Current Human Cell Research and Applications
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Molecular Diagnosis and Targeting for Gynecologic Malignancy

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Preface

The purpose of this book is to provide the recent advances in the complex field of gynecologic cancer, such as genetic engineering, cancer cell lines, signaling, or genomic profiling, focusing on their significance for our better understanding of gynecologic cancer biology, prevention and treatment, drug sensitivity, and cancer hereditariness. This book appeals to investigators, clinicians, residents, postdocs, and undergraduate medical students who are curious about new research on gynecologic malignancies and who desire more than the brief introduction to this field provided by most textbooks. It not only presents basic and translational research but also explores the generalizability of the evidence covering the interface between basic and clinical science. A number of the topics offer the basis for new ideas that have the potential to advance into the gynecologic malignancies. This book provides readers with state-of-the-art information that will help improve the lives of patients with these challenging diseases, and we hope that this book will serve a burgeoning array of young investigators.

This book devotes one chapter to each of the organs of the female genital tract or to timely specific topics of methodology or biology that may be responsible for clinical and subclinical variations. The organ-specific investigations were described by six experts. The topic of ovarian cancer was documented by Drs. Storu Kyo, Masashi Takano et al., and Nozomu Yanaihara et al.; endometrial carcinoma by Drs. Yoichi Kobayashi and Munekage Yamaguchi et al., and cervical cancer by Drs. Kei Kawana et al. For the organ non-specific topics, the topic of stem cells is argued by Drs. Tatsuya Ishiguro et al., xenograft models by Drs. Tomohito Tanaka et al., genomic profiling analysis by Drs. Michihiro Tanikawa et al., G protein-coupled receptor signaling by Drs. Hiroshi Yagi et al., signaling and drug resistance by Drs. Koji Yamanoi et al., and hereditary gynecological malignancy by Drs. Hideki Yamamoto et al. Taking together, these mostly cover the current scientific issues in gynecologic malignancies.

Any understanding of gynecologic science is always the result of a close communication between clinics and laboratories. Authors are all themselves hard-core scientists pursuing continuously the truth but are also dedicated in day-to-day clinics in each institution. They are particularly interested in the scientific findings in

their cases and wish to share their clinical and basic experiences with us. Ironically however, the world suffered from the explosive outbreak of the COVID-19 pandemic at the beginning of the Olympic year 2020, and more or less our authors are grappling with the pandemic as a clinical superintendent resulting in the unavoidable delay in the publication of this book.

We hereby gratefully acknowledge all the talented authors for their enthusiastic dedication and commitment to write manuscript of their outstanding works despite the ambient stress of this rough time. We appreciate the Japan Human Cell Society for planning this wonderful program; without that this book would not exist. We extend our thanks to the many people at Springer Nature for their diligent work during the editorial process. Finally, we hope this book will also be offered as a resource to inspire and assist those who wish to study the specialty of gynecologic science.

Minato-ku, Tokyo, Japan — Seiji Isonishi
Tokorozawa, Japan — Yoshihiro Kikuchi

Contents

Chapter 1
Cancer Stem Cells in Gynecologic Cancer

Tatsuya Ishiguro and Takayuki Enomoto

Abstract The survival rate of patients with advanced stages of gynecologic cancer including ovarian, uterine endometrial, and cervical cancer, remains poor. Cancer stem cells are responsible for tumor progression, metastasis, and drug resistance. Following the reports on leukemia stem cells, recent findings in solid tumors have begun to demonstrate the biological characteristics, molecular markers, and mechanisms of maintenance of cancer stem cells. In this issue, we provide an overview of cancer stem cells in different types of gynecologic cancers, including our recent results on the stable in vitro 3D cultivation method of ovarian and uterine endometrial cancer stem cells. Additionally, we focus on the molecular mechanisms exhibited by gynecologic cancer stem cells and discuss the future prospects for new therapeutic strategies targeting cancer stem cells.

Keywords Cancer stem cells · Cervical cancer · Endometrial cancer
Ovarian cancer · Targeted therapy

1.1 Introduction

Tumor tissues contain several types of cells including cancer cells, cancer-associated fibroblasts, endothelial cells, pericytes, and immune-inflammatory cells, and also functionally heterogeneous cells [1]. Additionally, the tumor niche and several other factors affect cancer cell propagation and metastasis [2]. Previously, cancer cell heterogeneity was recognized as a result of the accumulation of genetic instabilities during carcinogenesis, termed as the clonal evolution model [3]. However, there are

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different tumorigenic capacities among cancer cells; some of them contribute to propagation and metastasis.

In normal tissues, there is a hierarchy with tissue-specific stem cells at the top, and they have the ability of self-renewal and multipotency; these stem cells generate different lineages of progenitor cells and further differentiate into mature cells [4]. Like these normal adult stem cells, cancer cell heterogeneity has been refined by a hierarchy-based model. This model demonstrates that cancer stem cells can self-renew and differentiate to non-cancer stem cells; however, only the cancer stem cells possess tumorigenicity (Fig. 1.1) [5, 6]. Dick and colleagues identified cancer stem cells in human acute myeloid leukemia [7, 8]. Following this, several studies showed the presence of cancer stem cells in solid tumors including breast cancer, glioma, and colorectal cancer [9–11].

1.2 Cancer Stem Cell Research Model

Cancer stem cells are functionally defined as a cell within a tumor that possesses the capacity to self-renew and cause heterogeneous lineages of cancer cells that comprise the tumor [12], and identified with several functional assays. First, the most important function is the generation of a tumor with self-renewal and differentiation abilities. To confirm this ability, in vivo tumor-propagation assay after transplantation of these cells into immunocompromised mice was performed. Cancer stem cells can serially generate xenograft tumors that reproduce histological construction [4]. Second, in vitro three-dimensional (3D) spheroid cultivation is commonly applied to assess the capabilities of cancer stem cells. Because normal neural and

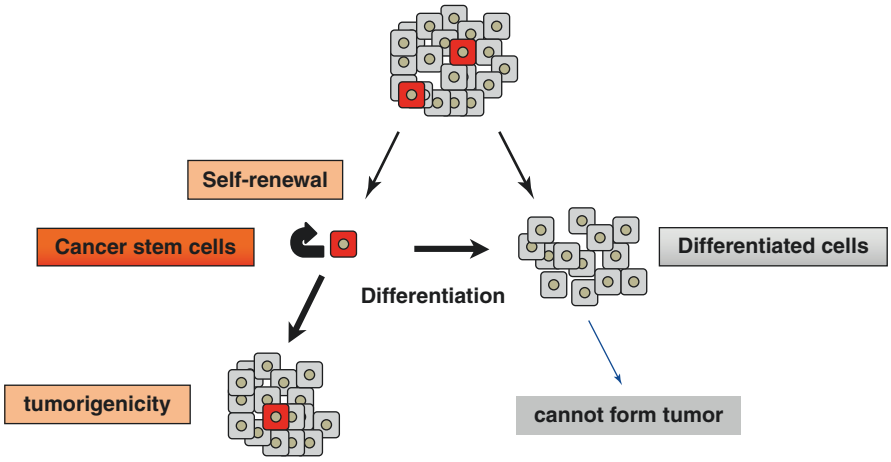

Fig. 1.1 Canonical cancer stem cell model. In the canonical cancer stem cell model, cancer cells are hierarchically organized and the cancer stem cells, which are tumorigenic, are at the top of the hierarchy

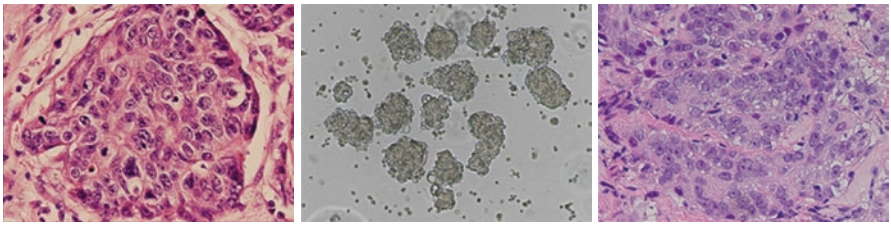

Fig. 1.2 3D spheroid cells derived from human ovarian cancer (drawn from [18]). Spheroid cells (center) can propagate under in vitro floating culture condition and generate xenograft tumor (right) which histologically similar to the original human tumor (left)

mammary stem cells can be propagated as 3D sphere-forming cells in vitro, as neurospheres and mammospheres [13], and stem cells propagate as a spheroid body [14], glioma and breast cancer stem cells were cultivated using the 3D spheroid cultivation method [15]. The tumor-derived spheroid cell cultivation method was employed in several other cancers to investigate stem cells [16]. Third, genetic-lineage tracing is a new technique, which relies on the identification of a marker gene. Although this method was restricted to the genetic mouse model only, CRISPR-Cas9 gene-editing technology has enabled the application of this method on human colorectal cancer organoid cells [17]. Herein, we specifically focused on gynecologic cancer stem cell research based on 3D human cancer cell cultivation. In fact, we could find a novel mechanism of cancer stem cell and therapeutic targets using established 3D ovarian and endometrial cancer stem cells from human cancer specimens (Fig. 1.2) [18, 19].

1.3 Gynecologic Cancer Stem Cells

Although research on gynecologic cancer stem cells has increased, most studies provide reports on experiments using conventional 2D cancer cell lines. Furthermore, reports on in vitro culture using 3D tumor-derived cancer spheroid cells from primary gynecological cancers are limited. Gynecologic cancer spheroid cells have been investigated as cells that have potential for metastasis and chemoresistance rather than as cancer stem cells [20, 21]. The morphology of in vitro spheroid cells is similar to that of floating aggregated cancer cells in ascites. These aggregated cells contain cancer stem cells [22], and play an essential role during specific metastasis; dissemination of cancer cells to the surface of the peritoneum and other organs within the peritoneal cavity is the frequent metastatic pattern of gynecologic cancer, especially in ovarian cancer. The cells detached from the primary tumor in ascites attach to the surface of other organs and form multiple disseminated tumors [23, 24]. Moreover, a specific environment of malignant ascites can promote spheroid formation [25].

1.3.1 Ovarian Cancer Stem Cells

Ovarian cancer research has made more progress in the field of gynecologic cancer stem cell research. Bapat and colleagues first reported the establishment of spheroid cells with a potential for cancer stem cell development using cells from ovarian cancer patients with ascites [26]. They maintained and propagated ovarian spheroid cells in vitro using 5% fetal bovine serum. Subsequently, Zhang and colleagues reported the culture of spheroid cells from primary ovarian cancer tissues using serum-free media [27]. Besides these reports, no other studies have analyzed the in vitro propagation of pure spheroid cells from ovarian cancer until our report [18], although modified cultivation methods have been reported. Previous studies have demonstrated the tumorigenicity of the established cells. Although we could establish long-time cultures of ovarian spheroid cells using rho-associated protein kinase inhibitor, most cancer cells died at the stage of initial cultivation and the propagated cells could be obtained only after several months; these results are consistent with those obtained by other researchers, with different cultivation methods. Any other unknown technical difficulties or physiological characteristics of ovarian cancer might have obstructed the establishment and progression of ovarian cancer spheroid cells.

Consistent with the previous reports on ovarian cancer stem cells involving the development of stable cell lines or short-lived spheroid cells from clinical samples [28, 29], aldehyde dehydrogenase (ALDH) was found to be a specific marker in ovarian cancer stem cells in our study [18]. In addition, ALDH activity was required for the proliferation of spheroid cells. In contrast, CD44, CD133, CD117 (commonly known as c-kit), CD24, and the epithelial cell adhesion molecule (EpCAM) are known markers of ovarian cancer stem cells [27, 30]. This discrepancy among observed markers may be due to the heterogeneity of ovarian tumor cells. Cells with these specific markers also expressed high levels of pluripotent stem cell markers including Nanog, Oct3/4, Sox2, Nestin, and Bmi-1 [18, 27, 30].

Recently, researchers have demonstrated the mechanism of maintenance of ovarian cancer stemness. Previously, we showed the unique mechanism of ovarian cancer stemness with a reciprocal regulatory relationship between ALDH1A1 and SRY-box transcription factor 2 (SOX2) [18]. Seo and colleagues revealed that hypoxia and Notch signaling increased SOX2 and ALDH expression [31]. Condello and colleagues showed that tissue transglutaminase/fibronectin/integrin β1 complex led to Wnt pathway activation, and β-catenin directly regulated ALDH [32, 33]. Wheeler and colleagues demonstrated that chromobox 2 upregulation, which plays a critical role in the activity of polycomb group complex, promoted ovarian cancer via induction of stem cell transcriptional profiles, specifically ALDH3A1 and anoikis escape [34]. Additionally, interleukin 6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling regulated ALDH expression and enrichment of ovarian cancer stem cells [35]. From these reports, ALDH is not only a specific ovarian cancer stem cell marker but also one of the important regulators for the maintenance of cancer stem cells. ALDH inhibition can lead to the suppression of

ovarian cancer stem cells. Disulfiram and N,N-diethylamino benzaldehyde (DEAB) are the major pan-ALDH inhibitors that suppress tumorigenesis [18]. Another ALDH inhibitor, 673A, which specifically inhibits ALDH1A1, ALDH1A2, and ALDH1A3, can induce necroptosis in ovarian cancer stem cells [36], and synergistically suppress ovarian tumorigenesis with cisplatin [37].

1.3.2 Uterine Endometrial Cancer Stem Cells

The presence of endometrial cancer stem cells were first reported in 2009 from clinical specimens [38, 39]. Rutella and colleagues reported that CD133-expressing cells had cancer stem cell ability. Although several groups showed that CD133 was a potential marker of endometrial cancer stem cells [39], other cell-surface markers (CD44, CD117, and CD126) and side populations have been assumed to be endometrial cancer stem cell markers [40–42]. Recently, we first reported the establishment of stable in vitro cultivation of 3D endometrial cancer stem cells from clinical cancer tissues, and ALDH activity was identified as a marker [19], consistent with previous reports using monolayer (2D) cell lines [42, 43]. We also found that ALDH mediated cancer stemness and paclitaxel resistance through glycolytic activation, and ALDH inhibitor was a potential new therapeutic reagent [19]. Other groups showed that IL6/Janus kinase/STAT3 signaling [42] and epithelial membrane protein 2 [43] functionally affect ALDH activity in endometrial cancer. Therefore, it can be suggested that ALDH is a key regulatory factor in endometrial cancer stem cells like ovarian cancer. Additionally, some regulatory signaling pathways play an essential role in the maintenance of endometrial cancer stemness. Lu and colleagues showed that secreted protein acidic and rich in cysteine-related modular calcium-binding 2 activated Wnt/β-catenin signaling, which specifically led to chemoresistance [44]. Furthermore, Kato and colleagues demonstrated that dual-specificity phosphatase 6-mediated extracellular-signal-regulated kinase and protein kinase B signaling contributed to the maintenance of cancer stemness [40].

1.3.3 Uterine Cervical Cancer Stem Cells

Sphere-forming cells with tumorigenicity were initially isolated in 2009 from primary cervical cancer [45]. Following this, several key factors that contributed to the maintenance of cervical cancer stemness were reported. Especially, the involvement of human papillomaviruses (HPV) has been clarified. HPV is a major cause of cervical carcinogenesis and possesses two well-known viral oncoproteins, E6 and E7, which affect the cell cycle by interacting with p53 and pRb, respectively. HPV-E6 induces the enrichment of CD71-positive or CD55-positive cervical cancer stem cell population [46, 47]. This oncoprotein also affects cervical cancer stemness

through upregulation of hairy and enhancer of split-1 [48], whereas the other oncoprotein E7 directly regulates Oct-3/4 [49].

The epidermal growth factor (EGF) pathway is one of the key regulatory signaling pathways for cervical cancer stem cells. Mucin 1-EGF receptor (EGFR)-IL6 signaling and EGF-phosphatidylinositol 3-kinase-SOX2 axis induce cervical cancer stem cell enrichment. The selective EGFR tyrosine kinase inhibitor, erlotinib, could suppress cancer stem cell enrichment [50, 51]. Wnt/β-catenin signaling might be another essential pathway for cervical cancer stem cells. MicroRNA-135a induced CD133-positive cancer stem cell subpopulation, and Wnt/β-catenin signaling enhanced the induction of CD133-positive cells [52]. In addition, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5)-Wnt/β-catenin signaling also contributed to cervical cancer stemness [53]. Furthermore, α-actinin-4 has been shown to regulate stemness and mediated the involvement of ATP-binding cassette family G2 involved in drug resistance [54].

1.4 New Therapy Targeting Cancer Stem Cells

Therapeutic strategies against cancer stem cells have been designed because it is thought that targeted therapy against stem cells can lead to complete tumor eradication. The first option is cancer stem cell ablation with specific inhibitors against functional cancer stem cell markers like ALDH [18, 19], and antibody–drug conjugates directed against cancer stem cell markers like CD133 and CD44 described as above [55].

The next strategy is the inhibition of critical cancer stem cell signaling pathways. Cancer stem cells exhibit several signaling pathways including Notch, Wnt, mammalian target of rapamycin complex 1, and Hedgehog signaling [31–33, 44, 56]. Inhibition of these signaling pathways could reduce cell viability and in preclinical models. Although there are a few clinical data available on cancer stem cell-targeted therapy, early phase trials with treatments targeting these signaling pathways have been examined in solid tumors including gynecologic cancer [57].

Cancer stem cell metabolism is another target. There is a specific correlation between glycolysis and oxidative phosphorylation, and metabolic plasticity in cancer stem cells. Our recent results showed that uterine endometrial cancer stem cells relatively depend on enhanced glycolysis for proliferation, and glucose transporter 1 inhibitors were potential new therapeutic reagents for endometrial cancer [19].

1.5 Future Prospects and Conclusions

Accumulating evidences support the existence of gynecologic cancer stem cells, and it makes sense to focus on cancer stem cell treatments because they aggressively expand during disease progression and lead to undifferentiated states in

clinical tumors [58]. However, some barriers obstruct the cancer stem cell-specific treatments. First, the above mentioned new therapeutic strategy may also affect normal cells. The stem cell signaling pathways are not activated specifically in cancer stem cells, and they also play an essential role in normal stem cells [59]. Cancer stem cell markers, CD44, CD133, Lgr5, ALDH, also act as normal stem cell markers. In addition, because cancerous and noncancerous stem cells preferentially use glycolysis and oxidative phosphorylation, there is no universal metabolic pattern specific for cancer stem cells [60]. Second, cancer stem cells dynamically change during cancer propagation. Recently, it has been thought that clonal evolution and cancer stem cell mechanisms may cooperate to cancer propagation and tumor heterogeneity [61], and this complex mechanism of propagation has been gradually elucidated. This intratumor heterogeneity is also attributed to the surrounding niche. Third, regeneration of cancer stem cell populations from non-cancer stem cells (cancer cell plasticity) might disturb complete cure (Fig. 1.3). Changes in tumor microenvironment, inflammation, and hypoxia coordinately promote plasticity. Moreover, there is a reciprocal regulation and switching between cancer stem cells and non-cancer stem cells, and anticancer therapies also induce dedifferentiation. Cancer stem cell-targeted delivery including nanoparticle-mediated strategies and oncolytic viruses might resolve these problems [56]. We should consider innovative therapeutic strategies from various perspectives to successfully eliminate cancer stem cells.

Disclosure Statement There are no conflicts of interest to declare.

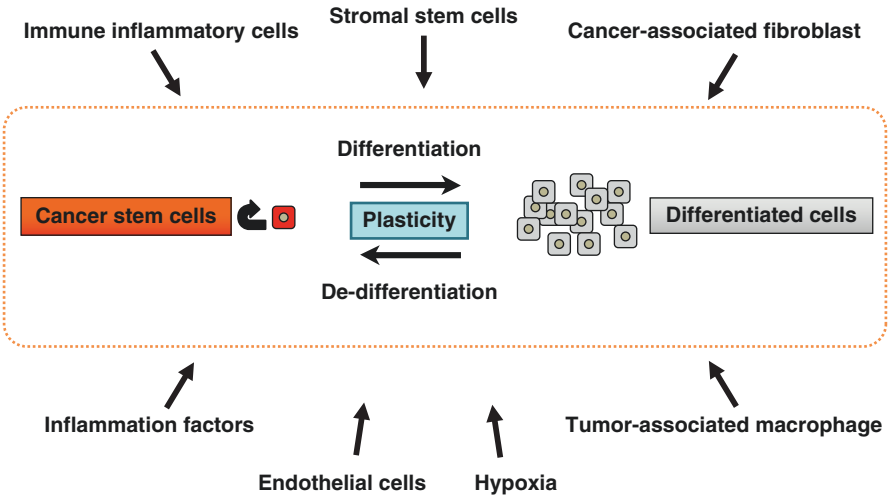

Fig. 1.3 Cancer cell plasticity and surrounding microenvironment. Cancer stem cells and non-cancer stem cells dynamically convert to each other, and this process is driven by several surrounding factors

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Chapter 2
Patient-Derived Xenograft Models in Gynaecological Malignancies

Tomohito Tanaka and Masahide Ohmichi

Abstract Established suitable models are important for cancer research. The recent progression of genomic or molecular analysis and precision medicine requires cancer models that reflect the characteristics of primary tumours. Patient-derived xenograft (PDX) models have been focussed on because of their similarity between PDX and primary tumours. PDX represents a promising tool for translational research since it closely resembles patient tumour features and retains molecular and histological features. Currently, PDX has been established in several types of cancer, including colon, stomach, breast, uterine, and ovarian cancers. However, several problems still exist. This review provides information on recent methods for the implantation and analysis of tumour characteristics in gynaecological cancers.

Keywords Gynaecological malignancy · Cervical cancer · Endometrial cancer Ovarian cancer · Patient-derived xenograft

2.1 Introduction

Molecular and genomic analyses have developed remarkably during the last decade. Several cell lines have been established and used for cancer research. Authentic, established cell lines express fixed gene arrangements and act in similar molecular

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pathways in certain situations, which has been useful in cancer research. However, researchers require more detailed gene information in each cancer cell and should be able to reproduce the tumour microenvironment.

Patient-derived xenograft (PDX) models, which are new animal models, are established by heterotopic or orthotopic grafting of fresh surgically resected tumour tissue into immune-deficient mice. The obtained tumour tissue is washed with saline and stored in a cell culture medium on ice, to reduce tissue metabolism. The tumour tissue should be implanted into animals as soon as possible, as prolonged implantation time has been associated with a lower engraftment rate [1]. Typically, the tumour fragment is cut into a size of 2–3 mm^3 and implanted in mice. Tumour growth is evaluated regularly until the tumour size reaches approximately 1000 mm^3. Then, the tumours are harvested and stored for the next stage. It takes about 3–6 months for tumour harvest [2].

PDX models reproduce the clinicopathological features of the original tumour and are used as experimental models for drug evaluation, biomarker identification, and precision medicine strategies. Current drug development has been mainly carried out using the cell line method; however, various studies have reported that drug responsiveness in the cell line methods is not sufficiently reflected in human patients [3]. The drug responsiveness in PDX models is more similar to clinically applied drug responsiveness because there are close similarities in genomic features and microenvironment status between PDX models and patient tumours [2, 4, 5]. By screening several anticancer drugs in PDX models, the most effective drug can be recommended prior to patient treatment. However, this approach is difficult because the generation of PDX models is not successful in all cases, and it takes several months to obtain the drug responsiveness data from PDX models [2]. For these reasons, a PDX cohort with genomic and drug responsiveness data has been suggested. The PDX cohort may be a powerful tool for drug development and treatment for patients with cancers [6–8]. Figure 2.1 shows a brief chart for using the PDX models. PDX models can be obtained from each type of cancer by grafting the original tumours into mice. Both the tumours of patients and the PDX models are preserved in a biobank, and several analyses have been performed, including pathological, genomic, and molecular analyses. Several anticancer drugs are evaluated using PDX models passaged from original tumours or those preserved in biobanks. These data are collected in databanks and are used for precision medicine in cancer patients in the future or for new drug development.

PDX models have been established in several cancers, including colon [9], stomach [10], breast [11], pancreas [12, 13], lung [14, 15], liver [16], kidney [17], bladder [18], uterus [8, 19–27], and ovary [28–32]; however, several questions remain unanswered. For example, what should be used as the starting material, tissue fragment, or tumour cells? Where should the materials be implanted? What is the success rate of each method? Is there any advantage or disadvantage in each method? Are there any alterations in retransplantation? This review complies with the information on the current methods of PDX in gynaecological cancers.

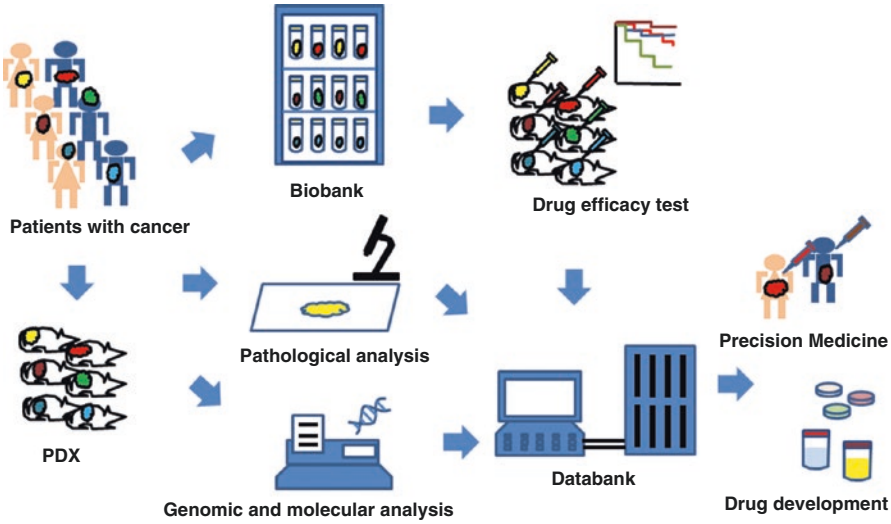

Fig. 2.1 The summary chart for using the patient-derived xenograft models. Patient-derived xenograft (PDX) models can be obtained from each cancer by grafting the original tumours into mice. These materials are preserved in biobanks and have undergone several analyses, including pathological, genomic, and molecular analysis. Several anticancer drugs are evaluated using PDX models. These data are collected in databanks and used for precision medicine for cancer patients in the future or for new drug development

2.2 Mouse Strains

The engraftment rate is important when there are limited funds and restricted specimens. It depends on several factors including the kind of recipient mice, site for implantation, method for transplantation, and size of the fragment. Nude mice, which have no thymus for mutation, were identified in 1962 by the appearance of their atrichia: they have no T cells [33]. It is easy to confirm subcutaneous engraftment since they are hairless. Severe combined immunodeficient (SCID) mice, which have no mature T and B cells due to their loss of protein kinase, DNA activated, and catalytic polypeptide (Prkdc), were identified in 1983 [34]. SCID-hu, which can be implanted in the foetal liver, thymus, and the renal capsule, contains human T cells [35]. Human T cells can increase in Hu-PBL-SCID with implanted human monocytes in their peritoneum. These mice containing human T cells contributed to the research on HIV; however, the rate and duration of implantation of human haematopoietic stem cells were unsatisfactory because SCID mice possess NK cell activity [36]. SCID–Beige is a hybrid of SCID with Beige, which has low NK cell activity; however, the rate and duration of implantation of human haematopoietic stem cells are not satisfactory [37, 38]. NOD mice have insulin-dependent diabetes mellitus (DM), where β cells in the pancreas are destroyed by T cells. They also possess low macrophage and dendritic cell activity [39]. NOD/SCID mice, which are a hybrid of NOD and SCID mice, do not show symptoms of DM because

of a deficiency of T cells; they are extremely immunodeficient [40]. NOG [41] and NSG [42] mice are hybrids of NOD/SCID with common γ-deficient mice; they do not show NK cell activity. NOG and NSG have achieved a satisfactory rate of implantation of human haematopoietic stem cells, monocytes, and malignancy. Recently, humanised mice have been used for the development of several immune checkpoint inhibitors. CD34-positive human haematopoietic stem and precursor cells were injected into NSG mice that received whole-body irradiation, resulting in reconstitution of immune cells. Humanised PDX models could be established in a partially human leukocyte antigen-matched allogeneic immune system [43–46].

2.3 Site of Transplantation

Several sites of transplantation, including subcutaneous, renal capsule, peritoneum, and orthotopic, have been reported. Subcutaneous transplantation is the most common because of the simple procedure, and it is easy to confirm the tumour implantation; however, metastasis to other organs rarely occurs (Fig. 2.2a). The minced tumour is injected subcutaneously, or the fragment of the tumour is placed subcutaneously directly after the skin is cut. The renal capsule may be used for tumours with low malignant potential or for normal tissue. The procedure is not simple; however, there is an increased blood supply for tumour growth in the renal capsule, and a high engraftment rate is expected. Mice are placed in the lateral position, and a 2-cm incision is made opposite the loin skin. The peritoneal cavity is accessed by an incision made in the abdominal wall overlying the kidney. After the kidney is exteriorised, the renal capsule and the space beneath the kidney capsule are opened. Following this, tumour fragments are inserted. Transplantation in the peritoneum is performed to examine ascites or metastasis to other organs. The minced tumour is injected into the peritoneum, or the fragment of the tumour is placed in the

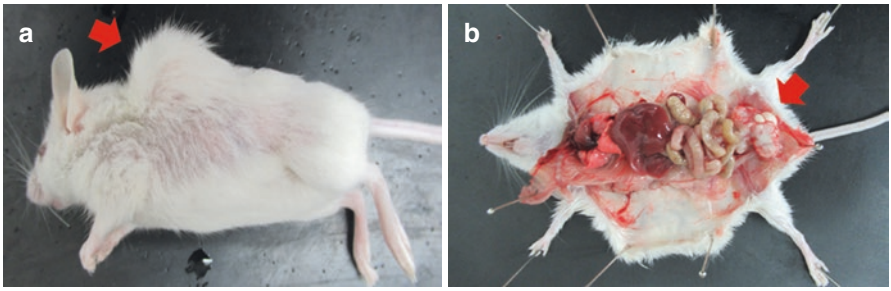

Fig. 2.2 (**a**) Subcutaneous patient-derived xenograft model. The minced tumour obtained from patients with cervical cancer were minced and injected subcutaneously into SCID mouse. After 4 months, the engrafted tumour was observed in subcutaneous layers of the mouse (red arrow). (**b**) Orthotopic patient-derived xenograft model. The minced tumour obtained from patients with cervical cancer were minced and injected into the uterine cervix of the SCID mouse transvaginally. After 4 months, the engrafted tumour was observed in the uterine cervix of the mouse (red arrow)

abdominal cavity directly below the skin cuts. The orthotopic xenograft is also common because the tumour environment can be reproduced more accurately. The minced tumour is injected into the uterus transvaginally, or the tumour fragment is placed on the primary site after the skin is cut, into the abdomen (Fig. 2.2b).

2.4 Cervical Cancer

2.4.1 PDX Procedure and Success Rate

Few reports exist on PDX models in cervical cancer. This may be a clinical feature for cervical cancer. Most patients with advanced-stage tumours receive radiotherapy or chemotherapy after biopsy. Surgical therapy may be performed at an early stage with a small lesion. Thus, it is difficult to obtain enough specimens for implantation. However, PDX models are advantageous for these patients because the tumour tissue can be expanded in mice to apply various experimental methods for tumour tissue analysis. Table 2.1 shows the previously reported literature on PDX models for cervical cancer. The engraftment rate varies from 0 to 75% [19–22]. The most important factor is probably the characteristics of each tumour, including infiltration and proliferation of each primary tumour. Hiroshima et al. implanted HER-2-positive cervical cancer, which was resected from one patient, subcutaneously and into the cervix of several nude mice with engraftment rate of 70–75% [20]. Chaudary et al. implanted a 1–2 mm tumour fragment that was resected from 33 cervical cancer patients to the cervix of SCID mice with an engraftment rate of 48%. On average, it took 3–4 months for the first palpable xenograft to arise following orthotopic transplant [21]. Hoffmann et al. implanted a 3–5 mm tumour fragment resected from six cervical cancer patients subcutaneously in SCID mice; however, no tumour engraftment was found. Then, they injected minced tumours resected from seven cervical cancer patients subcutaneously into SCID mice. The engraftment rate was approximately 70%. Palpable or visible tumours appeared 6–8 weeks after transplantation. No differences in engraftment were observed between squamous cell

Table 2.1 The engraftment rate of the patient-derived xenograft models for cervical cancer in the literature

	Mouse strains	Site of transplantation	Method of graft	Fragment size	Engraftment rate, %
Hiroshima et al.	Nude	Subcutaneous	Direct	3 mm^3	70 (7/10)
		Cervix	Direct	3 mm^3	75 (6/8)
Chaudary et al.	SCID, NOD SCID	Cervix	Direct	1–2 mm	48 (16/33)
Hoffmann et al.	SCID	Subcutaneous	Direct	3–5 mm	0 (0/6)
			Injection	Minced	70 (7/10)
Larmour et al.	NSG	Renal capsule	Direct	1 mm^3	71 (10/14)

carcinomas and adenocarcinomas [22]. Larmour et al. implanted a 1-mm^3 tumour fragment resected from 14 cervical cancer patients to the renal capsule of NSG mice with an engraftment rate of 71.4%. They also described that mouse stroma did not contribute to re-engraftment. The xenografting doses of 10^6 cells/kidney failed to generate tumours, irrespective of the presence of mouse cells. The size of the harvested xenograft limited the ability of re-engraftment. They also described that cervical dysplasia and normal tissue xenografted beneath the renal capsule could survive and grow [19].

2.4.2 Analysis of the Original Tumour and PDX

In most published literature on the PDX model of cervical cancer, there is a strong similarity of pathological findings between primary tumours and PDX. p16 overexpression, which is caused by the functional inactivation of Rb by human papillomavirus E7 protein, is peculiar in cervical cancer. Immunohistochemical findings of these proteins are also inherited from the primary tumour to PDX. Hiroshima et al. established a PDX model of HER-2-positive cervical cancer. They implanted the tumour fragment into the subcutaneous and cervix of nude mice. There were no cases of metastasis in subcutaneous PDX mice. In contrast, metastases, including peritoneal dissemination, liver, lung, and lymph node metastases, were found in the cervical orthotopic PDX model. They demonstrated that subcutaneous and cervical orthotopic xenograft tumours as well as metastases were stained by the ant-HER-2 antibody and recapitulated the histological structures of the original tumour [20]. Chaudary et al. evaluated the epithelial and stromal components of the original biopsy and xenograft models using two independent methods. The percentage of stroma tended to increase at early passages and decreased at later passages with a low value (<10%) after five passages. The decrease in stromal content in the later passage paralleled an increase in the growth rate of the tumours, as assessed by the mean time between passages. They also evaluated the epithelial and stromal components by immunostaining for SMA, collagen IV, cytokeratin, CD31, LYVE1, IFP, EF5, CA9, and Ki67. The expression of hypoxia markers (CA-9 and EF5) in the epithelial components of the tumour significantly increased with passage number. In parallel, there was a significant increase in vascular staining (CD31) in the stromal component. On average, there was also a significant increase in Ki67 staining in the epithelial component of the tumour and LYVE1 in both components. CD31 and LYVE1 levels were much lower in the epithelial component when compared to the stromal component, consistent with the finding that CA-9 and EF5 changed to a larger extent in the epithelial than in the stromal component. Ki 67 levels are much lower in the stromal component when compared to the epithelial component. A strong correlation was found between the passage 3 xenograft and primary biopsy for all markers, excluding collagen IV [21]. Larmour et al. described similar morphological features by H&E staining, and the immunostaining pattern for p16 and HPV were observed between primary tumours and several passaged xenograft

tumours. Hoffmann et al. reported that tumour markers such as EGF receptor and p16 were preserved after early and late tumour passage [19].

2.5 Endometrial Cancer

2.5.1 PDX Procedure and Success Rate

Hysterectomy is the main therapy for patients with endometrial cancer; it is easy for researchers to obtain sufficient specimens in this case. In contrast, most diseases are confined to the uterus in endometrial cancer; the prognosis does not differ in each disease. There are few studies on PDX models for endometrial cancer. Table 2.2 shows the previously reported literature on PDX models for endometrial cancer. The engraftment rate varies from 25 to 100% [8, 23–27]. Zhu et al. described PDX models using NOD/SCID mice in patients with high-risk endometrial cancer, including high-grade endometrioid carcinoma, serous carcinoma, clear cell carcinoma, and carcinosarcoma. They reported that the engraftment rate was 77.8% (14/18) regardless of the engraftment method. It was higher in the subrenal capsule models than in the subcutaneous models. The time to tumour formation varied from 2 to 11 weeks [23]. Unno et al. transplanted endometrial tumour tissue fragments to the renal capsule in NSG mice. The engraftment rate was 36.4% [24]. Depreeuw et al. reported PDX models from primary, metastatic, and recurrent type 1 and type 2 endometrial cancer patients. The engraftment rate was 60% with subcutaneous implantation using nude mice [25]. Cabrera et al. reported on PDX models from two endometrial cancers. First, they implanted tissue fragments into subcutaneous nude mice. After sufficient tumour growth, the tumours were mined and injected into the

Table 2.2 The engraftment rate of the patient-derived xenograft models for endometrial cancer in the literature

	Mouse strains	Site of transplantation	Method of graft	Fragment size	Engraftment rate, %
Zhu et al.	NOD/SCID	Renal capsule	Direct	$1 \times 1.5 \times 1.5$ mm^3	63 (16/19)
		Subcutaneous	Direct	$1 \times 1.5 \times 1.5$ mm^3	50 (9/18)
Unno et al.	NSG	Renal capsule	Direct	1.5×1.5 mm^2	36.4 (4/11)
Depreeuw et al.	Nude	Subcutaneous	Direct	8-10 mm^3	60 (24/40)
Cabrera et al.	Nude	Uterus	Injection	minced	90 (9/10)
Haldorsen et al.	NSG	Uterus	Injection	Cell suspension	100 (1/1)
Moiola et al.	Nude	Uterus	Direct	Small fragment	75–90
	Nude	Subcutaneous	Direct	5–10 mm^3	60–80
	Nude	Subcutaneous	Direct	8–10 mm^3	100
	NSG	Uterus	Injection	Cell suspension	25–100

uterus. The engraftment rate achieved was 90% and 78% of the patients that had pelvic implants [26]. Haldson et al. established patient-derived cell (PDC) models from tissues in patients with grade 3 endometrioid carcinoma. They obtained the cell suspension from the original tissue and injected them with Matrigel into the uterus of NSG mice [27]. Miola et al. described endometrial cancer PDX cohorts developed from primary tumours and metastasis covering all subtypes. In this cohort study, 124 patients with endometrial cancer were recruited from different centres across Europe. The tumour tissue fragments were implanted subcutaneously or orthotopically through a laparotomic incision into athymic nude mice. The engraftment rate of subcutaneous PDX varied from 60 to 80%; however, once the tumour was developed, the engraftment rate increased to nearly 100% in subsequent passages. It takes 3–5 months to engraft and develop the first generation. In contrast, the engraftment rate of orthotopic PDX model varied from 75 to 90% and also took 2–5 months to develop a palpable and transferable tumour. In a small study of five tumours from endometrial cancer patients, cell suspension from primary tumours with Matrigel were injected orthotopically into the uterus of NSG mice. For this type of model, the engraftment rate was lower, ranging from 25 to 100% in the first generation. Furthermore, the time of engraftment is slower; it takes an average of 10 months to develop an orthotopic PDX model [8].

