Review Article

Mechanisms of chemoresistance and poor prognosis in ovarian clear cell carcinoma

Hiroaki Itamochi,[1] Junzo Kigawa and Naoki Terakawa

Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishicho, Yonago 683-8504, Japan

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Clear cell carcinoma (CCC) accounts for 4% to 12% of epithelial ovarian cancer in Western countries and, for some unknown reasons, it comprises more than 20% of such cancers in Japan. CCC shows unique clinical features such as a high incidence of stage I disease, a large pelvic mass, an increased incidence of vascular thromboembolic complications, and hypercalcemia. It is frequently associated with endometriosis. Compared to serous adenocarcinoma (SAC), CCC is relatively resistant to conventional platinum, or taxane-based chemotherapy which is associated with its poor prognosis. However, the mechanisms underlying CCC's resistance to chemotherapy have not been understood. Although several mechanisms involved in drug resistance exist in CCC, including decreased drug accumulation, increased drug detoxification, and an increased DNA repair activity; however, no particular chemoresistance system has been identified. On the other hand, an *in vitro* study revealed that low cell proliferation may cause the insensitivity of CCC to cisplatin. The Ki-67 labeling index in CCC tumors was significantly lower than SAC. The Ki-67 labeling index for responders was significantly higher than that for non-responders in both tumor types. A multivariable analysis revealed that Ki-67 labeling index and residual tumor size were independent prognostic factors in CCC. Therefore, lower proliferation of the tumor cells may contribute to their resistance to chemotherapy. However, further investigation into the molecular biology and genetics of CCC is warranted. This review discusses the current state of knowledge of the chemoresistance mechanism in CCC and novel treatment strategies for CCC. (*Cancer Sci* 2008; 99: 653–658)

Clear cell carcinoma (CCC) of the ovary was originally termed 'mesonephroma' by Schiller in 1939 as it was thought to originate from mesonephric structures and resembled renal carcinoma.[1] However, the tumor was subsequently thought to be of Mullerian origin and designated as clear cell ovarian tumor.[2] Since 1973, CCC is recognized in the World Health Organization classification of ovarian tumors as a distinct histological entity, and its clinical behavior is also distinctly different from that of other epithelial ovarian cancers.[3] CCC constitutes about 4 to 12% of all epithelial ovarian cancers in the Western countries and, for some unknown reasons, it comprises more than 20% of such cancers in Japan.[4,5] Japanese living in the United States exhibit a markedly higher incidence of CCC than do Caucasians.[6] Its poor prognosis (median survival time for those with advanced disease is 12.7–24.0 months) may reflect the resistance of CCC to conventional platinum- or taxane-based chemotherapy.[4,5,7–10] Currently, the preferred treatment for CCC is a complete resection of the tumor, but this is difficult to accomplish when the disease is advanced. This review discusses the mechanisms of chemoresistance in CCC and some potential novel treatment strategies for advanced CCC.

Clinicopathological features

CCC tumors show distinctly different clinical behavior from that of other epithelial ovarian cancers. When compared with serous adenocarcinoma (SAC), CCC is often associated with a large pelvic mass and rarely occur bilaterally.[10,11] An increased incidence of vascular thromboembolic complications and hypercalcemia is also seen in patients with CCC.[5,10,12,13]

Histopathologically, the tumors were diagnosed to be CCC if 90% or more of all histological specimens show large cuboidal, hobnailed or flattened epithelial cells containing abundant clear cytoplasm lining tubules and cysts, and growing in solid/tubular or glandular patterns.[2] CCC is often associated with endometriosis (22–70%), whereas the hobnail cells bear a very strong morphological resemblance to endometrial Arias-Stella cells.[4,5,10,11,13–17] It has been reported that endometriosis frequently shows a sequential change to epithelial ovarian cancer, including CCC; therefore, atypical endometriosis is considered to be a precancerous change.[15,17]

Several reports have shown that CCC frequently appears during an early stage, especially stage Ic.[8,18,19] The percentage of patients with stage I was higher in patients with CCC than in patients with SAC, and among those patients stage Ic was observed at 32.2% to 38.8% in CCC and at 7.1% to 9.4% in SAC. On the contrary, the patients with advanced stage (III and IV) were lower in CCC than in SAC (Table 1). These findings suggest that the proliferation and progression of CCC are different from those of SAC.

