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Assessment of Hyperthermic Intraperitoneal Chemotherapy to Eradicate Intraperitoneal Free Cancer Cells[1]

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Abstract

OBJECTIVE: To assess the effect of hyperthermic intraperitoneal chemotherapy (HIPEC) to eradicate intraperitoneal free cancer cells and to explore the feasibility of cytological cure for peritoneal carcinomatosis (PC). *METHODS:* The peritoneal lavage fluid (or ascites) from 50 PC patients was collected before and after intraoperative HIPEC, respectively, for conventional cytology test, and conventional and real-time quantitative reverse transcript polymerase chain reaction detecting carcinoembryonic antigen (CEA) mRNA and cytokeratin-20 (CK20) mRNA. The blood samples 3 days before and 7 days after intraoperative HIPEC were also collected for detecting the serum tumor markers, including CEA, carbohydrate antigen (CA) 125, and CA19-9. *RESULTS:* The positive rate of conventional cytology test before HIPEC versus after HIPEC was100.0% versus 22.0% ($P = .000$). The positive rates of CEA mRNA and CK20 mRNA before HIPEC versus after HIPEC were 100.0% versus 86.0% ($P = .012$) and 100.0% versus 96.0% ($P = .495$), respectively. Moreover, after HIPEC, 18 (36.0%) patients had a decline in CEA mRNA ($P = .000$), and 17 (34.0%) patients had a decline in CK20 mRNA ($P = .000$). The positive rates of serum CEA, CA125, and CA199 before HIPEC versus after HIPEC were 52.0% versus 28.0% ($P = .014$), 52.0% versus 44.0% ($P = .423$), and 40.0% versus 28.0% ($P = .205$), respectively. *CONCLUSION:* HIPEC could effectively eradicate intraperitoneal free cancer cells and partially achieve cytological cure for PC.

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Introduction

Peritoneal carcinomatosis (PC) is one of the most frequent types of cancer recurrence after curative resection in gastrointestinal and gynecological cancers [1]. PC develops from micrometastasis, which originated from intraperitoneal free cancer cells (IFCCs). These cells first attach to the peritoneal surface and then migrate into the subperitoneal tissue, resulting in the formation of the peritoneal micrometastasis, which is thus thought to be the origin of recurrence [2].

Generally, PC patients have a very poor prognosis with less than 6 months of median survival [3]. The most widely accepted therapies for such patients are systemic chemotherapy, best support care, and palliative treatment, without any hope of cure. Over the past three decades, PC is no longer universally considered as terminal cancer metastasis but as regional tumor progression [4]. Moreover, aggressive cytoreductive surgery (CRS) plus hyperthermic intraperitoneal chemotherapy (HIPEC) has been developed as an integrated treatment package and has brought significant survival benefit in selected patients [5]. Major technical advantages of this treatment approach are to maximally reduce the visible tumor burden by CRS and to eradicate residual tumor nodules, micrometastases, and free tumor cells by HIPEC.

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In China, we have conducted a series of preclinical and clinical studies on the feasibility, efficacy, and safety of this multidisciplinary treatment approach in animal models [6] and in clinical settings [3] and established a designated CRS + HIPEC center. The survival benefits of this treatment package have been demonstrated in patients with PC that originated from gastric cancer [7,8] and colorectal cancer [9,10]. In this study, we collected the peritoneal lavage fluid (or ascites) and blood samples before and after intraoperative HIPEC of 50 PC patients and analyzed the changes of IFCCs, carcinoembryonic antigen (CEA) mRNA, cytokeratin-20 (CK20) mRNA, and serum tumor makers to assess the effect of HIPEC to eradicate IFCCs and the explore feasibility of cytological cure for PC.

Materials and Methods

Patient Selection

This study included 50 consecutive Chinese patients with PC that originated from gastric cancer, colorectal cancer, epithelial ovarian cancer, or appendix mucinous adenocarcinoma, all treated by CRS + HIPEC procedures from October 2014 to August 2015 at our hospital. The inclusion criteria were: 1) age 20 to 75 years old; 2) Karnofsky performance status >50; 3) life expectancy >8 weeks; 4) normal peripheral blood white blood cells count ≥3500/mm^3 and platelet count ≥80,000/mm^3; 5) acceptable liver function, with bilirubin ≤ 2× the upper limit of normal (ULN) and aspartic aminotransferase and alanine aminotransferase ≤ 2× ULN; 6) acceptable renal function, with serum creatinine ≤1.5 mg/dl; and 7) cardiovascular pulmonary and other major organ functions can stand major operation. Major exclusion criteria were: 1) age <20 years or >75 years; 2) any lung metastasis, liver metastasis, or prominent retroperitoneal lymph node metastasis during preoperative assessment; 3) serum bilirubin level >2× ULN; 4) liver enzymes >2× ULN; and 5) serum creatinine level >1.5 mg/dl. Informed consents were obtained from all patients, and the study was approved by the institutional review board and the ethics committee.