2.5.2 Analysis of the Original Tumour and PDX

Zhu et al. established the endometrial cancer PDX model and evaluated the pathological and immunohistochemical features between primary tumours and PDX. No significant differences were observed among the corresponding F1 and F3 PDX with regard to architecture and cytological features, as shown by H&E. No major differences in immunohistochemical features, including hormone receptor (oestrogen receptor and progesterone receptor), the status of cytokeratin, and P53 expression were observed among the original tumours and xenograft tumours. They also validated two high-risk endometrial cancer PDXs on genomic analysis, including DNA and RNA sequencing. F0 (original) and F4 tumour DNA mutation frequencies exhibit a significant linear correlation. On RNA sequencing, the expressions of F0 and F4 tumour genes exhibited a significant linear correlation; PDX exhibited high similarity with patient rumours [23]. Unno et al. evaluated the histological and immunohistochemical features of PDX models. Serous carcinoma, carcinosarcoma, and endometrioid carcinoma xenograft tissues were stained for hormone receptors, ER and PR, the proliferation marker, Ki67, endothelial cell marker CD31, and epithelial–mesenchymal transition (EMT) markers such as cytokeratin, vimentin, E-cadherin, P53, PTEN, uPA, and uPAR. The xenografts retained the characteristics of the original tumour and displayed features that were unique to type I and type II endometrial cancer [24]. Depreeuw et al. validated the established

PDX models histologically. In all models, irrespective of their classification, the tissue architecture and the epithelial component of the original tumour by H&E staining were preserved in the corresponding F1 and F3 PDX. They observed similarities in ER and PR staining between patients and xenograft tumours. They stained tumour sections for vimentin using two different antibodies: one specific for human vimentin (hu-VIM) and a second one binding both human and mouse vimentin (hu + mo-VIM). In all patient tumours, the stroma stained positive for hu-VIM, but the staining was negative in the PDX. The hu + mo-VIM was strongly positive for xenograft stroma, indicating that the human-derived stroma was lost and replaced by a reduced amount of murine stroma after tumour engraftment in mice. They also performed whole-exome sequencing on four models reflecting a different, more common and relevant subtype of endometrial cancer, including two endometrioid, one mesonephric, and one serous carcinoma without MSI or POLE mutations. They found an average of 57 non-silent mutations in the primary tumours and 77 of them in the xenografts. The majority of such mutations were common between primary tumours and xenografts (55%), while a minor fraction was unique either for the primary tumour (11%) or for the xenograft (34%). By studying the cancer consensus genes specifically, they observed that most of the mutations were common between primary tumours and xenografts. The copy number profiles were generated using low-covered whole-genome sequencing for both the primary tumour and xenograft in the endometrioid carcinoma model. On average, 90% of the genome had the same copy number between the primary tumour and xenograft [25].

2.6 Ovarian Cancer

2.6.1 PDX Procedure and Success Rate

PDX models have been more advanced in ovarian cancer than in other gynaecological malignancies. Table 2.3 shows the engraftment rate described in recently published literature on ovarian cancer PDX models. The engraftment rate varies from 8.3 to 100% [28–32]. Wu et al. injected the minced tumour fragment dissected from ovarian cancer patients into subcutaneously into SCID mice. The engraftment rate was 15.4% [28]. Dobbin et al. evaluated the engraftment rate of ovarian cancer fragments in several implanted sites using SCID mice. The engraftment rates were 85.3% in subcutaneous, 63.6% in MFP, 22.2% in IP, and 8.3% in renal capsules [29]. Weroha et al. injected the minced ovarian cancer fragment into the intraperitoneal cavity of SCID mice. The engraftment rate was 74% [32]. Eoh et al. injected minced ovarian cancer fragment into subcutaneous NOG mice with an engraftment rate of 53.4% [30]. Heo et al. injected a small fragment of ovarian cancer in the renal capsule of nude mice. The engraftment rate was 48.8% [31].

Table 2.3 The engraftment rate of the patient-derived xenograft models for ovarian cancer in the literature

	Mouse strains	Site of transplantation	Method of graft	Fragment size	Engraftment rate, %
Wu et al.	SCID	Subcutaneous	Injection	Minced	15 (4/26)
		Ovary	Direct	3 mm^3	100 (1/1)
Dobbin et al.	SCID	Subcutaneous	Direct	5 mm^2	85
		Mammary fat pat	Direct	Minced	64
		Intraperitoneal	Injection	Minced	22
		Renal capsule	Injection	3 mm^2	8
Weroha et al.	SCID	Intraperitoneal	Injection	Minced	74
Eoh et al.	NOG	Subcutaneous	Injection	Minced	53 (47/88)
Heo et al.	Nude	Renal capsule	Direct	2–3 mm	49 (22/45)

2.6.2 Analysis of the Original Tumour and PDX

Wu et al. performed immunohistochemical analyses for primary tumours and PDX. The tumour markers, including epithelial tissue marker (CK7), intestinal tissue marker (vimentin), nervous tissue marker (Syn), tumour protein p53 (P53), proliferating cell nuclear antigen (PCNA), Antigen KI-67 (Ki67), and nuclear factor erythroid 2-like 2 (NrF2) in PDX models tumours were in accordance with primary tumours; however, immunohistochemical scores in PDX models were higher when compared to those in primary tumours. The tumour-associated gene expression of the second-generation PDX model was also in accordance with the primary tumour. They also compared gene mutation and expression between PDX model tumours and primary tumours. Single nucleotide polymorphisms (SNPs), transition, and transversion of PDX model tumours were lower when compared to those of the primary tumour; however, the location of SNPs did not differ between the groups. Fusion gene analysis indicated that compared to the primary tumour, which includes three fusion genes, there was only one fusion gene in PDX model tumours in accordance with its primary tumour. Six new fusion genes were found in PDX model tumours. The alternative splicing of PDX tumour models was lower than that of their primary tumour. The consistent rate of expressed genes reached 87.2%. These results indicated that PDX model tumours were consistent with primary tumours on gene expression [28]. Dobbin et al. performed immunohistochemistry for tumour-initiating cell markers, including ALDH1A1, CD44, and CD133. The PDX models showed similar expression of ALDH1A1 and CD133. There was a significant change in the expression of CD44; however, it decreased from 5.5% to 2.4%. Moreover, immunohistochemistry for human HLA demonstrated the replacement of human stroma with murine cells. They performed an RT^2-PCR array, which quantifies mRNA levels of 84 targetable oncogenes. Most of the 84 cancer drug target genes had similar expression in the PDX and the original patient tumour [29]. Weroha et al. reported that the glandular characteristic of adenocarcinoma was

conserved between tumourgrafts and their primary tumours. The percentage of non-epithelial tissue area, which was negative for pan-CK expression, was similar between PDX and primary tumours. When patient and tumour tissue were evaluated for the expression of human vimentin using an antibody with no reactivity against mouse protein, the patient stroma stained strongly while the tumourgraft stroma did not. Array comparative genomic hybridisation revealed a marked overlap in genomic gains and losses between the patient tumour and the corresponding tumourgraft. In addition, commonly gained/lost genes are seen in ovarian cancer. They also evaluated the efficacy of platinum-based chemotherapy. Paclitaxel–carboplatin chemotherapy reduced tumour weight in PDX models, which were grafted from patients with platinum-sensitive tumours; however, it was not observed in models that were grafted from patients with platinum-resistant tumours. The gene expression showed two distinct patterns between platinum-sensitive and platinum-resistant tumourgrafts [32]. Heo et al. reported that H&E staining of primary patient tissue and PDXs after each passage revealed a similar architectural pattern of nesting configuration and comparable cytologic atypia in PDXs according to pathologic subtype. Interestingly, they found different histologic features in PDX tissue compared with the tissue of cell line xenografts. Short tandem repeat analysis showed an almost identical banding pattern between PDXs and primary patient tumours. They also evaluated the efficacy of chemotherapy and molecular target therapy. Paclitaxel–carboplatin chemotherapy significantly decreased the tumour weight in PDX grafted from a patient with high-grade serous carcinoma, which was sensitive to paclitaxel–carboplatin chemotherapy. Moreover, the EGFR inhibitor erlotinib significantly decreased tumour weight in the PDX model grafted from a patient with clear cell carcinoma, which strongly expressed EGFR [31].

2.7 Conclusions

Several aspects of human patient tumour tissue, including genomic and histological characteristics or sensitivity of anticancer drugs, are preserved in PDX models. These advantages could bring about drug development and appropriate treatment for precision medicine. PDX models are indispensable tools for precision oncology today.

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Chapter 3
Cancer Genomic Profiling of Gynecological Malignancies by Todai OncoPanel, a Twin DNA and RNA Panel

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Abstract Todai OncoPanel (TOP) has been established at The University of Tokyo and consists of DNA (version 3: 464 genes) and RNA panels (version 4: 463 genes).

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The University of Tokyo Hospital started TOP analysis in February 2017 as a research project approximately in 250 patients. Then, clinical sequencing for advanced solid tumors by TOP panel was performed as Advanced Medical Care Category B in 200 patients toward approval from the Ministry of Health, Labor and Welfare (Patient accrual was completed in December 2019). In this study, we performed TOP analysis in 54 gynecological malignancies and found various types of actionable somatic mutations, gene fusions, germline mutations (in *BRCA1*, *BRCA2*, and mismatch repair genes), as well as high tumor mutational burden. We describe the efficacy and the utility of TOP for gynecological malignancies, using our comprehensive analysis of the 54 gynecological malignancy cases. These findings will highlight the usefulness of cancer genomic profiling and shed light on precision medicine in gynecological malignancies.

Keywords Cancer genomic profiling · Gynecological malignancies · Precision medicine · Todai OncoPanel · Twin DNA and RNA panel

3.1 Introduction

Next-generation sequencing (NGS)-based tumor molecular profiling has become a fundamental component of precision medicine for cancer patients, which enables us to identify genetic alterations in genes and pathways for molecular-targeted therapies [1–3]. Several types of cancer genomic profiling (CGP) have proven their utility in cancer precision medicine, and two types of CGP (The OncoGuide™ NCC Oncopanel System and FoundationOne CDx Cancer Genomic Profile) were approved in Japan [4, 5]. However, most CGPs are based on analysis of genomic DNA of target genes, isolated either by polymerase chain reaction (PCR) or probe hybridization, enabling the detection of SNV, small insertions/deletions (indels), and CNV.

At The University of Tokyo, an original CGP assay, named Todai OncoPanel (TOP), was developed, which consists of a DNA panel and an RNA panel [6]. For the analysis, tumor DNA and RNA were prepared from FFPE tissues, and normal-paired DNA was extracted from peripheral blood collected from the same patients as a control to distinguish somatic and germline variants [6]. We analyzed >600 clinical samples since February 2017 under the approval of the institutional ethics committee. This clinical sequence assay consists of DNA and RNA hybridization capture-based next-generation sequencing panels that enable the comprehensive characterization of cancer-related genes. The TOP DNA panel can detect single-nucleotide variant (SNV), indels, and copy number variations (CNV) in 464 genes in version 3 (478 genes in version 4). Tumor mutational burden (TMB) and allele-specific copy number variations can be evaluated, and over 1000 microsatellite probes are included in the TOP DNA panel. The TOP RNA panel covers 463 genes

in version 4 (678 genes in version 5). The current version can detect gene fusions in 504 genes, as well as exon skipping (such as *MET* and *CTNNB1*) and provide gene expression profiling [6]. Fusion genes in the TOP RNA panel include *BCR-ABL1*, *EML4-ALK*, *RET*, *ROS1*, *NTRK1/2/3*, *FGFR1/2/3,* and *NRG1*, all of which can be (or are promising to be) candidates for specific molecular-targeted agents [7, 8]. Here, we describe the efficacy and the utility of TOP for gynecological malignancies, using our comprehensive analysis of 54 gynecological malignancy cases.

3.2 Patient Characteristics

Between February 2017 and April 2018, 54 gynecological cancer patients (with 79 FFPE tumor specimens) were analyzed by the TOP panel at The University of Tokyo Hospital. Tumor specimens were mainly obtained by surgically resected tumors. Under written informed consent, we returned the results to the patients. The number of patients in each tumor type is 6 in cervical cancer, 15 in endometrial cancer, 21 in ovarian cancer (as well as 2 synchronous endometrial and ovarian carcinomas), 9 uterine sarcomas, and 1 choriocarcinoma (Fig. 3.1a). Comprehensive analysis of all tumor types was reported previously [6].

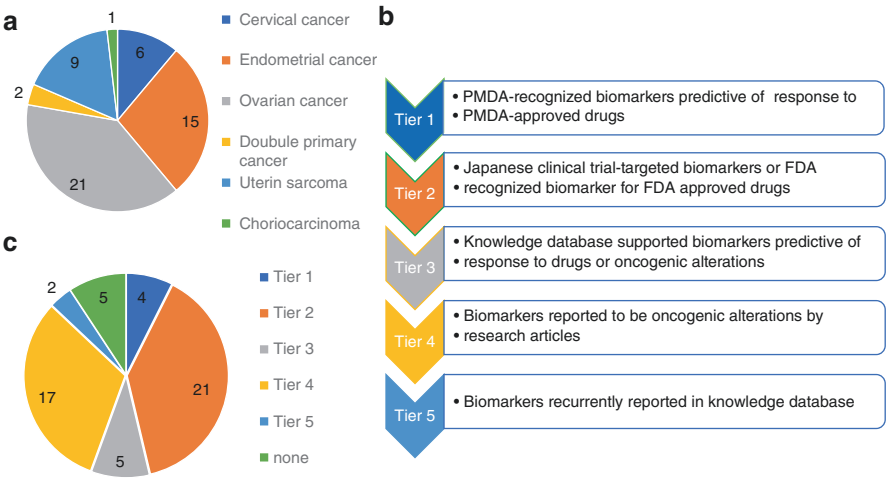

Fig. 3.1 Cancer types and evidence level classification. (**a**) Distribution of tumor types among the 54 gynecological malignancy patients. (**b**) TOP evidence level classification. The evidence level classification was used to annotated gene alterations in TOP between 2017 and 2018. (**c**) Clinical actionability of gene alterations detected in gynecological cohort by the TOP panel. The maximum evidence level for each case is listed

3.3 Clinical Annotations and Recommendation of Clinical Trials

We annotated somatic variants by using various types of public databases, including OncoKB, a curated knowledge database of oncogenic effects and treatment implications of gene alterations (http://oncokb.org); CIViC (Clnical interpretation of Variants in Cancer), a community-based curation database (http://civicdb.org), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and COSMIC (Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cosmic) to assess variants frequency in cancer. We additionally used specific databases for annotation of gene variants, including germline variants by BRCA Exchange (http://brcaexchange.org) for *BRCA1/2*, IARC TP53 (http://p53.iarc.fr) for *TP53*, and InSiGHT (https://www.insight-database.org/genes) for mismatch repair genes. We constructed our knowledge database using a website of the Pharmaceuticals and Medical Devices Agency, US Food and Drug Administration (FDA), and National Cancer Institute. We also included clinical trial databases, such as ClinicalTrials.gov and the Japanese clinical trial databases UMIN, JAPIC, and JMACCT.

Annotated variants were classified according to the level of evidence and drug availability [6] (Fig. 3.1b). In summary, Tier 1 was annotated to (pathogenic/likely pathogenic) variants with biomarkers to PMDA-approved drugs in the matched tumor type; Tier 2 was annotated to (pathogenic/likely pathogenic) variants with biomarkers applicable to clinical trials/FDA-approved drugs/PMDA-approved drugs in other tumor types. Tier 3 was for (pathogenic/likely pathogenic) variants, which were supported by knowledge databases for prediction of response to drugs or oncogenic alterations. Tier 4 corresponded to biomarkers, which were to be oncogenic. Tier 5 corresponded to biomarkers, which were recurrently reported in knowledge databases (Fig. 3.1b).

Recently, clinical and/or Experimental Evidence Levels have been standardized in Japan, which were defined by the Center for Cancer Genomics and Advanced Therapeutics (C-CAT) and Equivalent Evidence Levels in Other Guidelines [5].

3.4 Genetic Alterations in Gynecologic Malignancies by the TOP Panel

We evaluated the clinical utility of genetic alterations in each tumor in the TOP panel. All the tumors were subjected to both DNA and RNA panels. Each alteration in each case was discussed and annotated by the molecular tumor board at The University of Tokyo Hospital, which are currently applicable for various types of PMDA-approved CGPs. Therefore, information on clinical trials was confirmed between 2017 and 2018. Overall, 91% (49 out of 54) harbored one or more

clinically annotated alterations (Tier 1 to Tier 5), and 46% (25 out of 54) harbored one or more actionable variants (Tier 1 or Tier 2) in gynecologic cancers (Fig. 3.1c). The proportion of actionable variants in cervical cancer, endometrial cancer, and ovarian cancer was 50%, 73%, and 57%, respectively. In these 54 cancer patients, the most frequently mutated gene with clinical annotation was *TP53*, followed by *PIK3CA*, *PTEN*, *PIK3R1*, *KRAS,* and *ARID1A*.

3.5 Cervical Cancer

Among the six cervical cancer patients, actionable mutations (Tier 2) were identified in three cases (50%) (Fig. 3.2a). Two were somatic oncogenic variants of *PIK3CA*, which matched to the clinical trial for an AKT inhibitor in Japan. Another case harbored *the GOPC-ROS1* fusion gene (Table 3.1). *ROS1* is a proto-oncogene located on chromosome 6q and encodes a receptor tyrosine kinase involved in the regulation of cancer cell growth and differentiation. ROS1 is often involved in genomic rearrangements resulting in constitutively active kinases that stimulate multiple pathways such as JAK-STAT, PI3K-AKT-mTOR, and RAS-RAF-MEK-ERK [9–11]. Fusion products of ROS1 have been observed in a variety of types of cancer, including lung, gastrointestinal tract and hepatobiliary tract, and central nervous system [9]. The *ROS1* fusions are now considered as therapeutic biomarkers of crizotinib and entrectinib [12, 13].

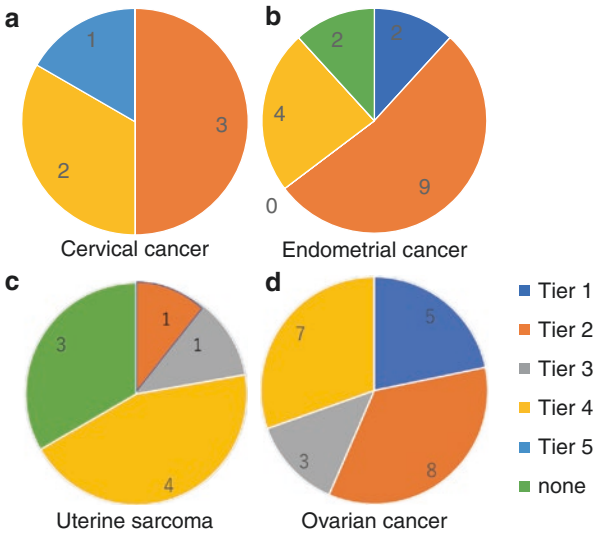

Fig. 3.2 Genetic alterations and clinical actionability by the TOP panel in gynecological cancers. (**a**–**d**) Detected gene alterations were annotated using the TOP classification. All the cases are assigned to the level with the most actionable alterations: (**a**) cervical cancer, (**b**) endometrial cancer, (**c**) uterine sarcoma, and (**d**) ovarian cancer. (**e**) Histological types of ovarian cancers. (**f**) Genetic alterations identified in 23 ovarian cancers

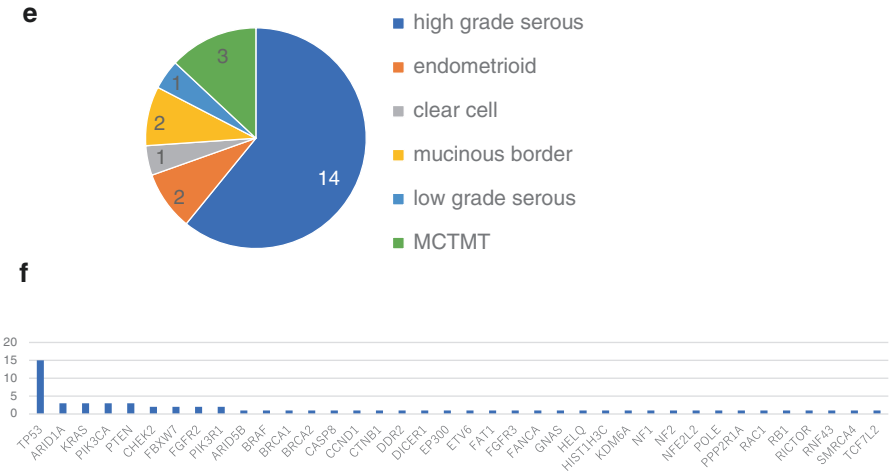

Fig. 3.2 (continued)

3.6 Endometrial Cancer

We analyzed 15 endometrial cancer cases and identified 18 actionable gene alterations in 11 patients (73%) (Fig. 3.2b). Somatic variants of the PI3K-AKT pathway are most frequent, with 6 pathogenic mutations of *PIK3CA*, 7 of *PTEN*, and 2 of *PIK3R1*. These were considered for clinical trials with AKT inhibitors. Pathogenic somatic variants of *BRCA1* were identified in one patient, which may be associated with homologous recombination deficiency (HRD). Thus, poly (ADP-ribose) polymerase (PARP) inhibitors were recommended in this patient, although no PARP inhibitors have been clinically approved in endometrial cancer. Pathogenic germline variants of MMR genes were identified in two patients (*MSH6* (p.F858Sfs*12) and *MSH2* (p.Q170*)) (Table 3.1). In addition to pathogenic variants in MMR genes, the primary tumors of these two patients were TMB-High, which also supports the recommendation of immune checkpoint inhibitors. Pembrolizumab was approved for microsatellite instability-high (MSI-High) solid tumors by FDA in 2017 and by PMDA in 2018 [14, 15].

3.7 Uterine Sarcoma

We analyzed nine uterine sarcomas and found that found no actionable mutations, except for one patient (Fig. 3.2c). No pathogenic alterations were detected in three patients (33%); however, two novel gene fusions were identified by the TOP RNA panel, which may be associated with tumorigenesis of uterine sarcomas. As *NTRK* gene fusions were identified at 4% in uterine leiomyosarcomas [16], the TOP RNA panel would be useful to identify actionable and/or novel gene fusions.

3.8 Ovarian Cancer

We enrolled 23 ovarian cancer cases. Totally, 15 actionable gene alterations were detected in 13 patients (Fig. 3.2d). Histological distribution was as follows: 14 (61%) with high-grade serous carcinomas, 2 with endometrioid carcinomas, 1 with clear cell carcinoma, 2 with mucinous borderline tumors, 1 with low-grade serous carcinoma, and 3 with malignant transformation of mature cystic teratomas (MCTMT) (Fig. 3.2e). The genome profile of ovarian cancers highly depends on histological types. For example, *TP53* is mutated in >90% of high-grade serous carcinomas. Pathogenic somatic variants are listed in Fig. 3.2f. *TP53* is the most frequently mutated gene, followed by *PIK3CA*, *PTEN*, *KRAS,* and *ARID1A*. Fourteen (12 high-grade serous carcinomas and 2 MCTMT) harbored pathogenic somatic variants of *TP53*. Pathogenic germline variants were identified in four patients (*BRCA1* in two and *BRCA2* in two patients, Table 3.1), and pathogenic somatic variant of *BRCA1* was identified in one patient, all of which could be targeted by PARP inhibitors. Pathogenic somatic variants of the PI3K-AKT pathway were identified in five patients, which were the candidate targets of AKT inhibitors. Actionable alterations in *FGF* and *FGFR* were identified in four patients, which led to the enrollment of a clinical trial with an FGFR inhibitor. Pathogenic *BRAF* somatic variant was identified in one case with low-grade serous carcinoma (Table 3.1).

3.9 Choriocarcinoma

We analyzed one choriocarcinoma and detected three clinical annotated genes (Tier 5). However, no actionable gene alterations were identified.

3.10 Germline (Secondary) Findings

In gynecological malignancy, the ratio of germline variants is expected to be high, due to the prevalence of hereditary breast and ovarian cancer syndrome (HBOC) and Lynch syndrome [17, 18]. While the primary purpose of CGP tests has been considered to identify pathogenic somatic variants, germline findings may also guide personalized therapy and may be clinically significant, especially in gynecological malignancies. Indeed, we found pathogenic germline variants of mismatch repair genes and *BRCA1/2* genes in endometrial and ovarian carcinomas, respectively (Table 3.1). The prevalence of pathogenic germline variants can be assessed by the TOP panel, as it analyzes paired-normal DNA from peripheral blood samples. The TOP panel basically includes cancer-related genes, which are recommended to report as germline (secondary) findings in clinical CGP [19, 20], and it is expected to contribute to find germline findings, which would be useful for both patients themselves and their relatives. As described above, TOP analysis of the 54

Table 3.1 Actionable mutations and candidate molecular-targeted drugs in gynecologic cancers

Primary	Drugs	Annotated variants
Cervical cancer	AKT inhibitor (Tier 2)	s*PIK3CA* (p.E545K), s*PIK3CA* (p.G1049A)
	ROS1 inhibitor (Tier 2)	GOPC-ROS1
Endometrial cancer	Immune checkpoint inhibitor (Tier 1)	g*MSH6* (p.F858Sfs*12), g*MSH2* (p.Q170*)
	AKT inhibitor (Tier 2)	s*PIK3CA* (p.E545K), s*PIK3CA* (p.R88Q), s*PIK3CA* (p.E545K), s*PIK3CA* (p.E545K), s*PIK3CA* (p.H1047R), s*PIK3CA* (p.H1047R)
		s*PTEN* (p.Y180fs*3), s*PTEN* (p.R130Q), s*PTEN* (p.T319Nfs*6), s*PTEN* (p.R130Q), s*PTEN* (p.R130Q), s*PTEN* (p.H93Tfs*5), s*PTEN* (p.D92E)
		s*PIK3R1* (p.L581Vfs*19), s*PIK3R1* (p.N564D)
	PARP inhibitor (Tier 2)	s*BRCA1* (p.R1699W)
Ovarian cancer	PARP inhibitor (Tier 1)	g*BRCA2* (p.P3039=), g*BRCA2* (p.Q356*), g*BRCA1* (p.E1257Gf*9), g*BRCA1* (p.Q396*), s*BRCA1* (p.E554*)
	AKT inhibitor (Tier 2)	s*PIK3CA* (p.E545K), s*PIK3CA* (p.G1049A)
		s*PTEN* (p.R130Q)
		s*PIK3R1* (p.V357Gf*7), s*PIK3R1*(p.Y580_M582del)
	FGFR inhibitor (Tier 2)	s*FGFR2* (p.Y375C), s*FGFR2* (p.S252W), s*FGF3* (amplification), s*FGF19* (amplification)
	BRAF inhibitor (Tier 2)	s*BRAF* (p.L597R)

individuals in this study revealed 6 pathogenic germline variants (2 in *BRCA1*, 2 in *BRCA2*, 1 in *MSH2*, and 1 in *MSH6*) (11%), all of which were disclosed to each patient by the physicians and certified genetic counselors. Two patients wished confirmatory single-site test and were found to be pathogenic.

3.11 Tumor Mutational Burden

The TMB representing the number of somatic variants per Mb is a biomarker to estimate the efficacy of immune checkpoint inhibitors. In 2019, Pembrolizumab was approved for adult and pediatric patients with TMB ≥10 [21]. In this study, we defined a threshold of 10 mutations/Mb as TMB-High and found that 7 cases (1 cervical cancer, 3 endometrial cancer, and 3 ovarian cancer) were TMB-high (Fig. 3.3). One cervical cancer patient exhibited TMB of 14.3/Mb. This patient received concurrent chemoradiation as primary treatment and the sample was obtained from the recurrent site in the heart. Careful caution is required to address TMB-high, as treatment by either chemotherapy or irradiation may increase the

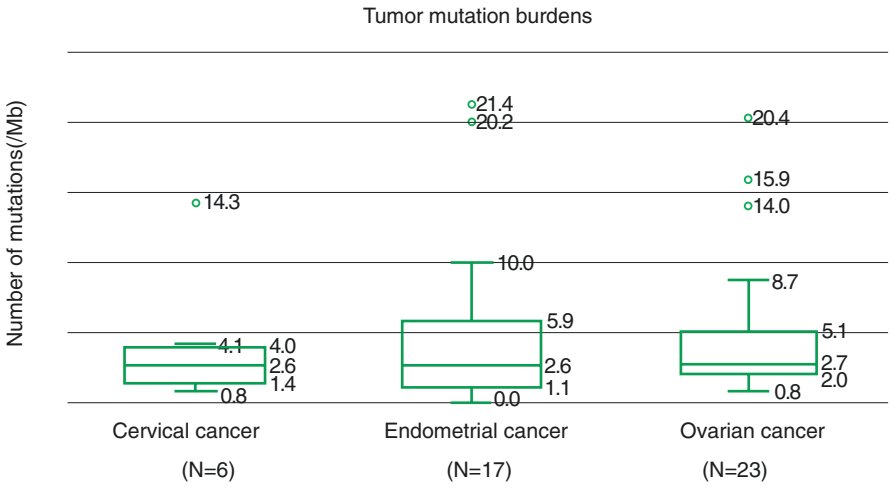

Fig. 3.3 Tumor mutational burdens in gynecological cancers. (**a**) Distribution of the somatic tumor mutational burden (TMB), defined as a number of coding mutations per megabase. The threshold for hypermutation is 10 mutations/Mb. A plot of 206.2/Mb in endometrial cancer is an ultra-mutated case with a variant of *POLE*

number of mutations. In endometrial cancer cases, three patients exhibited TMB-high (206.2/Mb, 21.4/Mb, and 20.2/Mb). One of the three patients harbored pathogenic germline variant of *MSH2*, one with a variant of uncertain significance (VUS) of *MSH2*, and the remaining one with somatic variants of *POLE* (206.2/Mb). In ovarian cancer, three cases were TMB-high. Two of them harbored somatic variants of *POLE*, which might induce hypermutation genotype, although one of the *POLE* variants was uncertain for pathogenicity. The remaining one patient with TMB-high (20.4/Mb) was initially diagnosed as double primary cancer (endometrial cancer and ovarian cancer) and harbored a germline VUS of *MSH2*. We confirmed that her sample from endometrial cancer also exhibited TMB-high with overlapping genotype. Therefore, we amended the diagnosis as endometrial cancer with metastasis to the ovary.

3.12 Discussion

Our study confirmed that NGS-based CGP, using the TOP panel, is useful to identify "actionable" mutations in gynecological malignancies. It has been uncertain which types of gynecological cancers are suitable for CGP, especially for the RNA panel. Here, we revealed by our cohort that the TOP panel, a twin-panel system of DNA and RNA, can efficiently detect molecular profiling of gynecological

malignancies, including identification of gene fusions. More than 90% of all patients harbored at least one clinically annotated gene alteration and 46% harbored actionable alterations (Tier1 or Tier2). The ratio of actionability is higher than that from the pan-cancer analysis (actionability rate at 32.2%) [6].

Allele-specific analysis of CNV is useful to identify homozygous deletion (and uniparental disomy) of tumor suppressor genes [6, 22]. Another merit of TOP is the RNA panel. The junction-capture method enabled us to accurately and cost-effectively detect hundreds of fusion genes, as well as aberrantly spliced transcripts [6]. The RNA panel can detect novel gene fusions if one of the constituent genes is targeted by the capture panel. Although the clinical actionability of the fusion gene may be limited in gynecological carcinomas [23, 24], detection of *ROS1* gene fusion in one cervical cancer suggested that exploring gene fusions may lead to the best-personalized therapy. In addition, the identification of fusion genes using junction-capture RNA sequencing may be useful for molecular diagnosis of sarcomas, which are characterized by various fusion genes [16, 25, 26]. We identified two novel gene fusions in uterine sarcomas in this study. Although these two alterations were not clinically actionable, the existence of a gene fusion was useful to differentially diagnose high-grade endometrial stromal sarcoma from undifferentiated uterine sarcoma.

By using the TOP panel, we constructed the infrastructure of a clinical sequencing laboratory (with ISO15189 certification) within The University of Tokyo Hospital and established an expert annotation team to properly assess the results of sequencing data and provide final reports to each patient. Building this in-house clinical sequencing system should be advantageous to further propel personalized medicine by the cutting-edge technology.

The low rate of clinical trial enrollment after CGP is still a major problem [1, 4, 6, 27]. In this study, only one patient with an oncogenic variant of *PIK3CA* was enrolled in the clinical of AKT inhibitor, although we included cancer patients regardless of the patients' status (both nonrecurrent patients and those who receive standardized treatment can be enrolled). Larger numbers of basket-type clinical trials are anticipated to provide more options for personalized therapy. We conducted the prospective TOP analysis as Advanced Medical Care Category B (Japanese medical system: Senshin-iryou B) in 200 patients toward approval from the Ministry of Health, Labor and Welfare from August 2018 (Patient accrual was completed in December 2019) (UMIN000033647). The data of the 200 patients will disclose the ratio of the actionability in patients who (almost) finished all the standardized treatments. We believe that the TOP panel system will accelerate personalized medicine and broaden cancer treatment options for cancer patients in the near future.

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Chapter 4
Investigating the Molecular Carcinogenesis of Ovarian High-Grade Serous Carcinoma

Satoru Kyo

Abstract Among various histological subtypes of epithelial ovarian cancer, high-grade serous carcinoma (HGSC) exhibits poor prognosis, especially with disseminated lesions. Approximately 5–10% of ovarian cancers are attributed to inherited germline mutations of susceptible genes, most of which involve mutations of *BRCA1* or *BRCA2*. The development of risk-reducing salpingo-oophorectomy (RRSO) for patients with *BRCA1/2* mutations has led to the discovery of various precursor lesions in the fallopian tubes, not the ovaries, such as serous tubal intraepithelial carcinoma (STIC), which is also known to be frequently associated with HGSC. Comprehensive genomic analyses have uncovered various genetic aberrations in STIC, and most are commonly observed in HGCS, suggesting that HGSC originates from STIC. Mouse studies and *in vitro* carcinogenesis models using immortalized fallopian tube cells have shown that three genetic hits are both necessary and sufficient for carcinogenesis, and that specific sets of driver mutations effectively contribute to tumorigenesis. These findings improve our understanding of the carcinogenesis of HGSC as well as further the development of novel molecular targeted therapies.

Keywords HGSC · Carcinogenesis · Driver gene · RRSO · Immortalization

4.1 Introduction

In Japan and the United States, ovarian cancer accounts for 3.1% and 2.5% of cancer diagnoses and is the ninth and fourth leading cause of cancer-related death, respectively [1, 2]. Among various histological subtypes of epithelial ovarian

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cancers, high-grade serous carcinoma (HGSC) exhibits poor prognosis, especially when disseminated lesions are present [3]. Recent progress in next-generation sequencing (NGS) has enabled comprehensive analyses of the genomic features of human cancers, and a variety of genomic aberrations have been uncovered in HGSC, including copy number alterations, somatic gene mutations, and epigenetic aberrations [4]. One major issue concerning the molecular carcinogenesis of the ovary is the cell-of-origin. Unlike other types of tumors, it has recently been postulated that ovarian cancer arises from extra-ovarian tissues, including the fallopian tubes and endometrium, depending upon the histological subtype [5]. Another important issue is to identify key driver mutations among the numerous genomic abnormalities detected by NGS. It is of great concern how many mutations are required and what their optimal combinations are for carcinogenesis. In the present chapter, we introduce recent progress in clarifying the molecular carcinogenesis of HGSC based on our own in vitro carcinogenesis model.

4.2 Characterization of Tubal Precursors

4.2.1 Discovery of Serous Tubal Intraepithelial Carcinoma

The idea that ovarian cancer arises from ovarian surface epithelial cells has changed over the past two decades due to the novel hypothesis that tubal fimbriae may be the origin of some types of ovarian cancers [5] as a result of the discovery of the *BRCA1* and *BRCA2* tumor suppressor genes. Around 10% of ovarian cancers are attributed to inherited germline mutations of susceptible genes, approximately 90% of which include *BRCA1* or *BRCA2* gene mutations [6–8]. The risk of ovarian cancer increases in such mutation carriers to 40–60% at the age of 70 years [9], which is far higher than the lifetime risk among the general population (1.3%) [10].

To prevent the occurrence of ovarian cancer, risk-reducing bilateral salpingo-oophorectomy (RRSO) has been recommended for patients with *BRCA1* or *BRCA2* gene mutations at the age of 35–45 years [11–13]. Histological examinations of resected fallopian tubes and ovaries at the time of RRSO have led pathologists to discover epithelial abnormalities of the fallopian tubes rather than the ovaries. These abnormalities are characterized by epithelial changes of carcinoma in situ and are thus named serous intraepithelial carcinoma (STIC) [14–17]. Thereafter, the Brigham and Womenaepithelial carcinomato extensively examine histological sections of resected fallopian tubes, called the Sectioning and Extensively Examining the FIMbriated end of the fallopian tube (SEE-FIM) protocol, for women with *BRCA* mutations or with a family history of breast and/or ovarian cancer [18]. Use of the SEE-FIM protocol increased the detection of STIC or early serous carcinoma and showed that about 2% of women with RRSO harbored them at the distal end of fallopian tubes around the fimbriae [19–21]. If STIC is a precursor of HGCS, there might be common genetic alterations between them. In fact, *TP53* somatic

mutations are frequently detected in both lesions, exhibiting p53 overexpression in immunostaining [21–23].