Several authors have discussed the prognosis of patients with CCC in comparison to that of patients with SAC.[5,8,11,13,18,19] In early stage (I and II), the prognosis of patients with CCC was not different significantly to that of SAC, while the recurrence rate of CCC was higher than that of SAC.[10] However, a high recurrence rate in patients with stage Ic CCC and lower survival rate of these patients was seen in comparison to those of SAC.[8] It has been thought that CCC in its advanced stages (III and IV) showed lower survival rate due to resistance to platinum- or taxane-based chemotherapy relative to SAC.[5,8,11,13,18,19] Although neither the estimated 3-year nor 5-year survival rates in patients with no gross tumor were significantly different between CCC and SAC, both survival rates in the patients with <2 cm residual disease and those with ≥2 cm residual disease were significantly lower in CCC than in SAC.[8] In addition, the overall response rate to platinum-based chemotherapy for CCC was significantly lower than that for SAC (11.1% vs 72.5%). Another author demonstrated that platinum-based chemotherapy did not appear to improve the survival of patients with CCC, in comparison to

[1]To whom correspondence should be addressed.
E-mail: itamochi@grape.med.tottori-u.ac.jp

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Table 1. Distribution by stage and histological type

Histology	No. of patients	Stage I (%)			Stage II (%)	Stage III (%)	Stage IV (%)
		a	b	c			
Sugiyama T *et al.*[8]							
Serous	235	6.4	0.9	9.4	5.5	61.7	16.2
Clear cell	101	10.9	0.0	37.6	9.9	30.7	10.9
Heintz APM *et al.*[18]							
Serous	3085	5.1	1.1	7.6	7.4	62.3	14.7
Clear cell	494	20.7	1.2	32.2	10.5	27.1	6.1
Mizuno M *et al.*[19]							
Serous	311	5.1	0.3	7.1	12.5	64.3	10.6
Clear cell	178	18.0	0.0	38.8	18.5	18.5	6.2

the survival from non-platinum-based chemotherapy.[12] CCC has a more aggressive course and a more malignant behavior than SAC. Therefore, a new treatment strategy for CCC, including alternative regimens of chemotherapy, should thus be established.

Mechanisms of drug resistance

It is generally accepted that CCC's insensitivity to conventional platinum-based chemotherapy tends to be followed by a poor prognosis. Drug resistance is an important factor in the poor prognosis of patients with CCC. Several mechanisms involved in drug resistance have been proposed, including decreased drug accumulation, increased drug detoxification, and increased DNA repair activity (Fig. 1).[20–25]

Drug efflux. Adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters, such as ABCB1 (also known as P-glycoprotein), ABCC1 (also known as multidrug resistance associated protein-1) and lung resistance protein, are known to affect intracellular drug concentrations and are important multidrug resistance factors.[26] ABCB1 and ABCC1 function as an ATP-dependent efflux pump for cytotoxic drugs, such as paclitaxel (PTX) and platinum agents.[26,27] The expression of the *ABCC1* gene in CCC cell lines has been associated with cisplatin (CDDP), etoposide (VP-16), and mitomycin-C (MMC) resistance.[28]

