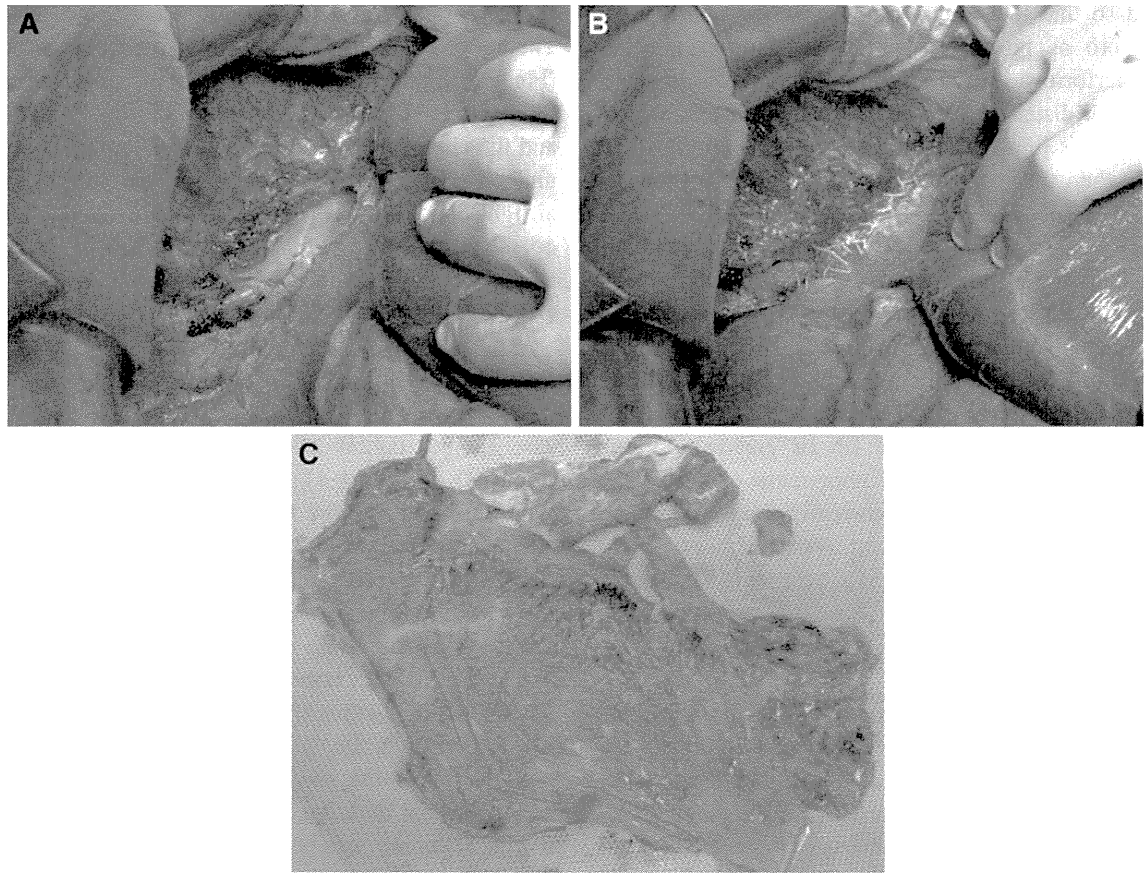

Fig. 2 **a** Diaphragm full-thickness resection completed. **b** The diaphragm is reconstructed using absorbable sutures. **c** This is a resected disease from the right diaphragm

<1 cm in five (15.6 %) and two (15.4 %) patients, respectively. The residual tumor site included the diaphragm, liver surface, mesentery, splenic hilum, and peritoneum (Table 2).

Discussion

When PDS for ovarian and/or peritoneal cancer is performed, it is important to accurately determine staging (surgical staging) based on systematic intraperitoneal tests and minimize the residual tumor to determine an appropriate treatment modality to improve prognosis. In recent years, opinions regarding optimal surgery have changed drastically. In our facilities, we recently adopted DS as a part of OS, although CS has been typically planned with DS since 2009.

With regard to the history of OS and CS, in 1994, Hoskins et al. [2] reported a correlation between residual tumor diameter and prognosis in primary laparotomy. Since then, the importance of primary laparotomy has been debated, and in 2002, Bristow et al. [3] performed a meta-analysis and concluded that for patients with stage III/IV ovarian carcinoma, tumor debulking is the most important factor impacting survival. However, this study provides the only evidence of surgery for advanced ovarian cancer, in which a residual tumor with a diameter of <1 cm indicated a significantly good prognosis and was considered for OS, which may be used as a target in primary surgery. In 2006, Chi et al. [4] divided 465 patients with stage IIIc ovarian cancer into five groups according to residual tumor diameter and comparatively examined patient prognosis. Similar to current reports, comparing patients with a residual tumor diameter of <1 vs. >1 cm, the former group had significantly better prognosis. Moreover, a significant difference in survival was found between patients without residual tumors and those with a residual tumor of <1 cm. These results may mark the beginning of a shift toward CS in primary laparotomy. Furthermore, IDS should be performed in cases in which chemotherapy following PDS reduces the tumor by more than 50 %. In cases with stable or progressive disease, IDS is contraindicated because it may worsen prognosis. When IDS is performed, CS should be targeted; however, as with PDS, it is important to

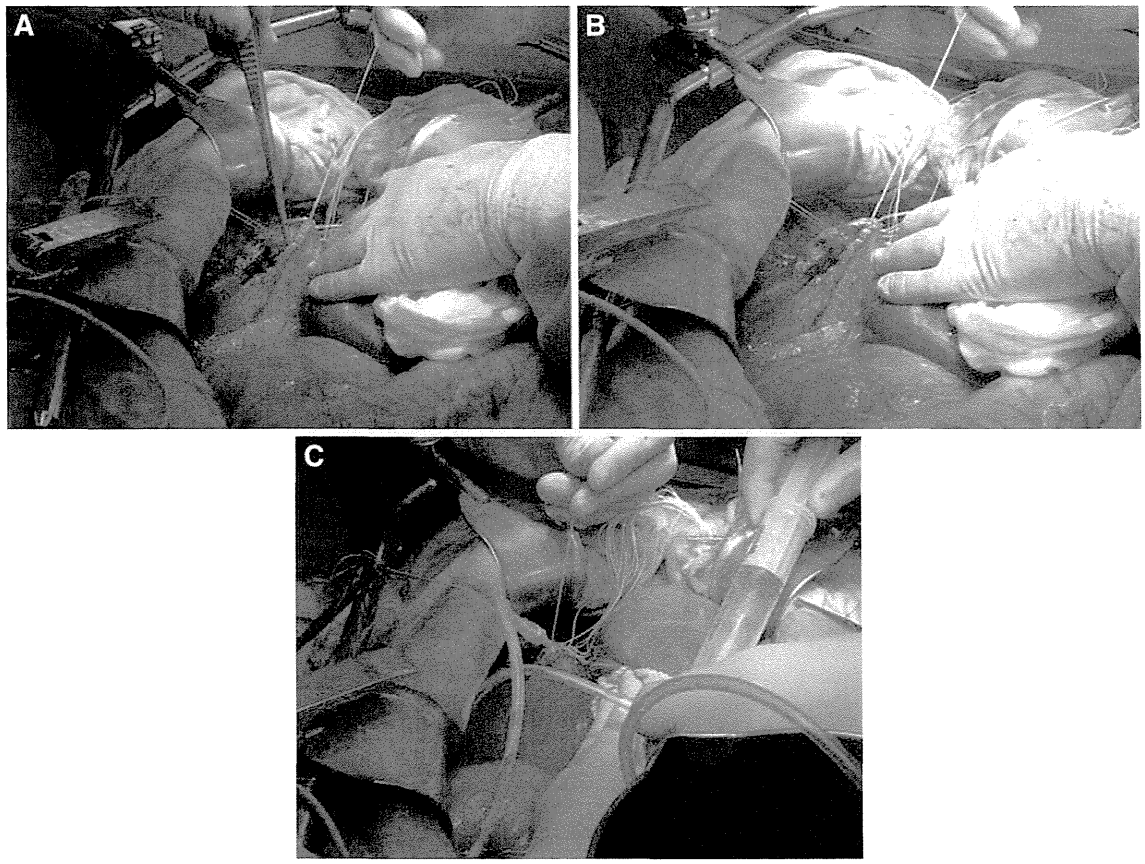

Fig. 3 **a**, **b** The diaphragm is reconstructed using absorbable sutures. **c** Deflation is performed using a narrow tube

choose the optimal surgical procedure for each patient. Qayyum et al. [5] reported that sites of tumor metastasis may impede CS (Table 3). In our study, unfortunately, we also found residual tumors in the diaphragm, mesentery, splenic hilum, and peritoneum as well as on the liver surface. We believe that the choice of a surgical procedure for cases with residual tumors and the ability to quickly and accurately intraoperatively determine the extent to which surgery should be performed needs to be addressed in the future.

Subsequently, metastatic dissemination in the diaphragm is observed in approximately 40 % or more of patients with advanced ovarian cancer. Zivanovic et al. [6] reported that the presence of metastatic epigastric lesions in ovarian cancer was significantly correlated with excessive accumulation of ascites and suboptimal cytoreduction. Moreover, in cases in which epigastric lesions may be managed by optimal cytoreduction, the recurrence risk is reportedly reduced to 28 % and the risk of death to 33 % [7]. Therefore, CS may help control the development of epigastric lesions.