4.2.2 The p53 Signature as a Precursor of STIC

Immunohistochemical analyses of fimbriae resected by RRSO have revealed that there exist small segments of epithelial cells with strong p53 expression in addition to STIC. Such p53-positive segments do not accompany morphological changes and are observed in women with wild-type *BRCA* as well [24] (Fig. 4.1). These p53-positive segments are called the "p53 signature" that is frequently observed in association with STIC [22]. Immunohistochemical studies have found that the p53 signature is observed in secretory cells of the fimbria and is frequently associated with γH2AX staining, a landmark of DNA double-strand breakages

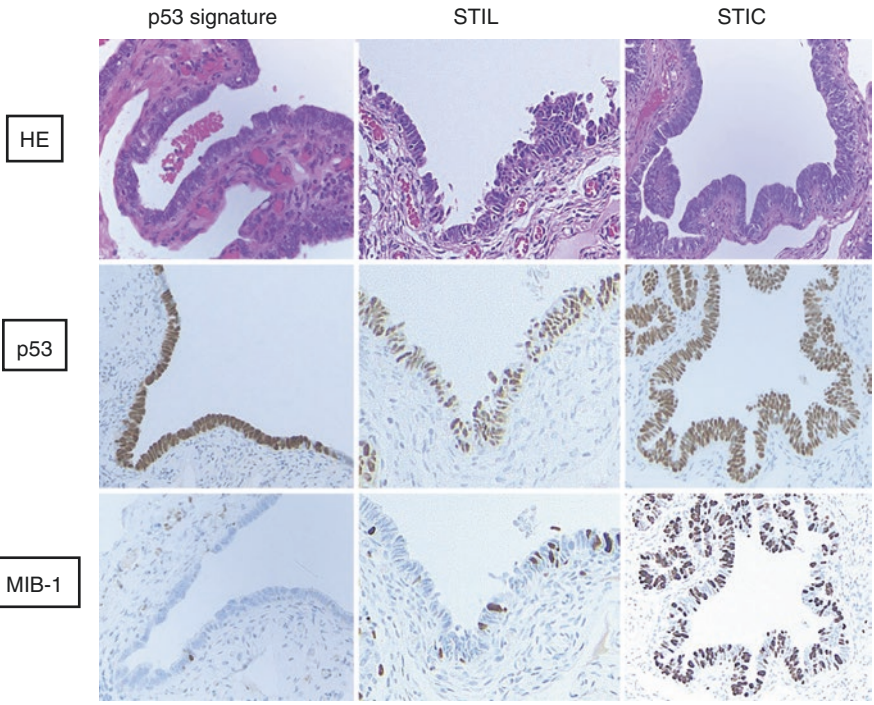

Fig. 4.1 Representative histological findings of the precursors of HGCS. The p53 signature has normal morphology and overexpresses p53 but lacks MIB1 expression. Serous tubal intraepithelial carcinoma (STIC) exhibits distinct cytological atypia as well as loss of polarity, with p53 overexpression due to gene mutation and with MIB-1 expression representing high proliferative activity. Serous tubal intraepithelial lesions (STILs) are characterized as intermediate lesions between the p53 signature and STIC, with transitional morphological findings and proliferative activity (Adapted from [24])

(DNA-DSBs) [22], showing that the p53 signature involves DNA-DSBs. Thus, genotoxic circumstances may play roles in the development of the p53 signature, possibly triggered by exposure to several oxidants contained in the follicular fluid during ovulation (as mentioned later) (Fig. 4.2). DNA mutational studies have revealed that the p53 signature accompanies frequent *p53* somatic gene mutations, probably caused by genotoxic stress [22]. Of particular interest is that patients with *p53* germline mutations (Li–Fraumeni syndrome) frequently exhibit a p53 signature in their fimbriae [25], suggesting that *p53* gene mutations play causative roles in the p53 signature.

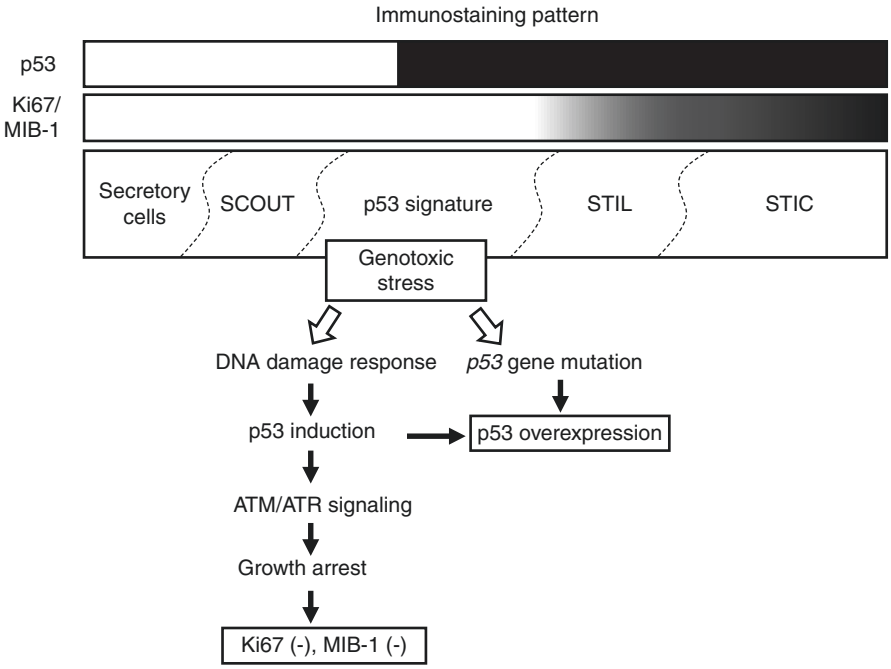

Fig. 4.2 Immunohistochemical features of tubal precursors during the process of carcinogenesis. Black and white boxes represent positive and negative status of immunostaining, respectively, while gray scales indicate transitional changes of the status during disease progression. Some of the secretory cells show expanded growth in the fimbria end, named the secretory cell outgrowth (SCOUT). By exposure to blood during retrograde menstruation or to follicular fluid at the time of ovulation, secretory cells or SCOUT undergo genotoxic oxidative stress that triggers DNA damage responses, leading to the onset of the p53 pathway and the subsequent activation of Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) signaling and cell cycle arrest, inducing the phenotype of the p53 signature. Eventually, the p53 signature exhibits p53 overexpression but lacks Ki-67 or MIB-1 expression. Continuous genotoxic stress induces or causes *p53* gene mutation, leading to the development of serous tubal intraepithelial lesions (STILs) and serous tubal intraepithelial carcinoma (STIC), with high proliferative activity as represented by Ki-67 or MIB-1 expression

4.2.3 Broad Spectrum of Tubal Precursors

There are two types of epithelial cells in the fallopian tube, secretory and ciliated cells. The secretory cells are thought to be in a less mature state compared to ciliated cells and are likely to be vulnerable to transformation. The p53 signature is observed in secretory cells that usually exhibit low proliferative activity, evaluated as low ki-67 or MIB1 expression (Fig. 4.1). This is a feature distinct from STIC, which has high proliferative activity. Interestingly, transitional lesions are often found between the p53 signature and STIC, and they show intermediate proliferative and morphological features. These lesions are called serous intraepithelial lesions (STILs) [26, 27] (Fig. 4.1). These findings are consistent with the concept that the p53 signature is a precursor of STIC (Fig. 4.2).

Recent studies have further reported a candidate precursor of the p53 signature, called secretory cell outgrowth (SCOUT), which is located at more proximal sites of the tube than the p53 signature. This lesion consists of a row of at least 30 secretory cells without the interruption of ciliated cells and is characterized by a pseudostratified appearance and low proliferative activity [28]. *p53* mutations are not usually detected in SCOUT, either by immunohistochemistry or DNA sequencing analysis [28], but there are some cases with a continuity of SCOUT, p53 signature, and serous carcinoma that share identical *p53* mutations. Therefore, SCOUT is a potential precursor of the p53 signature (Fig. 4.2).

4.3 Genetic Analyses of Tubal Precursors and HGSC

Sequencing studies have revealed a molecular relationship between the p53 signature and HGSC. Target sequencing analyses with laser-captured microdissection (LCM) in early studies identified approximately 50–60% of *p53* missense mutations in p53 signatures, while all STICs or pairs of STIC and ovarian cancer shared common *p53* mutations [22]. Recently, comprehensive genomic analyses by NGS have shown evidence that the p53 signature or STIC harbor the ancestral clone for the observed HGSC [29]. So-called cancer driver gene mutations, such as those of *p53*, *BRCA1*, 3*BRCA2*, or C*PTEN*, are commonly observed in STIC and HGSC, with additional genetic alterations present in HGSC, suggesting that STIC has daughter clones of HGSC [29]. Evolutionary analyses have demonstrated that p53 signatures and STICs are precursors of HGSC, in which a window of 7 years for development between STIC and HGSC has been suggested, and metastasis of HGCS is supposed to occur more rapidly thereafter [29].

The Cancer Genome Atlas (TCGA), with 489 clinically annotated stage II–IV ovarian HGSCs, has been used to uncover the genomic status of these tumors [4]. In this analysis, *p53* mutations were most frequently detected (96%) as expected, and mutations of *NF1, BRCA1, BRCA2, RB1*, and *CKD12* were additionally found at relatively low frequencies [4]. DNA copy number alterations were also observed in

CCNe1, MYC, and *MECOM*, and were highly amplified in more than 20% of tumors [4]. Furthermore, pathway analyses revealed frequent activation of RB-related (67%) and RAS/PI3K-related (45%) signaling pathways [4]. Homologous recombination defects (HRD) are found in approximately 50% of the tumors due to the inactivation of *BRCA1* via promoter hypermethylation or somatic or germline mutations [4]. Thus, HGCS is marked by a specific mutational spectrum, extremely frequent *p53* mutations as well as frequent activation of RB- or RAS/PI3K-signalings and HRD, distinct from other histological types of ovarian cancer.

4.4 How to Identify Driver Mutations of HGSC?

4.4.1 Mouse Model

Several mouse models have been developed to identify the molecular mechanisms of carcinogenesis of HGCS. One transgenic mouse model has used SV40 large T-antigen (*TAg*) as a transgene under the control of the Mullerian-specific *Ovgp-1* promoter [30]. The fallopian tubes of these mice exhibit precursor lesions as well as invasive lesions, analogous to HGCS (Table 4.1). This model seems to be consistent in that both p53 and Rb pathways are essential for carcinogenesis based on the established concept that the *TAg* cassette can inactivate both p53 and Rb. However, this model has a fundamental shortcoming in mimicking the carcinogenesis of HGCS, because the TAg is known to induce severe chromosomal instability and cause nonspecific genomic effects, rather than gene-specific effects, thereby making it unclear what the minimal genetic requirements for carcinogenesis are.

Conditional knockout mouse models seem to be preferable for identifying the specific genetic elements required for carcinogenesis. A conditional knockout of *BRCA, p53*, and *PTEN* has been established using the Cre-loxP system, and it enables tissue- and cell-type-specific knockdown in fallopian tube secretory cells (FTSECs) [31]. Mice with either $BRCA1^{mut}$ or $BRCA2^{mut}$ combined with $TP53^{mut}$ and $PTEN^{-/-}$ developed STIC and ovarian HGSC, while those with $TP53^{-/-}$ and $PTEN^{-/-}$ developed STIC but never HGCS, indicating that *BRCA* alterations are indispensable for the development of HGSC (Table 4.1). Mice with $BRCA2^{-/-}$ and $TP53^{mut}$ with intact *PTEN* showed STIC and peritoneal metastasis. However, tumorigenesis was not efficient in these mice and had much longer latency, underscoring that *PTEN* aberrations are essential for tumor development in cooperation with *BRCA* and *TP53* alterations.

4.4.2 Strategy to Immortalize Normal Human Cells

To more clearly understand human carcinogenesis, an in vitro multistep model originating from normal human cells might be ideal. In usual cultures of human primary cells, cells stop growing after several population doublings (PDs) [33, 34], the

Table 4.1 Genetically engineered model to identify potential driver genes for high-grade serous carcinogenesis

Transgenic mouse model [30]				
Trangene	Number of mice	p53 signature	STIC	Invasive adenocarcinoma
TAg	34	34/34 (100%)	34/34 (100%)	19/34 (56%)
TAg: SV40 T-antigen				
Knockout mouse model [31]				
Genotype	Number of mice	STIC	Ovarian metastasis	Peritoneal metastasis
BRCA1−/−, *TP53*MT, *PTEN*−/−	4	4/4 (100%)	1/4 (25%)	1/4 (25%)
BRCA1+/−, *TP53*MT, *PTEN*−/−	12	10/12 (83%)	6/12 (50%)	8/12 (67%)
BRCA2−/−, *TP53MT*, *PTEN*−/−	12	9/12 (75%)	9/12 (75%)	8/12 (67%)
BRCA2+/−, *TP53*MT, *PTEN*−/−	3	3/3 (100%)	3/3 (100%)	2/3 (67%)
TP53−/−, *PTEN*−/−	6	4/6 (67%)	0/6 (0%)	0/6 (0%)
BRCA2−/−, *TP53*MT	11	Hardly detectable due to widespread invasive tumors	NR	3/11 (27%) (much longer latency)
In vitro carcinogenesis model [32]				
Introduced genetic factors	Number of mice	Colony formation on soft agar	Tumor formation (subcutaneous)	Tumor formation (intraperitoneal)
DN-p53	4	0	0	0
DN-p53, KRAS MT	4	0	0	0
DN-p53, c-Myc	4	0	0	0
DN-p53, CA-AKT	4	+	0	0
DN-p53, KRAS MT *c-Myc*	4	+	4	4
DN-p53, KRAS MT, *CA-AKT*	4	+	4	4

Adapted from and with permission from [24]
MT mutation, *STIC* serous tubal intraepithelial carcinoma, *NR* not recorded, *DN-p53* dominant-negative form of p53, *CA-AKT* constitutively activated AKT

number of which is dependent on the cell type. In human epithelial cells, cells stop growing at several PDs, usually within PD 10 (Fig. 4.3). In contrast, human fibroblasts or stromal cells can usually continue to grow for a more extended number of PDs, usually up to 50 [33, 34]. Although both cell types stop growing at different PDs, the cells do not die and are metabolically still active, a state called cellular senescence. The status of senescence in each cell type is distinct. The senescence of human fibroblasts or stromal cells observed at later PDs is called replicative senescence and is characterized by shortened telomeres due to the considerable number of cell divisions [35] (Fig. 4.3). The early senescence observed in human epithelial cells is named premature senescence; in this case, some cell cycle regulators such as

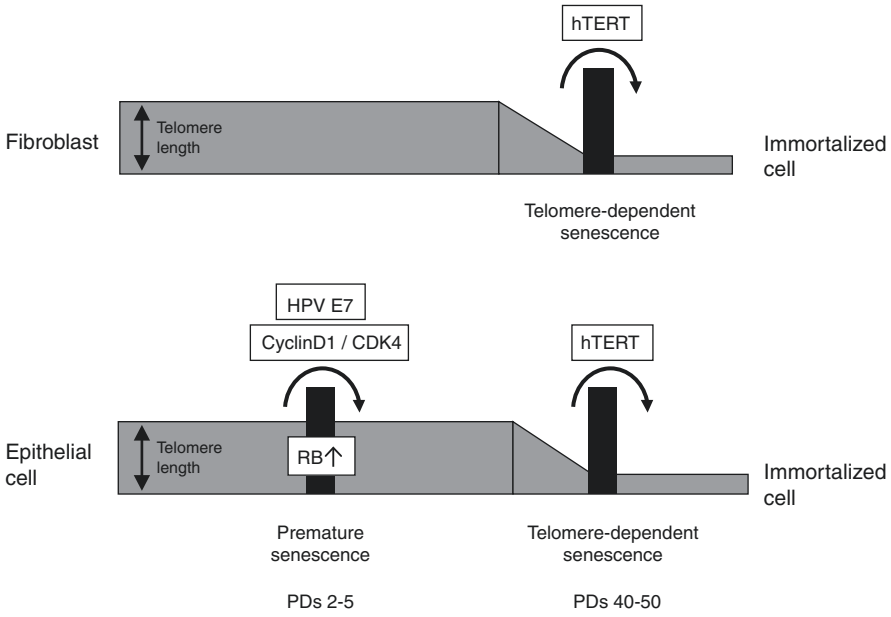

Fig. 4.3 Strategy to immortalize normal cells. In primary cultures, normal epithelial cells stop growing at population doublings (PDs) within 10, called premature senescence, that is caused by activation of the Retinoblastoma susceptibility protein (RB) pathway. In contrast to epithelial cells, normal fibroblasts usually escape from premature senescence. Oncogenic human papillomavirus (HPV) E7 overexpression or CyclinD1/cdk4 overexpression can both inactivate RB function, rendering epithelial cells able to overcome premature senescence. Subsequently, fibroblasts and epithelial cells encounter another type of senescence called telomere-dependent senescence, characterized by extensively shortened telomeres due to a considerable number of cell divisions. By human telomerase reverse transcriptase (hTERT) overexpression, the cells can overcome this type of senescence, leading to cellular immortalization. Black boxes represent two types of obstacles for immortalization, premature senescence, and telomere-dependent senescence. Gray boxes represent the changes in telomere length during the process of immortalization

Rb or cyclin-dependent kinase inhibitors are upregulated, resulting in cell cycle arrest [34]. This phenomenon is presumed to be a cellular response to artificial culture conditions, so called "culture shock" [34]. Even though epithelial cells can overcome premature senescence, they also undergo replicative senescence at later PDs, similar to fibroblasts [35]. Human fibroblasts or stromal cells are probably more resistant to artificial culture conditions and may spontaneously overcome premature senescence. Therefore, human epithelial cells must overcome two major obstacles, premature senescence and replicative senescence, for cellular immortalization, while human fibroblasts and stromal cells can be immortalized by overcoming only replicative senescence (Fig. 4.3).

To overcome premature senescence, several molecular techniques have been attempted. Initially, the human papillomavirus *E7* gene was introduced to inhibit Rb activity based on the knowledge that E7 specifically binds to Rb protein and

inhibits its normal function of repressing the cell cycle [33, 36, 37]. Subsequently, viral methods have been attempted to overcome premature senescence by activating upstream factors of Rb such as cyclin D1 and cdk4, both of which phosphorylate Rb, leading to the inactivation of its function [38]. A combination of cyclin D1 and cdk4 can inhibit Rb more effectively. Thereafter, replicative senescence can be overcome by the introduction of the human telomerase reverse transcriptase gene that confers reactivation of telomerase in human normal cells [39–41] (Fig. 4.3). This is based on the established concept that telomerase is inactivated in normal human cells due to the lack of expression of hTERT, which is a catalytic subunit of human telomerase and is a limiting factor of telomerase activity [42–44].

4.4.3 In Vitro Carcinogenesis Model with Immortalized Fallopian Tube Epithelial Cells

We established an in vitro model to study the carcinogenesis of HGCS with primary human tubal cells [32]. Fallopian tubes were surgically removed from patients operated on due to benign uterine disorders, and the epithelial cells of the fimbriae were isolated from stromal tissues, purified, primary cultured [32], and subjected to immortalization with overexpression of *cyclin D1/cadk4/hTERT* as previously reported [40, 41]. Successfully immortalized cells (named immortalized FTSEC) have a typical epithelial morphology with the expression of secretory cell markers (Fig. 4.4). We never selected secretory cells during epithelial isolation, but the immortalized cells were eventually composed of secretory cells, meaning that secretory cells may have a major growth advantage over ciliated cells during the process of immortalization.

Based on previous findings that *p53* mutations are extremely frequent in STIC and HGSC, we sought to mimic them by introducing a dominant-negative form of p53 (named DN-p53) that can efficiently inactivate normal p53 function into immortalized FTSEC (FTSEC-p53) [32]. No phenotypic changes were confirmed by the introduction of DN-p53, and these cells lack the ability to form tumors in immunodeficient mice (Table 4.1). We next sought to target RAS/MAPK and/or PI3K/AKT pathways based on findings of the TCGA [4]. Oncogenic mutant *KRAS* alleles or constitutively activated AKT (CA-AKT) were introduced into immortalized FTSEC-p53 to generate FTSEC-p53/KRAS or FTSEC-p53/AKT. Although the colony-forming ability on soft agar partly increased, these genetic manipulations did not lead to tumorigenicity in mice [32]. We then searched for a third hit as a candidate driver for mouse tumorigenicity and noticed that c-Myc amplification is frequently observed in ovarian HGSC in the TCGA [4]. We thus overexpressed c-Myc in FTSEC-p53/KRAS (FTSEC-p53/KRAS/Myc) and found that these cells efficiently formed tumors in mice [32] (Table 4.1). We further experimented with combinatorial introduction of oncogenic mutant *KRAS* alleles and constitutively

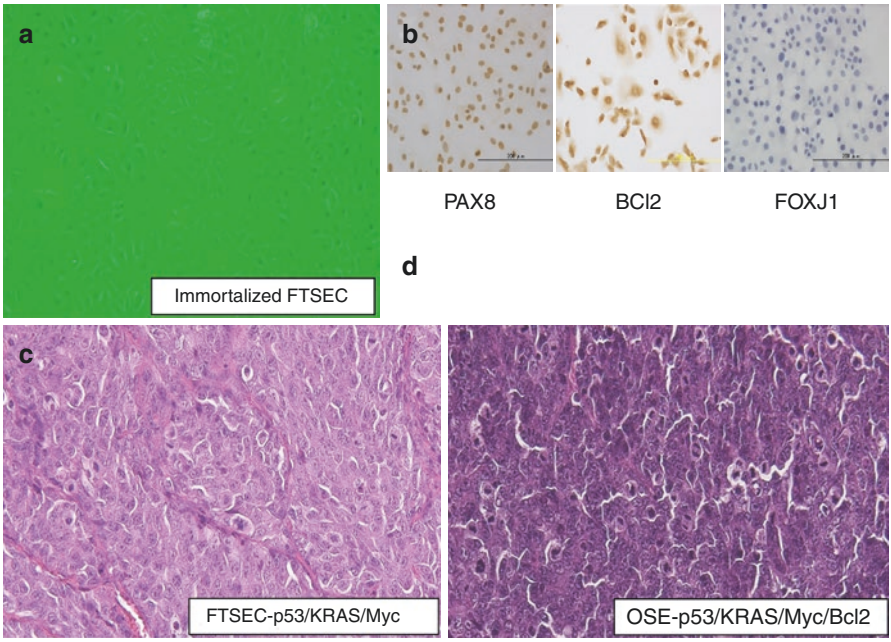

Fig. 4.4 Morphological features of immortalized and transformed fallopian tube secretory cells. (**a**) Fallopian tube secretory cells (FTSECs) immortalized with combinatorial overexpression of CyclinD1, cdk4, and hTERT exhibit round shapes with tight cell–cell connections, typical of epithelial cells. (**b**) Immunocytochemical analysis of immortalized FASECs, which are positive for PAX8 and Bcl2 expressions and negative for FOXJ1 expression. These expression patterns are typical for secretory cells, not ciliated cells. (**c**, **d**) Pathological features of subcutaneous mouse tumors formed with genetically engineered FASECs (**c**) or ovarian surface epithelial cells (OSEs) (**d**). Combinatorial overexpression of a dominant-negative form of p53 (DN-p53), oncogenic *KRAS* mutant allele, and c-Myc in immortalized FASECs successfully formed tumors in immunocompromised mice, while additional overexpression of Bcl2 was necessary for OSEs to form tumors. The morphologies of both tumors were distinct; the tumors derived from FASECs exhibited typical morphological features of high-grade serous carcinoma, with solid nests composed of round cells with a high nuclear/cytoplasmic ratio, cellular atypia, and tight cell–cell attachment (**c**). In contrast, tumors derived from OSEs showed morphologies reminiscent of undifferentiated carcinoma, with extremely high nuclear atypia with loose cell–cell attachment (**d**) (Adapted from [32])

activated AKT to simultaneously activate both RAS/MAPK and PI3K/AKT pathways (FTSEC-p53/KRAS/AKT). These cells also exhibited mouse tumorigenicity [32]. Mouse tumors were collected and subjected to pathological analyses. These tumors exhibited solid foci with nuclear atypia and tight cell–cell connections, typical characteristics of HGCS, clearly distinct from undifferentiated or sarcomatous tumors (Fig. 4.4). We thus concluded that a combination of oncogenic *KRAS* and c-Myc overexpression or AKT activation based on *p53* inactivation is a minimal requirement of the carcinogenesis of HGCS.

4.4.4 *In Vitro Carcinogenesis Model with Ovarian Surface Epithelial Cells*

Sasaki et al. previously established an in vitro carcinogenesis model using immortalized ovarian surface epithelial cells (OSEs) [41]. Several sets of genetic elements were introduced into immortalized OSEs, but any combinations including the three elements used for FASECs failed to create mouse tumors [41]. Bcl2 overexpression in addition to the above three elements was required to create mouse tumors (OSE-p53/KRAS/Myc/Bcl2) [41]. Using OSE-p53/KRAS/Myc/Bcl2 cells, we induced mouse tumors and confirmed their histology. The mouse tumors revealed typical features of undifferentiated carcinoma, not HGSC, representing excessive nuclear atypia and loose cell–cell attachment (Fig. 4.4). These findings are consistent with the idea that HGSC is not likely to arise from OSEs. Bcl2 is a protein that blocks apoptotic cell death [45]. Overexpression of Bcl2 is therefore presumed to contribute to tumorigenic phenotypes. It is notable that Bcl2 is constitutively expressed in fallopian tube secretory cells, not OSEs, and is used as a marker for secretory cells [28]. We speculate that fallopian tube secretory cells are more prone to carcinogenic development, compared to ciliated cells or OSE, via constitutive expression of Bcl2.

An advantage of our study is the use of human epithelial cells, not mouse cells, with isolated fallopian tube secretory cells, which is highly analogous to human HGCS carcinogenesis. Our study showed that three genetic hits are both necessary and sufficient for carcinogenesis. As shown in the TCGA, a number of frequently aberrant factors are involved in carcinogenesis, and the three factors that we detected are not the only ones. A variety of genetic elements can be tested in our system, being introduced into immortalized FTSECs, and other sets of potential gene mutations should be incorporated to identify novel sets of drivers.

4.5 Tumor Microenvironment of the Fallopian Tube

The fallopian tube is constantly exposed to blood during retrograde menstruation, which contains extracellular ferric ions tightly bound to an iron transporter protein, transferrin (Fig. 4.5). Transferrin is imported into cells via its receptor (transferrin receptor; TfR) and releases free ferric ions in the cytoplasm. While released free iron is utilized for the synthesis of heme and is sequestered in the form of ferritin, it catalyzes a Fenton reaction in the presence of hydrogen peroxide (H_2O_2) to generate hydroxyl radicals, a type of reactive oxygen species (ROS) [46–48]. Therefore, fallopian tube epithelial cells are exposed to an ROS-rich environment.

Besides exposure to menstrual blood, fallopian tube epithelial cells are in contact with the ovary during ovulation and are exposed to follicular fluid at that time. Human follicular fluids contain excess amounts of transferrin as well as H_2O_2,

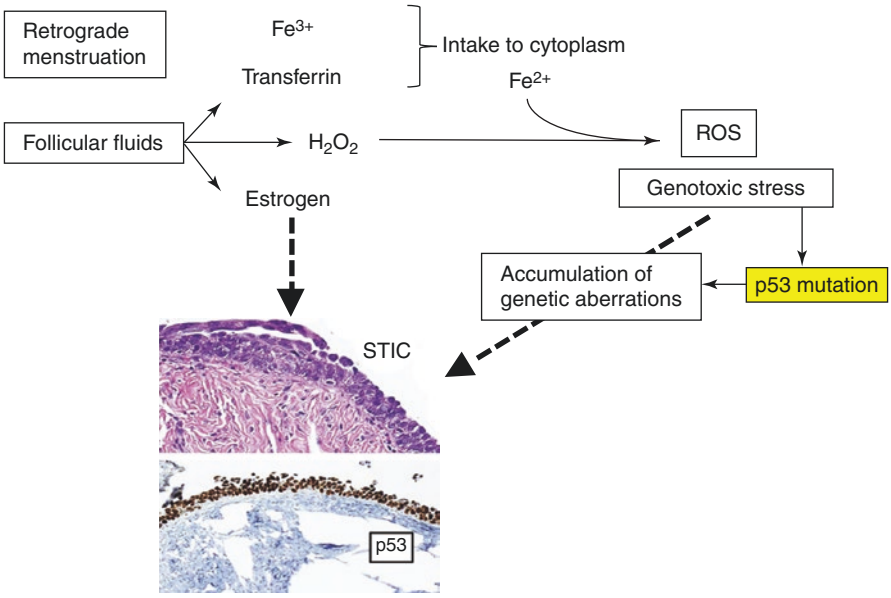

Fig. 4.5 The microenvironment of fallopian tubes may contribute to high-grade serous carcinogenesis. Fallopian tube secretory cells are exposed to the blood of retrograde menstruation containing extracellular ferric ions (Fe^{3+}) tightly bound to transferrin, which is imported into cells via its receptor, resulting in the release of free ferric ions (Fe^{2+}) via oxidative reduction in the cytoplasm. While released free iron is utilized for the synthesis of heme and is sequestered in the form of ferritin, it catalyzes a Fenton reaction in the presence of hydrogen peroxide (H_2O_2) contained in follicular fluids to generate hydroxyl radicals, a type of reactive oxygen species (ROS) that triggers genotoxic stress, causing a variety of genetic aberrations, including *p53* mutations. The mutation of *p53* further induces the accumulation of genetic abnormalities, generating STIC with p53 overexpression. In addition, various growth factors such as estrogen, enriched in follicular fluids, may stimulate the growth of fallopian tube epithelial cells at the time of ovulation, contributing to the development of STIC

adding to the ROS-rich environment of fallopian tube epithelial cells [49]. Experimental models have shown that treatment of fallopian tube secretory cells with transferrin induces the formation of γH2AX, a marker of DNA-DSBs, while knockdown of TfR by siRNA decreases transferrin-induced ROS [49]. Furthermore, TfR-dependent uptake of transferrin enhances the formation of DNA-DSBs in the presence of H_2O_2 [49]. These findings indicate that exposure to blood from retrograde menstruation and follicular fluid may cause increased DNA-DSBs in fallopian tube epithelial cells, consistent with the frequent γH2AX staining in the p53 signature or STIC, presumably leading to genomic instability and triggering carcinogenesis. In addition, it is known that follicular fluid contains high amounts of estrogen and other growth factors, which must function to promote carcinogenesis [50] (Fig. 4.5).

4.6 Do All HGSCs Arise from Fallopian Tubes?

One epidemiological study examined the effect of salpingectomy on reducing ovarian cancer risk using data on women who underwent surgery for benign diseases and compared them with an unoperated population [51]. As expected, a statistically significant decrease in risk for ovarian cancer was observed in women with previous bilateral salpingectomy (hazard ratio = 0.35, 95% confidence interval = 0.17 to 0.73, p = 0.0042) compared with the unoperated population. However, we have experienced a case of HGSC arising 3 years after bilateral salpingectomy [52]. A 51-year-old postmenopausal woman underwent an operation for uterine myoma (hysterectomy along with left oophorectomy and bilateral salpingectomy). Three years later, the patient was diagnosed as having ovarian cancer at stage IV and underwent primary debulking surgery. The pathological evaluation was HGSC of the right ovary, lacking evidence of STIC or any other cancerous lesions in the previously resected left ovary and bilateral fallopian tubes. Thus, we presumed that this case of HGSC developed from the right ovary.

A recent study using a genetically engineered mouse model assessed the tumor-forming properties of fallopian tubes and OSEs [53]. Combined inactivation of the RB family and p53 in fallopian tubes generated STIC that rapidly metastasized to the ovary, and engineering the same genetic elements into OSEs led to HGSC that had metastatic potential, despite low penetrance with longer latency [53]. RNA sequencing analysis (RNAseq) showed differences in the transcriptomes between fallopian tube- and OSE-derived HGSCs, indicating that differences in the cell-of-origin may contribute to distinct transcriptome patterns [53]. In contrast, both types of HGSC showed multiple differentially regulated genes compared to their respective cells of origin [53], which were supposed to be essential for the carcinogenesis of HGSC. These findings suggest that not only fallopian tubes but also OSEs are capable of developing HGSC with differentially regulated genes and with different efficacies and latencies.

4.7 Concluding Remarks

Our in vitro carcinogenesis model has found that three genetic hits into FTSECs, not OSEs, can induce mouse tumors with the typical morphological characteristics of HGSC, which is consistent with the three genetic hits theory for human carcinogenesis [54]. In addition, various genetic sets detected by the TCAG can be tested in our system, although p53 inactivation must be indispensable for the carcinogenesis of HGSC.

A recent NGS study showed that representative driver genes, including *p53*, *BRCA1*, 5*BRCA2*, or C*PTEN*, are commonly mutated both in STIC and HGSC, suggesting that STIC might be in the final stage of carcinogenesis as a daughter clone of HGSC [29]. Nevertheless, most HGSCs develop in the ovaries rather than

fallopian tubes. Different tumor microenvironments between both organs may promote tumorigenicity as HGSC. A recent mouse carcinogenesis model based on the combined inactivation of RB and p53 indicated that not only fallopian tubes but OSEs can form HGSC with different transcriptomes, suggesting that both organs can be the cell-of-origin but with distinct mechanisms.

Not many researchers in the field of gynecologic oncology have focused on fallopian tubes because tubal cancer has been a rare disease. Recent progress using NGS has uncovered a variety of molecular aberrations in tubal precursors, mainly caused by specific genotoxic environments. Therefore, the fallopian tube is now recognized as an important organ for both clinical and basic research. The use of organoids with improvements in gene editing technologies for the development of HGCS models is urgently required to more precisely understand carcinogenesis as well as to exploit novel therapeutic modalities.

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Conflict of Interest The author declares no conflict of interest.

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Chapter 5
The Role of G Protein-Coupled Receptor Signaling in Gynecologic Malignancy

Hiroshi Yagi and Kiyoko Kato

Abstract G protein-coupled receptors (GPCRs) are seven transmembrane receptors that represent the largest family of cell surface receptors. Ligand binding induces conformational changes in GPCRs, which lead to the activation of their associated heterotrimeric G proteins. GPCR signaling regulates diverse biological functions, including cell proliferation, migration, and angiogenesis. Cancer cells can co-opt the activity of GPCR signaling to proliferate autonomously, evade immune detection, increase their nutrient and oxygen supply, invade their surrounding tissues, and metastasize to other organs. Dysregulation of GPCR signaling, induced by elevated expression of GPCRs, G proteins, or their ligands as well as activating mutations of these genes, contributes to the progression of various human cancers. Although GPCRs are associated with cancer progression and represent one of the most druggable molecules, there are relatively few cancer treatments targeting these receptors. Therefore, by better understanding the molecular mechanisms underlying GPCR function in cancer, we can identify novel strategies for cancer diagnosis, prevention, and treatment. We present here our current understanding of many roles of GPCR signaling in the progression of gynecologic malignancy and the potential benefits of targeting GPCRs and signaling circuits in cancer treatment.

Keywords GPCR · G protein · Mutation · Inflammation · Angiogenesis Metastasis · LPA

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5.1 Introduction

G protein-coupled receptors (GPCRs) are seven transmembrane receptors that form the largest family among cell surface molecules. Currently, more than 800 GPCRs have been identified, accounting for >2% of the total genes encoded by the human genome [1, 2]. GPCRs regulate key physiological functions, including neurotransmission, hormone and enzyme release from endocrine and exocrine glands, immune responses, smooth muscle contraction, and blood pressure regulation. Dysfunction of GPCRs contributes to various human diseases including cancers. Cancer cells can hijack the normal physiological functions of GPCRs to proliferate autonomously, evade immune detection, increase their nutrient and oxygen supply, invade their surrounding tissues, and metastasize to other organs. Dysregulation of GPCR signaling, induced by elevated expression of GPCRs, G proteins, or their ligands as well as activating mutations of these genes, contributes to the progression of various human cancers [3]. Although GPCRs are associated with cancer progression and represent one of the most druggable molecules, representing approximately 35% of all therapeutics on the market, there are relatively few cancer treatments targeting these receptors [4, 5]. Therefore, by better understanding the molecular mechanisms underlying GPCR function in cancer, we can identify novel strategies for cancer diagnosis, prevention, and treatment.

5.2 G Protein and GPCR Signaling

Ligand binding on the extracellular side of the receptor induces a conformational change to GPCRs and alters the position of its transmembrane helices and intracellular loops [6–8]. This active form of GPCR couples to heterotrimeric G proteins, which promote the release of GDP from the Gα subunit, followed by loading of GTP and dissociation from Gβγ and the receptor. GTP-bound Gα and Gβγ stimulate their cognate effectors (Fig. 5.1). Both GTP-bound Gα and Gβγ subunit complexes stimulate multiple downstream signaling cascades, including the rapid generation of multiple second messengers. Conversely, regulators of G protein signaling (RGS) regulate GTPase activity of the Gα subunit to switch off signaling, leading to reassociation of GDP-bound Gα with Gβγ [2, 7].