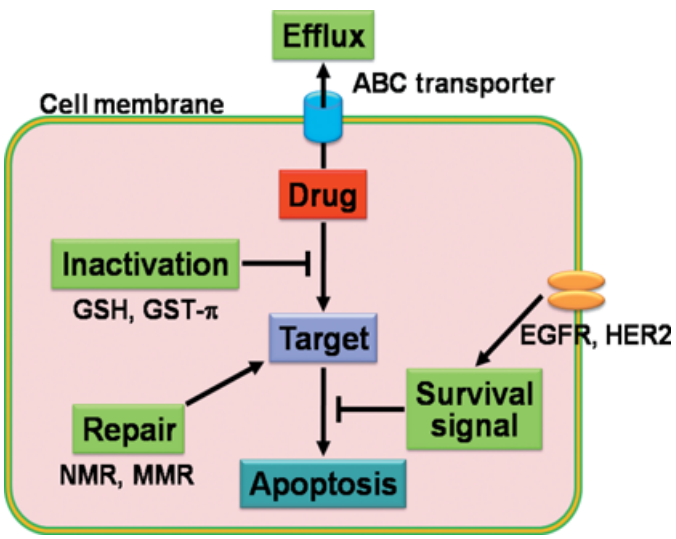

Fig. 1. Mechanisms of drug resistance. ABC transporter; ATP-binding cassette transporter; EGFR, epidermal growth-factor receptor; GSH, glutathione; GST-π, glutathione S-transferase-π; HER2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; NMR, nucleotide excision repair; MMR, DNA mismatch repair.

An immunohistochemical study of ABCB1 and ABCC1 expression in 41 patients with CCC and 90 patients with SAC who had measurable disease after initial surgery was conducted.[7] All patients underwent cytoreductive surgery followed by platinum-based chemotherapy and response rate to chemotherapy was 14.6% for CCC and 72.2% for SAC. The overall positive rates of ABCB1 and ABCC1 were 41.5% (17/41) and 19.5% (8/41) for CCC, and 46.7% (42/90) and 27.8% (25/90) for SAC, respectively. The expression of ABCB1 and ABCC1 did not differ between CCC and SAC. In addition, no significant differences were observed in the expression of ABCB1 and ABCC1 between responders and non-responders in both tumors. Therefore, these results suggest that these multidrug resistance proteins are not contributing factors to chemoresistance in CCC.

Ohishi *et al.*[29] examined the mRNA expression of ABCC superfamily members, *ABCC1*, *ABCC2*, and *ABCC3*, in tumors of CCC and SAC and found that only *ABCC3* gene expression in CCC was significantly higher than that in SAC. ABCC3 is known to transport various substrates, including anticancer drugs, and contributes to drug resistance against platinum agents.[26] Therefore, they concluded that increased expression of ABCC3 may be associated with, at least in part, the chemoresistant phenotype of CCC.

Tsuda *et al.*[30] found a significant increased in DNA and mRNA copy number of 12 genes in CCC tumors in comparison to those in SAC by genomic and gene expression array analyzes and focused on the ABC transporter F2 (*ABCF2*) gene on 7q35–36. The validation studies using real-time quantitative polymerase chain reaction (PCR) and immunohistochemical staining showed significantly higher *ABCF2* DNA and mRNA copy number and protein levels in CCC. Furthermore, ABCF2 cytoplasmic staining was significantly higher in non-responders to platinum-based chemotherapy than that in the responders for patients with CCC. These data suggests that ABCF2 protein could be a useful prognostic marker for CCC. However, the function of ABCF2 is unknown.

Drug inactivation. Several drug detoxification systems can also diminish the amount of intracellular drug activity. Cellular detoxification via the glutathione system is known to be involved in the metabolism of various cytotoxic agents, including the platinum agents, VP-16, and MMC.[22,23,27,31] Indeed, the glutathione concentrations of CCC cell lines was significantly increased after exposure to CDDP or MMC in comparison to before treatment.[28]

A gene expression study showed glutathione peroxidase 3 (*GPx3*), glutaredoxin (*GLRX*), and superoxide dismutase (*SOD2*) were highly expressed in CCC tumors and high levels of these and perhaps other antioxidant proteins may render these tumors more resistant to chemotherapy.[32]

