER immunoreactivity was 87.8% (43/49 cases) and 79.6% (39/49 cases) of endometrial high-grade serous carcinomas, respectively. WT-1-positive IHC staining was also present in 28.6% (14/49 cases), which is consistent with the typical immunohistochemical profile of endometrial serous carcinomas [46]. There were no significant differences of expression (Pearson's χ^2 test, $p > 0.15$) of these molecules within the endometrial high-grade serous carcinomas that were mutation-positive and -negative for *PPP2R1A*. Therefore, *PPP2R1A* mutations are independent of these already known molecular markers of endometrial high-grade serous carcinoma.

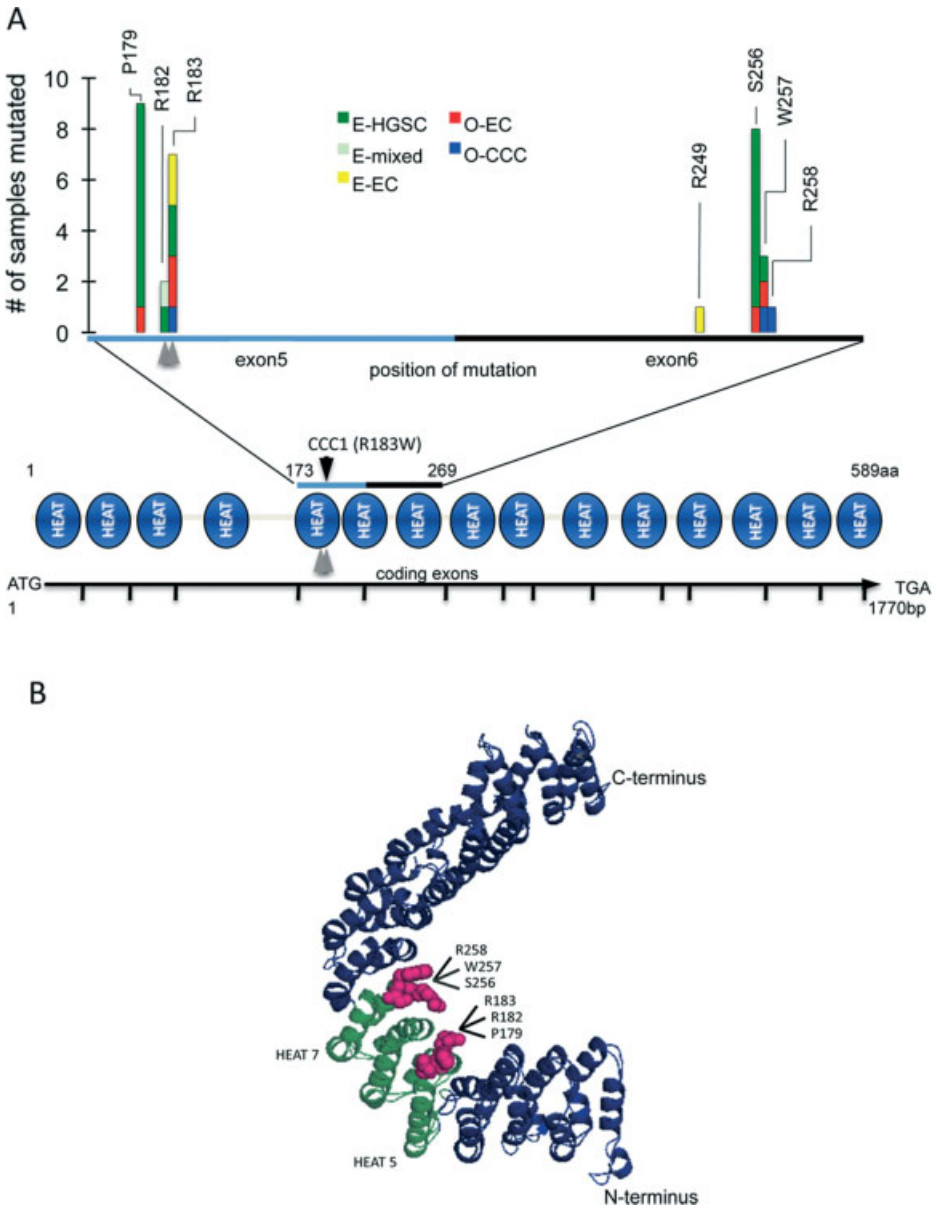

Figure 1. Schematic of mutations in PPP2R1A. (A) Lower panels represent the coding sequence and protein domain structure of PPP2R1A; grey arrows denote positions (corresponding to amino acids R182 and R183) of mutations described by Jones *et al.* [23], while the black arrow corresponds to the position of a single variant detected in whole-transcriptome sequencing data of ovarian clear cell carcinomas from Weigand *et al.* [24]. Upper panel denotes the position (amino acid residue), frequency and subtype of mutations found across the cohort of ovarian and endometrial malignancies as a histogram. (B) Three-dimensional protein structure of the PPP2R1A scaffolding Aα subunit of PP2A, showing 15 repeat HEAT motifs (model generated by SWISS-MODEL [45] and manipulated using PyMOL1.1). The HEAT 5 and 7 motifs coded by exons 5 and 6 are highlighted and amino acids affected by radical changes are annotated. Amino acid residues P179, R183 and W257 are known to interact with the regulatory B subunit of PP2A.

Discussion

High-grade serous endometrial carcinoma (uterine papillary serous carcinoma, or uterine serous carcinoma) is a well-recognized aggressive variant of endometrial cancer [27,28]. It has a high propensity for extrauterine disease at the time of diagnosis [29,31]. Five-year survival has been reported to be as low as 30% [32]. Unlike