DDRs in healthy and cancerous reproductive systems

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Abstract

DDRs are uniquely positioned to function as sensors for extracellular matrix (ECM) and to regulate a wide-range of cell functions from migration and proliferation to cytokine secretion and ECM homeostasis/remodeling. While activation of DDRs by ECM collagens is required for normal development and tissue homeostasis, aberrant activation and function of these receptors following injury or in disease is detrimental. Both DDRs are indicated to play key roles in development and metastasis of various types of cancer. In spite of this, the mechanisms whereby DDRs contribute to tumor formation and cancer progression are poorly understood. Among reproductive system cancers, epithelial ovarian cancer (EOC) and prostate cancer (PCa) result in high rates of morbidity and mortality. In EOC and PCa, atypical expression of DDRs are indicated to function in malignancy and metastasis. The molecular mechanisms underlying how DDRs contribute to these and other pathologies need to be understood to find new markers and for development of therapeutic agents for treatment of disease. This is particularly the case for EOC in which mechanisms explaining the atypically high levels of DDR1 at initial and late stages of disease have not been described. We first outline studies showing an essential role for DDR2 in steroidogenesis and gamete development in ovary and testes. We then focus on what is known on the role of DDR1 and DDR2 in PCa and DDR1 in EOC. Finally, we speculate on possible functions DDR1 could be playing in different stages of EOC disease. Interactions between ECM proteins and cell surface receptors are well-known to play key roles in regulation of cell behavior and determining physiological function. The switch from DDR2 expression in healthy ovaries to that of DDR1 in initial and late stages of EOC disease provides an experimental system to investigate to what extent ECM and DDR signaling enables malignant transformation.

Introduction

Many members of the receptor tyrosine kinase (RTK) family have been shown to play key regulatory roles in diverse critical cellular processes including growth, differentiation, metabolism and migration. Ligand-binding by RTK extracellular domains transduce environmental signals to the cell interior resulting in an appropriate cellular response. Ligand binding to specific RTK receptors is the basic mechanism for transduction of a wide range of environment signals. Ligand-dependent RTK activation leads to intracellular RTK auto/transphosphorylation resulting in formation of intracellular adaptor protein binding sites. Upon adaptor protein binding the signal is further propagated, ultimately leading to a cellular response. Mutation resulting in altered RTK mRNA formation or expression or RTK protein structure that leads to aberrant RTK signaling have been causally linked to progression of numerous inflammatory diseases and cancers as well as bone disorders.

The human genome encodes 58 RTKs, which are divided into 20 subfamilies based on modular architecture of their ectodomains [1]. The discoidin domain receptor (DDR) subfamily includes two members in vertebrates, DDR1 and DDR2 [2]. In humans DDR2 has only 1 isoform, while DDR1 exists in 5 isoforms (DDR1a–e) generated through alternative splicing [3]. Prototypical RTKs are activated by small soluble polypeptide growth factors, cytokines and hormones. DDRs are unique among RTKs in that they recognize and are activated by specific peptide sequences in collagen [4, 5], a major component of the vertebrate extracellular matrix (ECM) [6]. DDRs are thus attributed with having a direct role in RTK signaling with ECM [4, 5]. Prior to these studies with DDRs, the ECM had been considered to contribute to RTK signaling only indirectly by capturing and storing growth factors [7]. In this time, a signaling role for collagen had only been described for integrins and the platelet receptor, glycoprotein VI [8-10]. Importantly, DDR activation by collagen was shown to be independent of β1 integrins [11]. Both DDRs are activated by fibrillar collagens (e.g., types I, II and III), whilst their preferences for non-fibrillar collagens are distinct, with DDR1 and DDR2 being specific for type IV and type X collagen, respectively [4, 5, 12]. DDR activation strictly requires collagen to be in its native triple-helical conformation; heat-denatured collagen (gelatin) is not recognized by DDRs [4, 5].

Recent reviews discuss downstream signaling pathways and roles for DDRs in human disease [10, 13-15]. The biological outcomes of DDR activation and the roles they play in various human diseases are only partially understood. DDRs are widely expressed in mammalian tissues and regulate cell adhesion, migration, proliferation and differentiation [10, 13], as well as remodeling of ECM by matrix metalloproteinases (MMPs) [16, 17]. Mutation of DDRs leading to altered expression has been associated with many different kinds of cancers. The role DDRs may play in various cancers is complicated in that they may act as pro-tumorigenic or anti-tumorigenic receptors, and their effect is highly dependent on the type and stage of cancer. DDRs are thus recognized as potentially important targets for development of therapeutic agents in

cases where they promote tumorigenesis. This chapter focuses on the role of DDRs in cancers of the male and female reproductive system, with particular attention to ovarian and prostate cancers.

DDR2 in steroidogenesis and gametogenesis

We begin with a discussion of the role of DDR2 in normal healthy development of ovaries and testes. DDR2 is constitutively expressed in somatic interstitial cells of ovary and testis during postnatal development from prepuberty to adulthood [18]. A role for DDR2 in normal development of reproductive organs was shown with the isolation and characterization of smallie (*slie*), a spontaneous recessive mutation in mice [18]. The *slie* mutation was mapped to within a 2 Mb region in chromosome 1 in which a 150 kb deletion resulted in absence of expression of a single gene, *i.e.*, *Ddr2*.

Homozygous *slie* mice (lacking DDR2, $Ddr2^{slie/slie}$), are dwarf (30-40% reduced body weight and shortened long bones) and infertility results in both sexes suggesting that DDR2 is a newly discovered molecular player critical for reproduction [18]. Although the pituitary gland of prepubertal mutant mice was reduced in size compared with that of wild-type mice, gene expression levels for pituitary and hypothalamic-releasing hormones did not differ between $Ddr2^{slie/slie}$ and wild-type mice. This is in contrast with other spontaneous mutations in mice resulting in dwarfism in which neuroendocrine function is altered. Dwarf mutants of this type include Snell dwarf (*dw*), dwarf (*df*) and little (*lit*) mice [19-22]. Mutants *dw*, *df* and *lit* have played valuable roles in understanding the interplay of body size, reproduction and the neuroendocrine axis including the unraveling of the regulatory network of growth hormone underlying neuroendocrine organization and activation.