Although many recent reports have described the safety and usefulness of surgical procedures, such as stripping and full-thickness resection, in many cases, it is difficult to remove lesions that have disseminated throughout the diaphragm. In such instances, extensive lesions may fill the right triangular ligament of the diaphragm bordering the liver. Therefore, we recommend diaphragmatic stripping or diaphragmatic resection in such cases, although extensive diaphragmatic resection in cases with diaphragmatic permeation should be limited. Moreover, in cases that without extensive diaphragmatic permeation, diaphragmatic reconstruction is performed without soft tissue patch (GORE-TEX; W.L.Go re & Associates, Inc.,Tokyo, Japan) use in our hospital. Intraoperative procedural accidents include unexpected thoracotomy during stripping, whereas postoperative complications include pleural effusion. Terauchi et al. [8] reported that the incidence of unexpected thoracotomy and pleural effusion was 1.6 and 30.8 %, and 52.3 and 61.5 % in the PDS and IDS groups, respectively. At our hospital, unexpected thoracotomy occurred in 42.3 and 33.3 %, and pleural effusion in 43.8 and 53.8 %, in the PDS and IDS groups, respectively. Unexpected thoracotomy was common in the IDS group, which may be because of tissue fibrosis caused by the use of anticancer agents. The results at our hospital revealed that nearly half (42.3 %) of the patients in the PDS group required thoracotomy, which unfortunately may be considered resultant

Table 1 Patient characteristics

Characteristics	No. of patients	% of 45 patients
Age		
Mean age (years)	52.7	
Range	(37–73)	
Primary cancer		
Ovary	41	91.1
Peritoneal	4	8.9
Histological type		
Serous	31	68.9
Endometrioid	4	8.9
Clear	6	13.3
Mucinous	0	0
Mixed	3	6.7
Other	1	2.2
Stage		
IIIb	2	4.4
IIIc	33	73.3
IV	10	22.2
Surgical method		
PDS	32	71.1
IDS	13	28.9

PDS primary debulking surgery, *IDS* interval debulking surgery

Table 2 Intra- and post-operative data on DS

	PDS	IDS
Number of patients	32	13
Diaphragmatic surgery		
Stripping (%)	26 (81.3)	9 (62.2)
Full-thickness resection (%)	6 (18.8)	4 (30.8)
Intraoperative open chest (%)	17 (53.1)	7 (53.8)
Unexpected open chest (%)	11 (42.3 %)	3 (33.3 %)
Postoperative pleural effusions (%)	14 (43.8)	7 (53.8)
Pneumothorax (%)	0	0
Chest tube (%)	1 (3.1)	3 (23.1)
Side of diaphragmatic disease		
Right (%)	27 (84.3)	12 (92.3)
Bilateral (%)	5 (15.6)	1 (7.7)
Complete cytoreduction (%)		
Macroscopic residual tumor		
None	16 (50.0)	9 (69.2)
<5 mm	11 (34.4)	2 (15.4)
	Porta hepatis	Mesentery
	Mesentery	Small bowel mesentery
	Peritoneum	
<10 mm	5 (15.6)	2 (15.4)
	Lymphnode (PAN)	Liver surface
		Mesentery
	Diaphragm	
Mean intraoperative blood loss (mL)	4,090.8	2,847.9
Mean duration of the operation (min)	485.2	479.5
Mean hospitalization (days)	21.6	24.8

PDS primary debulking surgery, *IDS* interval debulking surgery

Table 3 Criteria for inoperable disease in newly diagnosed primary epithelial ovarian cancer

Peritoneal sites	Nodal sites	Other
Porta hepatis	Retroperitoneal	Hepatic metastases
Intersegmental fisure	(Above the renal hila)	Abdominal wall invasion
Subphrenic space	Celiac axis	
Gastrosplenic ligament	Supradiaphragmatic	
Lesser sac		
Small bowel mesentery		
Dome of the liver surface		

Aliya Qayyum et al. [5]

of competency. With respect to pleural effusion, there was no considerable difference between the two groups. Furthermore, Tsolakidis et al. [9] reported no residual tumor in 88.7 % of the PDS group and 94.6 % of the IDS group, which were relatively high rates. The blood loss volume, surgical duration, and length of hospitalization until aftercare in the PDS group were 2,177 mL, 355 min, and 21 days, respectively; in the IDS group, these values were 1,270 mL, 272 min, and 13 days, respectively. Among our patients, the values for complete cytoreduction were relatively low at 50.0 and 69.2 %, respectively. The blood loss volume, surgical duration, and length of hospitalization until aftercare in the PDS group were 4,091 mL, 485 min, and 21 days, respectively; in the IDS group, these values were 2,848 mL, 480 min, and 25 days, respectively. There were no significant differences observed other than the length of hospitalization in the PDS group. Beginning in 2009, only 5 years ago, many Japanese medical institutions adopted DS; therefore, major differences in complications, surgical duration, and blood loss volume may be attributed to inexperience. Because of the long learning curve due to the prolonged duration for legal approval of the epigastric method before it was applied to the diaphragmatic approach for liver surgery, the amount of blood loss associated with this procedure has continued to increase. In addition, switching to an open chest procedure was required in some cases, and several cases had postoperative hydrothorax. Moreover, the time from start of therapy and length of stay has increased.

Conclusions

The most important factor to establish a treatment plan for advanced cancer at the time of primary laparotomy and IDS is the extent to which a residual tumor can be eradicated. However, successful CS is dependent on patient age, performance status, and the presence or absence of complications. We believe that surgery should always be performed aiming for CS and should not markedly reduce the patient's quality of life. At our hospital, tumor debulking with DS resulted in excessive, but controllable blood loss, and OS was safely and successfully achieved in all patients, without any severe complications or delay in postoperative treatment. In 2009, du Bois et al. [1] reported that OS was considered to have very few benefits over CS; therefore, the success rate of CS should be improved while maintaining safety and, at the same time, gynecologic oncologists should improve their skills in surgical repair of the epigastric region.

Conflict of interest None.

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RESEARCH ARTICLE Open Access

MicroRNA-21 is a candidate driver gene for 17q23-25 amplification in ovarian clear cell carcinoma

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Abstract

Background: Epithelial ovarian cancer (EOC) is the most common cause of gynecological malignancy-related mortality. Ovarian clear cell carcinoma (CCC) has unique clinical characteristics and behaviors that differ from other histological types of EOC, including a frequent association with endometriosis and a highly chemoresistant nature, resulting in poor prognosis. However, factors underlying its malignant behavior are still poorly understood. Aberrant expression of microRNAs has been shown to be involved in oncogenesis, and *microRNA-21* (*miR-21*) is frequently overexpressed in many types of cancers. The aim of this study was to investigate the role of *miR-21* in 17q23-25 amplification associated with CCC oncogenesis.

Methods: We identified 17q23-25 copy number aberrations among 28 primary CCC tumors by using a comparative genomic hybridization method. Next, we measured expression levels of the candidate target genes, *miR-21* and *PPM1D*, for 17q23-25 amplification by real-time RT-PCR analysis and compared those data with copy number status and clinicopathological features. In addition, immunohistochemical analysis of PTEN (a potential target of *miR-21*) was performed using the same primary CCC cases. We investigated the biological significance of *miR-21* overexpression in CCC using a loss-of-function antisense approach.

Results: 17q23-25 amplification with both *miR-21* overexpression and PTEN protein loss was detected in 4/28 CCC cases (14.2%). The patients with 17q23-25 amplification had significantly shorter progression-free and overall survival than those without 17q23-25 amplification (log-rank test: $p = 0.0496$; $p = 0.0469$, respectively). A significant correlation was observed between *miR-21* overexpression and endometriosis. Both *PTEN* mRNA and PTEN protein expression were increased by *miR-21* knockdown in CCC cells. We also confirmed that *miR-21* directly bound to the 3′-untranslated region of *PTEN* mRNA using a dual-luciferase reporter assay.

Conclusions: *MiR-21* is a possible driver gene other than *PPM1D* for 17q23-25 amplification in CCC. Aberrant expression of *miR-21* by chromosomal amplification might play an important role in CCC carcinogenesis through the regulation of the *PTEN* tumor suppressor gene.

Keywords: Ovarian clear cell carcinoma, CGH array, microRNA-21, PTEN

Background

Epithelial ovarian cancer (EOC), a heterogeneous group of neoplastic diseases that arise from the epithelial cells of fallopian tubes, ovarian fimbria, ovarian surface epithelium, inclusion cysts, peritoneal mesothelium, or endometriosis, is the most lethal gynecologic malignancy in western countries and in Japan [1]. EOC can be classified into four major histological types: serous, mucinous, endometrioid adenocarcinoma, and clear cell carcinoma (CCC). CCC has unique clinical characteristics that differ from other histological types of EOC. CCC accounts for 5–25% of all EOC, depending on the population. The prevalence of CCC among EOCs in North America and Europe is 1–12%, while that in Japan is approximately 20% [2]. CCC is frequently associated with coexistent endometriosis and thrombosis, with 20% of patients

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developing deep venous thrombosis. Endometriosis has been identified in more than 30% of tumors and is reported to be a precursor of CCC as well as endometrioid adenocarcinoma [3]. The incidence of venous thromboembolic events was found to be significantly higher in CCC than in other epithelial ovarian cancers [4,5]. A greater proportion of CCC presents in the early stage as a large pelvic mass, which may account for their earlier diagnosis. However, CCC is generally refractory to standard platinum agent-based chemotherapy with a response rate of only 11–15%; therefore, this type of tumor typically has a poor prognosis, particularly in late stages. The survival rates of patients with CCC are significantly lower than those of patients with serous EOC [6]. Identifying novel therapeutic targets and establishing new treatment strategies for CCC is thus important.