The α subunits of G proteins are divided into four subfamilies, comprising $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$, and a single GPCR can couple to either one or more families of Gα proteins. Each G protein activates several downstream effectors. Typically, $G\alpha_s$ stimulates adenylyl cyclase and increases levels of cyclic AMP (cAMP) [9, 10], whereas $G\alpha_i$ inhibits adenylyl cyclase and decreases cAMP levels. $G\alpha_q$ binds to and activates phospholipase C (PLC), which cleaves phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol and inositol triphosphate (IP_3) [11, 12]. Gβγ subunits function as dimers to activate many signaling molecules, including phospholipases, ion channels, and lipid kinases [13, 14]. Besides the regulation of these classical second-messenger generation systems, Gβγ and Gα subunits such as $G\alpha_{12/13}$ and $G\alpha_q$ can also control the activity of small GTP-binding proteins of the Ras and Rho

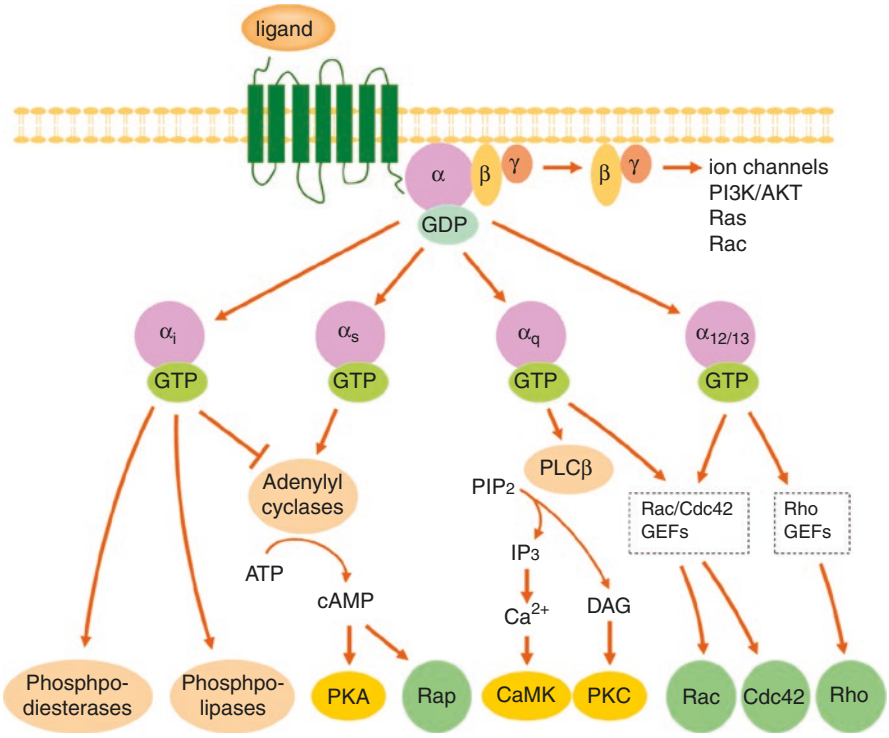

Fig. 5.1 G protein-coupled receptor signaling

families and members of the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), p38, and ERK5. Ultimately, the nature of downstream signaling by GPCRs is determined by its G protein-coupling specificity and the cellular environment, including the levels of the ligand and each G protein [9].

The activity of GPCR signaling contributes to various physiological conditions, including embryogenesis, tissue remodeling and repair, inflammation, angiogenesis, and normal cell growth. Indeed, cancer cells can co-opt the activity of GPCR signaling. Many cancers exhibit aberrant overexpression of GPCRs and G proteins, and various GPCR ligands are enriched in the tumor microenvironment, leading to enhanced or prolonged signaling, or both, and changes in the coupling specificity of GPCRs, thereby influencing cancer initiation and progression.

5.3 The Role of Lysophosphatidic Acid (LPA) in Ovarian Cancer Progression

Ovarian cancer, the most lethal of all gynecological malignancies, is characterized by a unique tumor microenvironment that enables specific and efficient metastatic mechanisms and mediates therapy resistance. The presence of ascites enables more

efficient tumor–stromal cell interactions and the transcoelomic spread of tumor cells to other pelvic and peritoneal organs. LPA is one of the most potent mitogens secreted to ascites by ovarian cancer cells and promotes growth, survival, and resistance to chemotherapy by stimulating LPA receptors (LPARs), which are frequently overexpressed in these cancer cells [15]. The LPAR family comprises $LPAR_1$–$LPAR_6$, which are coupled to distinct subsets of G proteins including $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$. Among them, $LPAR_{1–3}$ belong to the endothelial differentiation gene (Edg) family of GPCRs, whereas $LPAR_{4–6}$ belong to the purinergic P2Y family of GPCRs. In most cases, $LPAR_{1–3}$ promote tumor progression [16]. The role of $LPAR_{4–6}$ is less well understood when compared with that of $LPAR_{1–3}$ [17, 18]. Responses to LPA stimulation are determined mainly by the expression pattern of LPARs and their downstream signaling pathways in a given cell type. By acting on its receptors, LPA stimulates further LPA release and therefore establishing an autocrine loop that drives the uncontrolled growth of ovarian cancer cells. The activation of LPA receptors also increases the secretion of GROα, which is highly elevated in the plasma and ascites of ovarian cancer patients and contributes to the growth of tumor cells and their vascularization [19].

5.4 GPCR Signaling Links Inflammation to Cancer

Inflammation is often associated with the development and progression of cancer. Indeed, chronic inflammatory disease increases the risk of some cancers, and strong epidemiological evidence exists that nonsteroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, are powerful chemopreventive agents. Tumor microenvironments contain many different inflammatory cells and mediators. Among them, prostaglandins are a product of the cyclooxygenases COX1 and COX2, and their pro-inflammatory functions are initiated after binding to their cognate GPCRs that are expressed in many cells [20]. Treatment with NSAIDs that inhibit COX1 and COX2 can reduce the risk and incidence of various human cancers [21]. Among the COX2-derived prostaglandins, the contribution of PGE2 and its cognate GPCRs, EP1–EP4, to colon cancer progression has been reported in several studies [22].

EP1 is a $G\alpha_q$-coupled receptor that promotes calcium mobilization and PKC activation, whereas EP2 and EP4, which have a more prominent role in colon cancer, are coupled to $G\alpha_s$ and stimulate cAMP accumulation [23]. EP3 is coupled mainly to $G\alpha_i$ to suppress cAMP. COX2 overexpression and the activation of EP2 and EP4 by PGE2 released from tumor and stromal cells contribute to the aberrant growth, angiogenesis, and metastatic potential of various human cancers including colon, ovarian, and endometrial cancers [22, 24]. In gynecologic cancers, COX2 has been reported to be a negative predictor, contributing to carcinogenesis and progression. COX2 is expressed highly in human ovarian cancer tissues and promotes the proliferation and invasion of ovarian cancer cells. Furthermore, COX2 and its downstream gene PGE2 regulate the metastatic spread of ovarian cancer through the

regulation of metalloproteases (MMP2 and MMP9) and NF-κB. As for EPs, many studies focus on the function of the cAMP-linked EP2/EP4 signaling pathway, while the roles of EP1 and EP3 have not been fully clarified. Many clinical trials are currently being conducted to test the effect of COX2 inhibition in cancer prevention and as adjuvant therapy for early and advanced cancers [22]. Because of the potential cardiovascular complications of COX2 inhibitors, the direct inhibition of G protein-linked PGE2 receptors may serve as an alternative to COX2 inhibition as a means for cancer prevention and treatment.

5.5 The Key Role of GPCRs in Cancer Immunology

Numerous chemokines and their GPCRs have been implicated in intercellular communication between tumor cells and multiple immune cells. Among these chemokines, the role of CCL2 has been studied extensively for the recruitment of CCR2-bearing tumor-associated macrophages (TAMs), which play crucial roles in tumor vascularization and growth [25]. CCL5 has similarly been linked to macrophage recruitment. In contrast to macrophages, some immune cells can facilitate the killing of tumor cells. In this case, the tumor chemokine microenvironment may help evade the immune surveillance system, for example, by stimulating a less effective humoral response while inhibiting cell-mediated immune responses to tumor cells.

In the past few years, cancer immunotherapy has become one of the most exciting breakthroughs in cancer treatment. Recent revolutionary discoveries have highlighted the importance of the tumor microenvironment and its associated immune cells in cancer development and therapeutic resistance. Tumors can deploy multiple mechanisms to avoid immune recognition and an antitumor immune response, including the recruitment of myeloid-derived suppressor cells (MDSC) and conditioning of the surrounding microenvironment to become highly immune-suppressive by cytokines, such as IL-6, IL-10, and transforming growth factor β (TGF-β) [26]. This can lead to the accumulation of suppressive regulatory T cells (Tregs) and the polarization of macrophages toward an immune-suppressive phenotype, which is often referred to as the M2 or TAM phenotype [25]. A key emerging mechanism of tumor immunosuppression involves the induction of T cell exhaustion through activation of T cell checkpoints, including programmed death 1 (PD-1). The ligand to PD-1, programmed death-ligand 1 (PD-L1), is expressed by macrophages and some cancer cells, which can restrain T cell activation and induce immunosuppression [27]. Together, these conditions contribute to the suppression of cytotoxic CD8+ T lymphocyte recruitment, survival, and function, and ultimately to the loss of an effective antitumor immune response. Although the aberrant function and dysregulated expression of GPCRs are now beginning to be linked directly to tumors, the role of GPCRs on immune cells infiltrating tumors is not fully understood and grossly underappreciated. Given the diversity of GPCRs, current studies have only scratched the surface of delineating GPCRs on immune cells in cancer. The

importance of studying GPCRs in the context of cancer immunology is reflected by the multiple roles that this receptor family plays in inflammation, orchestrating immune cell trafficking, and regulating the tumor microenvironment. A crucial first step in antitumor immunity is the migration of cytotoxic cells recognizing tumor antigens to the tumor and this is mediated largely by chemokine receptors.

5.6 The Role for GPCRs in Cancer Metastasis

Many cancers metastasize to specific organs with an incidence much greater than would be expected from the circulatory pattern between the primary tumor and the secondary organs. This organ-specific metastasis is often caused by the aberrant expression of G protein-linked chemokine receptors in cancer cells concomitant with the release of chemokines from the secondary organs. Tumor cells express many chemokine receptors, which are activated by chemokines released into the tumor microenvironment by stromal cells, macrophages, tumor-infiltrating leukocytes, and even by cancer cells, thereby increasing the motility and survival of cancer cells in an autocrine and paracrine fashion [28]. Tumor cells ultimately gain, and for which they are selected, the ability to co-opt the potent pro-migratory activity of chemokines and their GPCRs to metastasize to regional and distant organs.

Among chemokine receptors, CXCR4 represents one of the best-established chemokine receptors driving cancer metastasis. Tumor cells frequently exhibit aberrant CXCR4 expression, which has proliferative, pro-survival, and pro-migratory effects, whereas the organs that are most frequent sites of metastasis, including lymph nodes, lungs, bone marrow, and liver, express its chemokine ligand, CXCL12/SDF-1 [29]. Although the role of the SDF-1-CXCR4 signaling axis has been reported in various human cancers and would represent an attractive target for therapeutic development, the use of CXCR4 inhibitors leads to the mobilization of stem cell progenitors from the bone marrow, thus limiting their use clinically for cancer treatment. However, targeting molecules involved in the regulation of CXCR4 expression on cancer cells or their downstream signaling may offer alternative approaches for therapeutic intervention. In this regard, CXCR4 activates Rac1 through P-REX1, which plays a central role in metastasis in most breast cancer types [30]. CXCR4 also couples to $G\alpha_{12/13}$ in basal-like breast cancer cells, where $G\alpha_{13}$ protein expression is highly upregulated, thus driving metastasis in a $G\alpha_{12/13}$-RhoA dependent manner, which can be considered potential targets for metastasis prevention and treatment (Fig. 5.2) [31].

SDF-1-CXCR4 signaling plays a role in the progression of gynecologic cancers including ovarian and endometrial cancers. Recently the contribution of CXCR4 to the progression of ovarian cancer through enhancement of tumor angiogenesis and an immunosuppressive network that regulates peritoneal dissemination has been demonstrated [32]. Several CXCR4 antagonists showed antitumor efficacy in ovarian cancer preclinical models. Additionally, upregulation of CXCR4 in human endometrial cancer tissues, as compared to endometrial hyperplasia or normal

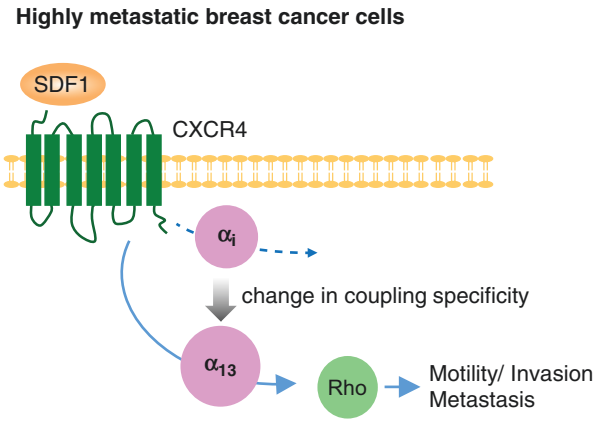

Fig. 5.2 The role of $G\alpha_{13}$ in breast cancer metastasis

endometrium, has been reported. Several in vitro and in vivo experimental models revealed enhanced migratory potential in a manner dependent on the SDF-1-CXCR4 signaling axis.

5.7 GPCRs in Tumor-Induced Angiogenesis

Solid tumors produce angiogenic factors promoting the migration and proliferation of endothelial cells, thus resulting in the formation of new vessels in response to the increasing nutrients and oxygen demands of tumor cells. Many angiogenic factors act on GPCRs expressed on endothelial cells, including thrombin, prostaglandins, SIP, and chemokines. Many chemokines, including CCL2, CCL5, and CXCL8/IL-8, recruit leukocytes and macrophages to the tumor site, which in turn can release VEGF and other angiogenic factors that contribute to the growth of new blood vessels. Furthermore, inflammatory cytokines released in the tumor microenvironment promote the expression of COX2 and the local release of PGE2, which increases the expression of VEGF, CXCL8, and CXCL5 by tumor and stromal cells [33]. Considering the vast and established evidence on the efficacy of VEGF inhibition in various human cancers including gynecologic cancers, understanding GPCR-regulated angiogenesis may provide a molecular framework for the development of novel approaches for therapeutic intervention.

5.8 Mutant GPCRs in Cancer

Large-scale genome sequencing analyses through multiple omics platforms of various cancer types revealed that GPCRs are mutated in approximately 20% of all cancers. Among all cancer cohorts, cancers arising in the gastrointestinal (GI) tract, including colon adenocarcinoma, stomach adenocarcinoma, and pancreatic

adenocarcinoma display the highest number of significantly mutated GPCRs and G proteins [34]. Additionally, mutations in GPCRs are more evident in metastatic sites rather than in the primary tumor of melanomas, or lung, prostate, large intestine, and pancreatic tumors. Despite the high frequency of mutations in GPCRs, most GPCRs do not harbor hotspot mutations. Interestingly, recently developed new bioinformatics approaches analyzing GPCR mutations considering three-dimensional structures and interaction partners revealed "hotspot structural motifs," including the DRY arginine motif, which is responsible for the intramolecular polar contacts that keep the receptor inactive until ligand binding, and ligand and G protein-binding sites [35]. However, contributions from GPCRs mutations to the initiation and progression of gynecologic cancers have not been clarified [34].

Surprisingly, mutational analysis of GPCRs in human cancers revealed high-frequency mutations in the coding sequence of members of the adhesion family of GPCRs, which remain poorly understood. A long N-terminal region that may have a role in cell–cell and cell–matrix interactions characterizes this family of GPCRs. Among them, GPR98 is the most frequently mutated GPCR across all cancer types. The physiological function and the ligand of GPR98 are poorly understood. GPR98 mutations cause febrile seizures and one form of Usher syndrome, which is characterized by combined blindness and deafness [36]. Although GPR98 mutations are associated with glioblastoma and lymphoblastic leukemia, the phenotypic and biological outcome of these mutations remains largely unknown, and thus these findings provide important information for the development of hypothesis-driven approaches to investigate their cancer relevance [37, 38].

5.9 The Emerging Role of Mutations in G Proteins in the Progression of Cancer

Although the contribution of mutant GPCRs to the initiation and progression of various cancers is still under investigation, the recent discovery of hot spot mutations in G proteins as oncogenic drivers in several cancers has accelerated research in this field. Among them, GNAS is the most frequently mutated G protein in human cancers. Recent analysis revealed that mutations in GNAS occur in various types of tumors, including growth-hormone-secreting pituitary tumors (28%), pancreatic tumors (12%), thyroid adenomas (5%), ovarian cancers (3%), and endometrial cancers (2%) [3, 39]. GNAS has been linked to pro-inflammatory functions because GNAS mediates the effect of inflammatory mediators such as cyclooxygenase 2 (COX2)-derived prostaglandin E2 (PGE2). The gain-of-function mutations in GNAS may induce pro-inflammatory gene expression, thus mimicking chronic inflammation leading to tumor development.

Mutations in GNAQ and GNA11 are considered to represent the driver oncogene of uveal melanoma, as 93% of patients harbor mutations in these genes encoding constitutively active $G\alpha_q$ family members [40, 41]. All cancer mutations in $G\alpha_q$ and $G\alpha_{11}$ occur at either glutamine 209 (Gln-209) or in arginine 183 (Arg-183). Mutated

residues impair GTPase activity, leading to prolonged signaling. A recent study revealed that activation of YAP regulated by the $G\alpha_q$-Rho signaling axis is essential for the uveal melanoma, thus identifying a druggable target downstream from mutated $G\alpha_q$ [42].

Mutations in GNA13 have been found at high frequency in bladder cancer and lymphomas, specifically Burkitt's lymphoma and diffuse large B cell lymphoma (DLBCL) [43–45]. In these lymphoma cases, mutations in GNA13 have been shown to be inhibitory, suggesting a tumor suppressor role for $G\alpha_{13}$; although, wild-type GNA13 overexpression has been implicated in many solid tumors, such as in gastric cancer, head and neck cancer, breast cancer, prostate cancer, and ovarian cancer [46–50].

Mutations in other Gα genes, GNAI1, GNAI2, GNAI3, GNAO1, GNAT1, GNAT2, GNA12, GNA14, GNA15, and GNAL have been found in cancer at much lower frequencies. In many cases, a detailed analysis of the relevance of these mutations to the progression of cancers is not possible because of the limited availability of sequencing data for these genes. Although the presence of activating hotspot mutations in GNAS, GNAQ, and GNA11 in cancer is established, further experiments are required to determine the oncogenic relevance of the less-frequently mutated G proteins.

5.10 Gene Copy Number Alterations and Expression of G Protein and GPCR in Cancer

In addition to mutations, gene copy number alterations of G proteins and GPCRs have been detected in human cancers (Table 5.1). Among G proteins, copy number gain of GNA12 is remarkably significant in ovarian cancer (Table 5.1) [34]. Ovarian cancer is characterized by few driver mutations and by the accumulation of a high concentration of LPA in ascites, which may work through $G\alpha_{12}$ to promote growth

Table 5.1 Most significant copy number variations (CNV) of G proteins (red) and GPCRs (black)

TCGA cohort	Genes encoding GPCRs and G proteins				
OV (579)	CCR1	DRD4	F2RL1	GPR146	GPR35
	GNA12				
UCS (56)	ADORA2B	CHRM5	LPAR6	FZD3	
	GNB1				
UCEC (539)	ADORA2B				
CESC (295)	GPR56	LGR4			
	GNB1	GNB5			

OV: ovarian serous carcinoma, UCS: uterine carcinosarcoma, UCES: uterine corpus endometrioid carcinoma, CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma

and metastasis [51]. As for GPCRs, F2RL1, the gene encoding protease-activated receptor (PAR) 2, was the most significantly altered gene in ovarian cancer. PAR2 has been linked to the migration of cancer cells and VEGF production by cancer cells [34, 52, 53].

$G\alpha_{12}$ and $G\alpha_{13}$, encoded by GNA12 and GNA13, respectively, are the GEP oncogene and implicated in tumor progression. The GEP oncogene is highly expressed in various human cancers including breast, oral, prostate, and ovarian cancers [49, 54, 55]. $G\alpha_{12/13}$ activates Rho by directly binding Rho guanine nucleotide exchange factor (Rho-GEF), and the $G\alpha_{12/13}$ signaling axis has a critical role in cancer progression. Rho activation promotes migration and invasion of cancer cells, leading to metastatic spread to distant organs, through the regulation of the actin cytoskeleton. Furthermore, recent studies revealed the critical role of the Hippo signaling pathway downstream of the $G\alpha_{12/13}$-Rho signaling axis [56]. The Hippo signaling pathway regulates organ size during development and regeneration [57]. The yes-associated protein (YAP), the core component of this pathway, functions as a transcriptional coactivator. The phosphorylation status of YAP is regulated by various upstream influences, including cell–cell contact, organ size sensing machinery, and other signaling pathways regulated by WNT, transforming growth factor-beta (TGF-β), and several GPCRs, especially $G\alpha_{12/13}$-linked GPCRs [58]. Phosphorylation of YAP at serine 127 represses its activity through the creation of a 14-3-3 binding site, which promotes cytoplasmic accumulation and ubiquitin-mediated proteolysis. In ovarian cancer cells, elevated expression of $G\alpha_{12/13}$ promotes cell proliferation and epithelial–mesenchymal transition through the regulation of the Hippo signaling pathway (Fig. 5.3) [49, 50]. Considering the critical role of aberrant expression of many wild-type G proteins and GPCRs in cancer even if not mutated,

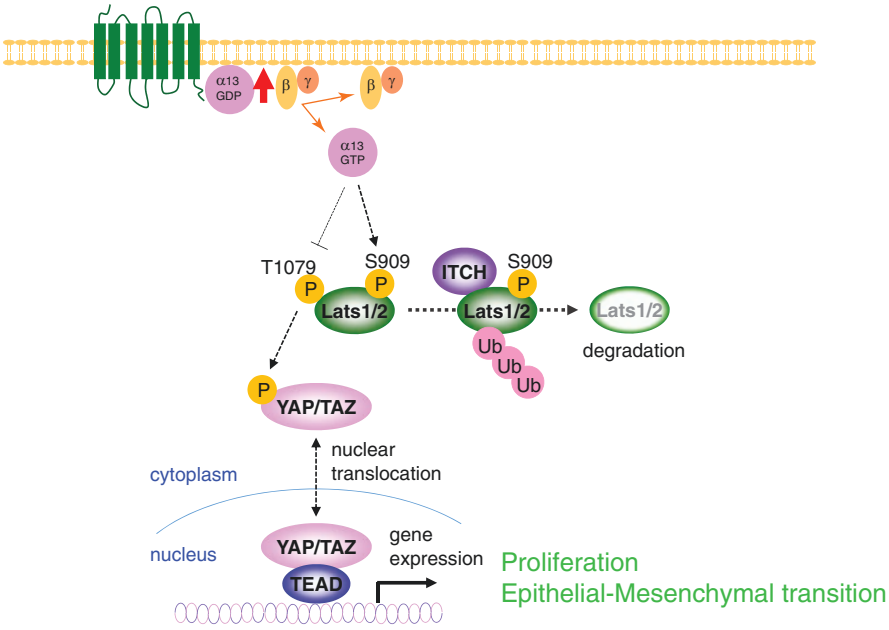

Fig. 5.3 Regulation of Hippo pathway by $G\alpha_{13}$ in ovarian cancer

determining the contribution of such alteration in cancer initiation and progression may be critical both for the discovery of driver oncogenic processes and for the development of novel targeted therapeutics.

5.11 Tumor Suppressor Functions of GPCRs

In certain malignancies, some GPCRs and G proteins may actually play tumor-suppressive roles and mutations may reflect inactivation of the respective genes. For example, inactivating mutations in the melanocortin 1 receptor (MC1R), which is important for pigment production, increase the risk of melanoma development [59]. CXCR3 ligands can indirectly mediate antiangiogenic effects to suppress tumor progression, whereas the cannabinoid receptors CB1 and CB2 display tumor-suppressive roles in several cancers, including gliomas and breast, colorectal, and skin cancer [60]. Additionally, the SIP2 receptor, which signals through $G\alpha_{13}$ in diffuse large B cell lymphoma (DLBCL), may exert tumor suppressor functions [61]. Although $G\alpha_{13}$ signaling has implications in tumor progression and metastasis, in the case of DLBCL, reduced expression or inactivating mutations in SIP2 and/or $G\alpha_{13}$ may instead enhance tumor progression. The GPR54/KiSS1-derived peptide receptor functions as a metastasis suppressor in melanoma and breast cancer cells [62]. Although GPR54 is coupled to $G\alpha_q$, the molecular basis of its antimetastatic signaling mechanisms remain unknown. These are certainly not the only GPCRs that may exhibit anti-tumorigenic effects in different cancers, and many, including the role in gynecologic cancers, are likely to be discovered in the future.

5.12 Conclusion

Activation of GPCRs elicits an array of signaling pathways including second messengers, GEFs, Ras and Rho GTPases, MAP kinases, PI3Ks, and their numerous downstream cytosolic and nuclear targets. These signaling pathways contribute to normal cell functions of growth, survival, differentiation, and migration. However, cancer cells exploit these pathways through aberrant expression and regulation of GPCRs/G proteins and their ligands to enhance tumor growth, promote angiogenesis, invade to surrounding tissues, metastasize to distant sites, and evade the immune system. Direct targeting of GPCRs or more selectively targeting of particular downstream signaling components provides many avenues for potential therapeutic development to treat cancer.

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Chapter 6
Tailor-Made Therapy According to Genetic Alteration in Epithelial Ovarian Cancers

Masashi Takano, Morikazu Miyamoto, and Tomoyuki Yoshikawa

Abstract Almost all primary epithelial ovarian cancers (EOC) respond to platinum-based chemotherapy; however, more than half of advanced cases develop recurrence. EOC has four major histologic subtypes: serous, endometrioid, clear cell, and mucinous tumors. Key genetic characteristics were determined according to these pathologic subtypes. High-grade serous carcinoma had four subtypes according to genetic profile. Clear cell and mucinous carcinomas had a lower response rate to platinum-based therapy. In the chapter, genetic analysis of drug sensitivity of EOC would be discussed for the clinical setting.

Keywords Ovarian cancer · Histology · Genetic alteration · Molecular profile Gene signature

6.1 Introduction

Tumors originating from the ovary are generally classified into three types: "superficial epithelial/interstitial tumor," "sex-cord interstitial tumor," and "germ cell tumor" [1]. The most common is "superficial epithelial/interstitial tumor," which is a lesion of cells covering the surface of the ovary, and is also called "epithelial ovarian tumor." This epithelial ovarian tumor accounts for 80–90% of all ovarian tumors. Moreover, ovarian tumors can be divided into three types" "benign tumors,"

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"malignant tumors," and "borderline malignancies." Eighty-five percent of ovarian tumors are benign and 15% are malignant. Epithelial ovarian cancer, which accounts for most of the malignant tumors, is roughly divided into four histological types: serous, mucinous, endometrioid, and clear cells. There are Grade 1, 2, and 3, according to the degree of differentiation, and Grade 1 has the highest degree of differentiation and the lowest degree of malignancy.

Specific genetic changes are observed for each of these histological types and degree of differentiation, and therapeutic methods targeting these changes are being implemented. In this chapter, we would like to consider these target gene abnormalities and candidate therapies for epithelial ovarian cancers.

6.2 Clinical Characteristics of Ovarian Cancers According to Histologic Subtypes

6.2.1 Serous Tumor

Serous tumor is the most common histological type of ovarian cancer and accounts for approximately 40% of all ovarian cancers in Japan [2]. Even with stage 1 cancer, one-third of cases have cancer cells in both ovaries. In addition, it is often already progressing when it is discovered, and it is said that 60–70% of cases have progressed to stage 3 to 4 at the time of initial surgery.

It is the fastest-growing cancer among epithelial ovarian cancer. Approximately, 30% occur in both ovaries. Lymph node metastasis is also frequently observed. Even in stage I, 30% has metastasized to the lymph nodes of the pelvis to paraaorta [3].

Chemotherapy is generally more sensitive, and the combination of surgery and chemotherapy helps improve prognosis (post-illness course). However, in terms of the 5-year survival rate, it is the histological type with the worst prognosis among ovarian cancers.

6.2.2 Endometrial Cancer

Endometrial carcinoma accounts for about 15% of all ovarian cancers, and nearly 30% of endometrial adenocarcinomas have cancer in both ovaries. It is common in the ages of 20s and 40s, but it can also be seen in the 60s. In addition, approximately all of endometrial carcinomas have endometriosis.

In fact, endometrial carcinoma may be found during the follow-up of endometriotic cysts. This type of tumor is characterized by developing from ovarian endometriosis (chocolate cyst).

It progresses relatively slowly and is considered to be the cancer with the best prognosis among ovarian cancers. It generally has a better prognosis than other histologic types of ovarian cancers [4]. Also, as with serous carcinoma, chemotherapy is relatively effective.

6.2.3 Clear Cell Cancer

Clear cell carcinoma has been on the rise in recent years, accounting for about 25% of all ovarian cancers in Japan [2]. The probability of having pathological endometriosis is as high as 50% or more, and it is often observed that clear cell carcinoma actually develops from endometriotic cysts.

Stage 1 cases account for 40–60% of all clear cell adenocarcinomas [5]. However, even in the same stage 1, the prognosis of clear cell carcinoma is slightly worse than that of other histological types of ovarian cancer. At this point, the effectiveness of chemotherapy is low, so the development of new drugs and treatments is awaited. Patients with clear cell carcinoma are at high risk of thrombosis and pulmonary infarction [6], and care must be taken not to develop these diseases.

6.2.4 Mucinous Cancer

Mucinous carcinoma is a cancerous and proliferating epithelial cell that produces mucus. It accounts for about 10% of all primary ovarian cancers. There are a few cases where both ovaries get cancer. It is common after menopause, but it can also be seen in people in their 20s and 30s. In addition, mucinous ovarian cancer generally has a better prognosis, because about half of mucinous adenocarcinomas are found in stage 1 cancer and there are many cases with low malignant potential (high histological differentiation). However, mucinous carcinoma that has spread outside the ovary may have a worse prognosis than serous adenocarcinoma [4]. Mucinous adenocarcinoma is often resistant to chemotherapy [7].

6.3 Genetic Alterations in Epithelial Ovarian Cancers According to Histologic Subtypes

In cancer cells, somatic mutations occur and accumulate at a rate significantly higher than in normal cells, a property referred to as “Mutator Phenotype.” Mutations in cancer cells cover a wide range of structural alterations in DNA, including changes in chromosomes copy numbers or chromosomal alterations [8].

Table 6.1 Characteristics of major histologic subtypes and targeted therapy in epithelial ovarian cancers

Histologic subtype	Endometrioid	Clear cell	Mucinous	Serous	
				Low-grade	High-grade
Dualistic classification	Type I				Type II
Mutations	PI3KCA PTEN MMR deficient	PI3KCA PTEN ARID1A	KRAS p53	KRAS BRAF ERBB2	p53 BRCA1/2
Intracellular signal Pathway involved	PI3K/AKT/mTOR		MEK/BRAF/KRAS		p53 DNA repair (double-strand)
Drugs for signal Pathway	mTOR inhibitor (Temsirolimus, Evelolimus, etc.)		MEK inhibitor (selumetinib, Trametinib), BRAF inhibitor (Dabrafenib)		PARP inhibitor (Olaparib, Niraparib)

PI3KCA phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *PTEN* phosphatase and tensin homolog deleted from chromosome 10, *MMR* mismatch repair, *ARID1A* AT-rich interactive domain 1A, *MEK* mitogen-activated protein kinase/Erk kinase, *BRAF* B-raf protein, *KRAS* K-ras oncogene, *mTOR* mammalian target of rapamycin, *PARP* poly(ADP-ribose) polymerase

Ovarian cancer has several specific genetic alterations according to histologic subtypes (Table 6.1).

6.3.1 Serous Tumor

Serous cancers are divided into two categories: high-grade serous carcinoma (HGSC), and low-grade serous carcinoma (LGSC), which have distinct biological characteristics. HGSC accounts for the overwhelming majority, and is often found as an advanced-staged cancer. Although the sensitivity to the chemotherapy is high, the frequency of recurrence is also high and the prognosis is poor. Both TP53 mutations and genomic instability are often found in HGSC [9]. The frequency of KRAS and BRAF mutations is low in HGSC, and germline or somatic BRCA1/2 mutations are found in about 20% of HGSC cases [10]. PARP inhibitors, such as Olaparib and Niraparib, are quite effective for HGSC in primary chemotherapy, in addition to second-line maintenance therapy for recurrent settings [11].

LGSC has a high frequency of bilateral occurrence and advanced cancer is not uncommon. Cases confined to the ovary have a good prognosis, but drugs for chemotherapy. Less susceptibility, disease-free survival was quite lower when residual

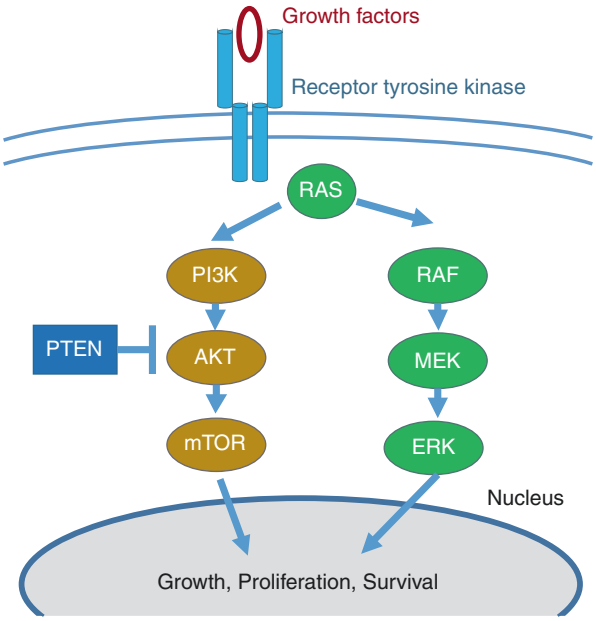

Fig. 6.1 PI3K/AKT/mTOR, and RAF/MEK/ERK pathways activated in ovarian cancers. PI3K/AKT/mTOR pathway is activated in endometrioid and clear cell carcinomas. mTOR inhibitors are candidates for those tumors. RAF/MEK/ERK pathway is activated in mucinous and low-grade serous carcinomas. MEK inhibitors and BRAF inhibitors are candidates for those tumors. *RAS* ras oncogene, *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase, *PTEN* phosphatase and tensin homolog deleted from chromosome 10, *AKT* protein kinase B, *mTOR* mammalian target of rapamycin, *RAF* Raf protein, *MEK* mitogen-activated protein kinase/Erk kinase, *ERK* extracellular signal-regulated kinase

tumor diameter exceeded 1 cm compared to those less than 1 cm. LGSC develops from serous borderline malignancies as precursor lesions. KRAS and BRAF mutations are frequently found, but TP53 mutations are not usually found in LGSC (Fig. 6.1). MEK inhibitors and BRAF inhibitors are candidates for the treatment of LGSC [12].

6.3.2 Endometrioid Carcinoma

Most endometrioid cancers are low grade and the cases with advanced stages are rare. It often develops from endometriosis. However, some cases are originated from progression from endometrioid adenofibromas. In addition to having PTEN, ARID1A, and PK3CA gene abnormalities, microsatellite instability is frequently observed (Fig. 6.1). Mammalian target of rapamycin (mTOR) inhibitors are candidates for endometrial cancers of the ovary [13].

6.3.3 *Clear Cell Carcinoma*

About half of clear cell carcinomas are diagnosed as stage I tumors, and the number of advanced cancers is small, but the sensitivity to chemotherapy drugs is quite low. Hypercalcemia and thrombosis are often observed in the patients with clear cell carcinoma of the ovary. Most of the cases developed from the background of endometriosis and almost half have mutations in ARID1A and PK3CA [14] (Fig. 6.1). mTOR (Mammalian target of rapamycin) inhibitors are candidates for clear cell carcinoma of the ovary [15].

6.3.4 *Mucinous Cancer*

Mucinous cancer progresses from mucinous adenoma to borderline malignant tumor, that is to say, "adenoma-cancer sequence." KRAS mutations are frequently seen [16]. It often forms large multilocular cysts that are unilateral and have a tumor diameter of more than 10 cm. Although few cases have advanced-stage disease, the sensitivity to chemotherapeutic agents is extremely low. Candidate molecular targeting agents included MEK inhibitors and BRAF inhibitors [17].

6.4 Molecular Profiling of High-Grade Serous Ovarian Cancers

High-grade serous ovarian cancer (HGSC) had a unique classification according to molecular profiling [18]. The gene signature "Classification of Ovarian Cancer" (CLOVAR) could recognize four types of tumors in HGSC: differentiated, immunoreactive, mesenchymal, and proliferative (Table 6.2).

Table 6.2 Subtypes of high-grade serous ovarian cancer

Subtype	Differentiated	Immunoreactive	Mesenchymal	Proliferative
High expression	Differentiated Marker (MUC1, MUC16, etc.)	PD-L1 MHC class I/II Chemokines	Stromal Markers	Proliferation marker
Low expression				Differentiated Marker (MUC1, MUC16, etc.)
Candidate therapy		anti-PD-1/PD-L1 (Nivolumab, Pembrolizumab, etc.)	anti-VEGF (Bevacizumab, etc.) Dose-dense TC	anti-VEGF (Bevacizumab, etc.)

PD-L1 programmed cell death 1 ligand 1, *PD-1* programmed cell death 1, *MHC* major histocompatibility complex, *VEGF* vascular endothelial growth factor, *TC* paclitaxel and carboplatin

Differentiated types are characterized by differentiated markers, such as MUC1 and MUC16, and immunoreactive types are by higher expression of PD-L1, MHC class I/II markers, and T cell chemokines. Mesenchymal type had higher expression of stromal markers, and proliferative type had high expression of transcription and proliferation markers. Clinical course is quite different according to CLOVAR classification [19]. Candidate treatment for mesenchymal and proliferative types included anti-VEGF drugs, such as Bevacizumab. On the other hand, the immunoreactive type would definitely respond to anti-PD-1/L1 drugs, such as Nivolumab. Mesenchymal type had higher efficacy of dose-dense therapy using paclitaxel and carboplatin [20].

6.5 Conclusions

The genetics of epithelial ovarian cancers according to histological subtypes are discussed in this chapter. Ovarian cancer is a heterogeneous and dynamic disease. In serous ovarian cancers, breakthrough therapies (PARP inhibitors) have been recently clinically available. However, there are no further targeting therapies for other types of histology. Therefore, the implementation of genotype-based therapy remains a challenge for the management of ovarian cancers.

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Chapter 7
Signaling and Drug Resistance

Koji Yamanoi and Masaki Mandai

Abstract Cervical cancer: Resistance to concurrent radiochemotherapy (CCRT) is strongly related to cancer stem cell (CSC) phenotype. Several pathways including Hedgehog, Wnt/β-catenin, and STAT3 pathways are involved in the acquisition or maintenance of the CSCs population.