The study of Kano et al. with $Ddr2^{slie/slie}$ mice, in contrast, indicates that gonad dysfunction in these mice is due to defects in hormone-responsive pathways in peripheral tissue [18]. As noted above, DDR2 is activated by collagens I, II, III and X (also V), but not by basement membrane collagen IV. Besides gonadal development discussed below, DDR2 is involved in remodeling the ECM during proliferation, development and differentiation as well as tissue repair. The shortening of long bones in $Ddr2^{slie/slie}$ mice may be due to the absence of DDR2 to interact with collagen X. This is the main collagen in chondrocyte ECM, and appears to be involved in signaling these cells to proliferate. Mice that do not have collagen X have reduced chondrocyte proliferation as well as stunted long bones [23]. It is thus speculated that interaction of collagen X and DDR2 may be involved in signaling chondrocytes to proliferate, but in $Ddr2^{slie/slie}$ mice this is not possible and normally longer bones were shorter in these mice.

Collagen I is indicated to affect steroidogenesis in the ovarian follicle [24], and to be an endogenous ligand for DDR2 in ovary [18]. There is overlap in distribution of collagen I and DDR2 in the ovary. Collagen I is found throughout the ECM of the mouse ovary

with higher concentrations in the stroma near surface epithelium and follicular compartments, consistently present during follicle maturation [25]. As the follicle develops, oocytes, the antrum and the granulosa cell layer express collagen I [25, 26]. DDR2 detected by indirect immunofluorescence in ovaries of adult wild-type mice was found in interstitial and thecal cells but not in cumulus, inner layer granulosa cells or oocytes. FSH (follicle stimulating hormone) initiates follicular growth, affecting granulosa cells during the initial phase of the menstral cycle. The subsequent surge in LH (luteinizing hormone) that triggers ovulation supports thecal interna cells, which express LH receptor (LHR), to provide precursor sex steroids to granulosa cells (for estrogen production, e.g.). After rupture of the follicle, these cells differentiate into theca lutien cells that secrete progesterone. In testis of adult wild-type mice, DDR2 receptors were detected primarily in somatic Leydig cells [18]. These cells are responsive to LH for testosterone production. DDR2 is expressed in both ovary and testis including interstitial cells, but not in germ cells. In addition *Ddr2* gene expression in gonads was not different between prepubertal and adult wild-type mice.

Follicular development in female *Ddr2*$^{slie/slie}$ mutants reach the Graafian stage, but luteinization of thecal cells was incomplete and corpora lutea were not formed [18]. This defect could not be restored with addition of exogenous LH, in which luteinization and steroid secretion from thecal cells remained deficient. The striking absence of corpora lutea in females and greatly reduced number of spermatids and presence of atrophy of spermatogonia, Serotoli and Leydig cells in males were the most significant differences found between *Ddr2*$^{slie/slie}$ and wild-type mice at 4 months. No gross abnormalities were found in *Ddr2*$^{slie/slie}$ mice in organs and tissues. These data imply that collagen I and DDR2 receptors share a common pathway facilitating steroidogenesis, i.e., steroid production necessary for maturity of structures and functions of the ovulatory cycle.

Ovarian transcriptome microarray analysis of wild-type and *Ddr2*$^{slie/slie}$ mice was compared to identify relative changes in gene expression throughout the mouse genome resulting from loss of DDR2 [24]. Decreases in relative expression of genes in several gene categories that likely regulate or affect ovary development, ovulation and steroid hormone production were found in *Ddr2*$^{slie/slie}$ mice ovaries compared to those of wild-type mice. Decreased expression of LHR and prostaglandin type E and F receptors was observed in *Ddr2*$^{slie/slie}$ ovaries. Surprisingly, MMP gene expression and protein production, which are typically activated in the canonical signaling pathway for DDRs, were not affected by loss of DDR2 in *Ddr2*$^{slie/slie}$ ovaries. Results were consistent with DDR2 signaling regulating classical endocrine pathways known to be critical in ovary development and the ovarian cycle, particularly LH-responsive gene expression [24].

In spite of the lack of detailed molecular or cellular mechanistic models for how DDR2 may interact with the LHR, co-regulated receptors or other molecules affecting LHR expression, it can be speculated that DDR2 may affect somatic cell proliferation in

response to the LH surge. It can also be speculated that DDR2 and LHR or associated gene products positively co-regulate each other to gradually induce remodeling of the follicular wall and release of the oocyte. Release of the oocyte during the LH surge is facilitated by contraction of smooth muscle cells of the theca externa (outer layer of theca folliculi), and involves increased levels of cAMP and progesterone PGF2α. In addition, *Ddr2* expression is increased by exogenous addition of LH in wild-type, but not in *Ddr2*$^{slie/slie}$ mutant ovaries further implicating an important interaction between these receptors. Lack of DDR2 signaling in *Ddr2*$^{slie/slie}$ mice may trigger anovulation by altering gene expression of *LHR,* and to a lesser extent, *Ptgfr* and *Ptger2.* Reduced expression of these receptors, in particular *LHR*, may lead to subsequent down-regulation of anti-apoptosis genes arising from impaired hormone signaling, and in turn drive follicular apoptosis, anovulation and ultimately infertility in *Ddr2*$^{slie/slie}$ mutants [24].

DDR2 is also essential for maintenance of adult male gametogenesis as indicated above. DDR2 is expressed primarily in Leydig cells, and procollagen I, precursor of collagen I is produced by interstitial cells on the outside of the seminiferous tubules [27]. In addition as in the ovary, there is overlap in DDR2 and collagen I localization suggesting a role for DDR2 in spermatogenesis. In a separate study, expression of several receptors, enzymes and proteins related to spermatogenesis was investigated in wild-type and *Ddr2*$^{slie/slie}$ mice at 10 weeks and 5 months of age [27]**.** Levels of LHR, StAR, P450c17, Hsd3b6 and Fshr were comparable for wild-type and *Ddr2*$^{slie/slie}$ mice at 10 weeks, but with Fshr levels appearing slightly higher in the mutant. Also, P450scc was somewhat lower in *Ddr2*$^{slie/slie}$ versus wild-type mice at 10 weeks. At 5 months, all of these proteins, except Fshr; were greatly reduced in *Ddr2*$^{slie/slie}$ versus wild-type mice. Fshr levels, in contrast, were significantly higher in *Ddr2*$^{slie/slie}$ mice compared to wild-type mice. At 10 weeks, a lower but sustained steroidogenesis was evident in Leydig cells of *Ddr2*$^{slie/slie}$ mice compared to wild-type mice. At 5 months a great decline in steroidogenesis in *Ddr2*$^{slie/slie}$ mice was evident that paralleled the lost of LHR. Testosterone was significantly reduced in *Ddr2*$^{slie/slie}$ mice at 5 months of age, but LH was similar in both types of mice at both 10 weeks and 5 months of age. Thus, there are interesting similarities with the functions of FSH and Fshr in spermatogenesis and follicle development in *Ddr2*$^{slie/slie}$. Ovarian follicles could develop only to the Graafian stage and at five months of age spermatogenegesis proceeded only to the round spermatid stage in *Ddr2*$^{slie/slie}$ mice.