The common molecular genetic alterations identified so far in CCC include mutations in *ARID1A* and *PI3K* as well as HNF1B overexpression. However, the molecular landscape of CCC oncogenesis remains poorly understood [7,8]. Since chromosomal aberrations are a cardinal feature of carcinogenesis, the identification of amplified or deleted chromosomal regions associated with CCC would elucidate its underlying pathogenetic mechanisms. Amplification at chromosome17q23-25 has been reported to occur with a frequency of approximately 40% in CCC [9]. The *PPM1D* gene (also known as *WIP1*) maps to the 17q23.2 amplicon and is amplified and/or overexpressed in various types of cancers, including CCC [10]. However, the frequency of *PPM1D* overexpression in CCC is reported to be only about 10%. In addition, the peak region of 17q23-25 amplification in CCC as assessed by GISTIC analysis maps adjacent to the *PPM1D* locus. Taken together, these findings suggest the involvement of undiscovered driver genes on 17q23-25 in CCC [11].

Recent evidence has shown that microRNAs (miRNAs) can have oncogenic or tumor suppressor functions and contribute to cancer biology [12,13]. Aberrant expression of miRNAs has been shown to be associated with oncogenesis. One of the most frequently overexpressed miRNAs in many types of cancers is *miRNA-21*, located on 17q23.2 within the intron of the *TMEM49* gene [14]. Protein expression of the *PTEN* gene, a target gene of *miR-21* [15], is absent in one-third of all CCC cases [16,17]. We thus hypothesized that *miR-21* is a potential candidate for 17q23-25 amplification and might play an important role in CCC oncogenesis through the regulation of PTEN expression.

Methods

Clinical specimens and ovarian cancer cell cultures

Tissue specimens were obtained from 28 patients with ovarian CCC who were treated at Jikei University Hospital from 2000 to 2010. The Jikei University School of Medicine Ethics Review Committee approved the study protocol (ethics approval number: 14-132) and informed consent was obtained from all patients. Most patients (27 of 28) underwent surgical resection followed by adjuvant chemotherapy with platinum-based regimens (platinum/paclitaxel, n = 12; platinum/irinotecan hydrochloride, n = 13; docetaxel/carboplatin, n = 2) as initial treatment. None of the patients had received chemotherapy or radiation therapy before the initial surgery. All samples were examined as hematoxylin–eosin-stained sections by a pathologist to confirm pure CCC histologically. Tumors were classified according to the World Health Organization classification system, and clinical stages were determined using the International Federation of Gynecology and Obstetrics (FIGO) staging system. Progression-free survival (PFS) was defined as the time from the date of primary surgery to the date of disease progression. Overall survival (OS) was calculated for the time from the date of initial surgery to the last follow-up visit or death. The mean age was 53 years (range, 37–81). FIGO staging was as follows: Stage I, n = 18; stage II, n = 2; stage III, n = 8. The median follow-up period was 45.7 months (range, 5.1–99.3). Coexistent endometriosis was found in 20 (71.4%) of 28 patients. The ovarian CCC cell lines JHOC-5 and JHOC-9 were obtained from Riken Bioresource center (Tsukuba, Japan). HAC-2 was kindly provided by Dr. Nishida (Tsukuba University, Ibaraki, Japan). RMG-I and RMG-II were provided by Dr. D. Aoki (Keio University, Tokyo, Japan). HAC-2, JHOC-5, and JHOC-9 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan). RMG-I and RMG-II were cultured in Ham F-12 medium (Sigma-Aldrich). Both media contained 10% heat inactivated fetal bovine serum, Penicillin-Streptomycin-Amphotericin B Suspension (×100) (Wako, Osaka, Japan). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

DNA and RNA isolation

All surgical samples were composed of at least 80% neoplastic cells and were immediately frozen after collection. For RNA isolation, the fresh clinical specimens were stored at 4°C for 24 hours in RNAlater (Ambion, Austin, Texas, USA) and were then frozen at −80°C in liquid nitrogen until further use. Using a commercially available DNA isolation kit (GentraPureGene kit; Qiagen, Tokyo, Japan), genomic DNA was extracted from stored frozen tumor samples following the manufacturer's instructions. Total RNA was isolated from tumor samples and cell lines with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA from the tumor samples was stored in RNAlater.

Candidate gene selection

Array comparative genomic hybridization (aCGH)

For this validation study, aCGH was performed using the Agilent Human Genome CGH 244AMicroarray Kit

244 K (Agilent Technologies, Santa Clara, CA, USA). DNA digestion, labeling, and hybridization were performed as recommended by the manufacturer. The test DNA (2 μg) and reference DNA (2 μg) were digested with Rsa I and Alu I (Promega). The digested tumor DNA and reference DNA were labeled with either cyanine (Cy) 5-deoxyuridine triphosphate (dUTP) or Cy3-dUTP using the Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). Labeled DNAs were purified using Microcon YM-30 filters (Millipore, Billerica, MA, USA). The hybridization mixture, containing Cy3-labeled test DNA and Cy5-labeled reference DNA, 2× Hybridization buffer (Agilent), 10× blocking agent (Agilent), and Human Cot-1 DNA (Invitrogen), was prepared in an Agilent SureHyb chamber. All microarray slides were scanned on the Agilent Microarray Scanner G2505B. Date was obtained using Feature Extraction software, version 10.7.3.1 (Agilent Technologies). Penetrance of aberrant chromosomal areas across the genome was demonstrated using Aberration Detection Method 2 (Agilent Genomic Workbench Lite Edition 6.5.0.18, Agilent Technologies), a quality-weighted interval score algorithm that identifies aberrant intervals in samples that have consistent gain or loss log ratios based on their statistical score. The $\log_2$ ratios for whole chromosomal number changes that were completely gained, lost, or had no change were evaluated. The threshold for determining amplification or deletion was defined as $\log_2$ ratio >0.5 or < −0.5.

Copy number assay for region 17q23–25 in the miR21 gene in CCC cells

The copy number for the 17q23–25 region was determined using commercially available and custom TaqMan Copy Number Assays (Applied Biosystems, Foster City, CA, USA). The *TERT* locus was used as an internal reference copy number. Genomic DNA was extracted from CCC cell lines (HAC-2, JHOC-5, JHOC-9, RMG-I, and RMG-II) using commercially available gDNA extraction and purification kits. Real-time genomic PCR was performed in a total volume of 20 μL per well containing TaqMan genotyping master mix (10 μL), genomic DNA (20 ng), and primers (20 ng each). Data were analyzed using SDS 2.2 sand CopyCaller software (Applied Biosystems). Copy numbers were assigned as follows: actual copy number <0.5, assigned copy number 0 (gene deletion); actual copy number ≥0.5 but <1.5, assigned copy number 1; actual copy number ≥1.5 but <2.5 , assigned copy number 2; actual copy number ≥2.5 but <3.5, and assigned copy number 3.

Quantitative reverse transcription-polymerase chain reaction

Reverse transcription (RT) of *miR-21* was carried out using the Taqman microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). cDNAs were synthesized from 2 μg of total RNA using the Superscript cDNA Synthesis Kit (Invitrogen) for *PPM1D* and *PTEN* mRNA detection. Real-time PCR Reactions with TaqMan Fast Advanced Master Mix (Applied Biosystems) were performed in 96-well plates using the Applied Biosystems StepOnePlus Real-time PCR System (Applied Biosystems). Each reaction was analyzed in triplicate. *MiR-21* expression was normalized to that of *U6* small nuclear RNA, and *PPM1D* and *PTEN* expression was normalized to that of *GAPDH*. The expression of *miR-21*, *PPM1D*, and *PTEN* were defined based on the threshold cycle (Ct); relative expression levels are presented as $2^{-\Delta\Delta Ct}$.

Immunohistochemical analysis

Immunohistochemical analysis of PTEN expression (1:100 dilution, Cell Signaling Technologies) was performed on 3-μm paraffin sections of formalin-fixed, paraffin-embedded tissues using the Ventana Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). After deparaffinization, antigen retrieval was carried out in CC1 buffer (Cell Conditioning 1; citrate buffer pH 6.0, Ventana Medical Systems). PTEN expression was scored independently by two investigators (Y. H. and N. Y.) based on stain intensity and extent. Immunohistochemical scoring was conducted in a manner entirely blinded to all clinical and biological variables. The intensity of positive staining was scored from 0 to 2 as follows: 0 (none), 1 (weak; intensity < positive control), 2 (strong; intensity ≥ positive control). Positive staining was assigned using a semi-quantitative, five-category grading system: 0, <5% positive cells; 1, 6–25% positive cells; 2, 26–50% positive cells; 3, 51–75% positive cells; 4, 76–100% positive cells. Addition of the two values gives the total score, and a score <4 was considered PTEN-negative.

Additional cohort

Additional cohort study was also approved by The Jikei University School of Medicine Ethics Review Committee (ethics approval number: 14-132). An additional cohort was analyzed using aCGH, realtime-PCR, and immunohistochemistry. This additional cohort was included to ensure association between miR21 overexpression and PTEN protein loss using 43 patients, with further confirmation in an additional 15 patients.

Western blot analysis

Western blot analysis was performed to detect PTEN protein expression (dilution of 1:2000, Cell Signaling Technologies, Danvers, MA, USA). CCC cell lines were washed in PBS and lysed in RIPA buffer containing 200 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 1% proteinase inhibitors, and 1% PMSF for 10 min on ice. Cell lysates were then sonicated

for 30 seconds, and cellular debris were removed by centrifugation at 14 000 rpm at 4°C for 30 min. Supernatants were collected and assayed for protein concentration using the BCA Protein Assay Kit (Invitrogen). Supernatants containing an equal amount of protein extract were supplemented with concentrated 4× LDS sample buffer (Invitrogen) and heated at 95°C for 5 min. Approximately 40 μg of lysate was loaded onto a 12.5% SDS-polyacrylamide gel. The supernatants were separated by SDS–PAGE, and proteins were transferred to Immobilon-P transfer membrane (Millipore, Milford, MA, USA). The transfer membrane was incubated with primary antibody in TBS with 0.1% Tween-20 and 5% bovine serum albumin overnight at 4°C. Anti-rabbit IgG-conjugated horseradish peroxidase (GE Healthcare) was used as the secondary antibody. The transfer membrane was incubated with secondary antibody in TBS with 0.1% Tween-20 and 5% skim milk for 90 min at room temperature. The proteins were visualized using the ECL-Plus Western blotting detection system and detected using the Image Quant LAS 4000 mini (GE Healthcare). The concentration of each target protein was normalized against beta-actin.