Endometrial cancer: It has been reported that Estrogen receptors or growth hormones are involved in acquiring chemoresistance in Type-I Endometrial cancer. Although the involvement of the PIK3/Akt pathway is also well known, the therapeutic effect of the monotherapy of PIK3CK inhibitor is insufficient. However, it might be expected in the case of mutation in CTNNB1. The STAT1 pathway and phosphorylation of ser727 are involved in chemoresistance in Type-II endometrial cancer. EGFR pathway is also important because we have the clinically available drug. A combination of HER2-target therapy with PIK3CA inhibitor is expected.

Ovarian cancer: High-grade serous ovarian cancer (HGSOC) is famous for its diverse mechanism of acquisition of chemoresistance. Epithelial to mesenchymal transition (EMT) is closely related to CSC functions and plays an important role in the acquisition mechanism. Various pathways such as TGF-β, STAT3, Hedgehog, and Wnt/β-catenin pathways are involved in the enhancement of EMT. TLE2 might be an important factor that can regulate multiple EMT-related pathways in common. A clinically important issue is that the mesenchymal subtype of HGSOC is relatively sensitive to paclitaxel. Ovarian clear cell carcinoma (OCCC) is also famous as a subtype with strong resistance to chemotherapy. HNF1β is specifically expressed in OCCC and is greatly involved in the chemoresistance ability of OCCC through alteration of metabolic pathway and regulation of cystine transporter expression.

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Hedgehog · TGF-β · HER2 · High-grade serous ovarian cancer
Endometrial cancer

7.1 Treatment Resistance in Cervical Cancer: Relationship with Cancer Stem Cell Phenotype

An important drug in the therapy for cervical cancer is cisplatin. Concurrent radiochemotherapy (CCRT), which combines cisplatin and radiation therapy, is a basic treatment strategy for advanced cervical cancer [1]. Sensitivity to both cisplatin and radiation is a factor that is greatly involved in the prognosis of cervical cancer patients.

The cancer stem cell (CSC) phenotype in cervical cancer has recently attracted attention as a factor related to treatment resistance. CSC is a theory originally proposed in hematological malignancies, in which a small population of cancer cells, known as CSCs, possesses several malignant phenotypes, including enhanced tumorigenicity and chemoresistance. Recently, it has been found that a CSC-like population exists in many kinds of solid tumors, including cervical cancer. Many researchers have also investigated specific markers to identify CSC-like cells. Thus far, several markers have been reported to identify CSCs, such as CD133-positive cells in Glioblastoma, LGR5-positive cells in colorectal cancer, and CD44-positive/CD24-negative cells in breast cancer. However, for cervical cancer, definitive CSC markers have not been detected. Therefore, studies have been conducted on markers that were reported in other carcinomas. CD133(+) cells, CD44(+)/CD24(–) cells, LGR5(+) cells, and SOX9(+) cells were recently reported as CSC-like cells possessing malignant potential in cervical cancer [2–6]. In addition, CSC usually possess high ALDH activity. Based on these aspects, we can consider ALDH-high populations as CSC-like fractions. Some researchers have investigated the role of these small populations in cervical cancer [7]. We summarize those reports in Table 7.1.

Thus, although we cannot define definitive CSC markers yet, there appears to be a small population of highly resistant therapeutic fractions for cervical cancer. In particular, such CSC-like fractions are either platinum-resistant or radiation-resistant. If we can elucidate the specific nature or signaling of those CSCs, we may be able to develop new treatment strategies to resolve resistance to platinum and radiation therapy. Several mechanisms have been related to controlling the CSC-like cell fraction. As for LGR5+-defined CSCs, inhibition of the Wnt/β-catenin pathway has been reported to control their malignant potential [2]. The Wnt/β--catenin pathway has also been reported to be involved in phosphorylation levels of eukaryotic translation initiation factor 4E (eIF4E) and controls the chemosensitivity of cervical cancer cells [20]. As for SOX9, cupper transporter protein 1 (CR1) has been reported to be regulated by the SOX9/miR-130a/CTR1 axis, controlling

Table 7.1 Summary of CSC-related reports in cervical cancer

Marker/ pathway	Main findings	References
LGR5 (marker)	Overexpression of LGR5 promotes CSC-like phenotype via Wnt/β-catenin pathway. LGR+ cells harbor multiple CSC characteristics including high in vivo tumorgenicity, asymmetrical division, and chemoresistance	[2]
SOX9 (marker)	SOX9 inversely regulates miR-130a through directly targeting the promoter of miR-130a, which regulates Copper transporter protein 1 (CTR1) and has a great influence on sensitivity to cisplatin	[3]
CD44+/ CD24− (marker)	Overexpression of cytosolic phospholipase A2α (cPLA2α) results in a CD44+/CD24− phenotype associated with mesenchymal traits, including increased invasive and migration abilities	[6]
	α-Actin-4 (ACTN4) knockdown suppresses sphere formation and CSC proliferation (CD44+/CD24− cell population). ACTN4-knockdown CSCs were sensitive to anticancer drugs, which was observed by the downregulation of the ABCG2 involved in drug resistance	[5]
SOX2, ALDH1A1 (marker)	Immunohistochemistry analyses reveal that low-$P16^{INK4A}$/high-SOX2 and low-$P16^{INK4A}$/high-ALDH1A1 groups had a worse prognosis. Depletion of $P16^{INK4A}$ promotes chemoresistance and radioresistance of cervical cancer cells, increased the expression of SOX2 and ALDH1A1, and exhibited higher self-renewal ability.	[4]
	Analyses using clinical samples show that increased expression of ALDH1 is related to poor response to NAC therapy	[7]
Hedgehog pathway (pathway)	Upregulation of the Hh pathway is observed in E-cadherin low cervical cancer cells, which is an in vitro EMT model. Inhibitors of the Hh pathway (cyclopamine and GANT58) inhibit invasiveness and apoptosis in E-cadherin low cervical cancer cells	[8]

cisplatin resistance [3]. As for CD44+/CD24-defined CSCs, cytosolic phospholipase A2a (cPLA2α) has been reported to control its fraction [6]. In addition, α-actin-4 (ACTN4), an actin-binding protein, is also involved in the fraction of CD44+/CD24− defined CSCs [5]. The sonic hedgehog pathway (sHh) is a famous pathway related to stemness and has been related to chemoresistance in cervical cancer. Inhibition of the sHh pathway, such as GANT58, may be potential strategies [8]. STAT3 pathway and Hippo pathway, which are well known to be related to CSC phenotype, are also reported to be related to CSCs in cervical cancer [21, 22]. However, identifying a novel important factor and developing a new treatment strategy are extremely difficult and time-consuming. Drugs that can be realistically considered as new treatment options are inhibitors of the PI3K/Akt and EGFR2 pathways.

The PI3K/Akt/mTOR signaling pathways are involved in HPV-related carcinogenesis in cervical cancer [23, 24]. From the viewpoint of cisplatin sensitivity, genetic variations in the PI3K/Akt pathway relate to chemotherapeutic sensitivity in squamous cell carcinoma, which is the most common subtype of cervical cancer [25]. Unfortunately, monotherapy with everolimus, a typical PI3K inhibitor, has not

improved prognosis thus far. However, when used in combination, it may enhance the sensitivity of cisplatin and further enhance the therapeutic effect. As for the EGFR pathway, there are several clinically available inhibitors. Erlotinib, an EGFR tyrosine kinase inhibitor (TKI), has been reported to overcome chemoresistance of MUC-1-positive cervical cancer [26]. Moreover, if HER2 amplification exists, TKI or antibody-HER2 may show some therapeutic effects. HER2 amplification can be found in approximately 5% of cervical cancers based on c-BioPortal analysis. Although HER2 amplification exists in only a few populations of cervical cancer, anti-HER2 therapies should be considered when present.

7.2 Chemoresistance and Signaling in Endometrial Cancer

Endometrial cancer is the most common gynecological malignancy in Japan. The number of patients has been increasing in recent years due to the influence of the spreading westernized diet. Endometrial cancer is generally classified into Type-I and Type-II based on pathological, molecular, and clinical backgrounds [27]. Type-I endometrial cancer is typically caused by long-term exposure to unopposed estrogen, sequentially developing via a precancerous condition known as atypical endometrial hyperplasia. Therefore, the carcinogenic process is strongly influenced by sex hormones, including estrogen and progesterone. Endometrioid adenocarcinoma Grade 1/2 is a typical pathological subtype. Type II, on the other hand, is not affected by unopposed estrogen and is said to develop de novo without a precancerous condition. Endometrioid adenocarcinoma Grade 3 and serous adenocarcinoma are typical pathological subtypes. It is also characteristic to possess a p53 mutation.

Type I accounts for approximately 80% of total endometrial cancers, and complete resection results in a good prognosis [27]. However, since chemosensitivity is relatively low, treatment is often difficult when surgery is no longer an option due to advanced stage or in case of recurrence. The estrogen receptor ERα controls the transcription of multiple genes and regulates the carcinogenesis and chemosensitivity of Type-I endometrial cancer. For example, the transcriptional coactivator NCOA6 plays an important role in ERα-activated growth-regulating estrogen receptor binding 1 (GREB1) activity [28]. This axis is involved in ERα-related carcinogenesis, and GREB1 status has been related to the chemoresistance of endometrial cancer. In addition, progesterone (P4) receptor membrane component 1 (PGRMC1) has been reported to be involved in cell growth and chemosensitivity [29]. Growth hormones can differentially modulate resistance to multiple chemotherapy, including doxorubicin, cisplatin, and paclitaxel in Type-I endometrial cancer cell lines [30].

The PIK3CA/mTOR pathway has also been associated with endometrial cancer because cross-regulation between ERα signaling and PI3K/Akt/mTOR pathways has been reported [31]. From this aspect, combination therapy using a PI3K inhibitor and hormonal therapy was conducted [32, 33]. Unfortunately, overall conclusions were negative, but there seem to be subpopulations where combination therapy might be effective in sub-analysis. Among recurrent cases of endometrioid

adenocarcinoma grade 1/2, the combination of everolimus and letrozole is effective for cases with CTNNB1 mutations. Although detailed mechanisms about the relationship between the CTNNB1 and PIK3CA pathways remain to be elucidated, PIK3CA inhibitors can be effective in some cases of endometrial cancer.

Type II accounts for approximately 20% of endometrial cancers [27]. They are known to have relatively high invasion/metastasis capacity, and their malignant potential is high compared to that of Type I. As the chemosensitivity is not high, the prognosis is even worse [34].

We have investigated detailed mechanisms of the malignant potential of uterus serous adenocarcinoma (USC), one of the major subtypes of Type-II endometrial cancer. Firstly, we found that the STAT1 pathway is highly involved in the malignant properties of USC, including platinum resistance [35]. Furthermore, among several phosphorylation sites in STAT1, we found that serine 727 is the most responsible phosphorylation site for platinum resistance [36]. Inhibition of its phosphorylation can resolve platinum resistance of USC. We are now trying to find a small molecule that can prevent phosphorylation of serine 727 in STAT1.

Other than the STAT1 pathway, the HER2-related pathway is interesting because we already have clinically available drugs, such as trastuzumab, anti-HER2 antibody, and lapatinib, an EGFR-TKI. Even though the frequency is low, HER2-positive populations can be found among tumors across many organs. From TCGA data analysis, HER2 amplification can be found in approximately 10% of type-II endometrial cancers. However, thus far, clinical trials using Lapatinib and Trastuzumab in HER2-positive endometrial cancer have not been very successful [37]. This may be because oncogenic pathways other than HER2 can coexist. For example, some reports have shown that the resistance to HER2-targeted therapy is caused by the coexistence of PIK3CA mutations, and the combination of PIK3CA inhibitors can confer this resistance [38, 39]. Although there are no clinical trials or case reports that use a combination of anti-HER2 therapy and other small molecule therapy at present, we expect future progress in this area.

7.3 Chemotherapy Is Particularly Important for Ovarian Cancer

Although there are fewer ovarian cancer patients than cervical and endometrial cancer patients, its prognosis is very poor. To improve its prognosis, we are seeking new treatment strategies. There are various histological types of ovarian cancer. Here, we discuss high-grade serous ovarian cancer (HGSOC) and ovarian clear cell carcinoma (OCCC).

HGSOC is the most common subtype of epithelial ovarian cancer in the world and in Japan [40, 41]. It is often found in advanced status, with multiple disseminations in the abdominal cavity at initial presentation [42]. For this reason, surgical treatment alone is often inadequate, and chemotherapy accounts for a very high proportion of treatments. Chemotherapy outcomes have improved since the advent

of platinum reagents, with most cases responding relatively well to initial chemotherapy with a combination of paclitaxel and carboplatin [43]. However, the tumor cannot be completely killed, and recurrence of the tumor occurs in many cases [44]. To make matters worse, as the recurrence is repeated, the resistance to chemotherapy increases. Tumors that have developed resistance to platinum are more likely to show resistance to other drugs simultaneously [43]. This acquisition mechanism is the main reason for the poor prognosis of HGSOC. Elucidation of the detailed acquisition mechanism is required because it can restore chemosensitivity and improve prognosis.

OCCC is the second most common ovarian cancer after HGSOC, especially in Asian countries [41]. OCCC is known to arise from endometriotic cells in endometriosis. From the viewpoint of chemotherapy, OCCC is characterized by its high resistance to chemotherapy, including platinum [45]. Therefore, especially in advanced stages, when chemotherapy is the main treatment, the prognosis is extremely poor [46, 47]. Recurrent tumors are more resistant to chemotherapy and are difficult to treat. OCCC clearly has limitations compared to HGSOC in current chemotherapy; thus, the elucidation of its resistance mechanism is a major goal.

7.4 Homologous Repair in HGSOC

When DNA damage occurs, there are several DNA repair mechanisms, one of which is homologous recombination repair (HR). BRCA1/2 is known to play an important role in HR and is known as a tumor suppressor. Thus, when there is a certain mutation or LOH in BRCA1/2, the risk of developing various malignant tumors is clearly high. HGSOC is one of those BRCA1/2-associated cancers [48]. Recently, it has been shown that BRCA-related cancers, including HGSOC, are selectively sensitive to the poly(ADP-ribose) polymerase (PARP) inhibitor PARPi [49–51].

PARP1, the major target of PARPi, is mainly involved in the repair of single-strand DNA breaks (SSBs). In the absence of BRCA1/2, SSBs caused by PARPi can be lethal. Recently, it has also been considered that PARPi may be sensitive to HR-defective cancers, even if BRCA1/2 is normal [52–55]. Clinical trials using the HR pathway as a marker for PARPi have begun. Unlike conventional anticancer agents, PARPi can be used as a maintenance therapy [49, 50]; thus, its clinical impact is very large.

However, from the perspective of chemoresistance, the role played by PARPi is limited. This is because the sensitivity to PARPi is usually positively correlated with that to platinum. That is, if the tumors become resistant to platinum, they are also resistant to PARPi [51]. Therefore, PARPi is considered refractory to tumors that have recurred repeatedly and become resistant to platinum [51, 56]. Conversely, PARPi could potentially be used if the mechanism for reversing resistance to platinum is elucidated. Therefore, it will be very useful to elucidate the key mechanisms of platinum resistance in HGSOC.

7.5 Role of Epithelial–Mesenchymal Transition in Malignant Potentials in HGSOC

A major feature of HGSOC is high copy number alterations [42]. As a result, morphological and genetic findings can differ greatly among samples. In 2011, the TCGA project announced for the first time that HGSOCs can be divided into four major subtypes: Immunoreactive (IR), proliferative (PG), differentiated (DG), and mesenchymal (MT) [57]. Clinically, the prognosis of the MT type was found to be particularly poor in comparison with the other three [58]. The MT type is a subtype characterized by activation of the epithelial–mesenchymal transition (EMT) pathway. The relationship between EMT and malignant potentials of cancer has been debated for a long time, and they are known to be closely related. Chemoresistance ability is also greatly related to EMT [59]. The chemotherapy regimen used in the first line of HGSOC is a combination therapy of paclitaxel (T) and carboplatin (C), well known as TC therapy. Among these, carboplatin, a type of platinum, is greatly involved in EMT. That is, tumors with elevated EMT are resistant to carboplatin [10, 60, 61]. Therefore, the elimination of EMT can restore the sensitivity to platinum. Thus, elucidating the factors controlling EMT in HGSOC is important.

In the era of single-cell sequencing, there are additional several reports that show an important role of EMT in HGSOC. Zhiyuan H et al. show that there is some heterogenicity in non-cancer fallopian tube epithelial cells [62]. Using the subtype molecular markers of non-cancer cells, they define a gene signature that robustly identifies a poor prognosis EMT–high subtype of HGSOC. They propose that they could make an accurate prediction of cancer behavior based on that signature. Tongtong Kan et al. investigated the relationship of disseminated cancer cells and their surrounding cells deeply using single-cell sequencing analysis [63]. They applied single-cell EMT-related transcriptional analysis and found that surrounding cells were heterogenous cellular units comprised of epithelial tumor cells, leukocytes, and cancer-associated fibroblasts. They also showed that cancer-associated fibroblasts induce EMT of tumor cells, resulting in the acquisition of malignant phenotype.

However, many pathways can cause EMT in HGSOC. We previously reported that TGF-β causes EMT via phosphorylation of Smad3C [64]. BMP2, a member of the TGF-β super-family, is also involved in the poor prognosis of ovarian cancer via phosphorylation of SMAD5 [65]. Recently, it was also reported that BMP2 is closely related to the proportion of CSC fractions characterized by ALDH-CD133+ and is associated with a poor prognosis [9]. STAT3 is also well known as an oncogenic transcription factor. It has been reported that, in HGSOC, the STAT3 pathway enhances EMT and is involved in various malignant factors, including acceleration of the cell cycle and chemoresistance, resulting in poor prognosis [60]. STAT3 is activated by stimulation with IL-6, which is a member of the interleukin family. Because IL-6 is also reported to be involved in platinum resistance in HGSOC by inducing CCL2 secretion in addition to the activation of STAT3 [66], anti-IL-6 antibody therapy might be effective. Recently, phase I clinical trials using IL-6 receptor

antibody have been conducted [67], and future progress is expected. Other than those pathways listed above, there are still other mechanisms reported to be involved in the malignant phenotypes of HGSOC, including the NF-κB pathway [10, 68]. Moreover, cancer stem cells (CSCs) are also known to be closely related to EMT [61]. There are also many pathways that are reported to be related to CSC in HGSOC including NFATC4 [69], ERK–RSK axis [70], and NAMPT [19]. We summarize several pathways recently reported to be related to EMT or CSC in HGSOC in Table 7.2.

Table 7.2 EMT- or CSC-related pathways reported in HGSOC

Pathway/ factor	Main findings	References
BMP2 pathway	BMP2 promotes ALDH+/CD133+ cell expansion while suppressing the proliferation of ALDH−/CD133− cells. BMP2 acts as a feedback mechanism promoting ovarian CSC expansion and suppressing progenitor proliferation	[9]
NF-κB pathway	Epithelial status exhibited higher resistance to cisplatin treatment. Pathway analysis revealed that activation of NF-κB downstream genes occurred by cisplatin	[10]
	HOTAIR, HOX transcript antisense RNA, expression results in sustained activation of DNA damage response after platinum treatment. Expression of HOTAIR induces NF-κB activation and includes acquisition of resistance to platinum	[11]
	Advanced ovarian cancers NF-κB signaling via RelB transcription factor supports tumor-initiating cell populations by directly regulating the cancer stem like associated enzyme ALDH	[12]
STAT3 pathway	High level of PBX1, a stem cell reprogramming factor, correlated with shorter survival in post-chemotherapy ovarian cancer patients. An analysis of genome-wide chromatin immunoprecipitation data indicated that PBX1 binds directly to the promoter of STAT3, positively regulating its transcription	[13]
	Deletion of STAT3 blocked cell proliferation and migration in vitro and suppressed tumor growth in mice. Deletion of STAT3 transcriptionally suppressed key genes involved in EMT	[14]
TGF-β pathway	Analyses using the microarray dataset show that TGF-β signaling pathway was activated in omental metastasis as compared to primary sites. A-83-01, an inhibitor of TGF-β signaling, has therapeutic effects in the mouse model of peritoneal dissemination	[15]
SOX9	Epigenome profiling of multiple cellular models of chemoresistance identified unique sets of distal enhancers, super-enhancers (Ses), and some EMT-related genes are involved in them	[16]
NFATC4	Nuclear factor of activated T cells cytoplasmic 4 (NFATC4) related to poor prognosis, associated with CSC in ovarian cancer	[17]
ERK1/2-RSK1/2 axis	Cisplatin and carboplatin induce ERK1/2-RSK1/2-EphA2-GPRC5A signaling. Inhibition of RSK1/2 prevented oncogenic EphA2-S897 phosphorylation and FphA2-GPRC5A co-regulation sensitized cisplatin-resistant ovarian cancer cells	[18]
NAMT	NAMPT inhibition suppresses senescence-associated cancer stem cells induced by platinum-based chemotherapy in ovarian cancer. A combination of the NAMPT inhibitor and cisplatin improved the survival in mice xenograft model	[19]

As shown, many factors are associated with chemoresistance in relation to EMT or CSC in HGSOC. This implies that the factors involved in EMT or CSC may differ from patient to patient. This is a clinically important issue. It may be possible to deal with each individual if markers can distinguish them easily and if inhibitors for each pathway are clinically available. However, at present, we do not have such methods or drugs. If some factors that affect several EMT-related pathways are shared, we can regard them as therapeutic targets that can act across multiple EMT-related pathways.

Therefore, we used functional screening using the shRNA library to identify such factors [71]. We conducted a functional screening focusing on CSC phenotype, which has been reported to be associated with EMT. In ovarian cancer, there is no consensus marker that defines the CSC-like population; thus, the side population (SP), which has high dye excretion ability, was used as a marker of CSC-like cells.

As a result, the expression of MSL3, ZN691, VPS45, ITGB3BP, TLE2, ZNF498 was closely related to the SP fraction individually. Downregulation of these six factors individually increased the SP fraction and vice versa. In addition to the proportion of SP fraction, it was greatly involved in the acquisition of resistance to multiple anticancer agents, including platinum and paclitaxel, the colony formation ability, and tumorgenicity in vivo. We then investigated the relationship between our six factors and the TGF-β, Wnt/β-catenin, Notch, and Hedgehog pathways, which have been reported to be closely related to EMT and stemness. We found that common alterations in the Hedgehog pathway occurred among all six factors. The specific mechanism by which these six factors are involved in the Hedgehog pathway remains unclear, but the Hedgehog pathway may be involved in a relatively large proportion of treatment resistance cases in HGSOC. In addition, among these six factors, TLE2 is a molecule of particular interest. Thus far, little is known about the functions of TLE2, other than as a corepressor of the Wnt/β-catenin pathway [72, 73]. In our study, when the expression of TLE2 was suppressed, the expression of more than 3000 genes was greatly altered, resulting in very large changes in cell function as well as morphology. Interestingly, deletions of TLE2 were found in more than 80% of HGSOC samples from TCGA data analysis. We believe that TLE2 clearly affects various pathways other than the Wnt/β-catenin and Hedgehog pathways and plays a very important role in HGSOC. In other cancer subtypes, N-myc downregulated gene 1 (NDRG1) has been reported to decrease TLE2 expression and is involved in the malignant phenotype [74]. It may be possible to establish strategies to increase TLE2 expression. Such treatments may possibly be novel therapeutic strategies for resolving chemoresistance in HGSOC.

7.6 Complementarity of Platinum Resistance and Paclitaxel Resistance

The above-described attempts to identify factors controlling EMT and search for therapeutic targets are inevitably time-consuming and cannot be clinically applied at present. Therefore, we searched for clinically available chemotherapies that were

particularly sensitive to MT type [75]. We analyzed multiple clinical data sets, including the reactivity to drugs, such as paclitaxel and carboplatin, and the comprehensive gene expression data of clinical samples, and we calculated the scores that can predict the drug sensitivity of each. As a result, the sensitivities of platinum and of paclitaxel had a complementary relationship; that is, the MT type had relatively low sensitivity to platinum, while the sensitivity to paclitaxel was maintained. As a clinical study, when comparing the effect of dose-dense TC (ddTC) therapy with increased paclitaxel dose and the effect of normal TC therapy in the MT type, ddTC contributed to the improvement of progression-free intervals [58]. In the present situation, where there is no specific therapeutic strategy for controlling EMT in HGSOC, the choice of ddTC for the MT subtype is a realistic method to improve its poor prognosis.

7.7 Various Mechanisms Relating to Acquisition of Chemoresistance in HGSOC

Various mechanisms other than EMT have been also reported to be involved in the acquisition of chemoresistance in HGSOC. For example, there are reports about focal adhesion kinase (FAK) and the acquisition of chemoresistance. Y397 phosphorylation of FAK has been observed in the process of chemoresistance acquisition, and this phosphorylation is related to β-catenin [76]. FAK inhibition sensitizes chemoresistant HGSOC cell lines to chemotherapy, and FAK inhibitors can be useful to sensitize chemoresistant HGSCO tumors.

In addition, several studies have evaluated the mechanism of tumor microenvironments and platinum resistance. It is known that tumors are exposed to a relatively hypoxic microenvironment, which favors the secretion of exosomes and chemokines. Under hypoxic conditions, it was found that cisplatin efflux via exosomes was significantly increased in HGSOCs [77]. Coculture of hypoxic ovarian cancer cell-derived exosomes (HEx) with tumor cells increased cell survival in response to cisplatin treatment. Hypoxic conditions also link invasion and immunosuppressive phenotypes, resulting in resistance to treatment. That is, improving hypoxia may be the key to resolving platinum resistance.

Intracellular metabolism is also involved in platinum sensitivity. Cellular metabolism is regulated by various enzymes and transporters, and metabolic reprogramming has been defined as a key hallmark of cancer cells. It was recently that subgroups of carbon resources show a preference for either aerobic glycolysis or oxidative phosphorylation (OXPHOS) [78]. HGSOC cells can also be divided into two groups: low-OXPHOS and high-OXPHOS. High-OXPHOS tumors are exposed to chronic oxidative stress and are sensitive to platinum. The PML-PGC-1α axis, which regulates OXPHOS metabolic processes in high-OXPHOS HGSOC, is greatly related to chemosensitivity via the production of oxidative stress.

Chromatin modification is also involved in platinum resistance. Bromodomain containing 4 (BRD4), a member of the bromodomain and extraterminal (BET) protein family, is involved in cancer cell proliferation and survival, including HGSOC. Inhibition of BRD4 is related to restored sensitivity to platinum via blocking HR [79]. Inhibition of BRD9, another member of the BET protein family, also inhibits HR via the RAD51–RAD54 axis and leads to sensitization of HGSOC to platinum [80].

There are also reports of fusion genes and acquisition of chemoresistance. In 2015, a study performed whole-genome sequencing of recurrent tumors and examined the process of drug resistance acquisition in detail [81]. According to the results, a fusion gene involving MDR1 occurred in recurrent tumors. This fusion gene was apparently associated with the acquisition of platinum resistance. MDR1 is an important transporter involved in drug excretion and is the cause of resistance to multiple chemotherapy, including platinum. The fusion gene relevant to MDR1 also plays some roles in the acquisition of chemoresistance in HGSOC.

Thus, various pathways are involved in platinum resistance in HGSOC, and the mechanism may differ from patient to patient. Rather than aiming to establish a novel treatment that can ubiquitously change platinum sensitivity in HGSOC, a personalized medicine-based approach may be an alternative way to search key drugs for chemoresistant tumors [82].

7.8 Chemoresistance in OCCC

Unlike HGSOC, OCCC is known to have low sensitivity to chemotherapy, including platinum and paclitaxel, from initial treatment [46, 47]. Accordingly, its prognosis is relatively poor compared to that of HGSOC [42, 45]. We are the first to find and report that there are several genes specifically related to OCCC, now referred to as the OCCC signature [83]. Among this signature, some famous oncogenic pathways, including the IL6-STAT3 axis, TAZ, and important members of the Hippo pathway, are included. In addition, there are several transcription factors that are strongly involved in cellular metabolism. Among them, we have focused on HNF1-β.

We previously revealed the role of HNF1-β in malignant characteristics of OCCC. Essentially, HNF1-β regulates the cellular metabolism of OCCC, and its downregulation changes metabolism from anaerobic glucose catabolism to aerobic glucose catabolism [84]. Aerobic glucose catabolism leads to activation of the TCA cycle and increases ROS production. At the same time, HNF1-β regulates the expression of rBAT, a cysteine transporter. Cystine is the source of glutathione that prevents ROS production. Suppression of HNF1-β decreased rBAT expression, resulting in increased ROS levels. Taken together, the production of ROS was significantly affected by alterations of HNF1-β expression.

Cisplatin can increase ROS production and results in cell death. In our research, suppression of HNF1-β increased sensitivity to platinum [84]. We believe that this

is caused by the acceleration of ROS levels via the downregulation of HNF1-β. Thus, HNF1-β seems to play an important role in platinum resistance in OCCC.

In OCCC, the loss of ARID1A, a member of the SWISS/Complex, a chromatin modifier, is also a common feature [85, 86]. The SWISS/Complex affects the activity of various pathways and also affects chemoresistance. For example, a reduction of ARID1A promotes the expression of SLC7A11, a cystine transporter, which increases glutathione production and contributes to platinum resistance by causing ROS resistance [87]. This can be another factor of chemoresistance in OCCC.

In OCCC, other than signaling pathways, cellular metabolism and cascades of ROS production are also key factors for chemoresistance [88]. We may therefore need to focus on factors other than signaling pathways to resolve chemoresistance in OCCC.

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Chapter 8
Molecular Perspective in Endometrial Carcinoma

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Abstract In endometrial cancer, risk evaluation has been made on such as histological types, tumor grade, muscular invasion, lymphovascular infiltration, and lymph node metastasis. But in recent years, molecular analysis of endometrial cancer has been advanced, and novel risk evaluation procedures have been proposed. Especially, The Cancer Genome Atlas (TCGA) and Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) could extract some high-risk groups in previously considered as low-risk groups of low-grade endometrioid endometrial cancer, and some preferable prognosis groups of high-grade endometrioid or type 2 non-endometrioid endometrial carcinoma which are considered as poor prognosis. These new classifications based on the molecular subtypes might be useful to decide the postoperative adjuvant therapy and might improve the quality of life of patients with endometrial cancer.

Keywords The Cancer Genome Atlas (TCGA) · Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) · POLE-ultramutated MSI-hypermutated · Copy-number-high · Copy-number-low · p53 abnormal p53 wild type

8.1 Introduction

Endometrial cancer is the fourth most common malignancy in the United States. Its estimated new cases and deaths were 61,880 and 12,160 in 2018, respectively [1], and both morbidity and mortality are increasing. In Japan, the same tendency is seen [2]. Endometrial cancer is classified into two types: type 1 and type 2 [3]. Type 1

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endometrial cancer is differentiated endometrioid tumors (grade 1/2) with usually early-staged, associated with estrogen, obesity, and diabetes mellitus, and its prognosis is generally favorable. On the other hand, type 2 endometrial cancer is mostly serous tumors with advanced-staged, and its prognosis is very poor. These distinct criteria according to the histopathology have been widely accepted, but approximately 20% of type 1 cancer cases arise from atrophic endometrium showed recurrence and poor clinical outcomes, then molecular genetic changes might occur in these type 1 cancers [4]. Still, other issues remain unclear in such as mucinous carcinoma, clear cell carcinoma, and mixed carcinoma [5]. So, a novel definition based on the molecular classification to predict prognosis should be developed.

8.2 The Cancer Genome Atlas (TCGA) and Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE): Novel Proposed Molecular Classifications

In 2013, The Cancer Genome Atlas Research Network (TCGA) reported that endometrial cancers could be divided into four groups of tumors based on the genomic analysis [6]. Group 1 is the "POLE ultramutated" subgroup with very high mutational load and mutations in the exonuclease domain of polymerase-ε(POLE). Group 2 is characterized by "microsatellite instability," frequently with MLH-1 promoter hypermethylation and high mutation rates ("hypermutated"). Group 3 is characterized by copy-number-low (CNL) subgroups with TP53-wild type and normal p53 expression ("endometrioid"), and group 4 is copy-number-high (CNH) with low mutation rates of TP53 mutations with aberrant p53 expression ("serous-like") [5–7]. According to these classifications, progression-free survival (PFS) of group 1 is most excellent followed by group 2/3, and group 4 is the worst [5, 6].

Although TCGA classifications could provide better clinical prognosis compared to histological classifications, easier and less expensive methods using immunohistochemistry has been developed (*Pro*active *M*olecular R*i*sk Classifier for *E*ndometrial Cancer; ProMisE) [8]. ProMisE classifications showed four molecular groups of endometrial cancer; POLE-mutated (POLEmt), MMR-deficient (MMR-D), p53-abnormal (p53abn), and p53-wild-type (p53-wt). In recent years, correlations of conventional histological classifications and molecular classifications of TCGA or ProMisE have been reported. Summary of these molecular classifications and prognosis is shown in Tables 8.1 and 8.2.

Table 8.1 Molecular classifications of endometrial cancers

The Cancer Genome Atlas (TCGA)	Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE)
POLE ultramutated	POLE-mutated (POLEmt)
MSI hypermutated	MMR-deficient (MMR-D)
Copy-number-low (endometrioid)	p53-abnormal (p53abn)
Copy-number-high (serous-like)	p53-wild type (p53-wt)

Table 8.2 Histological and molecular classifications and prognosis

	POLEmt	MMR-D	p53wt	p53abn
Low-grade EEC (grade 1,2)	◯	◯	◯	X
High-grade EEC (grade 3)	◯	△	△	X
Serous	◯	◯	X	X
Clear cell	◯	◯	X	X

Prognosis ◯, good; △, intermediate; X, poor; EEC, endometrioid endometrial carcinoma

8.3 Correlation of Histopathological Classifications and Molecular Classifications

8.3.1 Low-Grade Endometrioid Endometrial Carcinoma (EEC)

Low-grade EEC (grade 1/2), characterized by estrogen-dependent, usually develops from endometrial hyperplasia and is associated with obesity and diabetes mellitus. Generally, low-grade EEC is early-staged, and the prognosis is excellent. Overall, 5-year survival is about 95% after surgery without adjuvant therapies, but the subgroup of women with early-staged low-grade EEC are at increased risk of recurrence and death [9]. In conventional histological classifications, such "high-risk" low-grade EEC could not be selected. Moroney et al. reported CTNNB1 mutation, MMR-D, and MSI-H were significantly frequent in recurrent stage 1, grade 1 EEC compared to those without recurrence, and POLEmt was not found in recurrent cases but it was not significant [10].

8.3.2 High-Grade (Grade 3) EEC

In high-grade (grade 3) EEC patients, endocrine and metabolic disturbances are usually absent or occult, with deep myometrial invasion, frequent lymph node metastasis, and unfavorable prognosis [3]. So, grade 3 EEC with muscular invasion is classified as a high-risk group in ESMO Clinical Practice Guideline 2013 [11] and is treated with extended surgery including lymph node resection and adjuvant chemotherapy. In molecular analysis [6, 8], POLE-mutated tumors are endometrioid endometrial cancer (EEC), particularly grade 3 tumors with frequent mutation of PTEN, PIK3CA, PIK3R1, FBXW′, ARID 1A, KRAS, and ARID5B [5]. In cases of PORTEC-3 trial, molecular analysis was performed, and 12.4% of the cases were POLE-ultramutated. In these cases, 56.9% was grade 3 EEC, and although grade 3 EEC is worse histological grade, mainly were early-stage disease (76.4%) with excellent prognosis [12]. A systematic review by Travaglino et al. showed grade 3 EEC was higher prevalent in POLE-hypermutated, MSI, and CNH subgroups, but was lower in CNL subgroup than grade 1/2 EEC [7]. Although small series of study cases, Piulats et al. showed overall survival of high-grade EEC, and disease-specific

48 months survival rates were 100% in POLEmt, 82% in MSI, 77.8% in CNL, and 42.9% in CNH groups [5]. Boose et al. classified grade 3 EEC into four subgroups: P53abn, MMR-D, POLEmt, and no specific molecular profile (NSMP), and they showed 5-year recurrence-free survival rates was best in POLEmt (89%) and was worst in P53abn (47%) [13]. So, at least, grade 3 EEC with POLE-hypermutated could show a preferable prognosis like low-grade EEC. These cases might be over-treated, then those cases should be reclassified by POLE-mutated status in the future.

8.3.3 Serous Carcinoma

Endometrial serous carcinoma is a major component of type 2 endometrial cancers, usually occurs in older patients, and is not associated with estrogen or obesity. Most of serous carcinoma is classified as CNH (serous-like) subtype [14], and its prognosis is generally poor. Raffone et al. performed systematic review and meta-analysis based on ProMisE classifications, and the proportion of non-EEC was highest in the p53abn subgroup (73%), and ESMO 2013 high-risk category was also highest (84.7%) [15]. But in EEC with a "serous carcinoma" component <60 years, 16% of the cases showed MMR-D and 11% were diagnosed with Lynch syndrome, as well as 16% of the cases were POLEmt subtype. Overall survival of cases with MMR-D and POLEmt was significantly better than those without these features [14]. So, even though in serous carcinoma, MMR-D and POLEmt might be associated with preferable prognosis.

8.3.4 Clear Cell Carcinoma

Clear cell carcinoma (CCC) of endometrium accounts approximately for 3% of all endometrial cancers [16] and is classified as type 2 endometrial cancers with advanced stage. Molecular classifications of 52 cases of CCC according to ProMisE revealed 1 (1.9%) POLEmt, 5 (9.6%) MMR-D, 28 (53.8%) p53wt, and 18 (34.6%) p53abn [17], and CCC is molecular heterogeneous disease. Patients with POLEmut or MMR-D CCC had favorable outcomes and the worst in p53abn CCC [17, 18]. Patients with POLEmt or MMR-D subtype showed trends to younger age compared to P53abn subtypes. P53wt subtype accounts for about half of CCC patients [16, 17], but its prognosis was very poor, although the prognosis of other EEC tumors with p53wt is favorable [17, 19, 20].