Atrophy of Leydig cells was also evident in *Ddr2*$^{slie/slie}$ mice at 5 months. Mature sperm could be produced in younger *Ddr2*$^{slie/slie}$ mice but mature sperm production was not maintained at 5 months. There were no differences in the number of cells undergoing programmed cell death in testes of wild-type and *Ddr2*$^{slie/slie}$ mice at 10 days, but a significantly greater number were undergoing this process in *Ddr2*$^{slie/slie}$ mice at 5 months. The number of differentiated spermatozoa in seminal fluid was also significantly lower in *Ddr2*$^{slie/slie}$ mice at 5 months. The absence of DDR2 affects Leydig cells directly or germ cell communication indirectly. Absence of DDR2 expression alters fertility, spermatogenesis, Leydig cell response to LH and testosterone production in

older *Ddr2*$^{slie/slie}$ male mice. DDR2 thus provides essential functions for the maintenance of spermatogenesis [27].

Male reproductive system cancers

PCa disease development and DDR1

Prostate cancer (PCa) is a very common male malignancy, third in morbidity and sixth in mortality for cancer in males [28]. PCa is also one of the most heritable of all cancers with genetic factors estimated to account for 42% of risk [29]. The prostate secretes fluid that nourishes and protects sperm and requires testosterone to function properly. In PCa, cell proliferation becomes androgen independent. As outlined below, *DDR1* has been shown to be associated with genes clearly linked with risk for PCa. The importance of DDR1 in PCa disease development is implicated in that it functionally interacts with the major PCa antigen, PCA1 [30], and thus plays an important or perhaps an essential role in formation of malignant tumors.

High throughput genome-wide association studies (GWAS) have been highly successful in discovering susceptibility loci for disease [31], and more than 30 loci have been associated with risk for PCa [32]. GWAS of diverse populations representing different ethnicities including Chinese, European and Japanese have consistently identified loci encoding Hepatocyte Nuclear Factor-1, homeobox B (HNF1B, a POU homeodomain-containing transcription factor) as a risk locus for PCa [33-37] *HNF1B* variant rs4430796 (chromosome 17q12) was one of the first loci found to be associated with PCa in both European and Japanese men [33, 36]. Another *HNF1B* variant found to be strongly associated with PCa risk, rs11649743 (chromosome 17q12), was separated from variant rs4430796 by a recombination hotspot [38]. Large-scale fine mapping using single nucleotide polymorphism analysis showed that four other *HNF1B* variants together with variant rs4430796 provided the best model for PCa risk in this chromosomal region [32]. This same study also found variant rs11649743 to be associated with risk, as expected. The mechanism(s) by which variants at 17q12 may alter regulation or splicing of HNFB1 transcripts, e.g., to be associated with PCa risk is not known, and detailed structure-function studies are very clearly needed.

HNF1B, also known as transcription factor 2, was initially defined as a liver-specific factor of the homeobox-containing helix-turn-helix family, and is produced as one of three isoforms. Isoforms HNF1B(A) and HNF1B(B) seem to be transcriptional

activators, and isoform HNF1B(C) a transcriptional repressor [39]. More recently HNF1B was shown to play a regulatory role in nephron and pancreas development [40, 41]. Normally, HNF1B is believed to form homodimers or heterodimers that bind to enhancer and promoter sequences leading to either activation or inhibition of transcription of selected target genes.

A functional role for HNF1B in PCa remains unknown, and there are only vague suggestions on how it may function as a transcription factor. Analysis of expression profiles for 12,625 transcripts in prostate tumors from patients with different types of clinical outcomes showed that differential expression of *HNF1B* was associated with recurrence of the disease [42]. *HNF1B* was one of 14 genes associated with recurrence in this study. In addition, down-regulation of *HNF1B* expression has been associated with renal cell cancer progression [43]. There are also reported differences in *HNFB1* isoform expression comparing normal healthy versus cancerous prostate tissue in which isoform *HNFB1(B)* was higher and isoform *HNF1(C)* was lower in cancerous tissue [44]. A recent report complementing GWAS showing *HNFB1* to be a major risk gene in PCa used meta-analysis of gene expression data. This study showed that *DDR1* was one of only 12 genes associated with both *HNFB1* and PCa risk [45].

PCA1 encodes a predicted DNA alkylation damage repair enzyme having high levels of mRNA expression in prostate carcinoma. PCA1 is a marker for PCa in that it is expressed in a high number of both prostate carcinoma samples as well as atypical cells in high-grade prostatic intraepithelial neoplasias, but not in benign prostatic hyperplasia or normal adjacent tissues. PCA1 is considered to be the human counterpart of *Escherichia coli* AlkB in that *PCA1* transfected COS-7 cells had resistance to methyl methanesulfonate-induced cell death [46]. AlkB is a Fe (II)/2-oxoglutarate-dependent dioxygenase that removes methyl groups from 1-methyl adenines and 3-methyl cytosines in DNA. Knockdown of *PCA1* by small interfering RNA transfection induced apoptosis in reducing expression of anti-apoptotic Bcl-xl in androgen-independent PCa cell line PC3. In addition, silencing *PCA1* significantly down-regulated *DDR1* expression using *in vitro* matrigel and *in vivo* chorioallantoic membrane assays [30]. Transfection with *PCA1* increased levels of both Bcl-xl and DDR1 making cells more invasive with up-regulation of MMP9 in cell line DU145 [43]. Long-term culture of androgen-sensitive cancer cell line LNCaP in androgen-free medium resulted in increased levels of PCA1 as well as expression of Bcl-xl and DDR1. PCA1 and DDR1 were both found to be highly expressed in 169 prostate

carcinomas, including preneoplastic lesions, but no expression was found in normal epithelium. Interestingly, knockdown of DDR1 resulted in suppressed cancer cell invasion and also reduced cell survival in which the level of Bcl-xl expression was reduced to the same level as in knockdown of PCA1. Overexpression of PCA1 resulted in increased constitutive mRNA expression and gelatinolytic activity of MMP9 as well as enhanced cancer invasion *in vitro*, however these activities were strongly suppressed in DDR1 knockdown experiments. MMP9 is thus an effector molecule in PCA1-DDR1 signaling. This signaling pathway involving DDR1 is outlined in Fig. 1. Together, these results indicate that PCA1 and DDR1 are associated with establishment of a hormone-independent state and malignancy potential in PCa [30].