endometrioid-type endometrial cancer, high-grade serous carcinoma may present with paraaortic nodal or intraperitoneal metastases in the absence of myometrial invasion [29,30]. The identification of high-grade serous carcinoma has significant treatment implications, as these patients almost invariably receive adjuvant therapy, which often includes chemotherapy comparable to that used for high-grade serous carcinoma of the ovary. Despite multimodality therapy, they remain at high risk for relapse and death, which underscores the need for further research into high-grade serous endometrial carcinoma, including the identification of novel molecular targets for treatment.

We have identified a total of 31 missense mutations within exons 5 and 6 of *PPP2R1A*, with novel mutations most frequently (40.8%) found in high-grade serous endometrial carcinomas. No mutations were detected in either high-grade or low-grade serous

Table 1. Frequency of mutations in *PPP2R1A*

	Frequency (%)	Coding sequence changes (mutation occurrence)	Predicted amino acid changes
Endometrial carcinoma			
High-grade serous	20/49 (40.8)	c.536C>G (6)	p.Pro179Arg
		c.536C>T (3)	p.Pro179Leu
		c.544C>T (1)	p.Arg182Trp
		c.547C>T (2)	p.Arg183Trp
		c.767C>T (6)	p.Ser256Phe
		c.767C>A (1)	p.Ser256Tyr
		c.769T>G (1)	p.Trp257Gly
Clear cell	0/1 (0)	NA	NA
Endometrioid	3/60 (5.0)	c.548G>A (2)	p.Arg183Gln
		c.746G>A (1)	pArg249His
Mixed	1/3 (33.3)	c.544C>T (1)	p.Arg182Trp
Ovarian carcinoma			
High-grade serous	0/50 (0)	NA	NA
Low-grade serous	0/12 (0)	NA	NA
Clear cell	2/49 (4.1)	c.547C>T (1)	p.Arg183Trp
		c.771G>T[§] (1)	p.Trp257Cys[§]
		c.772C>T[§] (1)	p.Arg258Cys[§]
Endometrioid	5/41 (12.2)	c.536C>G (1)	p.Pro179Arg
		c.547C>T (2)	p.Arg183Trp
		c.767C>A (1)	p.Ser256Tyr
		c.769T>G (1)	p.Trp257Gly

Sequence coordinates are given relative to the coding sequence, or translation, within *PPP2R1A* transcript variant 1 (NM_014225.5); unique mutations observed for each subtype are listed (see also Figure 1A and Supporting Information Table S1).