8.3.5 Neuroendocrine Carcinoma

Neuroendocrine carcinoma (NEC) of the endometrium is a rare disease account for <1% of all endometrial carcinoma [21]. Howitt et al. reported that 15 cases of NEC were sequenced and were classified into four TCGA groups, and 50% of the cases

were in POLEmt (7%) or microsatellite instability/hypermutated groups (43%) [22]. Although the prognosis of NEC according to the molecular status is not yet elucidated, immune checkpoint inhibition may be a reasonable approach to the treatment of microsatellite instability subtype [22].

8.3.6 Endometrial Hyperplasia

Endometrial hyperplasia (EH), precursors of endometrial carcinoma, is classified for endometrial hyperplasia without atypia (non-atypical hyperplasia: NAH) and atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN) [23]. In a cohort of 7947 women diagnosed with EH, progression to endometrial carcinoma of NAH was 4.6% (95%CI, 3.3–5.8%) through 19 years of follow-up; meanwhile, that of AH/EIN was 27.5% (95%CI, 8.6–42.5%) [24]. Russo et al. reported mutations of PTEN, PIK3CA, and FGFR2 commonly detected in endometrial carcinoma were more frequent in EH progressing to endometrial carcinoma [25].

8.3.7 Lynch Syndrome

Lynch syndrome (LS) is an autosomal dominant inherited disease and is characterized by an increasing risk of colorectal and endometrial cancer [26]. Approximately 5% of endometrial cancer is a hereditary tumor, and LS accounts for the majority of inherited endometrial cancers. Lower uterine segment (LUS) cancer is often seen in LS. In a French multicenter study, 25% of the cases were involved LUS [27]. Germline mutations of mismatch repair genes (MMR): MLH1, MSH2, MSH6, and PMS2, are seen in LS, and in most endometrial cancer cases, germline mutations are in MLH1 and MHS2. The cumulative risk of LS-associated endometrial cancer has been reported to be 27–71% [26]. In a retrospective cohort study including 568 females already proven LS [27], 162 (28.5%) women were diagnosed with endometrial cancer, and mutations in MLH1, MSH2, and MSH6 were 53 (32.7%), 83 (51.2%), and 26 cases (16.0%), respectively. Women with MSH6 mutations presented with endometrial cancer at older ages than those with other mutations [28].

Whether the prognosis of LS-associated endometrial cancer is better or worse compared to sporadic ones is still controversial. In a prospective study of the Prospective Lynch Syndrome Database (PLSD), endometrial cancer cases diagnosed <65 years showed preferable 10-year survival rates (89%) [29]. Kim et al. reported women with MMR-D tumors had worse progression-free survival and higher recurrence rates compared with those with MMR-proficient tumors, but there was no significant difference in overall survival between mismatch repair groups [30]. Son et al. reported among all patients aged ≤60 years, MMR-D due to MLH1 methylation was associated with worse progression-free survival (48.6% vs. 83.3%, $p = 0.032$), and overall survival (56.5% vs. 90.0%, $p = 0.025$) [31]. In non-endometrioid endometrial cancer, patients with LS are associated with much better disease-free survival and overall survival than without LS [32].

8.4 Conclusion

Recent advances in molecular analyses could newly classify endometrial cancers for several types. These classifications could compensate for the problems and flaws of conventional pathological diagnosis and could avoid unnecessary adjuvant therapy for so-called "high-risk cancers" actually at low-risk. In the future, these molecular data should be accumulated to improve the prognosis and quality of life of endometrial cancer patients.

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Chapter 9
Molecular Landscape in Ovarian Clear Cell Carcinoma

Nozomu Yanaihara and Aikou Okamoto

Abstract Ovarian clear cell carcinoma (OCCC), one of five major histological subtypes of epithelial ovarian cancer (EOC), has unique clinical and molecular features. There is no specific targeted therapy for OCCC, and studies on translational genomics underlying OCCC pathogenesis are still ongoing. This chapter focuses on the molecular landscape in the OCCC tumor and its microenvironment. Our findings will help in the stratification of OCCC patients who may benefit from precision medicine for this unique histological subtype of EOC.

Keywords Clinical trial · Ovarian clear cell carcinoma · Molecular landscape Targeted therapy

9.1 Introduction

Ovarian clear cell carcinoma (OCCC) is one of five major histotypes of epithelial ovarian cancer (EOC). The clinicopathological and biological features of OCCC include hypercalcemia, thromboembolism, a close association with endometriosis, and a higher prevalence in Eastern Asian women [1, 2]. In addition, compared to other histological subtypes, OCCC patients present at a relatively younger age and an early stage [3]. The 5-year survival rate for stage I OCCC is ~90%, with differences depending on the substage: patients with stage IA or IC1 OCCC have a favorable clinical outcome, while patients with stage IC2 or IC3 have a statistically poorer prognosis [4–6]. In addition, advanced-stage OCCC is resistant to conventional platinum-based front-line chemotherapy, resulting in poor prognosis [6, 7].

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Because of the low prevalence of OCCC in Western countries, there is a lack of large randomized controlled clinical trials targeting OCCC with chemotherapy including molecular medicine. The current standard treatment is a one-size-fits-all approach, which includes debulking surgery with platinum agent + paclitaxel combination chemotherapy. A randomized phase III clinical trial conducted by the Japanese Gynecologic Oncology Group compared irinotecan and cisplatin (CPT-P) with paclitaxel plus carboplatin (TC) in stage I–IV OCCC patients. The authors reported no significant survival benefit in the CPT-P group [8]. In addition, studies on translational genomics underlying OCCC pathogenesis are ongoing [1, 9–12]. These findings highlight that other therapeutic approaches might improve survival in OCCC patients.

In this chapter, we reviewed recent advances in molecular profiling related to carcinogenesis and molecular targets of OCCC.

9.2 Mutational Landscape

Several studies on large-scale genome-wide gene profiling for OCCC have reported actionable gene alterations that could lead to the development of a target therapy for OCCC (Table 9.1) [13–20]. Both *AT-rich interactive domain 1A* (*ARID1A*) and

Table 9.1 Genetic alterations in OCCC

Alteration	Frequency (%)	Affected pathway	Reference
Somatic mutation			
ARID1A	40–70	SWI/SNF chromatin remodeling complex	[13–19]
PIK3CA	40–50	PI3K/Akt/mTOR	[14–16, 18–20]
PPP2R1A	10–20	Akt/MAPK	[16–19]
KRAS	5–20	Akt/MAPK	[14–16, 18, 19]
TP53	5–15	P53 pathway	[15, 17–20]
PTEN	5–10	PI3K/Akt/mTOR	[15, 16, 19]
Germline mutation			
BRCA1/2	2–6	DNA repair	[21, 22]
Copy number alteration			
ZNF217 (20q13.2 Amplification)	20–40	ZNF217	[23, 24]
MET (7q31.31 Amplification)	30	Akt/MAPK	[25]
AKT2 (19q3.2 Amplification)	20	Akt/mTOR	[25]
HER2 (17q12-q21 Amplification)	14	HER	[26]
PPM1D (17q23.2 Amplification)	10	P53 mediated apoptosis	[27]
CDKN2A/2B (9q21.3 Deletion)	9	CDK inhibitors (p15/p16)	[24]

phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) are most frequently mutated and often coexist in OCCC [19]. The coexistence of these mutations initiated OCCC tumor formation in a genetically engineered mouse model [28]. Since cancer cells are vulnerable to ARID1A deficiency, synthetic lethal approaches to *ARID1A* mutation in OCCC are of considerable clinical interest [1, 12]. *PIK3CA* and *phosphatase and tensin homolog deleted from chromosome 10* (*PTEN*) mutations highly activate the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signal [14–16, 18–20]. In addition, *KRAS* and *protein phosphatase 2 scaffold subunit A alpha* (*PPP2R1A*) mutations have been found in 5–20% and 10–20% of OCCC patients, respectively [14–16, 18, 19]. Notably, mutations of these genes, where the mitogen-activated protein kinase (MAPK) signal could be a target candidate, also often coexist [18]. Compared to high-grade serous ovarian cancer (HGSOC), the most common form of EOC, germline mutations of *germline breast cancer susceptibility 1* (*BRCA1*) and *BRCA2* are infrequent and are found in 2–6% of OCCC patients [21, 22], indicating that only a small percentage of OCCC patients might benefit from a newly innovated treatment strategy using poly-adenosine diphosphate (ADP)-ribose polymerase (PARP) inhibitors.

9.3 Copy Number Landscape

Several molecular technologies, including single-nucleotide polymorphism (SNP) array, comparative genomic hybridization (CGH) array, and exome sequencing, have revealed copy number alterations (CNAs) in OCCC. Frequent amplification has been observed in chr8q, chr17q, and chr20q loci, while deletion has been observed in chr9q, chr13q, chr18q, and chr19p loci [16, 23, 29]. Notably, the whole-arm-CNA ratio is higher in OCCC compared to HGSOC, although fewer CNAs are found in OCCC patients [29]. Interestingly, whole chr8q and chr20q13.2 amplification, including *zinc finger protein 217* (*ZNF217*), is more prevalent in Japanese OCCC patients compared to Korean or German OCCC patients [23]. Amplification or deletion of certain loci that contain several cancer-related genes might affect intracellular signals as potential therapeutic targets (Table 9.1).

9.4 Signaling Pathway Landscape

9.4.1 IL6/STAT3 Pathway

OCCC-specific expression signatures have been obtained using global gene expression assays. Compared to HGSOC, OCCC shows an enhanced interleukin 6 (IL6)/signal transducer and activator of transcription 3 (STAT3) pathway [30–32]. In addition, high tumor and serum IL6 levels are significantly correlated with poor

prognosis in OCCC patients [5, 31]. IL6 is a pleiotropic pro-inflammatory cytokine that mediates critical processes, including cell proliferation, angiogenesis, and chemoresistance. IL6 signal inhibition has antitumor effects in OCCC, indicating that this pathway is a promising therapeutic target [33]. Although anti-IL6 antibody (siltuximab) has shown clinical activity in a phase II clinical trial of 18 patients with platinum-resistant ovarian cancer (1 OCCC patient) [34], no clinical trials specific for OCCC-targeting IL6/STAT3 signals have been conducted.

9.4.2 Angiogenesis

Intertumoral hypoxia with high hypoxia-inducible factor 1-alpha (HIF-1α) expression, in which a malignant tumor commonly develops, leads to an increase in the activity of various angiogenesis-related signals. In OCCC, increased HIF-1α expression increases the intracellular glycogen content, causing cell chemoresistance [35]. In addition, in OCCC, IL6 signals via STAT3 activates the expression of downstream genes, including *HIF1A* [31]. Therefore, vascular endothelial growth factor (VEGF), induced by HIF-1α, is overexpressed in >90% of OCCC patients, and VEGF expression is correlated with the patient's survival [36]. Notably, bevacizumab, a monoclonal human VEGF antibody, has antitumor effects in OCCC both in vitro and in vivo.

On the basis of the findings that OCCC and renal CCC have similar gene expression profiles, one of which is characterized by the activated HIF pathway [37], researchers have focused on anti-angiogenetic therapy for OCCC (Table 9.2). The GOG-254 phase II study on sunitinib, which targets vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), for recurrent or persistent OCCC treatment reported a median progression-free survival (PFS) and overall survival (OS) of 2.7 and 12.8 months, respectively [38]. The NRG-GY001 phase II study on cabozantinib, which targets VEGFR, MET, and RET, for recurrent or persistent OCCC reported that a single administration of cabozantinib leads to a median PFS and OS of 3.6 and 8.1 months, respectively [39]. In addition, a phase II study on ENMD-2076, which targets Aurora A and VEGFR, for recurrent OCCC reported a median PFS of 3.7 months, and 22% of evaluable patients had a 6-month PFS, which did not meet the preset bar for efficacy [40]. Most of these trials showed limited efficacy. A randomized phase II international NiCCC (ENGOT-GYN1) study on nintedanib (BIBF 1120), which targets VEGFR, PDFGR, and fibroblast growth factor receptor (FGFR), versus chemotherapy in recurrent or persistent OCCC is ongoing (NCT02866370) [41].

9.4.3 PI3K/Akt/mTOR Pathway

The PI3K/Akt/mTOR pathway plays a crucial role in the malignancy of human tumors and is involved in OCCC pathogenesis via frequent genetic alterations [14–16, 18–20]. Notably, comprehensive genomic profiling of OCCC shows that ~70% of

Table 9.2 Clinical trials based on the molecular landscape in OCCC

Title	Drug	Targets	Condition	RCT	Phase	Trials identifier	Reference
Anti-angiogenesis							
GOG-254	Sunitinib	VEGFR, PDGFR	Recurrent or persistent	No	II	NCT00979992	[38]
NRG-GY001	Cabozantinib	VEGFR2, MET, RET	Recurrent or persistent	No	II	NCT02315430	[39]
A Study of ENMD-2076 in OCCC	ENMD-2076	VEGFR, Aurora A	Recurrent	No	II	NCT01104675	[40]
NiCCC (ENGOT-GYN1)	Nintedanib	VEGFR, PDGFR, FGFR	Recurrent or persistent	Yes	II	NCT02866370	[41]
Anti-PI3K/Akt/mTOR pathway							
GOG-268	Temsirolimus	mTOR	Stage III or IV	No	II	NCT01196429	[42]
Synthetic lethal approaches for ARID1A							
GOG-283	Dasatinib	Abl, Src, c-Kit	Recurrent or persistent	No	II	NCT02059265	[43]
Immune checkpoint blockade							
MOCCA	Durvalumab	PD-L1	Recurrent or persistent	Yes	II	NCT03405454	[44]
BrUOG 354	Nivolumab Ipilimumab	PD-1 CTLA4	Recurrent or persistent	Yes	II	NCT03355976	[45]

samples harbor mutations in at least one component of the PI3K/Akt/mTOR pathway [46]. In addition, inhibitors of the PI3K/Akt/mTOR pathway shows antitumor effects in OCCC cells with high pathway activity [47]. These findings indicate the potential benefits of therapies targeting the PI3K/Akt/mTOR pathway in OCCC patients.

The GOG-268 phase II study on temsirolimus, which targets mTOR complex 1, in combination with paclitaxel and carboplatin, followed by temsirolimus, as a first-line therapy in stage III–IV OCCC patients did not show an improved PFS compared to historical controls (Table 9.2; NCT01196429) [42].

9.4.4 HNF-1β Pathway

Hepatocyte nuclear factor 1β (HNF-1β), a transcription factor, is commonly expressed in OCCC and is therefore used as an OCCC diagnostic marker [1, 9, 11, 12]. A decrease in HNF-1β expression is associated with a favorable clinical

outcome in OCCC patients [48]. Epigenetic silencing via hypomethylation is one of the mechanisms underlying high HNF-1β expression [30]. In OCCC, HNF-1β plays an important role in cancer cell survival and chemoresistance by modulating glucose metabolism and internal oxidative stress [49, 50].

9.5 Synthetic Lethal Approaches for *ARID1A*

As mentioned before, cancer cells are vulnerable to ARID1A deficiency. Therefore, synthetic lethal approaches to targeting this vulnerability of OCCC cells are being developed. The small-molecule inhibitor of the enhancer of zeste homolog 2 (EZH2) methyltransferase (GSK126) inhibits growth in *ARID1A*-mutated ovarian cancer cells because ARID1A and EZH2 have an antagonistic association with regard to *PI3K-interacting protein 1* (*PIK3IP1*) expression that promotes apoptosis via negative PI3K/Akt signaling regulation [51]. As another epigenetic concept of ARID1A deficiency, modulation of histone deacetylase 6 (HDAC6) activity using the HDAC6 inhibitor (ACY1215) has a therapeutic effect in *ARID1A*-mutated tumors [52]. ARID1A transcriptionally represses *HDAC6*, so *ARID1A* mutation inactivates the apoptosis-promoting function of P53 via HADC6 upregulation. Notably, high HDAC6 expression, as shown by immunohistochemistry (IHC) assay, is correlated with unfavorable prognosis in OCCC with ARID1A loss [53]. In addition, HDAC6 inhibition can synergize with anti-programmed death-ligand 1 (PD-L1) immune checkpoint blockade in an ARID1A-inactivated genetic OCCC mouse model [54].

ARID1A-deficient tumors show therapeutic vulnerability to PARP inhibitors [55]. ARID1A is recruited to the site of double-stranded DNA breaks (DNA DSBs) via interaction with ataxia–telangiectasia and RAD3-related protein (ATR), in addition to facilitating DNA DSB end processing and sustaining ATR activation for DNA damage signaling. Therefore, impaired DNA damage checkpoint regulation in ARID1A-deficient tumors sensitizes cancer cells to PARP inhibitors. In addition, high-throughput RNA interference (RNAi) chemosensitization screening shows that ARID1A is a synthetic lethal partner of the ATR inhibitor [56]. ARID1A deficiency delays the cell cycle because of the inability to recruit topoisomerase II to chromatin, increasing dependency on ATR checkpoint activity. Therefore, ATR inhibition in ARID1A-deficient tumors induces premature mitosis, triggering genomic instability and cancer cell death.

A high-throughput drug screen targeting *ARID1A* synthetic lethal effects revealed dasatinib, a multitarget kinase inhibitor, as a clinically used selective drug for *ARID1A*-mutated OCCC cell lines [57]. The sensitivity of dasatinib in *ARID1A*-mutated cancer cells is characterized by G1 cell cycle arrest, followed by p21- and Rb-associated apoptosis. Studies focusing on cellular metabolism as a new concept of synthetic lethal approaches have shown that ARID1A-deficient tumors are vulnerable to glutathione (GSH) metabolism [58]. ARID1A maintains GSH homeostasis by modulating SLC7A11 expression (a transporter of cysteine, a key source for

GSH) and therefore maintains an intricate balance between GSH and reactive oxygen species (ROS). Inhibition of the GSH metabolic pathway using either APR-246 or buthionine sulfoximine (BSO, a rate-limiting enzyme in GSH synthesis) in ARID1A-deficient tumors collapses the GSH-ROS balance, followed by apoptosis by ROS.

The ARID1A deficiency status is used for OCCC patient stratification in both clinical settings and trials. OCCC with *ARID1A* mutation shows selective sensitivity to gemcitabine, although the underlying molecular mechanism is unclear [59]. Gemcitabine is commonly available for EOC treatment, so this finding might directly contribute to precision medicine for OCCC. In addition, a phase II retrospective study on dasatinib for recurrent or persistent ovarian and endometrial clear cell carcinoma to evaluate antitumor effects with regard to the ARID1A expression status is ongoing (NCT02059265) [43].

9.6 Immunological Landscape

Pembrolizumab is a humanized monoclonal antibody against programmed death-1 (PD-1) and is approved for any unresectable or metastatic solid tumor with microsatellite instability (MSI). MSI with a high tumor mutation burden arises from mismatch repair (MMR) deficiency caused by either germline mutations in MMR gene components in Lynch syndrome patients or somatic hypermethylation of the *MLH1* promoter region in tumors. Histological subtypes of EOC associated with Lynch syndrome include endometrioid carcinoma and OCCC [60]. The frequency of aberrant MMR expression, as assayed by IHC, is 6–13% in OCCC [61–63], and MMR expression and MSI status are correlated [63, 64].

In the KEYNOTE-100 phase II study on pembrolizumab in 376 recurrent EOC patients, the objective response rate (ORR) of OCCC was 15.8%, although the overall ORR was 8% [65]. Importantly, this study also showed that higher PD-L1 expression in tumors is correlated with a higher ORR; ~50% of OCCC patients showed positive PD-L1 expression regardless of the MMR status [62, 63], indicating that a large percentage of OCCC patients might benefit from this new therapeutic approach of immune checkpoint blockade. ARID1A deficiency induced impaired MMR via interaction with MSH2, followed by increased PD-L1 expression, in a syngeneic ovarian cancer mouse model [66]. In another phase II study on the anti-PD-1 antibody nivolumab in 20 platinum-resistant EOC patients, 2 patients (one with OCCC) showed a durable complete response [67]. Other ongoing clinical trials targeting immune checkpoint blockade for OCCC include the MOCCA phase II randomized study on durvalumab, which targets PD-L1, versus chemotherapy (NCT03405454) [44] and the BrUOG 354 phase II randomized study on nivolumab plus ipilimumab, which target CTLA4, versus only nivolumab (NCT03355976) [44] (Table 9.2).

9.7 Conclusion

The clinical need for OCCC treatment is still unmet. Alternative therapeutic strategies using targeted therapies based on the molecular characteristics of OCCC might significantly affect the clinical outcome in OCCC patients. Given its low prevalence, the proof-of-concept via adequately designed clinical trials with international collaboration on the basis of the OCCC molecular landscapes are required in order to develop precision medicine for OCCC.

Conflict of Interest Statement None of the authors have any conflict of interest to disclose.

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Chapter 10
Molecular Pathology and Clinicopathological Significance of Endometrial Carcinoma

Munekage Yamaguchi and Hidetaka Katabuchi

Abstract Endometrial carcinoma (EC) is rapidly increasing worldwide. In the twentieth century, it was believed that endocrine and metabolic disturbances might determine the biological features and clinical course of EC. The dualistic pathogenetic classification suggested in 1983 provided an understanding of a complex and heterogeneous disease, and thereafter, the immunohistochemical status of hormonal receptors provided a clue for discriminating EC based on two representative histological types, namely, endometrioid carcinoma and serous carcinoma. Molecular advancements in the 1990s revealed genomic alterations in the dualistic types of EC: the endometrioid type is primarily characterized by *KRAS* mutation, microsatellite instability, and *PTEN* mutation, and the serous type is characterized by *TP53* mutation. In 2013, integrated genomic transcriptomic and proteomic analyses classified EC into four categories: POLE, microsatellite instability, copy-number low, and copy-number high. The reclassification further contributed to a more comprehensive understanding of the molecular alterations and signaling pathways in EC and thus provides potential targeted approaches for the personalized treatment of EC.

Keywords Endometrial cancer · Dualistic classification · Gene alteration Carcinogenesis · Targeted therapy

10.1 Trend of the Incidence of Endometrial Carcinoma

Endometrial carcinoma (EC) is categorized as carcinomas of the uterine corpus that occur primarily in the endometrium. EC is the sixth most common carcinoma in the breast, colorectum, lung, uterine cervix, and thyroid in women worldwide, and

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382,069 estimated new cases and 89,929 deaths occurred in 2018 [1]. The prevalence of EC is increasing rapidly, and it has been estimated that this worldwide prevalence will increase by more than 50% by 2040 [2]. The incidence and mortality of EC vary across countries, and its incidence rates are generally higher in developed countries [3]. In the United States, it has been estimated that approximately 65,620 new cases of uterine corpus cancer and 12,590 deaths will occur in 2020 [4]. Continued declines in the fertility rate, as well as increased obesity, are believed to contribute to the continued increase in the incidence of uterine corpus cancer (1.3% per year from 2007 to 2016) [4]. In addition, the incidence rates of EC will continue to increase over the next 10 years, and the increases in the US total population and the rising proportion of older women are also believed to contribute to this increase [5]. In contrast, extreme increases are particularly pronounced in some Asian countries, including Japan [6]. In Japan, the estimated incidence of EC increased 16-fold over half a century from approximately 1000 in the 1970s to more than 16,000 in 2019, and EC is currently the most prevalent carcinoma of the female genital tract [7, 8]. Drastic changes in lifestyle, including a westernized diet, late marriage, and low birthrates, and the aging of the population, are believed to be responsible for this extreme increase in the frequency of EC in Japan [7]. This trend has not been found with cervical carcinoma and ovarian carcinoma, which indicates that the increase in the frequency of EC might be associated with a characteristic of EC known as hormone-dependent carcinoma. A similar increasing trend has also been observed with other hormone-dependent carcinomas, including breast carcinoma and prostate carcinoma, in Japan [9, 10], and these findings support the above-described theory. Accordingly, endocrine factors are important in discussions of the molecular pathology and clinicopathological significance of EC.

10.2 Dualistic Pathogenetic Classification of Endometrial Carcinoma

In the twentieth century, it had commonly been suggested that endogenous or exogenous estrogens play a role in the development of EC via endometrial hyperplasia [11]. It had also been suggested that late menopause, the frequent presence of uterine polyps and endometrial hyperplasia, a high incidence of feminizing ovarian tumors, concomitant fibroids, obesity, and diabetes are associated with EC [12], and these manifestations were believed to be caused by hyperactivity of the hypothalamic–pituitary axis [13]. The long-term disability of endocrine and metabolic functions might determine the biological features, clinical course, and prognosis of EC. Thereafter, a basis for the dualistic classification of EC was established through an evaluation of the histopathological findings, clinical backgrounds, and outcomes obtained for 366 EC patients with endometrioid histology. The first pathogenetic type of EC (type I) occurs in women with obesity, hyperlipidemia, and signs of hyperestrogenism, which includes anovulatory uterine bleeding, infertility, late

Table 10.1 Dualistic pathogenetic classification of endometrial carcinoma

Clinical features		Type I	Type II
Sings of hyperestrogenism	Anovulatory uterine bleeding Infertility Late menopause	Yes	No
Metabolic disturbance	Obesity Hyperlipidemia Diabetes mellitus Hypertension	Present	Absent
Other organs' status	Uterine myometrium	Myoma, internal endometriosis	No changes
	Ovaries	Hyperplasia of theca tissue Polycystic ovarian syndrome Feminizing tumors	Fibrosis
Histological features	Endometrial background	Hyperplastic processes	Atrophy
	Endometrioid differentiation	Differentiated	Poorly differentiated
	Myometrium invasion	Superficial	Deep
	Lymph node metastasis	Low	Not high
Hormone reaction	Sensitivity to progestogens	High	Not high
Clinical outcome	Prognosis	Favorable	Poor

menopause, and hyperplasia of the stroma of the ovaries and endometrium. The second pathogenetic type of EC (type II) occurs in women with no signs related to type I [14]. Type I, which accounted for 65% of cases, was associated with highly and moderately differentiated endometrioid histologies, superficial invasion of the myometrium, high sensitivity to progestogens, and favorable prognosis, whereas type II, which accounted for 35% of cases, was associated with poorly differentiated endometrioid histology, deep invasion of the myometrium, high frequency of lymph node metastasis, decreased sensitivity to progestogens, and poor prognosis (Table 10.1). From the 1990s onward, the advent of immunohistochemical, molecular, and genetic methods and further elucidation of the molecular pathology of non-endometrioid histology, primarily serous carcinoma, led to the development of the modern classification of EC from the previous dualistic pathogenetic classification of EC.

10.3 Hormonal Receptor

The hormone receptor status in EC has been evaluated by immunohistochemistry, and the findings according to the histological types have contributed to an improved understanding of the dualistic classification. It has been shown that increased

expression of estrogen receptor (ER) and progesterone receptor (PgR) in EC is associated with well-differentiated carcinoma, less myometrial invasion, and a lower rate of metastases and that these features independently predict better survival [15, 16]. This result suggests that the positivity of hormone receptors is consistent with the pathogenetic features of type I, suggested by Bokhman, which include well-differentiated endometrioid histology, less myometrial invasion, and favorable prognosis. In contrast, the loss of ER and PgR expression correlates with non-endometrioid and not endometrioid histology [17]. Serous carcinoma tends to show negativity for hormonal receptors [18], and their loss in serous carcinoma is associated with p53 overexpression [19]. Even in the endometrioid subtype, the loss of ER and PgR expression correlates with increases in the tumor grade and stage [20]. Concurrent ER/PgR loss is an independent predictor of EC recurrence [21], lymph node metastasis, and reduced disease-specific survival [22]. Therefore, the hormone receptor status has consistently been shown to be a relevant prognostic marker, even though the status shows an overlap between the dualistic pathogenetic types [23]. In addition, the hormone receptor status is of significant value to a subset of patients who will benefit from endocrine therapies.

10.4 Genetic Alterations and Endocrine Involvement

Based on an improved understanding of hormonal receptor expression in EC, the histological appearance and pathogenetic characteristics were integrated into the dualistic classifications of type I and type II EC. As result, type I frequently includes endometrioid histologies, and type II includes non-endometrioid histologies represented by serous carcinoma. Therefore, subsequent molecular studies have mainly focused on the distinct differences between endometrioid and serous carcinoma.

10.4.1 KRAS *Mutation*

Since v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) was successfully sequenced as an oncogene in 1982 [24], *KRAS* mutations have been detected in a broad range of malignant carcinomas. In the field of EC, the detection of *KRAS* mutations holds a prominent position as the beginning of the genetic study of EC. In 1990, Enomoto et al. first detected *KRAS* activity in 22% (2/9) ECs with endometrioid histology, even though these researchers did not observe *KRAS* activity in seven cervical carcinomas or four ovarian carcinomas [25]. *KRAS* mutations were also present in 12% (6/49) of ECs, which included five endometrioid carcinomas [26]. Thereafter, *KRAS* mutations have been found in 26% (15/58) of endometrioid carcinomas, which suggests that *KRAS* mutations are common in endometrioid carcinoma but not serous carcinoma [27].

10.4.2 Microsatellite Instability

Familial cancer syndromes have helped define the role of tumor suppressor genes, including mismatch repair genes, phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*), and *TP53*, in the development of EC. EC is the second most common carcinoma after colorectal carcinoma in women with hereditary nonpolyposis colon cancer (HNPCC), also known as Lynch syndrome, which is associated with microsatellite instability (MSI). Because MSI has been identified in 10–30% of sporadic colorectal carcinomas in addition to hereditary cases [28–30], sporadic ECs were also examined in the field of EC. MSI has been observed in 17% (6/36) of sporadic ECs, and all of these cases showed endometrioid histology [31]. Another study revealed that MSI can be detected in 20% (9/45) of sporadic ECs, and the study suggested that the mutation of *KRAS* might occur after MSI [32]. Accordingly, mismatch repair (MMR) genes have been considered responsible for sporadic EC associated with MSI as well as hereditary EC associated with HNPCC. Thereafter, Katabuchi et al. analyzed the association between sporadic EC with MSI and MMR genes but found that only 22% (2/9) showed somatic MMR gene mutations, which suggests that MMR genes might not be the main cause for sporadic EC with MSI [33]. After the discovery of cytosine methylation of the *MLH1* promoter region in a subset of colorectal carcinomas and cell lines of colorectal carcinoma and EC, which are negative for immunohistochemical expression with MLH1 [34], it was reported that *MLH1* is hypermethylated in 92% (12/13) of MSI-positive EC, and hypermethylation of *MSH2* has not been observed, which suggests that the hypermethylation of *MLH1* is associated with the MSI phenotype in sporadic EC [35]. Currently, the hypermethylation of *MLH1* rather than MMR gene mutations is perceived to be mainly responsible for EC with MSI, and the *MLH1* promoter is methylated in 74% (102/138) of endometrioid carcinomas with MSI [36]. MSI is found in 33% (147/446) of patients with endometrioid histology [36], which suggests that MSI is found frequently in type I. Tumor-infiltrating lymphocytes are characteristically prominent in EC with MSI, and this feature might be useful in the histological screening of tumors under consideration for MSI testing [37].

10.4.3 PTEN *Mutation*

Since the Rb gene, a tumor suppressor gene, was detected as a cause of hereditary retinoblastoma in 1986 [38], studies on loss of heterozygosity (LOH) at a specific locus in a tumor, which indicates the presence of a tumor suppressor gene in the corresponding lesion, have been performed on various types of carcinoma. LOH can be caused by the deletion of genomic DNA regions containing the normal copy numbers of tumor suppressor genes, and LOH on chromosome 10q has been identified in 40% of EC [39]. Moreover, in 1997, Cowden disease was identified as an

autosomal dominant cancer predisposition syndrome associated with an elevated risk for breast carcinoma, thyroid carcinoma, and skin carcinoma, and germline mutations in the tumor suppressor gene *PTEN* have been found to be linked to the disease [40]. Thereafter, Tashiro et al. reported that *PTEN* mutations are present in 61% (16/26) of endometrioid histologies and found no mutation in six serous carcinomas, which suggests that mutations in *PTEN* play a significant role in the pathogenesis of the endometrioid type [41]. Because 25% of atypical endometrial hyperplasia (AEH) show progression to EC, as determined during long-term observation [42], AEH was believed to be a precursor, and this finding was confirmed by subsequent studies on *PTEN* mutations. Loss of PTEN expression was detected in 83% (25/30) of endometrioid carcinomas and 55% (16/29) of precancer lesions [43], and 100% (65/65) and 22% (14/65) of *PTEN*+/− female mice develop endometrial hyperplasia and endometrial carcinoma, respectively [44], which suggests that the loss of *PTEN* is an early event in endometrioid carcinogenesis. A recent study suggests that *PTEN* mutations are frequently associated with other mutations in the phosphatidylinositol-3-OH kinase (PI(3)K)/AKT pathway, including mutations in *PIK3CA* and *PIK3R1* [45].

10.4.4 Involvement of Estrogen

The "unopposed estrogen" hypothesis has been used to explain the risk factors for type I; this hypothesis states that type I develops as a result of the mitogenic effects of estrogens and a deficiency in progesterone [46]. Accordingly, experiments using mouse models have been conducted to clarify the interaction between estrogen and *PTEN* loss in the development of EC. However, against the expectations, neonatal estrogenic exposure suppresses endometrial carcinogenesis in *PTEN*+/− female mice [47]. Similar findings were observed with *PTEN* loxP/loxP female mice with a conditional deletion in the uterus [48]. These results suggest that the development of type I EC through AEH frequently showing *PTEN* mutations might not be dependent only on estrogen [5, 49].

10.4.5 Involvement of Prolactin

Persistent hyperestrogenism and progesterone insufficiency are believed to be relevant to the pathogenesis of type I, but previous mouse experiments revealed that estrogen alone is not associated with the carcinogenesis of type I through *PTEN* mutations. Hyperprolactinemia is well known as a cause of infertility or anovulation, and these features strongly overlap with the risk factors for type I. An immunohistochemical analysis has shown that prolactin receptor (PRLR) expression in the endometrial glands is significantly higher in the proliferative phase than in the secretory phase and is correlated with ER expression during the menstrual cycle,

which suggests that the persistent secretion of estrogen and prolactin might induce abnormal proliferation of the endometrium in hyperprolactinemic women with anovulation [50]. Elevated levels of serum prolactin were recently identified in patients with EC [51, 52]. Approximately half of women with early-stage EC under 41 years of age showed hyperprolactinemia [53]. Hyperprolactinemia is significantly observed in women without polycystic ovary syndrome [54]. An analysis of all-aged patients with type I EC revealed that hyperprolactinemic women are significantly younger and that their insulin resistance is significantly lower than that of women without hyperprolactinemia [55]. In cell lines of both endometrial glands and low-grade endometrioid carcinoma, the expression of PRLR and ER is increased after the addition of prolactin, and increased proliferation can be induced by the addition of both prolactin and estrogen [50]. In cancer tissues of type I EC, the expression of PRLR is significantly higher, and the rate of loss of PTEN is significantly lower in hyperprolactinemic women than in women without hyperprolactinemia [55]. In conclusion, hyperprolactinemia might be an independent risk factor for young patients with type I EC who do not show obesity or insulin resistance. In these women, prolactin-PRLR signaling might play a crucial role in the progression of type I EC without involvement of the *PTEN* mutation.

10.4.6 **TP53** *Alterations*

TP53 was recognized as a tumor suppressor gene in 1989 [56], and germline *TP53* mutations have been identified primarily in patients with Li–Fraumeni syndrome, which is a complex hereditary cancer predisposition disorder that was associated with early-onset cancers in diverse tissues of origin in 1990 [57]. The rate of immunohistochemical overexpression of p53 in non-endometrioid histology is 38% (12/32), which is significantly higher than that of 13% (10/75) found in endometrioid histology. p53 overexpression is more frequent at advanced stages and is associated with positive peritoneal cytology, extrauterine metastases, and a negative PgR status [58]. These results suggest that *TP53* mutations might be associated with the carcinogenesis of non-endometrioid carcinoma, primarily serous carcinoma. To determine whether the alteration of the *TP53* gene was an early event in the carcinogenesis of EC, the *TP53* gene was examined in endometrial hyperplasia because endometrial hyperplasia is believed to be a precursor of EC. However, none of 117 endometrial hyperplasias were found to have mutations in the *TP53* gene [59], which suggests that endometrial hyperplasia might not be associated with an early event in non-endometrioid histology. In contrast, endometrial intraepithelial carcinoma (EIC), which is characterized by replacement of endometrial surface epithelium and glands by malignant cells that resemble invasive high-grade endometrial carcinoma, has been identified in 89% (34/38) of serous carcinomas and 6% (7/113) of endometrioid carcinomas, which suggests that EIC is a precursor of serous carcinoma [60]. Abnormal immunostaining for p53 was detected in 86% (24/28) of serous carcinomas and 79% (27/34) of EIC but 20% (9/45) of endometrioid

carcinomas [61]. To determine whether abnormal immunostaining for p53 correlates with *TP53* gene mutation, a subsequent gene analysis by Tashiro et al. revealed that *TP53* mutation was present in 76% (16/21) of serous carcinomas and 89% (8/9) of EICs. The presence of *TP53* gene mutations in EIC further suggests that *TP53* alteration plays an important role in the early pathogenesis of serous carcinoma and potentially accounts for its aggressive biological behavior [62].

10.5 The Cancer Genome Atlas Classification

The previous studies conducted in the 1990s and 2000s were limited to DNA sequencing based on the dualistic classification of type I and type II. In 2013, the Cancer Genome Atlas (TCGA) newly classified 373 ECs, including low-grade endometrioid, high-grade endometrioid, and serous carcinoma, through an integrated genomic and proteomic analysis. After sequencing the exomes, ECs were categorized into four groups based on a combination of somatic nucleotide substitutions, MSI, and extensive somatic copy number alterations (SCNAs): (1) an ultramutated group with unusually high mutation rates and a unique nucleotide change spectrum (POLE ultramutated), (2) a hypermutated group of MSI tumors, most of which exhibit *MLH1* promoter methylation (MSI hypermutated), (3) a group with a lower mutation frequency and most of the microsatellite stable (MSS) endometrioid carcinomas (copy-number low), and (4) a group that consists primarily of serous-like carcinomas with extensive SCNAs and a low mutation rate (copy-number high) [63].