PCa bone metastasis and DDR2

Dissemination and spread of aggressive PCas most frequently results in bone metastasis, and accounts for nearly 80% of PCa metastases. Metastasis of PCa cells to bone involves bone-cancer cell crosstalk mediated by various factors and cytokines [47, 48]. TGF-β secretion in the bone metastatic environment can promote active proliferation and dissemination abilities in PCa cells [49, 50]. It should be acknowledged that TGF-β, together with bone morphogenic protein (BMP), is also intimately involved in normal healthy osteoblast differentiation and bone formation [51]. PCa cells also secrete osteolytic factors, very likely the most important of which is parathyroid hormone-related protein (PTHrP), as it is found at elevated levels in 90% of PCa patients with bone metastasis. PTHrP produced by tumor cells binds to its receptor (PTHR1) on osteoblasts thereby stimulating expression of receptor activator of nuclear factor κB ligand (RANKL), which then induces osteoclast differentiation and activation [52-56]. Bone metastatic PCa cells also highly express bone-related genes that confer osteomimetic properties [57]. Runt-related transcription factor 2 (Runx2), an important transcription factor in osteogenic commitment significantly contributes to expression of genes conferring osteomimetic characteristics [58, 59]. Expression of osteomimetic characterisrics by these cancerous cells is considered to contribute to their survival in the bone matrix environment [57]. Runx plays critical roles in PCa bone metastasis in that it regulates RANKL expression, MMP2, 9 and13 that are involved in bone turnover and vascular endothelial growth factor (VEGF) [60, 61].

DDR2 plays an important role in ECM remodeling in that it regulates MMPs and stimulates cell proliferation, adhesion and migration. DDR2 normally controls osteoblast differentiation and bone formation by regulation of Runx2 phosphorylation and transcription activation dependent on ERK MAPK [62, 63]. Recently, Yan et al.

reported that DDR2 was highly expressed in bone metastatic PCa cells compared with normal healthy cells [64]. When these cancer cells were made to constitutively express DDR2, their mobility and invasiveness was significantly increased. Knockdown of DDR2, on the other hand resulted in corresponding decreases in proliferation and differentiation of osteoblast function. There was also a dramatic stimulation in osteoclast differentiation and bone resorption when DDR2 was highly expressed, whereas knockdown of DDR2 in PCa cells greatly reduced osteoclast differentiation.

Yan et al. proposed that the elevated levels of DDR2 in bone metastatic PCa cells regulates PTHrP expression and secretion as well as gene promoter activity by way of regulating phosphorylation and transcriptional activity of Runx2 to in turn affect RANKL expression in osteoblasts leading to osteoclast activation and resorption of bone. TGF-β stimulation of DDR2, which binds to type I collagen (most abundant in bone), mediated TGF-β-induced bone resorption. This signaling pathway involving DDR2 is outlined in Fig. 2. These authors previously showed that DDR2 activation leads to secretion and activity of MMP2 and 13, and may contribute significantly to early stages of bone metastasis. DDR2 expression on these cells should also target them to type I collagen, e.g., to potential bone metastatic sites. In the recent study [64], they indicate that PTHrP at these sites is stimulated by DDR2 activation in turn acting on osteoblasts to stimulate their own differentiation and bone formation as well as osteoclast differentiation and bone resorption (Fig. 2). This should result in release of stored TGF-β from the bone matrix to regulate DDR2 expression. In this way, DD2 can continue to act as mediator in a vicious cycle of bone destruction and metastatic cancer cell proliferation. DDR2 is thus involved at four important levels in progression and establishment of bone metastatic PCa cells, i.e., invasion, homing or targeting and promotion of both osteoblastic and osteoclastic activities.

DDR2 overexpression in PCa and testis carcinomas

Kim et al. recently reported that DDR2 is overexpressed in several cancerous tissues including those of lung, bladder, stomach and kidney as well as from prostate and testis [65]. The aim of their study was to better understand the potential function of the unusually long intracellular juxtamembrane region (IJR) of DDRs, compared with other RTKs, in collagen-dependent activation. They found that the central part of this region, ca. 50% of the IJR in DDR2, shared very little similarity with the same region in DDR1a. This distinct centralized 59 aa DDR2 IJR subregion, designated as JM2, was found to be an essential contributor to receptor dimerization, along with the transmembrane region (TM). JM2 was also required for efficient binding of collagen to the extracellular

discoidin (DS) domain, and to be required for activation after collagen binding. This study was focused experimentally on a lung cancer model rather than prostate or testis models.

Other cancers of the male reproductive system

Studies describing DDRs involved in cancers of epididymis, vas deferens and seminal vesicle have not been reported.

Female reproductive system cancers

Ovarian cancer

In contrast with normal healthy ovaries in which DDR2 plays an essential role in follicle development and steroidogenesis, analysis of a cDNA library of SKOV-3, an EOC cell line, showed that DDR1 was expressed [66]. They also reported that in various types of ovarian cancer cell lines DDR1 mRNA was expressed in moderately or poorly differentiated ovarian tumors, while benign or borderline tumors showed very little expression [66]. Another study showed that DDR1 mRNA expression was localized within cancerous ovarian epithelial tissue [67]. DDR1 protein expression in ovarian tumors was first reported by Heinzelmann-Schwarz, et al. [68]. High levels of DDR1 protein were found in all EOC histological types and in ovarian epithelial inclusion cysts, which are thought to be sites of metaplastic change within normal ovarian tissue. There was no significant correlation between DDR1 protein expression and prognosis [68]. DDR1 expression levels in ovarian cancer tissue samples were increased by an average of 6.7 fold compared with normal ovarian tissue. DDR1 protein was highly expressed in 69% (46/67) of serous ovarian cancer tissue samples whereas it was undetectable in normal ovarian surface epithelium. [69,70]. Recently, Testuri and Marco found, however, that high levels of DDR1 protein expression are present in both high grade as well as borderline EOC human tumors from all of over 50 patients using immunohistochemical methods [unpublished results].