Transfection

Twenty four hours before transfection, cells were seeded in plates and grown to 50% confluence. For inhibition of miR-21, RMG-II cells were transfected with *mir*Vana miRNA Inhibitors or a control (Ambion). Transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

Dual luciferase reporter assay

pGL3 wild-type *PTEN* 3′-UTR and pGL3 mutant-type *PTEN* 3′-UTR luciferase plasmids were obtained from Addgene (Cambridge, MA). RMG-II cells were seeded in 6-well plates (5×105 cells/well). After 24 h, the cells were transfected with pGL3 control vector (Promega), pGL3 wild-type *PTEN* 3′-UTR vectors, or pGL3 mutant-type *PTEN* 3′-UTR vectors using Lipofectamine 2000 reagent. Luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega) 24 h after transfection. Firefly luciferase activity was normalized to renilla activity for each sample. All the experiments were performed in triplicate.

MTS assay

MTS assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) following the manufacturer's protocol. Briefly, miR-21 inhibitor and negative control oligonucleotides were transfected at a final concentration of 200nM. After 24 hours transfection, RMG-II cells were seeded into 96-well plates at a density of 1×10^4 cells per well. MTS (20 μL) was added to each well 3 hours before the desired time points, and cells were incubated at 37°C. The absorbance was measured at 490 nm using a Microplate Reader (VersaMAx, Molecular Devices). All experiments were repeated three times. Values are presented as the mean ± standard deviation (SD).

Invasion assay

Cells were seeded into the top chamber of a 96-well matrigel-coated plate with 8-μm-pore polyethylene terephthalate membrane inserts (Corning). MiR-21 inhibitor and negative control oligonucleotides were transfected at a final concentration of 200nM.The bottom chamber was filled with 0.75 mL Ham F-12 medium with 10% FBS as a chemoattractant. The inserts were filled with 0.5 mL Ham F-12 medium with 1% FBS. After incubation for 48 h, the filter membrane was fixed with 100% methanol and stained with hematoxylin and eosin. The degree of invasiveness was quantified by counting the number of cells in 4 random fields of view per filter using 400× magnification. Data obtained from three separate inserts are shown as mean values.

Statistical analysis

All statistical analyses were performed using StatMate III software (ATMS, Tokyo, Japan). Comparisons between parameters were made using Fisher's exact test. For survival analysis, PFS and OS distributions were determined using the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. $P < 0.05$ was considered statistically significant.

Results

Chromosome 17q23-25 amplification, miR-21 expression, and PTEN protein expression in CCC

CGH array profiles of chromosome 17 in 28 primary CCCs revealed that 9 out of 28 patients (32%) showed 17q23-25 amplification that included *miR-21* (Figure 1). *MiR-21* and *PPM1D* mRNA expression were then measured by real-time RT-PCR analysis (Additional file 1: Figure S1). We defined standardized value as each median value of *miR-21* and *PPM1D* expression without 17q23-25 amplification. Overexpression of *miR-21* and *PPM1D* were found in 60% and 57% of these tumors, respectively. Seven of 9 tumors (77.7%) with 17q23-25 amplification showed *miR-21* overexpression, and 10 of 19 tumors (52.6%) without 17q23-25 amplification also showed *miR-21* overexpression. In addition, 6 of 9 tumors (66.6%) with 17q23-25 amplification showed *PPM1D* overexpression, and 10 of 19 tumors (52%) without 17q23-25 amplification showed *PPM1D* overexpression (Additional file 1: Figure S1). We next evaluated the relationship between 17q23-25 amplification and either *miR-21* or *PPM1D* overexpression. No significant correlation between

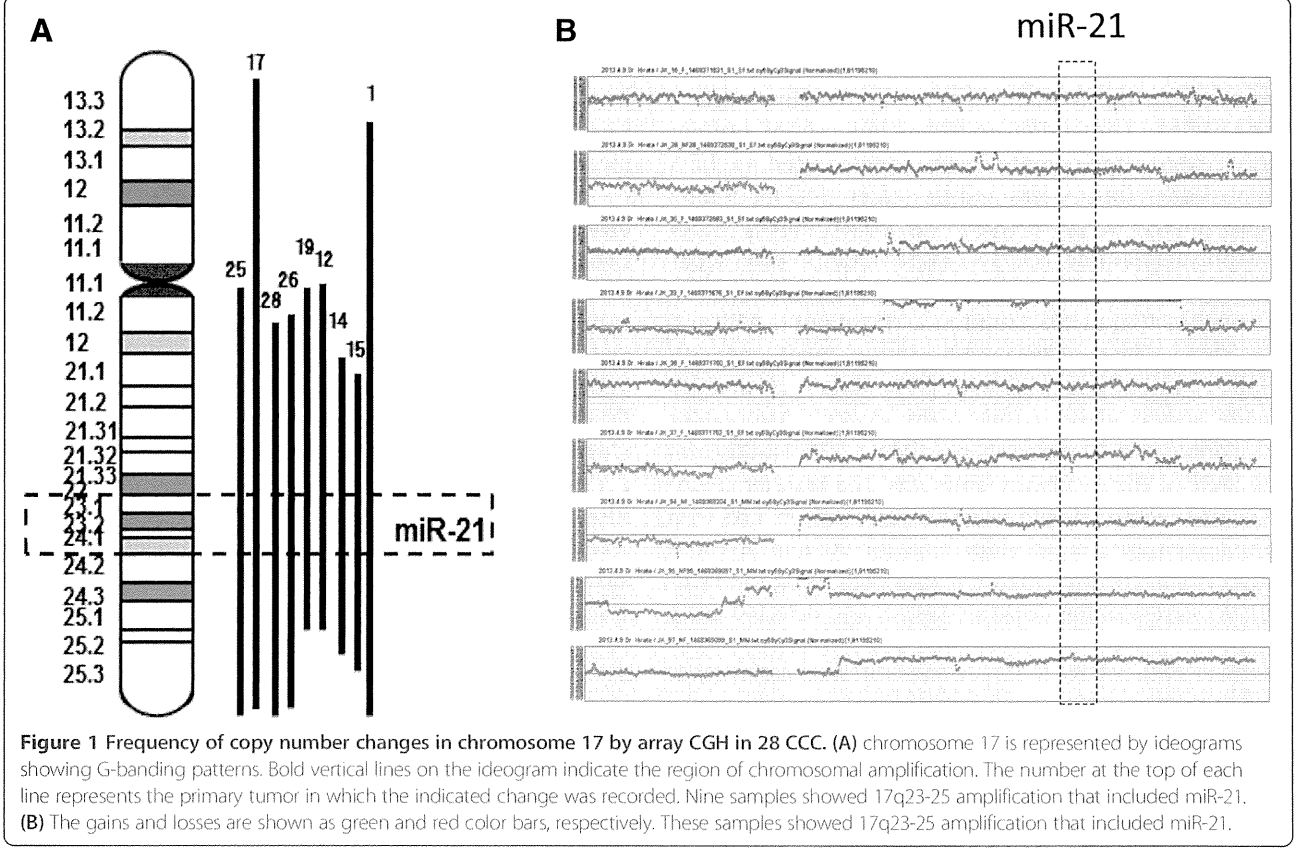

Figure 1 Frequency of copy number changes in chromosome 17 by array CGH in 28 CCC. (A) chromosome 17 is represented by ideograms showing G-banding patterns. Bold vertical lines on the ideogram indicate the region of chromosomal amplification. The number at the top of each line represents the primary tumor in which the indicated change was recorded. Nine samples showed 17q23-25 amplification that included miR-21. **(B)** The gains and losses are shown as green and red color bars, respectively. These samples showed 17q23-25 amplification that included miR-21.

the amplification and overexpression was observed for either gene. Next, immunohistochemical analysis of PTEN (a potential target of *miR-21*) was performed on samples from the same primary CCC patients. Loss of PTEN protein was observed in 13 of 28 patients (46.4%) (Additional file 2: Figure S2) and in 6 of 17 tumors (35.3%) with *miR-21* overexpression. No significant correlation was observed between *miR-21* overexpression and loss of PTEN expression. To further confirm these results, we added 15 CCC samples from an additional cohort, performing real-time RT-PCR of miR21 and IHC of PTEN. Again, no significant correlation was observed between *miR-21* overexpression and loss of PTEN expression (date not shown). In total, as shown in Figure 2, the occurrence of 17q23-25 amplification with both *miR-21* overexpression and PTEN protein loss was detected in 4 out of 28 CCC patients (14.2%) (Figure 2).

Associations between clinicopathological parameters and either 17q23-25 amplification, miR-21 overexpression, or PTEN protein loss

The relationship between clinicopathological parameters and genetic alterations including 17q23-25 amplification, *miR-21* overexpression, and decreased PTEN protein expression are summarized in Table 1. Interestingly, a significant correlation was observed between *miR-21* overexpression and endometriosis. Meanwhile, no correlations were observed between the other clinical parameters and any of the genetic alterations. According to survival analysis, patients with 17q23-25 amplification had significantly shorter progression-free and overall survival times than did those without 17q23-25 amplification (log-rank test; PFS, p = 0.0496; OS, p = 0.0469) (Table 2). On the other hand, the PFS and OS did not correlate significantly with *miR-21* overexpression or PTEN protein loss.