[§]Two somatic mutations were found in side-by-side nucleotides, resulting in two apparent codon changes in ovarian clear cell carcinoma sample OCCC2.

ovarian carcinomas; however, only a small number of the latter ($n = 12$) were studied. Previous PPP2R1A mutational analysis has suggested that the mutated amino acids P179, R182, R183 and W257 are involved in the interaction with different B subunit family members [20–22,25]. Mutation of residues 179, 182, and 183 to alanine results in variable loss (50–90%) of binding to B subunit family members and, of particular importance, a W257A mutation completely abolishes binding of B subunit family members [21]. The A subunit residues P179, R183 and W257 are known to interact with B subunit residues at N206, E214 and L107 of the B′ regulatory subunit B56γ1 (PPP2R5Cγ1), respectively [13,22], suggesting that all of these residues may influence interactions with the regulatory B subunits. Additional *in silico* computation, of these protein mutations using MutationAssessor [47], results in a medium functional impact score, adding further evidence to the importance of these amino acid residues. Furthermore, multiple studies have found polyomavirus middle and small tumour (T) antigens and SV40 small t antigens can displace the regulatory B subunits [34,35], resulting in increased phosphorylation of PP2A substrates and increasing cellular transformation [36]. Taken together, these factors may indicate that mutations of the above-noted regions of PPP2R1A could have a dominant-negative effect, modifying or eliminating proper interaction within the holoenzyme of regulatory B subunit family members. This may result in a change of the spectrum of the PP2A scaffold, catalytic and regulatory subunit combinations in cells, and possible destabilization of the PP2A complex.

A recent report from Jones *et al*. suggests that PPP2R1A may have pro-oncogenic functions [23]. This conclusion is largely based on the clustering of mutations in a small region, similar to the pattern of mutations seen in many well-described oncogenes. However, previous literature identifies the PP2A holoenzyme as a putative tumour suppresser [15,37,38]. The PP2A B subunits have been associated with negative regulation of multiple cancer-causing pathways, including c-MYC, BCL2, p53, ERBB2 and AKT (reviewed in [14]). PP2A plays a role in the regulation of MAP kinase signalling and WNT pathways, possibly by stabilizing β-catenin, which can lead to proliferation and tumorigenesis [14,39]. PP2A also plays a role in the G_2–M cell cycle transition through direct interaction and dephosphorylation of CHK2, leading to cell cycle arrest [38,40]. Additionally, the dysfunction of PP2A has been hypothesized to play a role in the progression of serous borderline tumour cell lines [41]. Although mutations of *PPP2R1A* appear at low frequencies, with only one missense mutation previously described in each of lung, breast and melanoma carcinomas and one insertion causing a frameshift in a breast carcinoma [42], the β-isoform of the A subunit, *PPP2R1B*, has been reported to be mutated in 15% of primary lung tumours, 6% of lung tumour cell lines and 15% of primary colon tumours [43].

Previous studies have highlighted the morphological similarities between high-grade serous carcinoma of the endometrium and ovary, starting with the first detailed description of serous carcinoma of endometrium [27]; however, this study demonstrates an important molecular difference between

them. Mutations in *PPP2R1A* were present in 40.8% of endometrial serous carcinomas, but were not seen in any high-grade serous carcinomas of ovary. Although this does not preclude the occurrence of low-frequency mutation in *PPP2R1A* in ovarian high-grade serous carcinomas, it does make them distinct. We have also shown IHC staining for the expression of p53, WT-1 and ER, which is consistent with previous reports of molecular profiling of endometrial high-grade serous carcinomas [48]. These results also confirm distinct expression differences of WT-1 between endometrial and ovarian high-grade serous carcinomas, further strengthening the argument to caution against the common practice of regarding these as equivalent tumours.

Certainly the role of PPP2R1A in cancer has not been fully resolved, and these new data and future studies of full *PPP2R1A* sequencing may reveal further evidence supporting a role for PPP2R1A in endometrial and ovarian cancers. We hypothesize that dysfunction of PPP2R1A, and thus the PP2A holoenzyme, may contribute to disease pathogenesis in a subtype-specific manner, serve as a molecular marker and, upon further study, yield molecular drugable targets for high-grade serous carcinoma of the endometrium.

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Author contributions

MM, MA, BG and DGH conceived and designed the study; MM, MA, AHM, WY, JS, CC, LMP carried out experiments: MM, MA, JM, MC, GM, JSK, SEK, SS, AMMM, BG and DGH collected data, performed data analysis and assisted in interpretation of data; MM, MA, NB, JSK, BG and DGH wrote the manuscript and created the figures; and AMMM and AOCS provided ovarian samples for assays. All authors reviewed and approved the manuscript.

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Table S1. PPP2R1A mutations by sample

Table S2. Endometrial and ovarian carcinomas dataset information