Another study of serous ovarian tumors found high levels of DDR1 protein in high-grade and advanced stage tumors compared with low-grade and early-stage tumors, and a significant correlation between DDR1 protein expression and poor outcome [71]. Thus, two independent studies [68, 71] suggest that DDR1 may be a useful target and biomarker for early [68] and advanced [71] ovarian cancer. DDR1 was expressed significantly more frequently in high-grade (79%) and advanced stage (77%) tumors

compared to low-grade (50%) and early stage (43%) tumors. Although the functional role for DDR1 in EOC remains to be understood, results indicate that DDR1 expression may serve as both a potential biomarker and a molecular target for advanced EOC [71].

Expression of DDR1 in EOC was described as predominantly cytoplasmic [68] suggesting that the receptor had been internalized to further propagate the activating signal [72, 73]. Alternatively, significant internalization may suggest adding another function in tumorigenesis not related to surface-mediated cell adhesion, such as receptor trafficking. These results also suggested that increased levels of DDR1 are correlated with early events in EOC development and has potential application for early detection of disease. Another study used short hairpin RNA (shRNA) interference to knockdown N-myc downstream regulated gene 1 (NDRG1), an important gene that in some cases may promote tumor invasion and in others act as a tumor suppressor. Results showed that DDR2 was one of ten genes up-regulated in common by an ovarian cancer cell line (HO-8910PM) and a cervical cancer cell line (CaSki). Interestingly, NDRG1 knockdown also resulted in reduction of MMP7 in both cell lines, which is considered to be a pro-invasion gene [74].

Other gynecological cancers

Endometrial cancer

Endometrial cancer (EC) is the most frequently found of invasive tumors in the female genital tract. Although usually detected in its initial stages, still 20% of patients are first diagnosed after advanced disease has developed. To date, no characterized molecular marker has been validated for diagnosis of EC. Two different studies on EC reported that DDR1 mRNA was significantly up-regulated in tumor tissues and in uterine aspirates compared with normal tissue. This suggests that DDR1 could serve as a novel diagnostic/prognostic marker for this type of cancer [75, 76]. Recently, somatically mutated RTK genes were sequenced from 112 EC tumors, and as well were evaluated for copy number alterations. This study showed the occurrence of low-frequency somatic mutations in DDR1 among serous, clear cell, and endometrioid ECs [77].

Cervical and other cancers

As discussed above using shRNA methods to suppress NDRG1 expression, the same strategy was used in CaSki cells (a metastatic cervical cancer cell line). Results showed that DDR2 was upregulated as well and was related to increased tumor cell adhesion, migration, and invasion activities but not cell proliferation using in vitro assays [74]. Also, the morphology of the cells was not affected by downregulation of NDRG1. However, there are also reports showing that NDRG1 may be a putative tumor metastasis promoter gene [74]. There are currently no other studies describing DDRs as being involved in cancers of other specific organs of the female reproductive system, i.e., uterus, Fallopian tubes, vulva or vagina.

EOC development and metastasis

Pathology of Primary Ovarian Tumor

While all cells within the ovary can give rise to malignancies, EOCs are the most common and lethal. Intraperitoneal dissemination is the primary metastatic route for EOC [78, 79]. Poor prognosis relates, in large part, to rapid progression of peritoneal metastasis compared to that of the hematological metastatic route, and distinct mechanisms involved contribute to devastating outcomes. EOCs are a heterogeneous group of cancers that may be categorized into two major groups [80]. Type I EOCs are low-grade, slow-growing tumors that are thought to arise from benign ovarian lesions and include all four major histotypes: serous, endometrioid, mucinous and clear cell. In contrast, type II EOCs frequently have p53 mutations and are thus genetically unstable. Histologically these tumors are high-grade serous, mixed epithelial, or undifferentiated carcinomas. These cancers are thought to seed the ovarian surface and pelvic peritoneum concurrently, which explains why they are rarely present as stage I disease [80]. See Table 1 for more detail in differences between EOC cancer types.

EOCs generally have an insidious onset. Due to the asymptomatic nature of early-stage disease, most patients are not diagnosed until after their tumors have metastasized intraperitoneally. At this point, their chance of surviving beyond 5 years is only about 25% [81], and this is largely due to the diffuse peritoneal lesions that impede surgical eradication. In fact, the completeness of surgical debulking is the best predictor of survival. Chemotherapy, while initially effective, ultimately fails to prevent disease progression because patients almost inevitably develop recurrent resistant disease [82, 83]. Intraperitoneal metastases can cause peritoneal organ adhesion and malfunction, massive ascites, and/or pleural effusions [84-86], leading to mortality.

A key factor contributing to the poor prognosis of intraperitoneally metastasizing cancers is the rapid, self-perpetuating, feed-forward cycle of seeding and growth that is fuelled by inflammation. Since the prognosis of patients with peritoneal metastases is tightly correlated with the completeness of surgical cytoreduction [87, 88], and widespread metastases are not amenable to surgery, the development of novel strategies to arrest metastatic progression is imperative (89). Steps in the progression of EOC are listed below.

Intraperitoneal dissemination of cancer cells

Cancer cells can freely disseminate in the peritoneal cavity after exfoliating from exposed primary intraperitoneal tumors: ovarian epithelia for type I EOC, fallopian tube epithelia for type II EOC. In either case, once suspended in the peritoneal fluid, single cells detach and then aggregate to form spheroids and then can establish as new tumors attached to the peritoneal membrane. In this way, cancer cells can resist anoikis, a specialized form of apoptosis triggered by a lack of attachment to other cells or to the ECM and evade clearance by peritoneal lymphatics. Accordingly, interactions between tumor cells and the peritoneum are key contributors to metastatic progression, which, if successfully blocked, should promote clearance or death of cancer cells.