DNA repair. Nucleotide excision repair is a multienzyme DNA repair pathway in eukaryotes that has been implicated in drug resistance in human tumor cells.[33] Reed *et al.*[34] examined the mRNA expression levels of two key genes, excision repair

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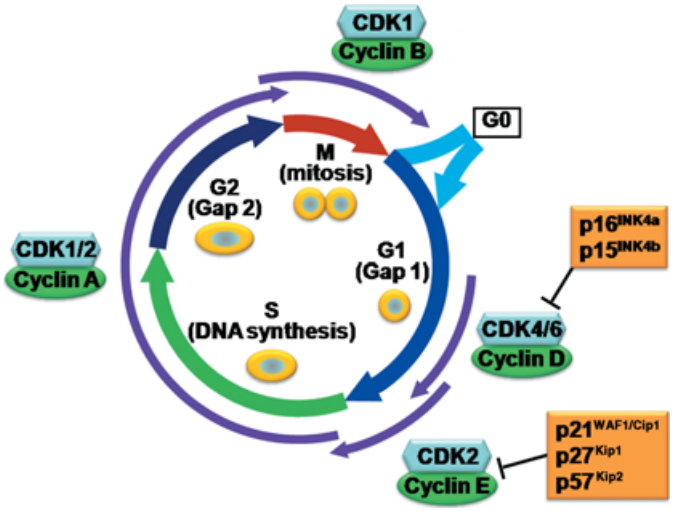

Fig. 2. The phase of the cell cycle and its regulation. Proliferation depends on the ability of the cell to successfully pass through the G1, S, G2, and M phases of the cell cycle. Such cell progression is controlled by cyclin-dependent kinases (CDKs), which themselves are regulated by cyclin binding, phosphorylation, and CDK inhibitors. The quiescence state is called G0 phase. There are two known CDK inhibitor families, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ inhibit CDK2 and $p16^{INK4a}$ inhibits CDK4 and –6.

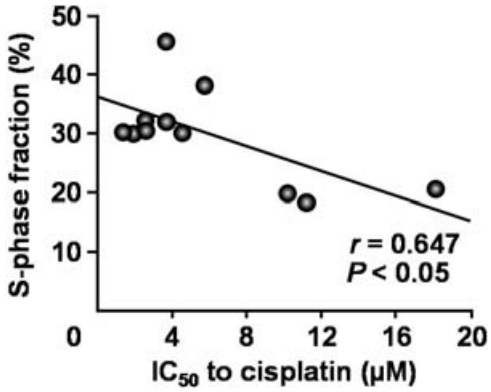

Fig. 3. Correlation between the S-phase fraction (SPF) and the IC_{50} to cisplatin (CDDP) in the clear cell carcinoma cell lines. There was a significant reverse correlation between the SPF and the IC_{50} to CDDP.

cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*) and xeroderma pigmentosum group B (*XPB*), involved in the nucleotide excision repair pathway in epithelial ovarian cancer tumors. The expression of *ERCC1* and *XPB* were higher in CCC tumors than in other histological tumor types, and this phenomenon may be related to *de novo* drug resistance against chemotherapeutic agents in CCC.

DNA mismatch repair systems (MMR), which correct errors occurring during DNA replication, also play a critical role in the sensitivity of DNA damaging agents. In experimental systems, MMR deficient cells are highly tolerant to the methylating chemotherapeutic drugs streptozocin and temozolomide and, although to a lesser extent, to CDDP and doxorubicin.[35] Loss of MMR may be caused either by a germline mutation of two major *MMR* genes, *hMLH1* or *hMSH2*, or by somatic *MMR* gene inactivation through epigenetic silencing via methylation of the *hMLH1* promoter. Cai *et al*.[36] reported that high expression of hMLH1 and hMSH2 protein are involved in the development of a subset of CCC, and there is a strong correlation between alterations in the expression of hMLH1 and hMSH2 and the presence of MSI in CCC tumors.