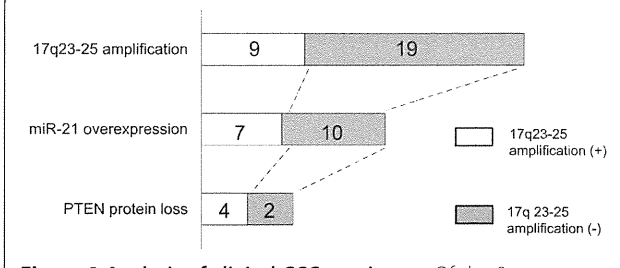

Figure 2 Analysis of clinical CCC specimens. Of the 9 tumors with 17q23-25 amplification, 7 (77.7%) showed *miR-21* overexpression. Of the 19 tumors (58.8%) without 17q23-25 amplification, 10 showed *miR-21* overexpression. Of all the 28 17q23-25 amplification cases, both *miR-21* overexpression and PTEN protein loss were detected in 4 (14.2%).

Table 1 Associations between clinicopathological parameters and either 17q23-25 amplification, *miR-21* overexpression, or PTEN protein loss

Variable	Cases (Total 28)	17q23-25 amplification		*miR-21* overexpression		Loss of PTEN protein expression	
		n = 9	P value	n = 17	P value	n = 13	P value
Age	6			4	>0.9999	5	0.0690
≧60	22	2	>0.9999	13		8	
< 60		7					
Stage			0.6464		>0.9999	8	0.1977
I-II	21	6		11		5	
III-IV	7	3		4			
Lymph node status	7				>0.9999	4	0.6702
Metastasis	21	3	0.6219	4		9	
No metastasis		6		11			
Endometriosis		8	0.2143	15		10	0.6859
Positive	20	1		2	0.0298	3	
Negative	8						
Thrombosis	3	1	>0.9999	2	>0.9999	2	0.5833
Positive	25	8		13		11	
Negative							

No correlations were observed between the other clinical parameters (age, stage, lymph node metastasis, thrombosis, and either 17q23-25 amplification, *miR-21* overexpression, or PTEN protein loss). A significant correlation was observed between *miR-21* overexpression and endometriosis. P-values were from two-sided tests and statistically significant when <0.05.

MiR-21 modulates PTEN expression

Based on the profiles of 17q23-25 copy number changes, *miR-21* expression, *PTEN* mRNA expression, and PTEN protein expression in 5 CCC cell lines, we selected RMG-II cells for further functional analysis. We considered this cell line to be ideal because the cells showed relatively 17q23-25 amplification, high *miR-21* expression with decreased PTEN protein expression (Additional file 3: Figure S3 and Additional file 4: Figure S4).

To investigate the regulation of PTEN expression by *miR-21* in CCC, we used a loss-of-function antisense approach in RMG-II cells. Knockdown efficiency was confirmed by real-time RT-PCR analysis of *miR-21* (Figure 3A). In RMG-II cells, we found that *miR-21* knockdown caused a significant increase in PTEN protein expression as indicated by Western blot analysis, along with increased *PTEN* mRNA expression (Figure 3A). However, suppression of *miR-21* expression did not inhibit cell proliferation or invasion (date not shown). We next investigated the direct binding of *miR-21* to the 3'UTR of PTEN mRNA by luciferase assay using a pGL3 plasmid harboring either the wild- or mutant-type *PTEN* 3'-UTR. The activity of the luciferase reporter was significantly decreased when fused to the wild-type *PTEN* 3'-UTR. Deletion mutations in the *miR-21*–interacting seed region rescued the luciferase activity. Taken together, these data suggest that *PTEN* is a direct functional target of

Table 2 Proportional hazard regression analysis of single predictors for PFS and OS in CCC

Parameters	PFS		OS	
	95%CI	P-value	95%CI	P-value
Age (≦60 vs. >60 years)	0.289–1.656	0.3371	0.244-–1.965	0.3337
Stage (I, II vs. III, IV)	0.289–1.234	<0.05	0.289–1.168	<0.05
Endometriosis	0.153–2.834	0.2384	0.154–2.684	0.2156
Residual tumor ≦2 VS. >2 cm)	0.3440–2.484	<0.05	0.1332–2.408	<0.05
17q23-25 amplification	0.1768–1.684	0.0496	0.154–1.756	0.0469
miR-21 overexpression	0.441–1.168	0.3141	0.441–1.645	0.3204
PTEN protein loss	0.4422–1.980	0.6393	0.3771–1.465	0.7067

PFS, progression-free survival; OS, Overall survival; CI, Confidence interval.
For survival analysis, PFS and OS distribution was determined using the Kaplan–Meier method. The patients with 17q23-25 amplification had significantly shorter PFS and OS than that did those without 17q23-25 amplification in CCC tumors Meanwhile, PFS and OS did not show significant correlations in *miR-21* overexpression, PTEN protein loss, or clinicopathological date.

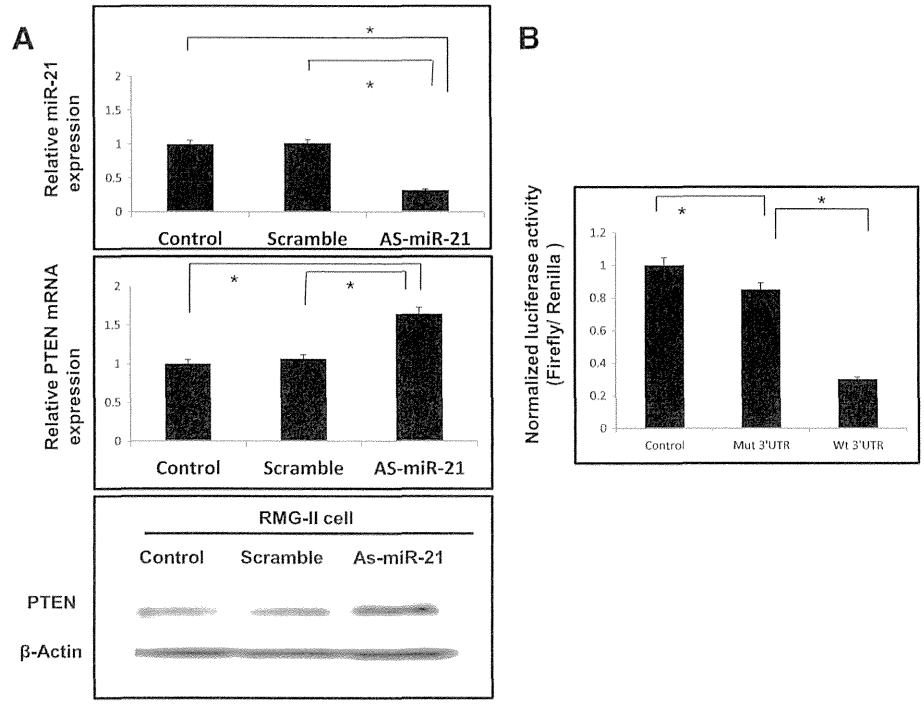

Figure 3 *miR-21* modulates PTEN tumor suppressor gene expression. To evaluate the biological significance of *miR-21* overexpression in CCC, we used a loss-of-function antisense approach. An antisense *miR-21* oligonucleotide (ODN) was used to knock down *miR-21* expression in RMG-II cells. **(A)** Efficiency of RMG-II cell transfection was confirmed by real- time RT PCR. *PTEN* mRNA expression was increased by knockdown of *miR-21* in RMG-II cells. Western blot analysis showing that PTEN expression was increased in RMG-II cells upon inhibition of *miR-21*. **(B)** *MiR-21* directly targets the 3'-UTR of *PTEN* mRNA. The activity of luciferase in the pGL3 wild-type *PTEN* 3'-UTR was downregulated compared to pGL3 mutant-type *PTEN* 3'-UTR and the pGL3 control in RMG-II cells. P <0.05 according to the t-test.

miR-21, and its expression is regulated by *miR-21* in CCC (Figure 3B). Several potential miR21 targets that could have implications in CCC were identified using web-based computational approaches to predict gene targets (miRBase Targets BETA Version 1.0, PicTar predictions, and TargetScan). Three putative target genes, PDCD4, SMARCA4, and SPRY2, were predicted by 3 different programs. This result indicates that tumor suppressor genes are potentially regulated by miR21. Therefore, we performed real-time RT-PCR for PDCD4, SMARCA4, SPRY2 in the miR21 knockdown experiments in RMG-II cells. We found that *miR-21* knockdown increased the expression of these mRNAs (Additional file 5: Figure S5). To investigate the regulation of PTEN expression by *miR-21* in JHOC9 cells, we overexpressed miR21 using miR21 mimics in JHOC9 cell. Quantitative real-time PCR analysis confirmed the level of miR21 was significantly overexpressed. As expected, the level of PTEN mRNA was downregulated in JHOC9 cells. Expression of PDCD4, SMARCA4, and SPRY2 mRNA was also decreased by the overexpression of miR-21 in response to miR-21 mimics in JHOC9 cells (Additional file 6: Figure S6).

Discussion

DNA copy number aberrations are a frequent event in many malignant tumors, leading to altered expression and function of genes residing within the affected genome region. Such genomic abnormalities can harbor either oncogenes or tumor suppressor genes depending on the original gene function and whether the copy number is amplified or deleted. Previous studies have identified a high frequency of copy number amplifications in CCC, including 17q23-25 (18-40%), 20q13 (22-25%), and 8q21q- 24q. Additionally, deletions at chromosome 9q and 19p have been also reported in CCC [9,18-20]. Of the chromosomal alterations associated with CCC, 17q23-25 is one of the most frequently amplified regions and is reported to be associated with patient outcome [9]. So far, *PPM1D* and *APPBP2* have been identified as potential targets of 17q23-25 amplification in CCC. However, a recent report suggests there might be new driver genes other than *PPM1D* and *APPBP2* in this region [11]. More than half of miRNAs have been aligned to genomic fragile sites or frequently deleted or amplified regions in several malignancies [21,22]. MiRNAs are a class of small, non-coding RNA molecules that regulate gene expression

through translational repression or cleavage of target mRNA. Among them, *miR-21*, located on 17q23.2, is unique in that it is overexpressed in many cancers as an oncogene. Previous studies have revealed several significant *miR-21* targets that might be related to carcinogenesis. Based on this evidence, *miR-21* is a potential candidate for 17q23-25 amplification in CCC oncogenesis.