Cellular and molecular properties of the peritoneum

The peritoneum is comprised of a single layer of mesothelial cells and its associated underlying ECM, which cover the vast surface of the abdominal and pelvic cavities, as well as visceral organs. Mesothelial cells apically secrete glycosaminoglycans, surfactants and proteoglycans to provide an anti-adhesive peritoneal surface that ensures the appropriate gliding of the abdominal viscera and prevents intra-abdominal organ fusion. Mesothelial cells regulate entry of leukocytes and inflammatory cells into the peritoneal cavity. In response to injury or insult, these cells release chemokines MCP-1 and IL-8 and upregulate cell surface adhesion molecules ICAM-1 and VCAM-1, to which leukocytes attach [90]. The ECM underlying the peritoneal mesothelial cells is rich in collagen I and fibronectin, with thin deposits of laminin and collagen IV lying directly beneath the mesothelium [91]. The ECM is, for the most part, concealed by the flattened squamous-like mesothelial cell layer; however, it is periodically exposed at lymphatic portals through which the peritoneal fluid drains into the venous circulation. These lymphatic portals are particularly abundant on the omental and sub-diaphragmatic peritoneal surfaces and are commonly referred to as “milky spots”

because of their whitish appearance that results from the accumulation of resident lymphocytes participating in immune surveillance [90].
Ultrastructural analysis reveals the absence of a basement membrane at milky spots. Instead, the collagen I-rich stromal matrix is exposed. The adjacent mesothelial cells have a cuboidal morphology with disruptions and intercellular gaps that further expose the peritoneal ECM.

Cancer cell attachment to the peritoneum

A variety of different adhesion molecules can mediate attachment of cancer cells to mesothelial monolayers. For example: The ovarian cancer biomarker CA125/MUC16 is a transmembrane mucin that binds to mesothelin, a GPI-linked protein expressed by mesothelial cells [92]. Attachment of cancer cells to ECM, on the other hand, is mainly mediated by integrins. The β1 integrin subunit is key as it can pair with a variety of α-integrin subunits to confer binding to most ECM substrata. Blocking β1 integrin inhibits EOC cell attachment and migration on ECM substrata relevant to the peritoneum [93, 94]. β1 integrins also participate in cancer cell attachment to mesothelial monolayers [94-97], which could indicate that cancer cells are binding to mesothelium-associated ECM and/or to mesothelial cell surface VCAM-1.
Many studies have been designed with the assumption that peritoneal metastasis
Is dependent on cancer cell attachment to mesothelial cells. However, other studies indicate that cancer cells have a much greater affinity for the peritoneal ECM, which is consistent with the clinical pattern of metastatic spread.

Metastasis

The clinical pattern of disease progression underscores the concept that cancer cells preferentially attach to areas where the mesothelium is disrupted. During the initial stages of peritoneal metastasis, cancer cells attach to milky spots where the collagen-rich connective tissue matrix is exposed [98-101] (Fig. 3). Resident immune cells of milky spots are not able to prevent tumor growth [50, 54, 98, 102]; instead, their production of pro-inflammatory cytokines promotes cancer growth and dissemination. The impact of inflammatory cytokines on peritoneal metastasis is profound and transforms the initial pattern of dissemination, which is limited to milky spots, into widespread peritoneal metastasis [99, 103]. This transformation is triggered by increased exposure of the sub-mesothelial ECM, driven by inflammation, and inflammatory cytokines causing the protective mesothelial cells to retract, exposing the previously obscured underlying ECM. Since cancer cells preferentially attach to the

ECM, widespread cancer cell attachment ensues [99, 103] (Fig. 3). While activated mesothelial cells may be less efficient in creating a barrier than a quiescent mesothelial monolayer, in either case, these cells protect against cancer cell adhesion by concealing the underlying connective tissue matrix to which cancer cells preferentially attach. Knowing that cancer cells prefer areas where the mesothelium is absent and the peritoneal ECM is exposed highlights the importance of targeting cancer cell interactions with the ECM while simultaneously preventing mesothelial cell retraction.

Suggested roles for DDRs in EOC

The precise molecular events that occur during development, progression/invasion, and formation of secondary tumors in EOC metastasis are poorly characterized and understood. In addition, contribution the local microenvironment at secondary sites in tumor progression has been recognized as highly relevant in several metastatic cancers [104-106], however no information on EOC in this area is available. Establishing a potential role of DDR1 in EOC metastasis is vital to clarifying molecular mechanisms involved progression of this disease. This in turn may enable identification of novel and more effective treatment strategies against advanced disease.

DDR1 in cancer metastasis

DDR1 is widely expressed in epithelial cells of both fetal and adult organs. Highest expression is found in brain, lungs, placenta and kidneys. DDR1 is also found at low levels in cells such as melanocytes and those of various adult organs including heart, liver, skeletal muscle, pancreas, and ovaries [14]. Although the physiological functions of DDR1 are not fully understood, DDR1 signaling is essential for cerebellar granule cell differentiation [107], arterial wound repair [108], and mammary gland development [109]. It is clear that DDR1 is involved in cell interactions with the ECM involving adhesion. DDR1, however, was found to be overexpressed in breast, brain, colon and lung cancers, thus suggesting that this receptor may play a role in tumorigenesis of these epithelial cancers [110-113]. In breast cancer, DDR1 was overexpressed in both primary breast tumor samples and lymph nodes containing metastatic tumors [114]. DDR1 protein levels were elevated in 100% of patients with primary and metastatic brain tumors [111], in 61% of patients with non-small cell lung cancer and in 64% of patients with invasive lung adenocarcinoma [115]. Thus, DDR1 expression appears to be elevated in a variety of human cancers. Consistent with studies of DDR1 in these

solid tumors, elevated levels of DDR1 were seen in serous ovarian cancer, noted above. Elevated levels of DDR1 were also found for all stages of serous and mucinous ovarian cancers as noted above [Testuri and Marco, unpublished results].

Analysis of DDR1 isoforms in mouse embryonic and adult tissues revealed that DDR1b is the major isoform [116]. However, DDR1a is found in high levels in breast and glioma tumor cell lines [116,117]. No information is currently available on which DDR1 isoforms are associated with EOC. Additional information on which DDR1 isoforms may contribute to EOC oncogenesis and predicted role in binding collagen I in the submesothelial ECM of the peritoneum remain unknown.

As noted above, integrins are important molecules specifically involved in signaling or adhesion of cancer cells allowing for metastatic establishment. DDRs are similarly implicated to play related roles in some types of cancer, but how they may collaborate in some way with integrins in EOC is also not known. Some hypotheses, however, can be suggested based on current information. The most relevant issue is an apparent regulated “switch” in expression that occurs from that of DDR2 (in normal ovary epithelial cells) to DDR1 (in EOC cells). As discussed above, DDR2 is required for reproductive functions in ovaries, particularly steroid production needed to complete the ovarian cycle.