10.5.1 POLE (Ultramutated)

The major catalytic and proofreading subunits of the Polε DNA polymerase enzyme complex involved in nuclear DNA replication and repair are encoded by polymerase epsilon (*POLE*) [64]. The TCGA newly identified 7% (17/232) of a subset of endometrioid carcinomas exemplified by an increased C-to-A transversion frequency, all with mutations in the exonuclease domain of *POLE*, and an improved progression-free survival. This subtype is also characterized by a robust intratumoral T cell response, which is considered a cause of an enrichment of antigenic neopeptides [65]. The prevalence of POLE mutations and the frequent mutation sites in EC vary among races [66]. This subtype is strongly associated with high-grade endometrioid histology [67–69] and is frequently accompanied by AEH [66, 70, 71]. *TP53* mutations occur in 35% of the subtype, even though they are associated with an excellent prognosis [72]. However, the subtype frequently shows multiple *TP53* mutations and immunohistochemical features of subclonal abnormal p53 patterns, which reflects its heterogeneity [73]. It is believed that a *POLE* mutation might act as a driver mutation in the carcinogenesis process, and subsequent *TP53* variants that do

not affect biological behavior might likely occur as a passenger event [66, 73], even though *TP53* might act as a driver gene in serous carcinoma [61]. Accordingly, in clinical practice, when determining the therapeutic management of patients with high-grade endometrioid carcinoma with abnormal p53 expression, it would be desirable to discriminate patients in this subtype from those in the copy-number high subtype because patients in both subtypes will exhibit considerably different prognoses [66].

10.5.2 Microsatellite Instability (Hypermutated)

The TCGA showed that MSI was found in 40% of endometrioid carcinomas, including grade 1–3, and 2% of serous carcinomas. Twenty-eight percent (65/232) of EC with all histological types were classified into a microsatellite instability subgroup, in which *MLH1* mRNA expression was decreased, probably due to its promoter methylation. These results were consistent with those obtained in previous studies, as shown in the preceding paragraph. The MSI endometrioid tumors had a mutation frequency that was approximately tenfold higher than that of MSS endometrioid tumors, few SCNAs, and few mutations in *TP53*. *ARID5B*, a member of the same AT-rich interaction domain (*ARID*) family as *ARID1A*, was more frequently mutated in this group (23%) than in the other groups. In addition, mutations in *PIK3CA* and *PIK3R1* in the PI(3)K/AKT pathway cooccurred with *PTEN* mutations in this subtype [63].

10.5.3 Copy-Number Low (Endometrioid)

Thirty-eight percent (90/232) of EC cases were classified into a copy-number low subgroup, which shows MSS and includes more than half of low-grade endometrioid tumors. The subtype also shows increased PgR, which is suggestive of responsiveness to hormonal therapy. Fifty-two percent of *CTNNB1* is mutated, and this gene exhibits the highest mutation frequency in the subtype. Moreover, mutually exclusive alteration patterns of gene networks contained *CTNNB1*, *KRAS*, and *SOX17*, which suggests that WNT signaling is activated in this subtype. In addition, the concomitant mutation of *PTEN* and genes related to the PI(3)K/AKT pathway was also observed in this and the MSI subtype, which suggests that both subtypes are approximately equivalent to type I in the dualistic classification scheme [63].

10.5.4 Copy-Number High (Serous-Like)

Twenty-five percent (60/232) of EC cases were classified into a copy-number high subgroup, which contained the cases of serous carcinoma and 25% of grade 3 endometrioid carcinoma cases. In addition to the frequent mutation of *TP53*, *FBXW7*, and *PPP2R1A* mutations were frequently observed, which is consistent with the mutation patterns reported in serous carcinoma but not endometrioid carcinoma [74]. Decreased levels of phosphor-AKT, which are consistent with downregulation of the AKT pathway, were found in the subtype. This subtype also exhibited few DNA methylation changes, low hormonal receptor expression, and the poorest outcome among the four subgroups, which suggests that the copy-number high subtype is approximately equivalent to type II in the dualistic classification scheme.

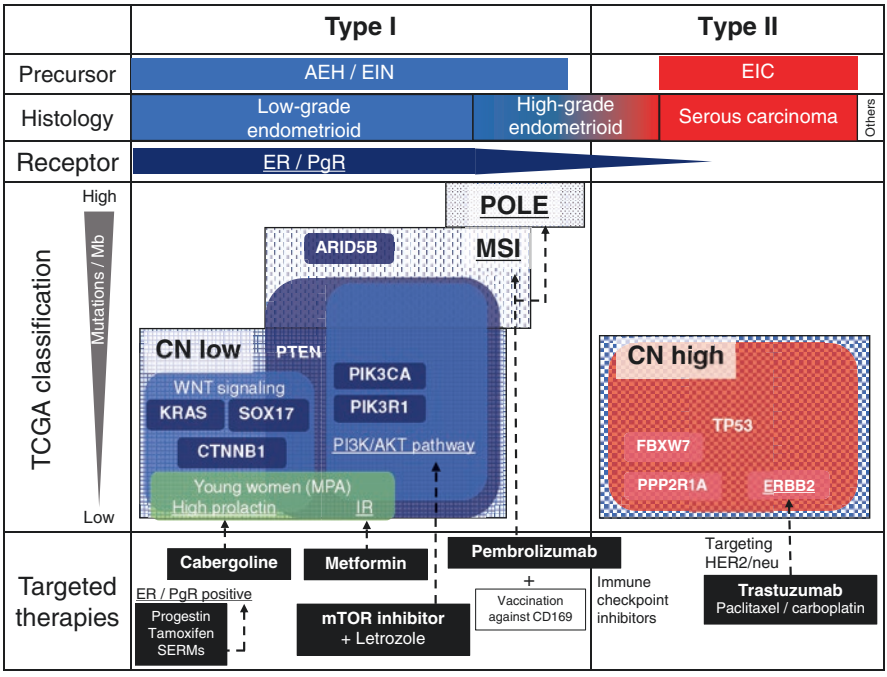

Fig. 10.1 Dualistic types, TCGA classification, and potential targeted approaches in endometrial carcinoma. Underlines indicate therapeutic targets. AEH, atypical endometrial hyperplasia; EIC, endometrial intraepithelial carcinoma; ER, estrogen receptor; PgR, progesterone receptor; CN, copy-number; IR, insulin resistance

10.6 Potential Targeted Therapies

Clinicopathological studies using molecular pathology methods that were conducted over the past 30 years have gradually contributed to clarifying the diversity of EC, which had not been previously elucidated based only on histological studies. A more comprehensive understanding of the molecular alterations and signaling pathways in EC has recently led to the establishment of novel targeted approaches for the personalized treatment of EC (Fig. 10.1).

10.6.1 Immune Checkpoint Inhibitors

MMR deficiency and MSI-high tumors are associated with increased somatic mutation and higher neoantigen loads, which results in increased tumor infiltration by cytotoxic T cells. Accordingly, a large number of somatic mutations due to MMR defects are thought to be susceptible to immune checkpoint blockade [75]. It has been reported that a large proportion of mutant neoantigens in MMR-deficient carcinomas are sensitive to immune checkpoint blockade, regardless of the type of carcinoma [76]. In 2017, the Food and Drug Administration approved pembrolizumab, an anti-PD-1 drug, for the treatment of recurrent MMR-deficient or MSI-high tumors. This approval provides a benefit peculiarly to patients with EC because the rate of MMR deficiency is highest in patients with EC compared with patients with various other types of carcinoma [76]. A similar response is also expected in the POLE subtype of EC due to its ultrahigh mutation burden [77].

Tumor-infiltrating lymphocytes, which as known as a feature of EC with MSI, are positively correlated with an increased number of CD169-positive sinus macrophages in the pelvic regional lymph nodes, which act as antigen-presenting cells to stimulate the antitumor immune response [78]. Vaccination against CD169, which might induce a carcinoma antigen-specific T cell response, combined with immune checkpoint inhibitors is a potential strategy for the induction of clinical reactions [79].

10.6.2 Endocrine Therapies

Hormonal therapies, including progestin, tamoxifen, and selective estrogen receptor modulators, are frequently used in the treatment of advanced EC, particularly in the treatment of patients with low-grade and ER- and/or PgR-positive disease. A systematic review and meta-analysis concluded that hormonal therapies are associated with modest objective response rates in the treatment of advanced EC and that the greatest benefits are observed in ER- and/or PgR-positive tumors [80].

The increasing incidence of EC in young women remains another issue to be resolved because these individuals desire to preserve their uterus. Progestin therapy, including medroxyprogesterone acetate (MPA), has been reported to be effective for young women with early-stage EC who desire to maintain their fertility; however, the risk of disease relapse and exacerbation during the observational period remains high [81, 82]. Accordingly, novel strategies are needed. Dienogest, which was developed as a fourth-generation progestin for endometriosis, has demonstrated anticancer activity against endometrial neoplasms that is equivalent to that of MPA in a mouse model [83]. Metformin has been found to be a potent inhibitor of cell proliferation in EC cell lines by inhibiting the mammalian target of the rapamycin (mTOR) through the activation of AMP-activated protein kinase [84]. Fertility-sparing clinical trials to evaluate the efficacy of progestin with metformin are currently in progress worldwide [85, 86]. A retrospective study showed that the combination of MPA and cabergoline, an anti-prolactin drug, contributes to extending the estimated mean period until hysterectomy in young hyperprolactinemic patients with early-stage EC [53], but further studies are necessary to establish the efficacy of the therapy.

10.6.3 Other Targeted Therapies

The mTOR pathway in coordination with PI(3)K/AKT pathway plays a pivotal role in endometrial carcinogenesis. Therefore, the efficacy of mTOR inhibitors, including everolimus, temsirolimus, and ridaforolimus, continue to be examined. A multicenter, single arm, phase II study recently reported the effectiveness of everolimus, letrozole, and metformin for women with recurrent endometrioid EC particularly with the expression of PgR [87].

The TCGA showed that the *ERBB2* oncogene, which encodes human epidermal growth factor receptor 2 (HER2/neu), was focally amplified by protein overexpression in 25% of serous or serous-like tumors. A multi-institution prospective randomized phase II clinical trial demonstrated that the addition of trastuzumab, a humanized, recombinant monoclonal antibody that binds to HER2, to carboplatin–paclitaxel therapy increased the progression-free survival of patients with advanced or recurrent uterine serous carcinoma [88].

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Chapter 11
Novel Approach for Therapeutics of Cervical Cancer Based on HPV-Associated Carcinogenesis at the Cervix

Kei Kawana, Osamu Kobayashi, Takahiro Nakajima, Takehiro Nakao, Yuji Ikeda, Mikiko Asai-Sato, and Fumihisa Chishima

Abstract High-risk human papillomavirus (HPV) is associated with the carcinogenesis of not only cervical cancer but anal, penile, vulvar, vaginal, and oropharyngeal cancers. Although molecular biological mechanisms of high-risk HPV (HR-HPV)-associated carcinogenesis is well studied, it remains unclarified why cervical cancer is the most common among these HPV-associated cancers. Two major causes are that the cervix is a susceptible site to viral infection because of its immune deficiency to protect allogenic sperm in reproductive function and that the squamocolumnar junction (SCJ) where cervical neoplastic diseases develop is composed of tissue stem cells with self-renewal and pluripotency. This specific environment of the cervix allows HPVs to be persistently infected into the cervical epithelial cells, followed by the immortalization of the HPV-infected cells. We here focused on the carcinogenesis specific to the cervix as novel therapeutic strategies for cervical cancer, targeting cancer stem cells and mucosal immunotherapy.

Keywords Cervical cancer · Human papillomavirus · Cancer stem cell Squamocolumnar junction · Mucosal immunity · HPV therapeutic vaccine

11.1 Epidemiology of HPV-Associated Cancers and Cancer Prevention

Cervical cancer is the second most common cancer in women worldwide. Over 95% of cervical cancer is caused by high-risk human papillomavirus (HR-HPV) infection. High-risk HPVs are also reported to be the cause of about 90% of anal cancer,

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40% of vaginal cancer, 60% of pharyngeal cancer, 40% of vulvar cancer, and 50% of penile cancer [1].

In Japan, the age-adjusted mortality rate of various cancers, including the five major cancers, has been decreasing in the last two decades, but that of only cervical cancer is increasing since about 10,000 women per year develop and 2500 women die from cervical cancer. The incidence of cervical cancer in Japan is increasing although the HPV vaccine is implemented into the national immunization program. HPV vaccine can protect against sexual transmission of HPV16 and 18, the most oncogenic types and prevention of high-risk HPV infection is the most fundamental cancer prevention. HPV vaccine is implemented in 2007 worldwide and recently HPV vaccine has been reported to have a great population impact on the prevention of HPV-associated cancers [2]. In the subjects of clinical trials of the 4-valent HPV vaccine, the precursor or precancer lesions of cervical cancer are not found during 14 years from 2007 [3]. World Health Organization (WHO) has declared at the board in 2019 that it will eliminate cervical cancer in the world by 2060 [4]. However, it will be 40 years later that cervical cancer will be eliminated even if the HPV vaccine is implemented worldwide. Low-income countries have not yet implemented the HPV vaccine due to the limitation of financial issues.

Furthermore, as for Japan, the Ministry of Health, Labor and Welfare has suspended proactive vaccination of HPV vaccine because of the reporting of various adverse events after vaccination. Japanese citizens hesitant to vaccinate the HPV vaccine due to this government policy. Although the safety and efficacy of the HPV vaccine was confirmed by epidemiological studies in Japan, the government has not changed its position on HPV vaccination [5].

Taken together, the development of a new therapeutic agent for precursor lesions of cervical cancer, cervical intraepithelial neoplasia (CIN), is a medical need worldwide even after the implementation of HPV vaccination.

11.2 Mechanisms and Feature of HPV-Associated Carcinogenesis

Genital HPVs infect various mucosal sites, including the cervix, penis, vagina, vulva, anus, and oropharynx, by sexual transmission and are widespread worldwide regardless of gender. Among genital HPVs, about 13 genotypes are high-risk (oncogenic) HPVs that can transform the infected cells to malignant cells [6]. Therefore, HPV-associated cancers can develop anywhere on the infected sites; cervical, penile, vaginal, vulvar, anal, and oropharyngeal cancers.

Numerous molecular biological studies have demonstrated the mechanisms by which HPV infection occurs carcinogenic change in the infected epithelium [6]. When HPV E6 and E7 oncogenes of HR-HPV are expressed strongly and ubiquitously in the infected cell, the function of p53 and Rb anti-oncoproteins is suppressed, hTERT is inactivated, and the cell cycle is accelerated. These actions suppress apoptosis and promote the immortalization of the infected cells. The epithelium consisting of the immortalized cells is histologically diagnosed as

high-grade intraepithelial neoplasia, a precancer lesion, with disorder of polarity and proliferation of undifferentiated cells. The immortalized cells can acquire malignant features by chromosomal instability and finally invasive cancers develop. It is a feature of HPV-associated carcinogenesis that molecular biological change correlates to pathological change of the epithelium.

Among HR-HPVs, HPV types 16 and 18 are the viruses with the highest risk of cervical cancer. The odds ratio of developing cervical cancer is 434 times higher in women infected with HPV16 and 248 times higher in women with HPV18, compared with HPV-negative women [7]. Seventy to ninety percent of HPV-associated cancers are caused by HPV16 or 18. Notably, 90% of cervical cancer patients of 20–40 years old are caused by HPV16 or 18 [8], meaning that HPV16 and 18 infected cells are rapidly immortalized and easy to transform into malignant cells. We have estimated the fate of CIN using the Markov model, an epidemiological predictive simulation model, and a large scale of retrospective cohort of CIN patients and we found that HPV16-positive CIN patients most frequently had a progression to the invasive cancer when compared with other HR-HPVs [9].

11.3 Specificity of HPV-Associated Carcinogenesis to the Cervix

Cervical cancer develops most frequently and most quickly after infection among HPV-associated cancers although the epithelium of cervix, vulvar, vagina, penis, anus, and oropharynx are similarly exposed to HR-HPVs. This suggests that the cervix has unique characteristics in HPV-associated carcinogenesis, which allow persistent infection with HPV and result in the infected cells becoming more susceptible to tumorigenesis. CIN and cervical cancer arise from transformation zone (TZ) including the squamocolumnar junction (SCJ) of the cervix as shown in Fig. 11.1. SCJ is the unique site consisting of tissue stem cells, called “reserve cell,” which retain embryonic features such as self-renewal and pluripotency. Herfs and Crum et al. examined immunologically the features of the embryonic cervix of fetus at 16, 18, and 20 gestational weeks and showed that the stem cell markers p63, keratin 5, and keratin 7 were strongly expressed throughout the TZ. Like the fetal cervix, it was shown that these markers remained in the cervix of adult females, consistent with SCJ [10]. This indicates that SCJ retains the properties of embryonic stem cells even in adulthood, and has the ability to differentiate into “squamous” and “columnar” epithelium (pluripotency) and self-renewal (Fig. 11.1b). Interestingly, the most susceptible and favorable site to HPVs is the TZ and SCJ among the cervix. The proliferative infection of HPVs is dependent on squamous differentiation [11]. The tissue stem cells in the SCJ can spontaneously differentiate into squamous epithelium, which is called squamous metaplasia, and infection of HPVs to the stem cells seems to promote the differentiation [10]. In contrast, the squamous differentiation provides DNA replication of the HPV genome [11], and thereby SCJ is a really suitable site for the proliferation of HPVs.

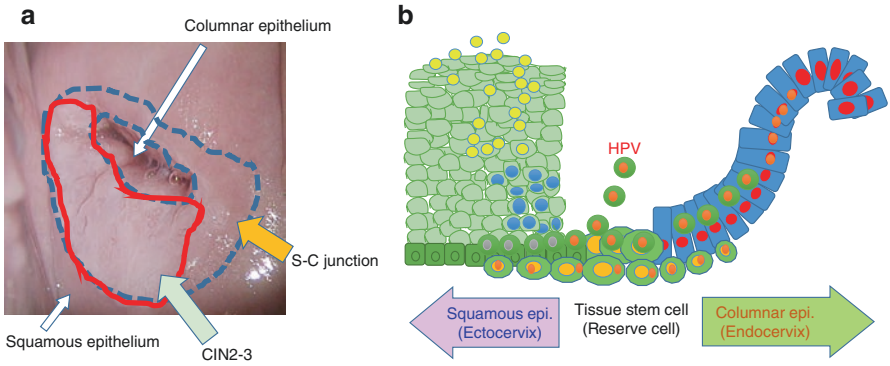

Fig. 11.1 (**a**) Squamocolumnar junction (SCJ) and cervical intraepithelial neoplasia (CIN); a picture shows a representative CIN lesion (white epithelium) that arises from the SCJ. The SCJ is the border region between the squamous and columnar epithelium on the mucosa of the cervix. CIN is likely to arise in the SCJ. (**b**) Tissue stem cells (called reserve cells) and HPV infection in the SCJ; Cell populations, called reserve cells, in the SCJ maintain the stem cell features expressed from embryonic stages [5]. Reserve cells have the pluripotency to differentiate into squamous (ectocervix) and columnar (endocervix) epithelium, and they are also capable of self-renewal. This is where HPVs prefer to infect, as they can use the stem cell features to maintain their own persistent infection of HPVs

Since the tissue stem cells locating at the SCJ possess the self-renewal potential, HR-HPVs that once infected into the cells can persistently retain viral genome there and HPV viral genes are expressed when SCJ move toward differentiation to squamous metaplasia for proliferation. These mechanisms lead to persistent infection of HPVs at the cervix alternating latent and proliferative infections, by which HPVs can evade immunological clearance.

The above-mentioned HPV-associated carcinogenesis begins when the oncogenes are accidentally integrated into the host genome in the proliferative infection and over-expressed in a disorderly manner [6]. Interestingly, Hu et al. demonstrate by whole-genome sequencing that the overexpression of oncogenes occur without integration in some cases [12]. It is confirmed that persistent proliferative infection, which is persistently positive for HR-HPV DNA, is the most critical risk factor for the development of cervical cancer and SCJ is a unique site to favor such infection and transformation toward cancer.

11.4 A Possible Therapeutic Strategy Targeting Cancer Stem Cells of Cervical Cancer

Heterogeneity is an important theory for understanding cancer behavior and biology of all cancers and is demonstrated in various gynecologic cancers at the cell line level [13, 14]. On the other hand, cancer stem cells (CSC) are focused on various cancers. Numerous studies on CSC have demonstrated that CSC possess specific characteristics distinct from cancer cells: stem cell-like features such as self-renewal

and pluripotency, malignant features such as metastasis/recurrence, therapy-resistance, anti-apoptosis, and specific metabolism [14]. CSC is thought to be a unique population among the "heterogeneity" of cancer.

In CSC research of cervical cancer, cultured cells positive for some stem cell markers (ALDH1 and CD44 variant 6, etc.) or sphere-forming cells (spheroid) derived from cervical cancer cell lines are often used as a substitute for CSC or cancer stem-like cells. We have demonstrated sphere-forming cells of cervical cancer cell lines are positive for ALDH1 [15]. On the other hand, we focused on the development of cervical cancer from SCJ as mentioned above (Fig. 11.2). To study on CSC of cervical cancer, we have first generated a novel cervical tissue stem cells locating at SCJ, called "reserve cells," from induced pluripotency stem (iPS) cells [16, 17]. The iPS cell-derived reserve cells we have generated (called induced reserve cells: iRCs) have pluripotency to differentiate into squamous and columnar epithelium and express the cervical stem cell markers in SCJ described above. Furthermore, by transducing HPV16 or 18 oncogenes, E6 and E7, into the iRC cells, the iRCs were immortalized by these oncogenes (called 16E6/E7-iRC and 18E6/E7-iRC). Since the stem cells of the SCJ mimicked by the iRCs are likely to transform into CSC of cervical cancer by HPV oncogene expression, E6/E7-iRCs we generated may show the original features of CSC derived from the SCJ (Fig. 11.2). Then, by using HPV16/18 E6/E7-iRCs, we plan to address the

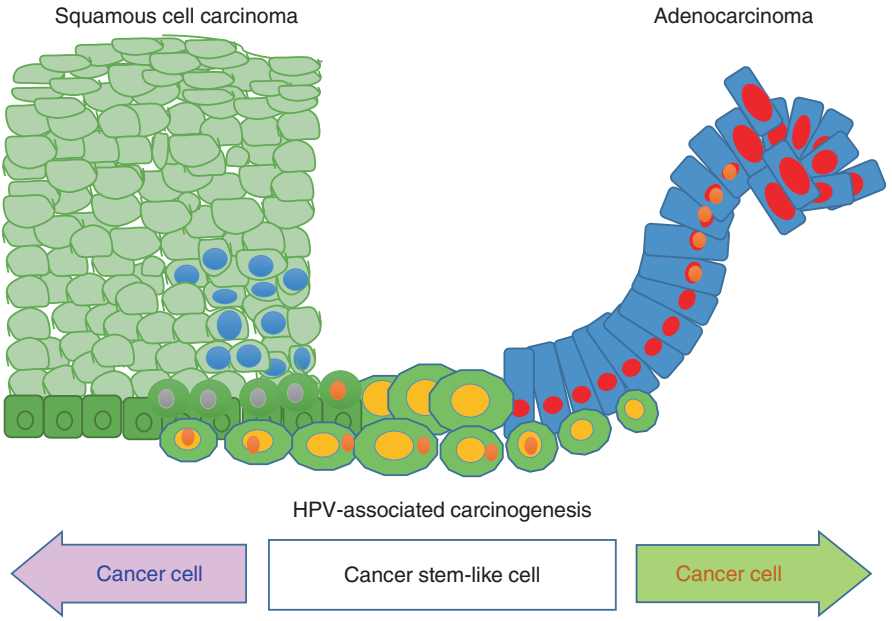

Fig. 11.2 Hypothesis of cancer stem cells (CSCs) of cervical cancer derived from HR-HPV-infected reserve cells; when HR-HPV-infected reserve cells become immortalized and transform to malignant cells, they become cancer cells with stem cell features. Unlike the process of CSC formation from cancer cells, the direct formation of CSCs by HR-HPVs may be related to the rapidity of cervical cancer carcinogenesis

candidates for the development of therapeutic agents targeting the original CSC of cervical cancer. It is well known that HPV18 causes cervical cancer with the highest risk ratio and is most likely to cause adenocarcinoma among HR-HPVs [7]. These clinical features of HPV18-associated cancer might be explained by the characteristics of HPV18 different from those of other HR-HPVs including HPV16. We now try to examine the difference between HPV16- and 18-carcinogenesis using HPV16 and 18 E6/E7-iRCs and focusing on stem cell-like features. The E6/E7-iRCs provides new insights to explore therapeutic strategy targeting CSC of cervical cancer.

We have approached comprehensive gene expression of each E6/E7-iRCs using RNA sequencing and some interesting genes that overexpress in 18E6/E7-iRCs but 16E6/E7-iRCs are not found. Furthermore, the TCGA database shows some genes of interest are expressed higher in HPV18-associated cervical cancer tissue compared with HPV16-associated one (unpublished data). Interestingly, the gene is barely expressed in human keratinocytes immortalized by HPV18 E6/E7, suggesting the gene expression is enhanced by HPV18 E6/E7 only in the stem cell-like cells (iRCs). The suppression of the gene expression by the siRNA method downregulated cell proliferation of E6/E7-iRCs. The gene might be a target gene against CSCs of cervical cancer.

11.5 Therapeutic for Precancer Lesion of Cervical Cancer Is an Unmet Need

Preventing the infection of HR-HPVs is the most fundamental cancer prevention, and HPV vaccines for the prevention of infection are having a major impact. Although it is expected that HPV vaccines will be able to eradicate cervical cancer in the future, at present, HPV vaccination rates are limited in many countries and regions (due to cost issues), and it will take some time before the global elimination of cervical cancer and to eradicate cervical cancer worldwide. The development of therapeutics to treat precursor lesions (CIN) is still necessary even now that the HPV vaccine has been implemented worldwide.

Surgical resection is the only treatment that can be given for early cervical cancer and its precancerous lesions (CIN2-3), which peak in the 20s and 30s. At present, there are no pharmacologic treatments. Hysterectomy terminates fertility, and cervical and conization worsen obstetrical outcomes in subsequent pregnancies. In other words, the risk of preterm birth is approximately three times higher at the time of pregnancy after conization, and the rate of cesarean delivery and low birth weight are also increased approximately threefold [18]. Since the age at which CIN2-3 develops in a woman coincides with the age at which she becomes pregnant and gives birth, the worsening of obstetrical outcomes through cervical incompetence due to partial resection of the cervix is a major issue for the reproductive health of young women. Therefore, the development of a therapeutic agent for CIN as a nonsurgical treatment for CIN2-3 is an unmet medical need.

11.6 Immunotherapy Targeting HPV Molecules Is a Promising Therapeutic Strategy

The ubiquitous and overexpression of HR-HPV oncoproteins, E6 and E7, in cervical epithelial cells leads from CIN2-3 to cervical carcinoma. E6 and E7 are essential viral proteins for the progression of CIN to cervical cancer and maintenance of HPV-associated cancer [6]. On the other hand, E7 is known to be highly immunogenic in humans while E6 is less likely to induce immune responses in humans. Therefore, HR-HPV E7 is not only a viral protein but a "tumor antigen" of HPV-associated cancer, suggesting that E7 is the most definitive tumor antigen and the target molecule of immunotherapy in the case of HPV-associated cancer, including cervical cancer.

Previous prospective cohort studies on the natural history of CIN have demonstrated that CIN regresses spontaneously by host immune responses. Matsumoto et al. reveal that approximately 70% of CIN1 and 50–60% of CIN2 patients will spontaneously regress within 2 years of follow-up [19]. Another study shows about 20% of CIN3 regresses within 2 years of follow-up with no intervention [20]. Since CIN1 expresses HPV E2 protein whereas CIN2-3 expresses HPV E7 and E2 and E7 are immunogenic for humans, these antigens are recognized by host immune cells and TH1 immune responses occur followed by immunological clearance. This process of spontaneous regression could be used to develop a novel noninvasive therapeutic strategy for CIN, referred to as cancer immunotherapy or HPV therapeutic vaccine. Immunization with CIN patients by various vaccine carriers expressing HPV molecules (E7, E6, and E2) can elicit HPV-specific TH1 cellular immunity to eliminate CIN or cervical cancer.

A number of clinical trials (Phase I–III trials) of various HPV therapeutic vaccines have been conducted for treatment of CIN2-3 since the 1990s, the majority of which have used HPV E7 as the target molecule (Table 11.1) [21]. Immunologists and gynecologists have considered that the immunotherapy targeting HPV E7 is a promising therapeutic agent to treat CIN based on its natural history. In earlier trials, vaccine antigens were administered either intramuscularly or subcutaneously to induce E7-specific cell-mediated immunity (E7-CMI) in the peripheral blood of immunized patients. However, their immune responses do not always correlate with clinical efficacy, and none have been applied clinically at this time. The U.S. phase III trial (VGX-3100, Invio) and our phase I/II trial (IGMKK16E7) are ongoing (bold in Table 11.1). Trimble et al. have reported that the plasmid DNA vaccine, VGX-3100, is intramuscularly administered to 167 patients with HPV16-positive CIN2-3, in a randomized placebo-controlled trial [22]. The regression to normal was observed in 48% of the VGX-3100 group and 30% of the placebo group with a significantly higher regression rate in the VGX-3100 group ($p = 0.034$). However, CIN2 patients are enrolled in 30% of the VGX-3100 and 26% of the placebo group in this trial. Since CIN2 is likely to regress spontaneously compared with CIN3, the difference in patients background might influence the result. Although there was a significant difference in clinical efficacy, adverse events at the inoculation site occurred in 98% of cases due to intramuscular injection. The VGX-3100 is currently in a phase III trial in the USA.

Table 11.1 Previous and ongoing clinical trials of HPV therapeutic vaccines: Since the 1990s, many clinical trials of HPV therapeutic vaccines have been conducted, but no agents are available clinically. Two clinical trials are ongoing in 2020

Phase	Target molecule	Vaccine carrier	Route	Disease	Developers
Ph-I/II	L1, E7	Chimera-VLP	Subcutaneous	CIN2-3	NCI
Ph-II	E7	Hsp-fusion protein	Subcutaneous	CIN2-3	Stressgen
Ph-I/II	E6, E7	Vaccinia virus	Subcutaneous	Cervical cancer	Xenova
Ph-II	L2, E6, E7	L2E6E7 fusion protein	Intramuscular	CIN2-3	Xenova
Ph-IIb	E6, E7	Plasmid DNA	Intramuscular	CIN2-3	Zycos
Ph-IIb	E7	Vaccinia virus	Intramuscular	CIN2-3	Roche
Ph-I	E6, E7	Plasmid DNA	Intramuscular	CIN2-3	VGX
Ph-IIb	E6, E7	Plasmid DNA	Intramuscular	CIN2-3	Inovio
Ph-III	**E6, E7**	**Plasmid DNA: VGX-3100**	**Intramuscular**	**CIN2-3**	**Inovio**
Ph-I/IIa	E7	Lactobacillus: GLBL101c	Oral	CIN3	Authors
Ph-IIb	E7	Lactobacillus: GLBL101c	Oral	CIN2	Authors
Ph-I/II	**E7**	**Lactobacillus: IGMKK16E7**	**Oral**	**CIN2-3**	**Authors**

11.7 Development of a Novel Therapeutic Agent Targeting HPV E7 Using Mucosal Immunity

Noting that CIN is an intraepithelial lesion locating at the cervical mucosa, we hypothesized that mucosal immunity to HPV E7 must be induced in order to immunologically eliminate the mucosal lesion. In the mucosal immune system, the Peyer's patches (GALT: gut-associated lymphoid tissues) or mesenteric lymph nodes are known to be the inductive sites for the genital mucosa including the cervical mucosa (Fig. 11.3). Gut-derived mucosal lymphocytes are recruited and activated at GALT and mesenteric lymph nodes home to the genital mucosa via the peripheral blood. The mucosal lymphocytes have a unique surface antigen called integrin β7 which binds to its natural ligands (MadCAM) expressed at the endothelial cells of the mucosal vessels, and infiltrate into the mucosa. The integrin β7 also binds to E-cadherin expressed at the cervical epithelium and mucosal lymphocytes can accumulate the epithelium. We have previously revealed that approximately 20–40% of CD3+ T cells in the cervical epithelium of CIN patients were integrin β7+ T cells that are gut-derived and furthermore, we found that CIN was more likely to regress when the content of integrin β7+ T cells was high [23]. Thus, we considered that memory helper and killer T cells educated in the gut mucosa can infiltrate into CIN2-3 lesions, and TH1 immune cells activated by HPV E7 antigen will recognize CIN2-3 cells and induce TH1 immune responses to eliminate the lesion.

We have developed a new HPV16 E7-targeting therapeutic vaccine using *Lactobacillus casei* (*L. casei*) that is known to have an adjuvant effect on TH1

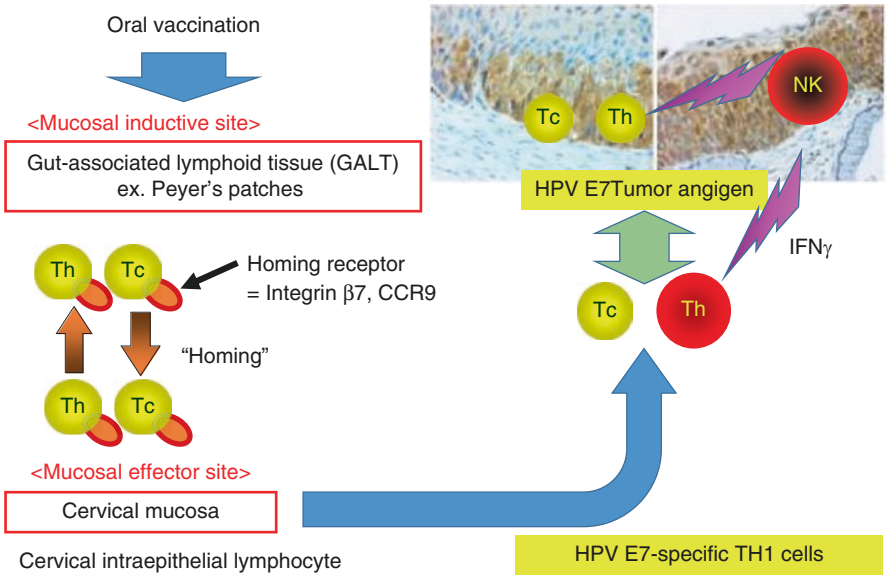

Fig. 11.3 Mechanism of HPV E7-targeting mucosal immunotherapy to treat CIN2-3; Oral administration of E7-exposed lactobacillus-based therapeutic vaccine elicits E7-specific mucosal Th1 cells at the mucosal inductive site (GALT: Peyer's patches etc.). E7-specific TH1 cells home through the peripheral blood to the mucosal effector site (cervical mucosa) and infiltrate the mucosal epithelium and recognize and activate E7-overexpressed CIN2-3. This leads to an E7-specific TH1 immune response and immunological clearance of CIN2-3

immune responses. Our concept of the therapeutic vaccine is that oral administration with the agent provides induction of mucosal TH1 immune response to HPV16 E7 at the GALT and the mucosal T cells infiltrate into the CIN2-3 lesions followed by immunological clearance by E7-specific TH1 immune responses including natural killer activity (Fig. 11.3). *L. casei* is safe because it is already used as a lactic acid-based beverage and the immunization route is oral administration of capsule tablets, which is a completely different route of administration than the other HPV therapeutic vaccines mentioned above.

The first lactobacillus-based vaccine generated was GLBL101c which expressed the HPV16 E7 gene on the cell surface [24]. But the number of E7 molecule expressed on the cell surface was not optimized. Then, we generated a second-generation lactobacillus-based vaccine on which the maximum amount of HPV16E7 molecules is expressed, called IGMKK16E7. Oral immunization of mice with these agents have demonstrated that the ability of IGMKK16E7 to induce the number of IFNγ-producing cells in response to E7 was approximately four times greater than that of GLBL101c [25].

After these preclinical studies, we conducted an exploratory Phase I/IIa clinical study with the approval of the Research Ethics Review (IRB) Committee. Patients were treated with GLBL101c once a day for 5 days a week for 1, 2, 4, and 8 weeks

in CIN3 patients with HPV16 positive. In all 17 patients, there were no grade 2 or higher adverse events and none of the grade 1 adverse events were causally related to GLBL101c. The regression rate of CIN3 to CIN1/normal was 38.4% in the first 12 months of treatment, which was clearly higher than the rate of spontaneous regression (about 10% per year). In addition, the group that regressed to CIN2 or less clearly had a higher induction of mucosal E7-specific IFNγ-producing cells into cervical intraepithelial lymphocytes than the non-regressed group [26]. Next, we conducted a randomized, placebo-controlled double-blind Phase IIb clinical trial of GLBL101c to treat HPV16-positive CIN2. Compared to the placebo group, there was no difference in adverse events and safety was confirmed although the trial did not show the significant clinical efficacy on regression of CIN2 (in preparation for submission).

Since the first generation GLBL101c used in these two exploratory clinical studies was considered to have limited pharmacological efficacy, we developed a next-generation agent, IGMKK16E7, mentioned above [25]. Then, in June 2019, a phase I/II physician-initiated clinical trial of IGMKK16E7 began in HPV16-positive CIN2-3 in four groups; placebo, low dose, medium dose, and high dose (1:1:1:1). This was a multicenter study conducted at our hospital and other university hospitals, with a target enrollment of 164 patients (124 with CIN3 and 40 with CIN2). The primary endpoint was pathological remission (CR = normal, PR = CIN1, SD = CIN2-3, PD = invasive cancer), and the protocol was designed to assess efficacy at 16 weeks from the start of treatment [27]. We plan to conduct a Phase III trial after proving the efficacy of IGMKK16E7.

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Chapter 12
Hereditary Gynecological Malignancy and Molecular Features

Hideki Yamamoto and Akira Hirasawa

Abstract Hereditary gynecological malignancy constitutes a group of women's cancer syndromes caused by constitutional genetic variants, which carry inherited susceptibility to certain pelvic epithelial malignancies, such as endometrial and ovarian cancers, including primary peritoneum and fallopian tube cancers of synchronous or metachronous onset. The most common inherited gynecological malignancy is Hereditary Breast and Ovarian Cancer syndrome (HBOC), which carries increased lifetime risks of breast and ovarian cancers, including other types of malignancies, such as pancreas, male breast, and prostate cancers. The next leading cause of inherited gynecological malignancy is Lynch syndrome (LS), a hereditary cancer syndrome predisposing individuals to various organ malignancies, including gynecological (endometrium is the most common) and non-gynecological (colonic or extracolonic) cancers, including stomach, urinary tract, brain, small intestine, hepatobiliary, and pancreatic cancers, which harbor impaired DNA mismatch repair due to germline disorders of *MLH1*, *MSH2*, *MSH6*, or *PMS2*, or the deletion of *EPCAM*, a gene epithelial cell adhesion molecule. HBOC and LS have communal aspects, which provide effective information for the treatment of symptomatic patients (probands), as well as for at-risk family members or relatives in surveillance and the prevention of malignancies. Cowden syndrome (CS) and Peutz–Jeghers syndrome (PJS), which are inherited hamartoma tumors, or polyposis syndrome are also associated with gynecological malignancies. As CS and PJS are much rarer and have lower malignancy risks, HBOC and LS are discussed as representatives of the hereditary gynecological cancer predisposition syndromes in this chapter.