We analyzed DNA copy number alterations at chromosome 17 in a panel of 28 primary CCCs using CGH array. In our data set, 17q23-25 amplification was observed at a frequency similar to that of previous reports. In addition, we confirmed that 17q23-25 amplification correlated negatively with patient prognosis, suggesting that the chromosomal alteration might result in the overexpression of genes that contribute to the genomic instability of CCC. Although we did not find a statistical correlation between *miR-21* overexpression and amplification of this region, overexpression of *miR-21* was observed in 60% of the CCC cases examined.

Targets of *miR-21* in cancer include *PTEN*, *PDCD4*, *LRRFIP1*, *RECK*, *TIMP-3*, *TPM1*, *BTG2*, and *Sprty2* [23]. *PTEN* can restrict growth and survival signals by limiting the activity of the phosphoinositide 3-kinase (PI3K) pathway. A decrease in PTEN might cause activation of the PI3K pathway, including Akt and mTOR, which leads to tumor development [24]. The prominent role of PTEN inactivation in CCC is thought to involve multiple mechanisms. In our study, loss of PTEN protein was observed in 46% of CCC patients. On the other hand, low of PTEN copy number was not indicted by CGH array (data not shown). Furthermore, no significant correlation was observed between *miR-21* overexpression and loss of PTEN expression in our date set. Therefore, we suggest the involvement of another epigenetic mechanism, such as *PTEN* mutations, promoter methylation of *PTEN*, loss of heterozygosity at the *PTEN* locus other miR are infrequent in CCC. Although there was no statistical correlation between PTEN loss and *miR-21* overexpression, the occurrence of 17q23-25 amplification along with both *miR-21* overexpression and PTEN protein loss was detected in 14% of CCC cases. Thus, this oncogenetic mechanism might play a prominent role in CCC. Additionally, we showed that *miR-21* inhibition significantly increased PTEN expression in vitro. Moreover, the results obtained from the dual luciferase reporter assay supports the idea that *miR-21* directly targets the *PTEN* gene, regulating the protein expression. It is therefore possible that miRNAs such as *miR-21* modulate PTEN expression by transcriptional regulation or target degradation in CCC.

Finally, we found a significant correlation between *miR-21* overexpression and endometriosis in CCC. Endometriosis-related CCC is thought to be a chronic inflammatory disease, characterized by increased production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, and TNF-α [25]. We recently reported that CCC showed a dominant Th-2 cytokine expression pattern driven largely by *IL-6* expression [26]. In addition, IL-6 induces *miR-21* expression through a STAT3-dependent pathway [27]. We also confirmed that IL-6 induces *miR-21* overexpression in RMG-II (data not shown). In our study, *miR-21* overexpression was observed in 60% of the CCC cases, regardless of 17q23-25 amplification status, suggesting another mechanism might regulate *miR-21* expression. *miR-21* might contribute to inflammation-induced

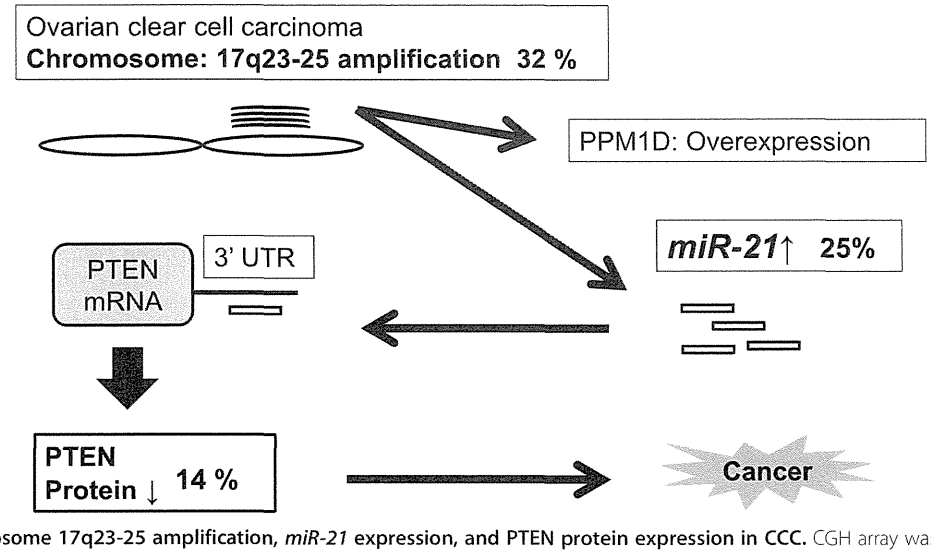

Figure 4 Chromosome 17q23-25 amplification, *miR-21* expression, and PTEN protein expression in CCC. CGH array was performed to evaluate chromosomal alterations in 28 primary CCC tumors. Nine out of 28 patients (32%) showed chromosomal amplification in the 17q23-25 region that contains *miR-21*. Seven of 9 tumors (77.7%) with 17q23-25 amplification showed *miR-21* overexpression. 17q23-25 amplification with both *miR-21* overexpression and PTEN protein loss was detected in 4/28 cases (14.2%).

carcinogenesis in CCC with endometriosis. We need to further analyze miR21 expression using in situ hybridization in the endometriotic lesions of CCC specimens. The correlation between miR21 and endometriosis observed in our study indicates a role for miR21 in precursor lesions of ovarian CCC.

Conclusions

This study is the first to indicate *miR-21* as the gene of interest in 17q23-25 amplification associated with CCC (Figure 4). Aberrant expression of *miR-21* by chromosomal amplification might play an important role in CCC carcinogenesis through regulating the *PTEN* tumor suppressor gene. Moreover, the modulation by *miR-21* overexpression of genes other than *PTEN* should not be overlooked in determining the oncogenic mechanism of CCC.

Additional files

Additional file 1: Figure S1. *MiR-21* and *PPM1D* mRNA expression located on 17q23- 25. Black dots indicate a cluster with 17q23-25 amplification, and white dots indicate a cluster without 17q23-25 amplification. We measured the median expression of *miR- 21* and *PPM1D* mRNA and set a transverse line as standard value. Seven of 9 tumors with 17q23-25 amplification showed *miR-21* overexpression. Six of 9 tumors with 17q23-25 amplification showed *PPM1D* overexpression.

Additional file 2: Figure S2. Immunohistochemical analysis of PTEN that might be a potential target of *miR-21* was performed using the same primary CCC cases. The intensity of positive staining was scored from 0 to 2, while the extent of positive staining was scored from 0 to 4. Addition of the two values gives the total score; scores >4 were considered PTEN-positive. (A) Typical image of a PTEN-negative case. (B) Typical image of a PTEN-positive case. Loss of PTEN protein was observed in 13 of 28 patients (46.4%).

Additional file 3: Figure S3. Frequency of copy number changes in Chr 17q23-25 region by copy number assay in 5 CCC cell lines. We found the copy number was increased in RMG-I and RMG-II cells.

Additional file 4: Figure S4. *MiR-21*, *PTEN* mRNA, and PTEN protein expression in CCC cell lines. (A) (B) Relative expression of *miR-21* and *PTEN* mRNA were detected with real-time RT-PCR, and the relative amount of *miR-21* was determined using $2^{-\Delta\Delta}$CT. (C) PTEN protein was measured by western blotting. The RMG-II cell line was selected for further analysis, because it had the most prominently overexpressed *miR-21* and decreased PTEN protein of the CCC cell lines.

Additional file 5: Figure S5. Three putative target genes, PDCD4, SMARCA4, and SRY2, are potentially regulated by miR21. (A) (B) (C) Real-time RT-PCR for PDCD4, SMARCA4, SPRY2 in the miR21 knockdown experiments in RMG-II cells. *miR-21* knockdown caused an increase in mRNA expression of these genes by real-time RT PCR in RMG-II cells.

Additional file 6: Figure S6. Mir21 modulates PTEN expression in JHOC9 cell. To investigate the regulation of PTEN expression by *miR-21* in JHOC9 cells, we overexpressed miR21 by miR21 mimics in JHOC9 cells. Quantitative real-time PCR analysis confirmed miR21 was significantly overexpressed. As expected, the level of PTEN mRNA was downregulated in JHOC9 cells. PDCD4, SMARCA4, and SPRY2 mRNAs were also reduced by the overexpression of miR-21 in response to miR-21 mimics in JHOC9 cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YH performed experiments and analyzed data. YH and NY drafted manuscript YH, MU, and AO carried out bioinformatics analyses of the CGH data. YH and MS carried out the molecular genetic studies. YN, MN, SM, YM, and YH participated in the design of the study. All authors contributed to data analysis, interpretation, and final approval of the manuscript.

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Gynecologic Cancer InterGroup (GCIG) Consensus Review for Clear Cell Carcinoma of the Ovary

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Abstract: Clear cell carcinoma of the ovary (CCC) is a histologic subtype of epithelial ovarian cancer with a distinct clinical behavior. There are marked geographic differences in the prevalence of CCC. The CCC is more likely to be detected at an early stage than high-grade serous cancers, and when confined within the ovary, the prognosis is good. However, advanced disease is associated with a very poor prognosis and resistance to standard treatment. Cytoreductive surgery should be performed for patients with stage II, III, or IV disease. An international phase III study to compare irinotecan/cisplatin and paclitaxel/carboplatin as adjuvant chemotherapy for stage IIV CCC has completed enrollment (GCIG/JGOG3017). Considering the frequent *PIK3CA* mutation in CCC, dual inhibitors targeting PI3K, AKT in the mTOR pathway, are promising. Performing these trials and generating the evidence will require considerable international collaboration.