Our laboratory has evidence that such a switch from DDR2 to DDR1 expression occurs in cells at the beginning of malignant transformation in serous and mucinous border line ovarian tumors [unpublished results]. It is known that DDRs initiate signaling pathways in a context and cell type-dependent manner. The primary or secondary EOC tumor microenvironment might play a role in switching DDR expression, as the tumor microenvironment has been suggested to play an active role in cancer initiation and progression [104, 118]. Stromal–epithelial cross talk could provide a mechanism for switching since this microenviroment can be very different from that of normal health tissue. This might involve interaction with any number of stromal components including different collagens, of other high molecular weight ECM components as well as growth factors, hormones, cytokines and chemokines present in the secondary tumor microenvironment that facilitate progression of EOC that may include upregulation of DDR1. Some evidence suggests that the switch to DDR1 could by the secretion of cytokines by stroma cells [119] or by stimulation by collagen I, normally present in ovary stroma [120].

Detailed studies on signal transduction pathways activated by DDR1 and DDR2 in different cell types and their transcriptional targets have not yet been described. Similarly, very little is known about the regulation of DDR expression, and so far, only a few inflammatory mediators including TNF-α, IL-1β, and LPS have been shown to increase DDR1 expression [121]. Characterization of regulatory mechanisms governing DDR expression involving detailed structural studies has not been done. A recent study in primary human lung fibroblasts suggested a mechanism of induction for DDR1 expression is through collagen I, DDR2 and a JAK2-ERK1/2 pathway [121].

In addition, DDR1 was reported to be able to both promote and inhibit EMT in a ligand- and cell type-dependent manner. In pancreatic cancer cells, DDR1, together with integrin α2β1, promotes cell scattering on collagen I and results in increased expression of mesenchymal marker, N-cadherin [122]. Thus, DDRs can interact with multiple proteins and these interactions result in complex signaling processes that vary between cell-type and can be ligand-based RTK activity dependent or independent. Together, these studies suggest that switching between DDR2 and DDR1 could be associated with EMT and possibly MET described above.

DDR cross-talk with other molecules

In addition to mediating direct collagen-dependent signaling, DDRs can also modulate signaling pathways initiated by other matrix receptors (e.g., integrins), cytokines (e.g., TGF-β) and transmembrane receptors (e.g., insulin receptor and Notch1). Cross-talk between DDRs and integrin is complex and influences multiple processes including cell adhesion and differentiation. DDR1 can both potentiate and inhibit integrin-mediated signaling. DDR1 cooperates with integrin α2β1 in maintaining mouse embryonic stem cells undifferentiated via activation of selective collagen–DDR and collagen–integrin mediated signaling pathways that ultimately converge to the self-renewal controlling molecule, Bim-1 [123]. Moreover, overexpression of DDR1 or DDR2 in cells expressing collagen binding receptors integrins α1β1 and α2β1 results in enhanced integrin-mediated adhesion to collagen due to increased integrin activation rather than increased integrin expression levels [124]. In contrast, DDR1 has also been shown to counteract integrin-mediated signaling when promoting epithelial cell differentiation [125]. Thus DDR1-integrin cross-talk is highly dependent on the type of integrins expressed on a given cell type.

DDRs can also modulate signaling initiated by growth factors. Cross-talk between

DDR1 and TGF-β is critical for proper growth and patterning of mouse mammary gland. TGF-β negatively regulates mammary gland ductal extension and lateral branching by promoting Wnt5a expression and DDR1 phosphorylation [126]. In human mammary cells, Wnt5a acts as an upstream regulator positively affecting collagen-induced DDR1 phosphorylation. In addition, levels of Wnt5a are directly associated with increased cell adhesion and reduced cell migration on collagen [127]. This suggests that Wnt5a might control two important cell functions in regulating the phosphorylation and activation of DDR1. Recently, cross-talk between DDR2 and the insulin receptor and between Notch1 and DDR1 was proposed. Stimulation of cells with collagen I and insulin showed that insulin can act to enhance collagen I-dependent signaling by increasing the activity of DDR2 [128]. Finally, it has been proposed that collagen-stimulated DDR1 promotes survival of cancer cells by binding to and activating Notch1 thus promoting the activation of the two transcription factors Hes1 and Hey2 [129]. In conclusion, cross-talk of DDRs with various receptors is critical for regulation of cell survival, migration, and differentiation in development as well as in pathological conditions. DDR1 signaling modified by growth factors has not been explored in EOC.

It is not clear why or how DDRs can act as either pro-tumorigenic or anti-tumorigenic receptors. This behavior is highly dependent on the type and stage of cancer. Consistent with a pro-tumorigenic action of DDR2, DDR2-null mice show reduced primary tumor-associated angiogenesis and reduced lung colonization following tail vein injection of melanoma cells [130]. Normally, lung cancers involve DDR1. Studies in breast cancer have shown involvement with either DDR1 or DDR2. A recent study of DDR1 and DDR2 expression in breast cancer patients, e.g., showed a six-fold increase in DDR2 levels in tumor vs. normal tissue, while DDR1 expression was decreased in tumor tissues [131]. In EOC, in contrast, it seems that DDR1 expression contributes to the progression of the disease. This seems to be more similar to what is described for non-small cell lung cancer where DDR1 overexpression is suggested to play a role in this type of cancer [132].

Perspectives

DDR1 overexpression is found in a wide variety of different human cancers as mentioned above suggesting that it has a function in tumor progression. As shown in Fig. 3, DDR1 may have a critical role in adhesion, migration, invasion of EOC metastasis, but additional study is needed. As a diagnostic tool, elevated DDR1 protein expression levels may be predictive for EOC [68]. However, using DDR1 levels as a

marker for EOC is not necessarily specific, and may indicate various other malignancies. DDR1 expression levels in body fluid or serum may have clinical prognostic value as a biomarker for cancer in a more general sense. The shedding of the DDR1 ectodomain by membrane-type MMP may add another level to regulate as well as to detect these receptors [133]. These biomarkers could be found in serum of EOC patients. Such understanding is critical for developing selective DDR inhibitors or treatments that can alter activity indirectly to result in novel and safe therapies for the treatment of a broad range of diseases including cancer.

Figure and table legends

Fig. 1 Schematic presentation of interaction of PCA-1 and DDR1 in mediating signals leading to androgen independence (resistance) and cancer progression in PCa. PCA-1 and downstream DDR1 and Bcl-xl or MMP 9 signals promote cancer invasion and survival. PCA-1-mediated signals are amplified and associated with hormone resistance and enhance malignant potential of androgen-independent prostate cancer cells. Adapted from Shimada et al. (30).