Growth-factor signaling. Epidermal growth-factor receptor (EGFR) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2; HER2) are cell-surface-receptor tyrosine kinases and can activate both the mitogen-activated protein kinase and phosphatidylinositol 3′-kinase (PI3K) –Akt signaling pathways.[37] Activating these pathways leads to phosphorylated Bc1-2 antagonist of cell death (BAD) and B-cell leukemia/lymphoma (Bcl)-2, thereby inhibiting chemotherapy-induced apoptosis.[38] Amplified PI3K and activated Akt have been found in 30–40% of ovarian carcinomas and they could thus represent a mechanism of drug resistance in patients with ovarian cancer.[39]

An overexpression of EGFR has been associated with chemoresistance and poor prognosis in ovarian cancer. An immunohistochemical study showed that EGFR was detected in 61% of CCC tumors.[40] Molecular analyzes of various types of ovarian tumors showed HER2 to be overexpressed in CCC relative to other major histological types of epithelial ovarian cancer.[41] The *HER2* proto-oncogene encodes a 185-kDa transmembrane growth factor receptor tyrosine kinase.[42] Several types of tumors that overexpress HER2 have shown poor sensitivity to conventional anticancer agents and poor prognosis.[43,44] In ovarian cancer, HER2 protein is overexpressed as a consequence of *HER2* gene amplification in 20 to 25% of cases and is a marker of poor prognosis.[45,46]

Cell-cycle control. Cell proliferation depends on the ability of the cell to successfully pass through the G1, S, G2, and M phases of the cell cycle (Fig. 2). Such cell progression is controlled by cyclin-dependent kinases (CDKs), which themselves are regulated by cyclin binding, phosphorylation, and CDK inhibitors (e.g. p16, p21, and p27).[47] p53, known as a tumor suppressor protein, also up-regulates p21 expression and causes cell cycle arrest at G1. Changes in the p53 gene are seen in 50 to 70% of cases of advanced serous adenocarcinoma.[48,49] In contrast, the p53 mutation is rare in CCC, and immunohistochemical staining shows that CCC tends to express little or no p53 protein.[50]

Cytotoxic drugs are primarily effective against proliferating cells; therefore, quiescent cells show a degree of drug resistance relative to cycling cells.[51] Dimanche-Boitrel *et al*.[52] reported that intracellular drug accumulation decreases in resting cells. The proliferation activity and CDDP sensitivity of 11 CCC and 5 SAC cell lines were examined, and this showed that the doubling time for CCC cells was significantly longer than that for SAC cells (61.4 vs 29.8 h).[53] There was a significant reverse correlation observed between the S-phase fraction and the response to CDDP (Fig. 3). These findings may relate to the high incidence of stage I patients with CCC (Table 1), and also suggests that the resistance of CCC to CDDP may be caused by low cell proliferation. In addition, Ki-67, a nuclear antigen, which is expressed in all states of the cell cycle except in resting cells in G0, has a significantly lower labeling index in CCC than in SAC (Fig. 4).[7] Furthermore, a significantly higher Ki-67 labeling index is observed in responders than in non-responders in both CCC and SAC tumors. A multivariable analysis revealed that the Ki-67 labeling index and residual tumor size were independent prognostic factors. Other authors also reported that the immunohistochemical staining of CCC revealed a low expression of Ki-67, p53, and cyclin A and significantly increased expression of both p21 and cyclin E, which are other histological subtypes.[54] These results suggest that CCC had low tumor proliferation activity and that this low proliferation activity in CCC could be associated with chemoresistance.

What is an effective agent against clear cell carcinoma of the ovary?