Key Words: Clear cell carcinoma of the ovary (CCC), Deep venous thrombosis, Glycogen, Hepatocyte nuclear factor-1ß, *WT1*, Pulmonary embolism, Ethnics, *ARID1A*, *PIK3CA*, *PPM1D*, *PPP2R1A*, *KRAS*, Cytoreductive surgery, Paclitaxel, Platinum, Irinotecan hydrochloride, Cisplatin, PI3K/AKT/mTOR

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Ovarian cancer is made up of several different histological subtypes, and it is clear that these represent different diseases with distinct biology, pathogenesis, and clinical behavior. However, to date, they have all been treated in the same way. As understanding of the differences increases, this is no longer a rational approach. Most women included in clinical trials have high-grade serous (HGS) ovarian cancer, and it cannot be assumed that the results of these trials are applicable to women with other histotypes. Clear cell carcinoma of the ovary (CCC) is more likely to be detected at an early stage than HGS cancers, and when confined within the ovary, the prognosis is good. However advanced disease is associated with a very poor prognosis and resistance to standard treatment.[1] Histotype-specific trials and treatment protocols are required. Performing these trials and generating the evidence will require considerable international collaboration.

EPIDEMIOLOGY

The CCC is a histologic subtype of epithelial ovarian cancer with a distinct clinical behavior. There are marked geographic differences in the prevalence of CCC. In North America and Europe, CCC is the third most common histologic subtype of epithelial ovarian cancer, with an estimated prevalence of 1% to 12%.[2] Recent Surveillance, Epidemiology, and End Results data revealed that the incidences of CCC in women living in United States were 4.8% in whites, 3.1% in blacks, and 11.1% in Asians.[3] In Japan, the prevalence of CCC is higher than in western countries, although the reason for this remains unknown.[1] The annual report of the Japanese Gynecologic Cancer Committee showed an increasing incidence of CCC as a proportion of all epithelial ovarian cancers (Fig. 1),[4] now making up more than 25% of epithelial ovarian cancers in Japan.

The incidence of thromboembolic complications in CCC, such as deep venous thrombosis and pulmonary embolism, is reported to be higher than other epithelial ovarian cancers (16.9%–27.3% vs 0%–6.8%) and is considered as an independent prognostic factor.[5,6]

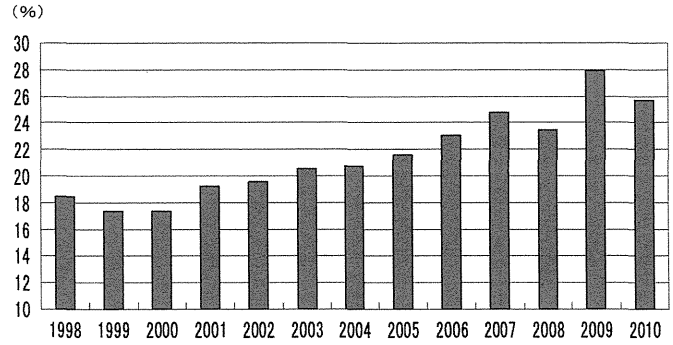

FIGURE 1. The rate of CCC among all epithelial ovarian cancers in Japan (annual reports on Japanese Gynecologic Cancer Committee).

PATHOLOGY

Gross

Most CCCs are unilateral. Typically, the sectioned surface of the tumor reveals a unilocular cyst with 1 or more solid, yellow nodules protruding into the cyst. Cysts may contain watery, mucinous fluid or brownish "chocolate-colored" fluid. Multilocular cysts are less common, and occasional tumors are predominantly solid. The mean size of CCC is 15 cm.

Microscopic

The CCC is composed of glycogen-containing cells with abundant clear cytoplasm and hobnail cells. Many tumors also contain cells with granular eosinophilic cytoplasm. Nuclei are often eccentrically placed, with rounded-to-angulated contours. Hobnail cells have scant cytoplasm and enlarged, bulbous, hyperchromatic nuclei that protrude into tubule and cyst lumens. Bland and flattened cuboidal cells may line cysts or glands. It may arise within an endometriotic cyst, and benign endometriotic lesions or atypical endometriosis may be seen adjacent to CCC. Occasionally, CCCs also present in association with adenofibromatous, clear cell borderline tumors.

The CCC exhibits high-grade nuclear features, although a spectrum of nuclear atypia may be present. The CCCs have traditionally been considered to be of high grade, but mitotic figures are relatively uncommon compared with other ovarian carcinomas.

Architectural patterns include tubulocystic, papillary, solid, and mixtures of them. Tubulocystic areas include tubules and cysts that are lined by flat-to-cuboidal cells with variable atypia and scattered hobnail cells (Fig. 2A). Papillary areas contain papillae that are small and round in comparison with those in serous carcinoma (Fig. 2B). The fibrovascular cores may be filled with either fibromatous, myxoid, spherulelike mucoid, or hyalinized basement membrane-type material. Solid areas are composed of sheets of polyhedral cells with clear cytoplasm (Fig. 2C).

Mixed subtypes of epithelial carcinomas are found. However, these should be considered as HGS tumors.[7]

Immunohistochemistry

The differential keratin profile is CK7+/CK20−, although CK7 may be focal in approximately 10% of cases.[8] In general, CCCs are negative for estrogen receptor, progesterone receptor, and *WT1*. Hepatocyte nuclear factor-1ß is a relatively new marker that is positive in CCC.[9] WT1 is useful in distinguishing CCC from mixed serous/clear cell tumors as it is typically positive in the latter.

Molecular Biology and Genetics

Unlike HGS tumors, CCCs are generally p53 wild type and have a lower frequency of *BRCA1* and *BRCA2* mutations.[10] The most frequent alterations are *ARID1A* and *PIK3CA* mutations.[11,12] *ARID1A* encodes the protein *BAF250a*, which is integral in the SWI-SNF chromatin remodeling complex. *ARID1A* mutations are seen in 40% to 60% of CCCs, but not in HGS carcinomas. In general, loss of *BAF250a* expression correlates with mutational status. *PIK3CA* mutations are seen in approximately 40% of clear cell tumors. Amplification and overexpression of the antiapoptotic protein, *PPM1D*, is seen in 10% of CCCs,[13] and mutation of *PPP2R1A* has been reported

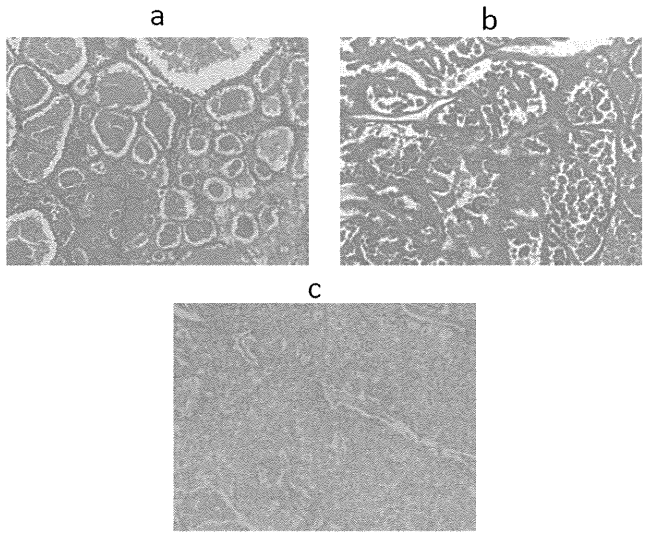

FIGURE 2. Microscopic findings of CCC. Tubulocystic areas include tubules and cysts that are lined by flat-to-cuboidal cells with variable atypia and scattered hobnail cells (A). Papillary areas contain papillae that are small and round in comparison with those in serous carcinoma (B). The fibrovascular cores may be filled with either fibromatous, myxoid, spherulelike mucoid, or hyalinized basement membrane-type material. Solid areas are composed of sheets of polyhedral cells with clear cytoplasm (C).

in 7%, whereas KRAS has been reported in 5%.[14] The CCCs are not a uniform group. Tan et al demonstrated groups with distinct patterns of copy number aberration in a comparative genomic hybridization analysis,[15] which seems to have prognostic significance.

Gene Expression Analysis

Yamaguchi et al[16] identified the gene signature that distinguishes CCC from other types of ovarian cancer using a microarray data set of ovarian cancers. The signature consisted of 437 genes and was designated as the CCC signature, which is specific for CCC. A categorical analysis demonstrated that genes belonging to 3 categories—stress response, sugar metabolism, and coagulation—are frequently involved in this signature.

INITIAL TREATMENT

Appropriate surgical treatment, followed by systemic chemotherapy, is recommended as an initial treatment for patients with CCC. The standard surgical treatment for patients with CCC is the same as for other epithelial ovarian cancers and includes hysterectomy, bilateral salpingo-oophorectomy, omentectomy, pelvic and para-aortic lymphadenectomy, and cytoreductive surgery. The recommended regimen of postoperative chemotherapy is paclitaxel (175 mg/m^2) combined with carboplatin (AUC 5–7.5), given every 3 weeks for 6 cycles.