Fig. 2 A schematic model depicting molecular mechanisms underlying DDR2-mediated prostate cancer cell bone metastasis. TGF-β in tumor microenvironment stimulates expression of DDR2, which is responsible for Runx2 activation and subsequently PTHrP secretion. In bone metastasis sites, PTHrP promotes osteoblast proliferation, differentiation and function, at the same time DDR2 stimulates RANKL expression in osteoblasts, leading to the activation of osteoclasts and enhanced osteoclastogenesis. Furthermore, DDR2 facilitates specific binding of PCa cells to collagen I, which is the main collagen of bone. Adapted from Yan et al. (64).

Fig. 3 A working model for metastatic spread in progression of EOC. EOC cells growing on the surface of the ovary undergo EMT to attain motile functions required for cancer metastasis. Rupture of the ovarian tumor results in shedding of tumor cells into the peritoneum where they survive as cellular aggregates/spheroids which secrete cytokines. The surrounding mesothelial and infiltrating blood cells facilitate invasiveness of carcinoma spheroids. In secondary sites, cancer cells initially attach to milky spots where the stromal matrix is exposed, providing direct access to a preferred substrate, collagen I. With disease progression and in response to increasing concentrations of inflammatory mediators, mesothelial cells retract and detach. The resulting exposure of underlying ECM, with a discontinuous basement membrane,

facilitates widespread peritoneal metastasis. TGF-β, released by cancer and inflammatory cells, stimulates myofibroblast transdifferentiation. The spheroids attach to and invade the peritoneal matrix. The combination of their contractile and proteolytic capacities remodels the collagen I-rich matrix to facilitate stromal implantation and invasive growth.

Table 1. Hystologic and associated genetic differences between EOC cell types. Adapted from Nik et al. (134).

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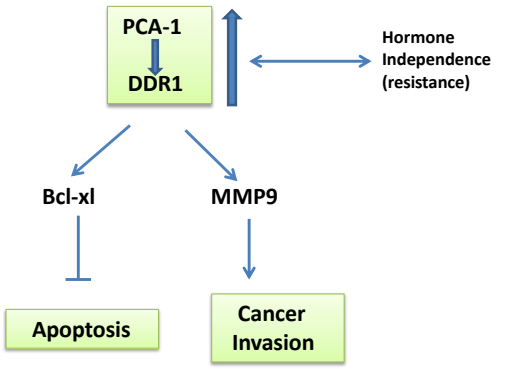

Figure 1

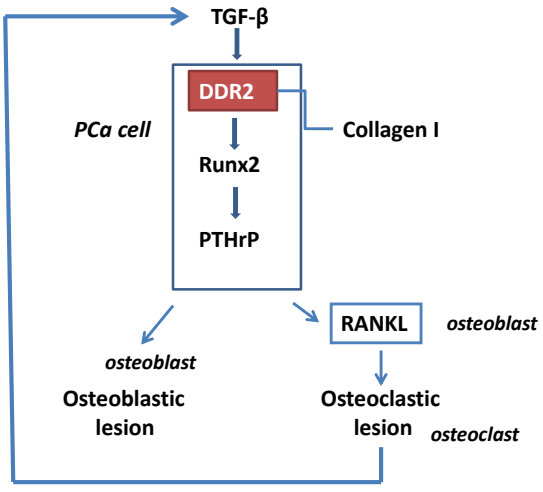

Figure 2

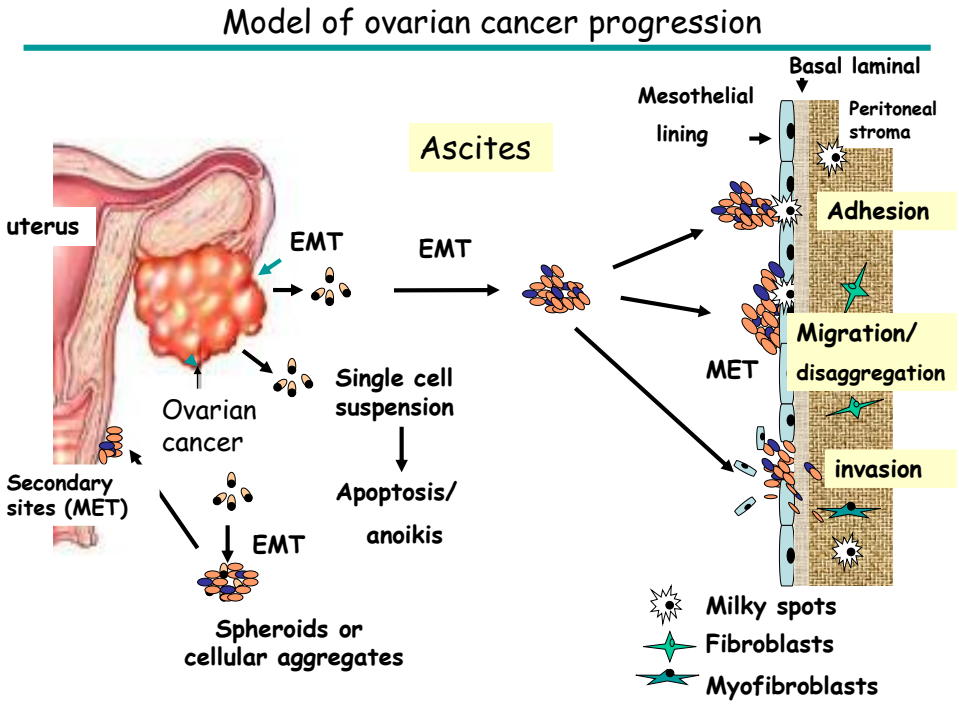

Figure 3

Table 1: Type I and type II ovarian carcinoma

	Putative precursor	Most frequent mutation(s)
Type I tumors		
Low-grade serous carcinoma	Serous borderline tumor	*KRAS, BRAF, ERBB2*
Low-grade endometrioid carcinoma	Endometrioma	*CTNNB1, PIK3CA, PTEN, ARID1A*
Clear cell carcinoma	Endometrioma	*PIK3CA, ARID1A, FBXW7*
Mucinous carcinoma	Mucinous borderline tumor	*KRAS*
Type II tumors		
High-grade serous carcinoma	Fallopian tube epithelium	*TP53, BRCA1/2*
High-grade endometrioid carcinoma	Not recognized	*TP53*
Undifferentiated carcinoma	Not recognized	Unknown
Carcinosarcoma	Not recognized	*TP53*

Table 1