The clinicopathological features of CCC are provocative and suggest a new strategy for chemotherapy in CCC should be

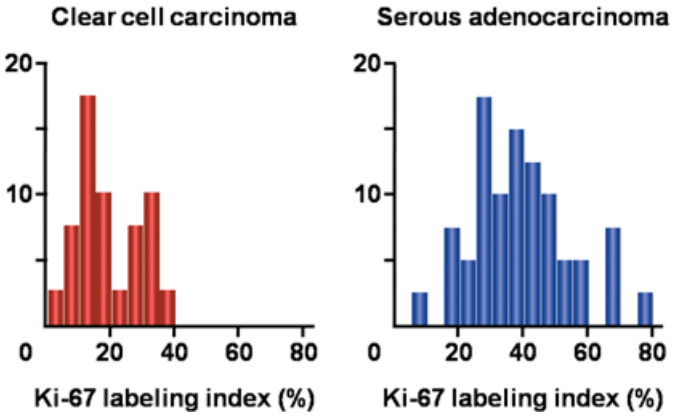

Fig. 4. Histograms of the Ki-67 labeling index for clear cell carcinoma and serous adenocarcinoma. The Ki-67 labeling index for clear cell carcinoma was significantly lower than that for serous adenocarcinoma (mean ± standard deviation, 18.4 ± 9.9 vs 38.8 ± 15.0%; $P < 0.01$).

adopted. Cloven *et al.*[55] examined *in vitro* drug response profiles for 102 CCC and 2660 SAC tumors, and found higher sensitivity to PTX, cyclophosphamide and doxoruvicin in CCC cells in comparison to SAC cells, with no significant differences in sensitivity observed for topotecan, carboplatin or CDDP. In another study, three out of five CCC cell lines were sensitive to 7-etyl-10-hydroxycamptothecin (SN-38), which is an active metabolite of irinotecan (CPT-11), and PTX.[28] All five cell lines were resistant to MMC and VP-16, and only one cell line responded to CDDP. Thus, the combination treatment of PTX and SN-38 could control four of five cells, thus indicating that these agents might be an effective treatment for CCC.

Irinotecan (CPT-11) inhibits topoisomerase (topo) I by forming stable topo I-DNA cleavable complexes, and topo I activity relates to sensitivity to CPT-11.[56,57] There is a collateral sensitivity between CDDP and SN-38, and synergistic enhancement of CDDP cytotoxicity by SN-38 for CDDP-resistant cells.[57,58] Combination chemotherapy of CPT-11 and CDDP (CPT-P) has also been reported to be effective in patients with CCC.[59,60] However, only a small number of patients were investigated in these studies, and no prospective randomized controlled trials were performed. An international randomized clinical trial of the Gynecologic Cancer Intergroup/Japanese Gynecologic Oncology Group 3017 with PTX/carboplatin and CPT-P for the patients with CCC is ongoing and the results of this trial are eagerly awaited.

Novel treatment strategies and future directions

CCC has unique biological and clinical characteristics from other types of epithelial ovarian cancer. Therefore, novel treatment strategies based on molecular biology should be established. Recently, many new agents with specific molecular targets, which are present in tumor cells, have been developed. These targeted agents offer the potential for improvement in long-term disease control with fewer toxicities.

v-erb-b2 erythroblastic leukemia viral oncogene homolog 2. Among those molecular targets, HER2 has been found to be overexpressed in CCC relative to SAC and important disease progression.[32] Two molecules thought to target HER2: trastuzumab and adenovirus type 5 *E1A* were investigated.[61] Trastuzumab is a recombinant anti-HER2 monoclonal antibody that has antiproliferative activity against HER2-overexpressing cells.[62] Human adenovirus type 5 *E1A* is an early viral gene that codes for two major proteins by alternative splicing of two exons. The two early viral proteins (243 and 289 amino acids in length) can activate or repress transcription of several viral or cellular genes and thereby regulate the cell cycle.[63] E1A acts as a tumor suppressor by down-regulating *HER2* transcription.[64] HER2 protein was detected at various levels by Western blotting in all 10 cell lines used in this study. Trastuzumab did not inhibit the proliferation in any of the four CCC cell lines tested. However, transfection with *E1A* reduced colony formation in all 10 CCC cell lines regardless of the HER2 expression level. Infection of CCC cells with an adenoviral vector encoding *E1A* led to significant suppression of proliferation and enhancement of cell death. This effect required the stabilization of p53 protein and was associated with the up-regulation of Bax and the cleavage of caspase-9. Treatment with E1A prolonged survival in a CCC xenograft model.[61] Therefore, *E1A* gene therapy is worth exploring as a treatment modality for women with ovarian CCC.