Surgery

Lymphadenectomy is important to detect whether lymph nodes are involved in CCC because the presence of lymph node metastasis is an independent prognostic factor[17,18] and may guide the need for adjuvant therapy in early disease. Several authors have examined the therapeutic role of lymphadenectomy in therapy for this disease. In the Multicenter Italian Trials in Ovarian Cancer (MITO 9) retrospective study, disease-free survival in patients who underwent lymphadenectomy was longer than in other patients ($P = 0.0001$), in both early (I/II) ($P = 0.0258$) and advanced (III/IV) ($P = 0.037$) stages.[18] Lymphadenectomy also prolonged overall survival (OS) in patients with advanced stage ($P = 0.0039$). However, previous other reports have failed to show a therapeutic benefit from lymphadenectomy.[17,19] Further study will be required to identify the impact of lymphadenectomy on a patient's outcome from CCC.

Cytoreductive surgery should be performed for patients with stage II, III, or IV disease. Takano et al[20] reported no significant prognostic difference between the patients who underwent optimal cytoreduction (<1 cm) and those who had residual disease of greater than 1 cm. Complete surgery with no residual macroscopic disease was the only independent prognostic factor (median progression-free survival, 7 vs 5 vs 39 months, respectively). In a study by the Gynecologic Oncology Group, the markedly poor prognosis of CCC was observed even when patients have small-volume disease.[21] These findings suggest that a maximal effort should be made to remove all gross disease in patients with CCC.

Unilateral salpingo-oophorectomy preserving contralateral normal ovary and uterus should be considered for patients desiring to remain fertile. Several studies have examined outcomes of fertility-sparing surgery in patients with stage I CCC.[22,23] A total of 23 IA patients underwent fertility-sparing surgery, and all patients, excluding one (4%), were alive without recurrence. In contrast, 6 (25%) of the 24 patients at stage IC relapsed after surgery. Therefore, fertility-sparing surgery should only be offered for patients with stage IA disease.

Adjuvant Therapy

All patients with CCC have traditionally received postoperative systemic chemotherapy. However, observation may be considered for patients with surgical stage IA disease, because survival for these women is greater than 95%.[20,22,23]

It is generally accepted that CCC is resistant to conventional platinum-based chemotherapy compared with HGS ovarian cancer. The variation in reported response rates may reflect heterogeneity in patients included with some older studies including those with mixed tumors that would now be considered to be HGS tumors. Combination chemotherapy with paclitaxel plus platinum (TC) is thought to yield a higher response rate than conventional platinum-based chemotherapy (22%–56% vs 11%–27%) and improved survival in patients with advanced CCC, especially for those with optimal cytoreduction,[24,25] although the addition of a taxane was not an independent prognostic factor in the MITO 9 study. Nevertheless, responses remain much lower than with HGS, and there is an urgent need for more effective therapies.

In a randomized phase II study, the Japanese Gynecologic Oncology Group compared irinotecan hydrochloride plus cisplatin (CPT-P) with TC.[26] Both regimens were tolerated well, and progression-free survival between the 2 groups was

similar. An international phase III study to compare CPT-P and TC as adjuvant chemotherapy for stage I-to-IV CCC has completed enrollment (GCIG/JGOG3017). The results will be reported in 2014.

Adjuvant radiotherapy may have a role in the adjuvant treatment of early stage CCC. A retrospective series, which included 375 women with CCC, endometrioid and mucinous, found a benefit in survival for women treated with adjuvant chemotherapy plus whole abdominopelvic radiotherapy compared with chemotherapy alone.[27] The benefit seems to be mainly in high-risk stage 1C (positive peritoneal cytology and ovarian surface disease) and stage II disease.[28] These findings require confirmation in a prospective randomized trial.

METASTATIC DISEASE AND RELAPSE

Pattern of Relapse

The prognosis of recurrent CCC is very poor, even compared with that of recurrent serous adenocarcinoma with 5-year OS rates of 22.5% and 32.4%, respectively (P = 0.0007) and median OS of 25.3 versus 42.0 months. The 5-year postrecurrence survival rate is only 13.2% for CCC compared with 18.2% for HGS ($P < 0.0001$), with a median postrecurrent survival of 10.0 versus 18.9 months.[29] Recurrence rates of CCC were 29%, 30%, 62%, and 73% for stages I, II, III, and IV, respectively, with a median time to recurrence for the stages I and II of 12.2 months.[1] The prognosis is particularly poor relative to HGS tumors, where there is residual tumor after initial surgery.[1,29]

The pattern of recurrence also differs from HGS with higher rates of relapse in the lymph nodes (pelvic, para-aortic, and other lymph nodes) (40% vs 7%, $P < 0.001$) and parenchymal organs (liver, lung, bone, spleen, brain and others) (40% vs 13%, $P < 0.01$).[30]

Treatment

Treatment of recurrent CCC has, to date, followed the same protocols as are used for other recurrent ovarian epithelial carcinomas.[31] However, recurrent CCC is very resistant to chemotherapy with response rates of less than 10%.[32] Unlike in ovarian cancer in general, platinum-free interval does not seem to predict for further chemotherapy sensitivity. Despite evidence that recurrent CCC is resistant to subsequent chemotherapies,[32] no correlation between an efficacy of the second-line chemotherapy and histopathological types has been yet revealed.[33] However, this may be due to the small numbers of clear cell cancers included in trials of recurrent disease. For example, in a phase III study of paclitaxel plus carboplatin versus liposomal doxorubicin plus carboplatin in platinum-sensitive recurrence, the proportion of serous adenocarcinoma was 72%, whereas that of clear cell adenocarcinoma was only 2.8%.[34] In a phase III trial, liposomal doxorubicin was compared with gemcitabine in platinum-resistant recurrent cases; the proportion of serous adenocarcinoma was 80%, whereas that of clear cell adenocarcinoma was only 6.5%.[35]

Given the limited benefit from cytotoxic drugs, there is now great interest in the development of molecular targeted therapy for the treatment of CCC.

FUTURE DIRECTIONS

Table 1 lists the potential therapeutic targets in CCC. The continued development of targeted agents in the treatment of CCC requires investigation of a number of areas in the laboratory. The mechanisms of resistance to targeted agents are largely unknown, but an understanding of these will be essential for clinical development and application.

The lack of a mouse model of CCC is another important issue. For the deeper understanding of the pathogenesis of CCC as well as the more accurate evaluation of the antitumor activity of particular agents against CCC, a mouse model of CCC needs to be developed in the future.

Intelligently designed clinical trials are essential for the clinical development of novel therapies. There are a number of promising agents, but the optimal way to sequence and/or combine them needs to be established. Tumors require a vascular blood supply to grow beyond 2 to 3 mm; thus, subclinical ovarian tumors that develop after complete clinical response to

TABLE 1. Potential therapeutic targets in CCC

Targets	Roles in Tumor Development	Comments
Phospho-AKT	Proliferation/survival	Frequently in CCC and SAC
Phospho-mTOR	Proliferation/angiogenesis/metabolism	Frequently expressed in CCC
HIF-1α	Angiogenesis, adaptive response to hypoxia	Frequently expressed in CCC and SAC
VEGF	Angiogenesis	Frequently expressed in CCC and SAC
HNF-1β	Detoxification, chemoresistance, and survival	Frequently expressed in CCC
Annexin A4	Detoxification, chemoresistance	Frequently expressed in CCC
Osteopontin	Survival/migration/invasion	Frequently expressed in CCC and SAC
UGT1A1	Detoxification, irrinotecan resistance	Frequently expressed in CCC
IGFBP-1	Proliferation/survival	Frequently expressed in CCC
IGF2BP3	Translation, migration	Frequently expressed in CCC and SAC
IL-6/STAT-3	Proliferation/antiapoptosis/angiogenesis	Frequently expressed in CCC

SAC, serous adenocarcinoma.

first-line chemotherapy require angiogenesis for continued proliferation. Considering the significant antiproliferative and antiangiogenic activities of targeted agents, the activity of these agents as a maintenance therapy for preventing or delaying the development of recurrent disease needs to be investigated.

As the PI3K/AKT/mTOR signaling pathway is hyperactivated in CCC, strategies aimed to inhibit this pathway may have therapeutic activity for CCC.[36] Not only using them as a monotherapy, combination treatments may also be an attractive strategy to investigate in trials. For example, patients might start on mTOR-targeting therapy and then switch to VEGF-targeting therapy on progression. Other avenues of research include sequencing mTORC1 inhibitor initially and then switching to an mTORC1/2 inhibitor. Considering the frequent *PIK3CA* mutation in CCC,[12] dual inhibitors targeting PI3K, AKT in the mTOR pathway, are also promising. The efficacy and toxicities of simultaneous inhibition of different signaling pathways should be investigated. Another potential avenue is the combination of targeted agents with effective cytotoxic agents, as targeting agents are generally cytostatic. Intriguing preclinical results suggest that trabectedin is the most effective of the existing cytotoxic agents against CCC,[37] and this may be enhanced by the addition of an mTOR inhibitor. This combination warrants further investigation in future clinical studies.

Translating preclinical findings into successful treatment for patients is challenged by issues that are common to studying targeted agents in any tumor and studying any therapy in rare tumors. First, the method of efficacy evaluation of targeted agents needs to be reassessed. As many of the targeted agents have cytostatic rather than cytotoxic effects, the traditional criteria applied to cytotoxic agents, such as RECIST, might be less applicable when determining the clinical benefit of targeted agents. Second, identifying biomarkers that can be used to predict a patient's sensitivity to the targeted agents is a critical issue. The identification of surrogate markers to monitor the activity of targeted agents is also necessary. For this purpose, future clinical studies of targeted agents must incorporate translational research.

Finally, given the rarity of and the geographical difference in the prevalence of CCC, international collaboration, mediated by the Gynecologic Cancer InterGroup, may be essential to obtain adequate patient numbers. These efforts will allow selection of the best treatment for investigation in larger-scale clinical trials. Moreover, these challenges will aid in the development of optimal, personalized targeted therapies for CCC.

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