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12.1 Epidemiology and Risks of Hereditary Susceptibility to Ovarian Cancers; Hereditary Breast and Ovarian Cancer Syndrome (HBOC)

It is conventionally known that ovarian cancer is associated with inherited factors [1, 2]. The strongest risk factor for ovarian cancer is a family history of breast or ovarian cancer, and a quarter of all ovarian cancers are caused by heritable conditions [3]. *BRCA1/2* germline pathogenic variants are representative of these heritable factors, leading to an increased lifetime risk of ovarian cancer ranging from 39 to 63% with *BRCA1* variants and 16.5–27% with *BRCA2* variants, both of which are significantly higher risks of ovarian cancer than that in the general population. The accumulated risk of breast cancer is 38% or more, which is estimated to be over 80% by 70 years of age [4–8] (Table 12.1). The overall prevalence of *BRCA1/2* variants is estimated to be 1 out of 400–800, which varies depending on ethnicity; a higher prevalence of 1 in 40 is observed in the Ashkenazi Jewish [7]. A multicentric cohort study showed that the cumulative ovarian cancer risk by 80 years was 44% for *BRCA1* and 17% for *BRCA2* variant carriers, of which the corresponding relative risks are 35–40 times that of women in the general population [9] (Fig. 12.1). Hirasawa et al. demonstrated that *BRCA1/2* is the most frequent germline pathogenic variant in Japanese ovarian cancer patients, with prevalence rates of 8.3% for *BRCA1* and 3.5% for *BRCA2* [10]. A multicenter study also showed that the overall prevalence of germline *BRCA1/2* variants was almost 15%, with germline *BRCA1* variants (9.9%) and *BRCA2* variants (4.7%) in ovarian cancer patients in Japan [11].

12.2 Epidemiology and Risks for Hereditary Susceptibility to Endometrial Cancer; Lynch Syndrome (LS)

Lynch syndrome (LS), alternatively termed as hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant inherited multiple organ malignancy due to a germline variant in one of four DNA mismatch repair (MMR) genes, *MSH2*, *MLH1*, *MSH6*, or *PMS2,* or the deletion of *EPCAM*. The estimated prevalence in the population ranges from 1 in 250 to 1 in 3000, depending on the country and ethnicity, or whether the individual carries founder variants or not [13]. Colorectal carcinoma is generally the most common, followed by endometrial carcinoma in women with LS. Three percent of all new cases of colorectal cancer are attributable to LS in the USA [14]. According to various studies, women with LS are estimated to carry higher risks of endometrial cancer than colorectal cancer [15–17]. Two to four

Table 12.1 Lifetime risks of gynecological and other malignancies

	BRCA1 variant (%)	*BRCA2* variant (%)	LS (%)	General population (%)
Breast cancer	46–87	38–84	–	>12
Ovarian cancer	39–63	16.5–27	4–12	1–2
Endometrial cancer	–	–	25–60	2.7
Male breast cancer	1.2	Maximally 8.9	–	0.1
Prostate cancer	8.6 (up to 65 years), 20 (whole lifetime)	15 (up to 65 years)	–	6 (up to 69 years)
Pancreatic cancer	1–3	2–7	–	0.5

Adapted from GENEReviews® (http://www.genereviews.org) [Internet] Bookshelf ID: NBK1211 (https://www.ncbi.nlm.nih.gov/books/NBK1211) and [Internet] Bookshelf ID: 1247 (https://www.ncbi.nlm.nih.gov/books/NBK1247) accessed in August 2020. © 1993–2020 University of Washington

	Cumulative risk for diagnosis through age 80 years old				Cumulative risk for diagnosis through lifetime
	MLH1 (%)	*MSH2* (%)	*MSH6* (%)	*PMS2* (%)	General population (%)
Colorectal cancer	46–61	33–52	10–44	8.7–20	4.2
Endometrial cancer	34–54	21–57	16–49	13–26	3.1
Ovarian cancer	4–20	8–38	≤1–13	3	1.3
Prostate cancer	4.4–11.6	3.9–15.9	2.5–11.6	4.6–11.6	11.6
Breast cancer (female)	10.6–18.6	1.5–12.8	11.1–12.8	8.1–12.8	12.8

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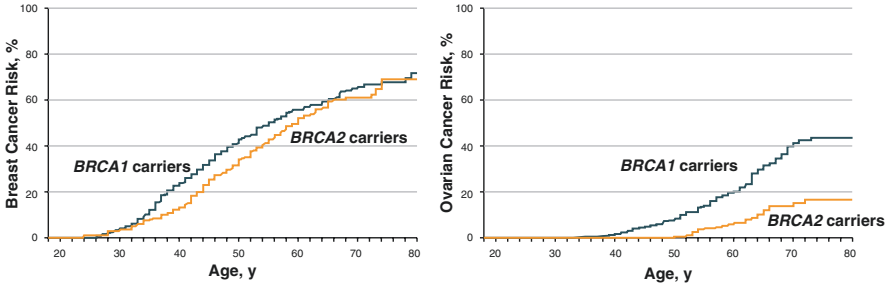

Fig. 12.1 Estimated cumulative risk of breast cancer (left panel) and ovarian cancer (right panel) among *BRCA1* and *BRCA2* variant carriers. Adapted from Kuchenbaecker et al., *JAMA*. 2017; 317(23):2402–2416. doi: 10.1001/jama.2017.7112 © 2017, American Medical Association

percent of endometrial cancer before 70 years, and nearly 5% of endometrial cancer onset between 20 and 54 years old, is estimated to be attributed to LS or to those with LS-associated family history [18–20]. Loss of function of any of the *MMR* genes is associated with microsatellite instability, a type of genomic instability, and increased risk of LS-associated cancers. The lifetime risk of endometrial cancer in women with LS is 25–60%, which is comparable to the lifetime risk of colorectal cancer in women with LS [16, 21]. The accumulated penetrance rate of ovarian cancer during the lifetime of women with LS is 6–13%, which is significantly higher than the 1–2% risk of ovarian cancer in the general population [22, 23]. The estimated lifetime endometrial cancer risks in women with LS are dependent on the causative genes. For women with *MLH1* or *MSH2* variants, the lifetime risk of endometrial cancer is reportedly 34–54% with *MLH1* variants and 21–57% with *MSH2* variants. The lifetime risks for ovarian cancer are 4–20% with *MLH1* variants and 8–38% with *MSH2* variants [24–26].

According to the International Society for Gastrointestinal Hereditary Tumors (InSiGHT) database, which records variants identified in over 3000 of LS cases, *MLH1* and *MSH2* are dominantly responsible genes for LS, while the remainder, such as *MSH6* and *PMS2*, are less frequently identified in LS. The proportions of LS attributed to pathogenic variants are 42% in *MLH1* and 33% in *MSH2*, while the smaller population is attributed to *MSH6* (18%) and *PMS2* (7.5%) [27]. *EPCAM* deletion is observed in 1–3% of the population with LS [28, 29]. Although *MSH6* variants are less commonly observed in LS than *MLH1* or *MSH2* variants, *MSH6* is a dominant causative variant gene in LS-associated endometrial cancer and in an older age onset of LS-associated colorectal cancer [17]. The cumulative risk for endometrial cancer by 80 years in women with *MSH6* variants ranges from 17 to 44% [17, 30], while the risks for endometrial carcinoma carrying *PMS2* variants or *EPCAM* deletions are reported to be less than 15 or 12%, respectively [31, 32].

The age of cancer onset in the population with LS is younger than that of the general population; the mean age at the time of diagnosis of endometrial cancer is 48–62 years and the average age for ovarian cancer is 42.5 years in women with LS [8].

According to a cohort study by Win et al., women with a diagnosis of endometrial cancer carrying an *MMR* variant had significantly higher risks in other cancers, such as colorectal cancer, breast cancer, or urological cancers, such as the ureter, urinary bladder, kidney, and renal pelvis cancers, during the 20-year follow-up visits of endometrial cancer patients [33, 34]. Based on this evidence, endometrial cancer can be termed a "sentinel cancer," a preceding cancer which is first detected among a series of primary cancers developed in women with LS.

12.3 Molecular Features and Diagnosis of *BRCA*-Associated Hereditary Gynecologic Malignancy

The germline pathogenic variants of *BRCA1/2* account for the majority of hereditary breast and ovarian cancers showing an autosomal dominant predisposition to those diseases [7]. Both *BRCA1* and *BRCA2* were identified by positional cloning in the early 1990s as genes responsible for susceptibility to breast and ovarian cancers

[35, 36]. The locus of *BRCA1*, which encodes a predicted protein consisting of 1863 amino acids, is at 17q21.31 in the long arm of chromosome 17 (OMIN#113705). *BRCA1* is expressed in numerous tissues, including the testis, thymus, breast, and ovary [35]. *BRCA2* is located at 13q13.1 in the long arm of chromosome 13 (OMIN#600185) and encodes 3418 amino acids [36]. Although there is no structural homology in *BRCA1* and *BRCA2*, these two genes share communal functions as caretakers in the maintenance of genomic integrity and homologous recombination (HR) during DNA damage repair of double-strand breaks [37]. The loss of function of either *BRCA1* or *BRCA2* causes serious disruption in the open reading frame of the transcription unit. BRCA1 protein functions by interacting with several proteins. BRCA1-associated RING domain 1 (BARD1) binds to the RING-finger domain near the N-terminus of BRCA1, both of which carry nuclear exporting signal (NES). BRCA1 creates three different types of complexes exclusively with phosphorylated Abraxas (ABRA1), BRCA1-associated C-terminal helicase (BACH1), or CtBP-interacting protein (CtIP) through the BRCT domain near the C-terminus of BRCA1 [38, 39]. As a consequence of interacting with these different proteins, BRCA1 plays distinct roles in DNA damage resistance, ubiquitination, gene transcription, and cell cycle progression, such as G(2)-M checkpoint control [40]. *BRCA2* plays a role in genomic integrity maintenance through the DNA repair process and facilitates HR. BRCA2 protein functions to prevent nascent DNA degradation and promote HR-mediated prevention in replication fork stalling by loading RAD51 on DNA breaks and gaps [41, 42]. BRCA2 can form a complex with BRCA1 through PALB2 mediation (Fig. 12.2).

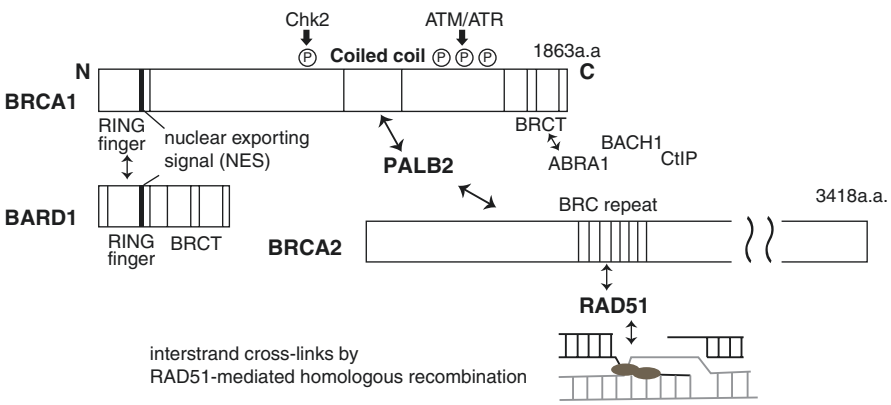

Fig. 12.2 Structures and Functions of BRCA1 and BRCA2 on DNA double-strand breaks. BRCA1 (1863a.a.) contains several recognizable protein motifs, such as a RING-finger domain near the N-terminus for binding with BRAD1 to function as a nuclear exporting signal (NES), coiled-coil domain on exon 11 for interaction with PALB2, and a BRCT domain at the C-terminus. BRCA2 (3418a.a.) contains eight BRC repeats of 30–40 residue motifs found in exon 11, which mediate the binding of BRCA2 to RAD51. RAD51 functions in homologous recombination through interstrand cross-links. Referenced from Sedukhina A et al. Seikagaku 84(7), 529–538, 2012. ©2012, The Japanese Biochemical Society

More than 1600 or 1800 variants have been identified in *BRCA1* and *BRCA2*, which leads to loss of function due to frameshift deletions, insertions, or premature truncation of transcripts, suggesting the significant functions of *BRCA1* and *BRCA2* as tumor suppressor genes [7, 43, 44]. The loss of function of *BRCA1* or *BRCA2* increases the sensitivity to poly(ADP-ribose) polymerase (PARP), which is referred to as synthetic lethality, resulting in vulnerability to PARP inhibitors [45, 46]. The locus of variants in *BRCA1* and *BRCA2* is also known to affect cancer risk. In the analyses of over 400 families carrying a *BRCA2* variant with the presence of ovarian cancer and other malignancies, families with ovarian carcinoma or breast cancer were more likely to harbor variants in the ovarian cancer cluster region of exon 11 of *BRCA2* than families with variants elsewhere in *BRCA2* [47].

12.4 Genetic Testing of *BRCA1/2*

Genetic testing using blood samples is applicable for clinical diagnosis not only for symptomatic patients (probands) with breast and/or ovarian cancers but also for at-risk relatives as predisposition testing. Distinct testing strategies, for example, targeting analyses, comprehensive analyses, or large genomic rearrangement tests, are provided by Myriad Genetic Laboratories (Salt Lake City, UT, USA). Target analyses may be used for the detection of population-specific founder variants, such as *BRCA1* c.68_69delAG (185delAG), *BRCA1* c.5266dupC (5382insC), or *BRCA2* c.5946delT (6174delT), which are detected at frequencies as high as 1 in 40 individuals of Ashkenazi Jewish heritage [48]. Comprehensive analysis is useful for the evaluation of the predisposition of at-risk individuals through combined methods to detect common *BRCA1/2* variants and five specific large genomic rearrangements in *BRCA1*, which are ethnic-specific or family-specific variants. Further complementary analysis is conducted as a large rearrangement test of the above and beyond the common five rearrangements of *BRCA1*, such as large genomic rearrangements in *BRCA1/2* [7] (Table 12.2).

Table 12.2 Genetic testing methods for *BRCA1* and *BRCA2*

Methods	Population	Mutation detected	Mutation detection frequency (%)
Comprehensive analysis	At-risk individuals	*BRCA1* and *BRCA*2 sequence variants and five specific large genomic *BRCA1* rearrangements	~88
Large rearrangement test	At-risk individuals	Large genomic rearrangements in *BRCA1* and *BRCA2*	3–4
Targeted mutation analysis	Ashkenazi Jewish heritage	*BRCA1*: 185delAG *BRCA1:* 5382insC *BRCA2*: 6174delT	90

Adapted from Petrucelli et al. *Genet Med* 2010: 12 (5): 245–259. doi: 10.1097/GIM.0b013e3181d38f2f. © 2010, The American College of Medical Genetics

Clinical *BRCA1/2* testing in Japan is covered by the social insurance service as companion diagnostics to determine the indication of PARP inhibitors, as well as for the diagnosis of HBOC only for symptomatic patients (probands) with breast and/or ovarian cancers so far in 2020. Other comprehensive or specific germline cancer panels/analyses including *BRCA1/2* are provided by several diagnostic companies, such as LabCorp (Burlington, NC, USA), Ambry Genetics Corporation (Aliso Viejo, CA, USA), and ACT Genomics (Taipei City, Taiwan). These may be used for the investigation of other related disorders of probands as well as for at-risk relatives and for differential diagnosis.

The analysis of genetic testing is reported in three variant categories: a positive, a negative, or an inconclusive, termed as a variant of uncertain significance (VUS), in clinical pathogenicity. It is estimated that up to 20% of *BRCA1/2* variants are reported as a VUS [49–52]. In the large rearrangement tests and the family-specific variant tests for at-risk relatives, the testing results will be shown in one of two categories: absent (negative) or present (positive). Even if negative results are obtained, careful interpretation is indispensable because negative results do not necessarily eliminate the possibility of a hereditary susceptibility to cancer. There is also a possibility that cancer in the family might be associated with unknown hereditary factors that are undetectable by the genetic test performed. When VUS results are obtained, further analysis using samples from additional family members might be a clue to examine whether the variants co-segregate with cancer in the family [7].

12.5 Relationships Between *BRCA1/2* Variants and Histological Properties of Hereditary Gynecological Malignancies

The prevalence of germline *BRCA1/2* variants is known to be associated with frequencies of specific types of histology [11]. The most common histology type of ovarian cancer carrying *BRCA* variants is high-grade serous carcinoma, comprising about 70–80% of women with *BRCA1/2* variants, while it is approximately 50% in sporadic controls or women without *BRCA1/2* variants [53–58]. Endometrioid and mucinous carcinomas of the ovary account for a smaller population, which is a maximum of 6–12% among women carrying *BRCA1/2* variants. In contrast, approximately 10–20% of people in the general population with wild-type germline *BRCA* present with these carcinomas. It is estimated that approximately 10–15% of women with pelvic serous carcinoma have pathogenic germline *BRCA* variants.

Serous carcinoma of pelvic malignancies is generally high-grade, a clinically aggressive type, and characterized as a type II tumor, which is frequently bilateral and is often found on the peritoneal surfaces at diagnosis [7, 59]. In *BRCA*-associated ovarian cancers, distinct molecular pathways of carcinogenesis, which are different from sporadic ovarian cancers, are associated with unique histopathologic subtypes [60]. According to the accumulated evidence through careful histopathologic

analyses, such as of the resected fallopian tubes at the risk-reduction salpingo-oophorectomy from patients carrying *BRCA* germline variants, a hypothesis was established that the distal fimbria end may be a potential site for early-stage tubal carcinoma leading to advanced tumorigenesis of pelvic malignancies, including primary peritoneal carcinoma [61–65]. Many studies have clarified that noninvasive carcinoma arising in the fallopian tube is potentially able to metastasize without invading into the substantial stroma of the distal salpinx, and this character is analogous to superficial serous carcinoma of the endometrium [66]. Such early stages of serous carcinoma, termed as serous tubal intraepithelial carcinomas (STICs) in fimbria, are observed with ovarian carcinoma in over 70% of sporadic ovarian and peritoneal malignancies of high-grade serous carcinoma [67]. Therefore, it is hypothesized that STICs, which are detected in fimbria, would be a source of high-grade serous carcinoma in pelvic malignancies, regardless of the status of germline *BRCA1/2*.

Occult malignancy, a small in situ carcinoma, was originally described by Colgan et al. in the study of salpingo-oophorectomy specimens at a prevalence rate of 8.3% in 5 of 60 high-risk women carrying *BRCA1/2* variants [61]. The incidence rate of occult carcinoma varies to some extent in study groups. Leeper et al., for example, reported that the occult carcinoma is observed in 17% (5 patients of 30) of *BRCA1/2*-variant positive women [68]. Paley et al. studied two *BRCA1*-positive patients with occult carcinoma in fallopian tubes and with positive peritoneal cytological malignancy, suggesting the micro-implantation potential of malignant cells in the peritoneum [69]. Another study by Agoff et al. showed that two of four cases of early fallopian tube carcinoma were positive for peritoneal cytology [70]. This leads to the central hypothesis that the inherited *BRCA* status is included as part of the cancer spectrum associated with STICs, which develops into fallopian tube carcinoma with a high potential to metastasize [66]. Depending on the location and the speed of tumor growth, the tumor might be mistakenly presumed as primary carcinomas of the ovary, peritoneum, or fallopian tube [60]. These are significant research outcomes by analyzing salpingo-oophorectomy specimens from pathogenic *BRCA1/2* variant-positive women. These findings are also supported by the finding that almost all STICs showed positive staining for p53, which is similar to that of high-grade serous carcinoma. Small linear p53-positive foci, termed the p53 signature, are commonly detected in the distal fimbria of both *BRCA*-variant positive women and sporadic groups with early tubal cancer [71]. Another study analyzing 29 cases of pelvic serous carcinoma showed that STICs and concordant high-grade serous carcinoma were identical to the *TP53* variant of ovarian carcinoma, which supports a clonal relationship between STICs and *TP53* [72]. Although these data are not necessarily relevant to the germline status of *BRCA1/2*, this suggests that the p53 signature would be an early precursor of high-grade serous carcinoma [66]. The accumulated evidence obtained from analyses of fallopian tubes from *BRCA*-variant-positive women has strengthened the fact that the fimbria end may be an origin of pelvic malignancies. What is more obvious from this evidence is that *BRCA* variants are susceptible factors for a subset of serous carcinomas, which has a strong connection with the distal end of the fallopian tube.

12.6 Molecular Features and Diagnosis of Gynecological Malignancies with LS

Microsatellite instability (MSI) and DNA mismatch repair deficiency (MMR-D) are basic tools for screening LS. Latham et al reported that LS was identified in 16.3% of patients with MSI-high (MSI-H). Immunohistochemical staining of LS-positive MSI-H, including MSI-intermediate (MSH-I) tumors, demonstrated MMR-D in 98% of patients [74]. MSI-H status is generally concordant with a high tumor mutational burden (high TMB), but the converse is not always true. According to the study by Chalmers et al., 16% of high TMB (> 20 mutations/Mb) was classified as MSI-H, of which concordance is dependent on malignancy type [73]. High TMB and MSI-H are rarely detected in lung and skin carcinomas, while those two statuses are matched frequently in cases of gynecological malignancies, such as endometrial carcinoma including endometrioid carcinoma of the uterus [73] (Fig. 12.3).

The initial step of clinical screening for gynecological malignancies with LS is the selection of patients and families based on the revised Amsterdam criteria II, a clinical diagnostic criterion of hereditary nonpolyposis colorectal cancer (HNPCC)-associated malignancies, covering colorectal, endometrial, renal pelvic, ureteral, and small intestinal cancers [12] (Table 12.3). Endometrial and ovarian cancers are also listed as LS-associated tumors in the revised Bethesda Guidelines, which is a clinical screening criterion for individuals with HNPCC who should be tested for microsatellite instability (MSI) [75]. MSI is caused by mismatch repair deficiency

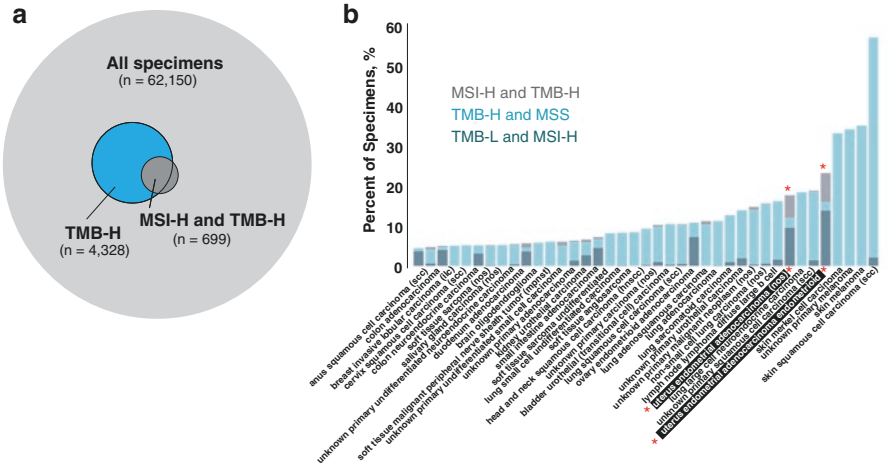

Fig. 12.3 The relationship between tumor mutation burden (TMB) and microsatellite instability (MSI). A majority (83%) of MSI-high (MSI-H) samples also had high TMB (TMB-H), while a smaller population (16%) in TMB-H was classified as MSI-H (panel **a**). Co-occurrence of MSI-H and TMB-H was observed at high incidence in gynecological malignancies, such as endometrial carcinoma of the uterus (panel **b**, *asterisks*). Adapted from Chalmers et al., *Genome Medicine* (2017) 9:34. doi: 10.1186/s13073-017-0424-2 © Zachary R. Chalmers et al.

Table 12.3 Revised ICG-HNPCC Criteria (Amsterdam Criteria II, 1999) [12]

There should be at least three relatives with a Lynch syndrome/HNPCC-associated cancer (cancer of the colorectum, endometrium, small bowel, ureter, or renal pelvis); the following criteria should be met:
1. One should be a first-degree relative of the other two
2. At least two successive generations should be affected
3. At least one of the relatives with cancers associated with HNPCC should be diagnosed before the age of 50 years
4. Familial adenomatous polyposis should be excluded in the CRC case(s) if any
5. Tumor diagnosis should be confirmed by histopathological examination

CRC, colorectal cancer

and is characterized by insertion/deletion or alteration in the lengths of repetitive regions within DNA. Polymerase chain reaction (PCR)-based MSI screening is widely conducted using the Bethesda panel, a five-marker panel comprising two mononucleotide repeats, BAT25, BAT26, and three dinucleotide repeats of D2S123, D5S346, and D17S250, of which application is recommended by the National Cancer Institute, USA [76, 77]. If two or more of five markers show instability, for example, variable shifting in the wave patterns in capillary electrophoresis of fluorescent-adjunct and amplified fragments from tumor and unaffected tissue, those MSI statuses are called high-frequency MSI (MSI-H). In contrast, if a single or no marker out of five shows instability, it is termed as low-frequency MSI (MSI-L) or microsatellite status stable (MSS) [76]. In recent years, PCR-based methods using five mononucleotide markers (BAT25, BAT26, MONO27, NR21, and NR24, instead of the Bethesda panel) as well as next-generation sequencing (NGS) techniques have been developed to detect MSI status with high sensitivity and specificity. Quasi-monomorphic variation range (QMVR), in which PCR products from normal DNA are almost confined regardless of ethnicity, is applied to MSI testing using five mononucleotide markers. MSI status can therefore be determined using the mononucleotide marker panel without normal DNA analysis [78]. In the NGS technique by FoundationOne® CDx (Foundation Medicine, Inc., Cambridge, MA, USA), a tumor sequencing used in cancer precision medicine, the MSI status is designated based on the genome-wide analysis of 95 microsatellite loci. Approximately 90% of LS-associated endometrial cancers are estimated to show MSI-H [79], while nearly 30% of sporadic endometrial cancer cases are presumed to show MSI-H [80]. In contrast, in ovarian cancer, the MSI-H population ranges from 3 to 13%, while the prevalence rate of LS in ovarian cancer is estimated to be almost the same or less at 0.9–2.7% [74, 81–83].

Immunohistochemical (IHC) analysis of MMR protein expression has been conventionally and universally performed as the second step in the diagnosis of LS. IHC analysis is advantageous in the direct visual detection of altered MMR. MSH2 functions as a heterodimer with MSH6, forming a major MutSα complex or with MSH3 to form a minor MutSβ complex. MLH1 and PMS2 proteins function as stable

heterodimers by forming a MutLβ complex that detects the short insertion–deletion loop of the mismatch structure. Loss of expression in both MSH2 and MSH6 is typically caused by germline variants. In contrast, loss of expression in both MLH1 and PMS2 indicates a germline alteration of *MLH1* or somatic methylation of *MLH1* promoter in sporadic cancers [14, 84]. To rule out sporadic MSI-H carrying epigenetic methylation of *MLH1*, *BRAF* V600E testing is applied based on the evidence that *BRAF* V600E is positive in approximately 40% of sporadic MSI-H colorectal cancers, while it is rarely observed in LS-associated colorectal cancer [85–87]. It must be noted that this is the case with colorectal cancer but not with endometrial cancer. The *BRAF* V600E test is not applicable to endometrial cancer in clinical practice [88, 89]. It should also be noted that the majority of MSI-H in gynecological malignancy is due to hypermethylation of the *MLH1* promoter rather than germline variants of *MMR* genes.

The majority of LS-associated endometrial cancers are endometrioid carcinomas, most of which are found as Grade 1 of the International Federation of Gynecology and Obstetrics (FIGO). Other non-endometrioid carcinomas including clear cell carcinoma, serous carcinoma, and carcinosarcoma, which is known as malignant mixed Mullerian tumor (MMMT), have also been reported [90]. Mesenchymal tumors, such as leiomyomas, leiomyosarcomas, and other stromal tumors, are not associated with LS. The location of endometrial cancer in patients with LS is likely to be in the lower uterine segment (LUS), which is a rare site for sporadic endometrial carcinoma [91, 92].

12.7 Risk Assessment, Surveillance, and Prevention of Hereditary Gynecological Malignancies

While ovarian cancer is a relatively rare type of malignancy, in which the prevalence rate in the general population is as low as 1.3% (1 of 78) according to the statistics in the USA, the lifetime incidence of ovarian cancer increases to 39–58% in women carrying germline pathogenic *BRCA* variants, and it rises to 9–12% in women with germline *MMR* pathogenic variants according to a study in the USA [93]. Population-based, single institutional, or nationwide multicentric studies showed that the positive rates for germline *BRCA* variants are 11–15% in invasive ovarian cancer patients regardless of ethnicity [10, 11, 55, 94]. It is as much as 2% in invasive ovarian cancer cases, which are positive for germline *MMR* variants [83]. The positive rates may be increased in patients with early onset of ovarian cancer (before 40 years of age) [95]. Due to the genes inherited in an autosomal dominant manner, each child of a person with HBOC or LS carrying germline pathogenic mutations in *BRCA1/2* or *MMR* genes has a 50% chance of having inherited causative variants, irrespective of gender. Appropriate surveillance following proper genetic testing at the right time point is important for the prevention of cancer development associated with inherited cancer syndromes.

12.7.1 Guideline Overview on BRCA1/2*-Associated Gynecological Malignancies*

Identifying women at high lifetime risk for ovarian cancer, due to germline variants relevant to the inherited syndromes, provide asymptomatic women with prevention opportunities, such as surveillance, chemoprevention, and risk-reducing surgery, by using a systematic tailored screening strategy [96]. Some medical societies recommend germline genetic testing for all women diagnosed with ovarian cancer. According to the American Society of Clinical Oncology (ASCO) Guidelines, published in 2020, it is strongly recommended that all women diagnosed with epithelial ovarian cancer should have germline genetic testing for *BRCA1/2* and other ovarian cancer susceptibility genes, irrespective of their clinical features or family cancer history [97]. It is moderately recommended that women diagnosed with clear cell, endometrioid, or mucinous ovarian cancer should undergo somatic tumor testing for mismatch repair deficiency (dMMR) [97]. The National Comprehensive Cancer Network® (NCCN®) (NCCN) regularly updates its clinical practice guidelines in oncology (NCCN Clinical Practical Guidelines in Oncology® (NCCN Guidelines®)) which can link to NCCN.org. According to Version 1.2020 of NCCN Guidelines® for Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic, focusing on *BRCA1/2* variant-positive management, clinical breast examination should be performed every 6–12 months, starting at the age of 25 years. Genetic counseling on risk-reducing mastectomy should include a discussion regarding the degree of protection, reconstruction options, and risks [98]. Risk-reducing salpingo-oophorectomy (RRSO) is recommended, which is typically performed between 35 and 40 years of age and upon completion of childbearing [98]. There is also a description regarding the management of RRSO, depending on the variant status of *BRCA1/2*. For patients with *BRCA2* pathogenic/likely pathogenic variants, RRSO can be reasonably delayed until the age of 40–45 years, since the onset of ovarian cancer in patients with *BRCA2* pathogenic/likely pathogenic variants is an average of 8–10 years later than in patients with *BRCA1* pathogenic/likely pathogenic variants [98]. Salpingectomy alone, which is based on the detection of precursor lesions, including serous tubal intraepithelial carcinomas (STICs) in fimbria, is not standard of care for risk reduction [98]. Clinical trials of interval salpingectomy and delayed oophorectomy are ongoing. As a discretion option, transvaginal ultrasound (TVUS) combined with serum CA125 level may be considered for ovarian cancer screening for patients who have not elected RRSO [98]. In any case, education regarding signs and symptoms of cancers, especially those associated with *BRCA* pathogenic/likely pathogenic variants, is important for women (ovarian cancer) and men (male breast and prostate cancers) carrying these variants [98]. Symptoms of pelvic or abdominal pain, bloating, and increased abdominal girth are associated with ovarian cancer development. The US Preventive Services Task Force recommends that primary care clinicians assess women with a personal or family history of breast, ovarian, tubal, or peritoneal cancer or who have an ancestry associated with *BRCA* variants with an appropriate brief familial risk assessment tool [99]. Women with a positive result on the risk assessment tool should receive genetic counseling, and genetic testing at the indicated time points thereafter [99].

12.7.2 Surveillance and Prevention Strategy for LS-Associated Gynecologic Malignancies

A majority (e.g., 67% in Cancer Statistics 2017) of endometrial cancer patients show symptoms, such as vaginal bleeding, and are diagnosed at an early stage with disease confined to the uterus [100, 101]. The NCCN Guidelines® for Genetic/Familial High-Risk Assessment: Colorectal Version1.2020 recommend that women should be educated regarding the importance of prompt reporting and evaluation of any abnormal uterine bleeding or postmenopausal bleeding [26]. Endometrial biopsy is included as an option for the evaluation of these symptoms; a screening via endometrial biopsy every 1–2 years starting at age 30–35 years can be considered [26]. Hysterectomy may be considered as a risk-reducing surgery for endometrial cancer in at-risk women [26]. Schmeler et al. demonstrated that prophylactic hysterectomy with bilateral salpingo-oophorectomy is effective for preventing endometrial and ovarian cancer in women with LS [102]. Consideration and discussion on the risks and benefits of these risk-reduction agents, as well as patient education regarding the early symptoms of endometrial cancer, are important. Genetic counseling, which is a critical component in cancer risk assessment and helping clients make informed decisions, covers those procedures. As for ovarian cancer, there is no effective screening strategy so far. Transvaginal ultrasound for ovarian cancer screening with or without serum CA125 is not a routine recommendation since those modalities have not been shown to be sufficiently sensitive or specific as the screening of ovarian cancer. To conduct the referral to the genetic counseling at the right time point, including genetic testing, information regarding significant family history of the involved disease will be very important, especially for ovarian cancer.

12.8 Cancer Susceptibility Gene to Gynecological Malignancies Is Presumed Through Tumor Genomic Sequencing

Somatic genomic testing using next-generation sequencing (NGS) is becoming a common practice in clinical oncology, such as for the care of patients with advanced or metastatic cancer. The analysis of tumor genomes also has the potential to uncover germline variants as the underlying background information, called germline findings [103]. *BRCA1/2* and *MMR* genes are important presumed germline genes among the minimum 59 listed genes whose disclosure is recommended by the statement of the American College of Medical Genetics and Genomics (ACMG) [104, 105]. The goal of the disclosure of those presumed germline pathogenic variants (PGPVs) is to identify and manage risks for selected highly penetrant genetic disorders that can be prevented and of which morbidity and mortality can be reduced through established interventions after confirmation as pathogenic germline variants (PGVs) [105]. Both *BRCA1/2* and *MMR* are high-actionable cancer susceptibility genes (CSGs), which confer a predisposition to specific tumor types, such as

breast, ovarian, or colon cancer. Even though pathogenic CGCs are detected in organs in which elevated risk of cancer is generally not conferred by those genes, the pathogenic variants of *BRCA1/2* or *MMR* genes should be regarded as germline origin [106]. The NCCN Guidelines® for Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic Version 1.2021 introduces that *BRCA1/2* germline genetic testing should be considered if a pathogenic or likely pathogenic variant is found through tumor profiling [76, 98]. The homologous recombination deficiency (HRD) status, due to deleterious variants of *BRCA1/2*, can be used for response prediction to poly(ADP-ribose) polymerase (PARP) inhibitor. Detection of somatic variants and subsequent confirmation as PGVs allows for a higher likelihood of responses to PARP inhibitors, as well as the effective chance of surveillance to prevent ovarian cancer, colon cancer, or other associated cancers in patients and relatives.

12.9 For Understanding of Hereditary Gynecological Malignancies

Web-based resources and links for HBOC with *BRCA1* and *BRCA2* variants and LS with *MMR* variants:

- GeneReviews®: https://www.ncbi.nlm.nih.gov/books/NBK1116/
- National Comprehensive Cancer Network (NCCN) Guidelines® for Detection, Prevention, & Risk Reduction: https://www.nccn.org
- American Society of Clinical Oncology Guidelines: https://www.asco.org/research-guidelines
- European Society of Medical Oncology (ESMO) Clinical Guidelines: Gynaecological Cancers: https://www.esmo.org/guidelines/gynaecological-cancers
- U.S. Preventive Services Task Force Recommendation Statement: BRCA-Related Cancer: Risk Assessment, Genetic Counseling, and Genetic Testing: https://www.uspreventiveservicestaskforce.org/uspstf/recommendation/brca-related-cancer-risk-assessment-genetic-counseling-and-genetic-testing

Book

- Hereditary Gynecologic Cancer: Risk, Prevention and Management edited by Karen H. Lu, published in 2012 by Informa Healthcare, UK.

12.10 Conclusion

BRCA1/2-associated ovarian cancer and LS-associated endometrial cancer are representatives of hereditary gynecological malignancies. A better understanding of these symptoms as well as attention to significant family history provides women

with opportunities to identify HBOC or LS, leading to the early detection of asymptomatic stages of ovarian cancer and prevention of secondary cancers. Owing to the progress of analytical technologies and risk-reducing modalities, we have unveiled the detailed mechanisms by which ovarian carcinogenesis and development occur. By identifying specific germline variants associated with gynecologic malignancies, unaffected family members, and relatives also have the opportunity to undergo predictive testing and surveillance. Recent advancements in cancer genomic analytical technology using next-generation sequencing are becoming a common modality that provides us with another opportunity to consider pathogenic variants presumed as germline origin, as well as other potential cancer susceptibilities, called germline findings. The utilization of inherited information, which is estimated through somatic genomic testing and germline analysis, is becoming more common and important in the management of gynecological malignancies.

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