Epidermal growth-factor receptor. EGFR is known to be detected by immunohistochemical staining in 61% of CCC tumors and may be a therapeutic target for patient with CCC.[40] Gefitinib, an EGFR tyrosine kinase inhibitor, is now used for patients with non-small cell lung cancer. Fujiwara *et al.*[40] reported that gefitinib decreased the growth and invasion of three CCC cell lines *in vitro* and inhibited the growth of xenografts of the CCC cell line RMG-I *in vivo*. They also showed that severe combined immunodeficient mice bearing RMG-I xenografts treated with geftinib survived for longer than the untreated control group, and concluded that geftinib may thus offer a new and effective treatment for CCC.

Mitogen-activated protein kinase and phosphatidylinositol 3′-kinase–Akt signaling pathways. Other signaling pathways, such as the mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase kinase (ERK) signaling cascade and a PI3KAkt pathway, which are proliferation and cell-survival pathways, are also potential targets for modulating drug resistance. Combining PTX with either MEK inhibitor (PD98059 [PD]) or PI3K inhibitor (LY294002 [LY]) had an additive effect on cell-growth inhibition in five ovarian cancer cell lines.[65] In contrast, a synergistic effect was observed when PTX was combined with both PD and LY. Furthermore, treating nude mice with PTX and PD and LY prolonged survival in an ovarian cancer xenograft model, this indicating that the inhibition of both the MEK and PI3k signaling pathways can help to overcome the drug resistance of CCC.

Vascular endothelial growth factor. Neovascularization is essential for tumor growth and it is induced by several growth factors, including vascular endothelial growth factor (VEGF).[66] Overexpression of VEGF have been shown to be a poor prognostic factor in ovarian cancer, and VEGF is thought to be one of the most promising targets of therapy.[67] Three antiangiogenic drugs, bevacizumab, sunitinib malate, and sorafenib, have been approved by the United States Food and Drug Administration for treatment patients with specific types of cancer. Bevacizumab is a humanized monoclonal antibody that selectively binds and neutralizes all isoforms of VEGF-A. In phase II studies, bevacizumab showed improved survival of patients with ovarian cancer.[68] Phase III trials of bevacizumab in combination with first-line chemotherapy in ovarian cancer are ongoing. Both sunitinib and sorafenib are orally administered, small molecule inhibitors of multiple receptor tyrosine kinases, including VEGF receptors and platelet derived growth factor receptors, implicated in tumor growth, angiogenesis, and metastatic progression.[69]

A wide range of therapeutic strategies based on molecular biology should be evaluated to improve survival in patients with CCC. These include developing of novel cytotoxic drugs, small molecule inhibitors, monoclonal antibodies, and gene therapy strategies.

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Conclusion

CCC has a unique molecular biology and clinical characteristics from other epithelial ovarian cancers; therefore, CCC should be studied separately. Although several mechanisms involved in drug resistance are observed in CCC, the lower proliferation of tumors may be a behavior of CCC that contributes to its resistance to chemotherapy. Further investigation into the molecular biology and genetics of CCC is warranted. Several molecular targeted agents have recently been developed and may modulate resistance to chemotherapy in CCC. Among those targets, growth-factor signaling pathways, such as HER2, EGFR, and VEGF, enhance tumor growth, and inhibition of these pathways is promising to improve survival in patients with CCC.

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