CRS Plus HIPEC Procedure

All CRS + HIPEC procedures were conducted by a designated team focusing on PC treatment. In brief, the abdominal exploration was performed through a midline xiphoid-pubic incision after general anesthesia, and peritoneal cancer index (PCI) was evaluated according to the Sugarbaker criteria [11]. Then, maximal CRS was performed. The extent of CRS was determined by the Sugarbaker criteria [12] on the completeness of cytoreduction (CC). A score of CC-0 indicates no residual peritoneal disease after CRS; CC-1, less than 2.5 mm of residual disease; CC-2, residual tumor between 2.5 mm and 2.5 cm; and CC-3, more than 2.5 cm of residual tumor or the presence of a sheet of unresectable tumor nodules.

The HIPEC was implemented by the open Colliseum technique, and with paclitaxel 120 mg and lobaplatin 100 mg each dissolved in 6 l of heated saline with temperature of 43.0°C ± 0.5°C. The total HIPEC time was 60 minutes, with a flow rate of 400 ml/min, after which gastrointestinal anastomoses or stoma was made. After operation, the patient was delivered to the intensive care unit for recovery. When the condition stabilized, the patients were transferred to the surgical oncology ward.

Peritoneal Lavage

Ascites fluid (50 ml) was collected from the deep abdominal cavity (spleen fossa, Douglas pouch, paracolic sulci) at laparotomy. In patients with scanty ascites, 50 ml of saline was introduced into the abdominal cavity at the beginning of the operation and aspirated after general stirring. After HIPEC, 50 ml of peritoneal washing fluid was aspirated. These washes were equally divided into three parts, and all were centrifuged at 2000 rpm at 4°C for 10 minutes to collect intact cells. One part of each peritoneal wash was examined cytopathologically after conventional Wright's staining, and the other two parts were stored at −80°C until use for reverse transcriptase-polymerase chain reaction (RT-PCR).

Conventional Cytology Examination

The cell sediments were smeared onto a glass slide, stained by Wright's method, observed with Olympus BX51 microscopy, and photographed by Olympus DP72 image collecting system. Experienced cytopathologists performed cytological evaluation. The smears were classified according to their cytological features, as follows: 1) IFCC positive: Cells were arranged mainly in loose clusters with the occasional presence of floating cells. Nuclear cytoplastic rate was disturbed. Nuclei were usually eccentric, with a thick nuclear membrane, large prominent nucleoli, and coarsely granular chromatin. 2) IFCC negative: Normal cells are present, or cells showonly milder changes of chromatin. 3) Cancer cell cytolysis: Cancer cells lost cell membrane integrity and with karyolysis, pyknosis, or karyorrhexis. Cell debris are dispersing into extracellular space.

Total RNA Extraction and cDNA Synthesis

Frozen samples were thawed, and total RNA was extracted using RNAsimple Total RNA kit (TIANGEN, Beijing, China) by a guanidinium-isothiocyanate-phenol-chloroform–based method. cDNA was generated with a first-strand cDNA synthesis Kit (Thermo, Waltham, MA) using the protocol recommended by the manufacturer. Extracted total RNA (up to 5 μg) was preincubated with 50 ng of oligo $(dT)_{18}$ primer in 9 μl of solution for 5 minutes at 65°C. After chilling on ice, 4 μl of five-fold synthesis buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM dithiothreitol), 1 μl of 20 U/μl RiboLock RNase inhibitor, 2 μl of 2.5 mM each dNTP, and 1 μl of 200 U/μl RevertAid- M-MuLV reverse transcriptase were added. The reaction mixture was then incubated for 60 minutes at 42°C and then heated at 70°C for 5 minutes, and the resultant first-strand cDNA was immediately used for PCR solution.

Conventional RT-PCR

The integrity of isolated RNA was proven by RT-PCR analysis of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Only samples with positive bands for GAPDH underwent the steps described below. Each series of RT-PCRs included RNA-negative samples as negative control, and mRNA from MGC 803 cells, which are well known to express high amounts of CEA and CK20, as a positive control. CEA-specific oligonucleotide primers for

Table 1. Primers Sequences in This Study

ID	Primer Sequences	Product Size
CEA	A: 5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′ B: 5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′ C: 5′-GGGCCACTGTCGGCATCATGATTGG-3′	131 bp
CK20	Forward: 5′-CAGACACACGGTGAACTATGG-3′ Reverse: 5′-GATCAGCTTCCACTGTTAGACG-3′	370 bp
GAPDH	Forward: 5′-CAAGGTCATCCATGACAACTTTG-3′ Reverse: 5′-GTCCACCACCCTGTTGCTGTAG-3′	496 bp

the nest RT-PCR reported by Gerhard et al. [13] and CK20-specific, GAPDH-specific primers were listed in Table 1. All the primers were synthesized by Sangon Biotech (Shanghai, China). All the PCRs were carried out in a 25-μl reaction mixture containing 12.5 μl of 2× Taq MasterMix (CWBIO, Beijing, China), 0.4 μM primers, and 1 μl of template cDNA. For CEA, 20 rounds of amplification were performed with primers A and B in a thermal cycler (Applied Biosystems, Grand Island, NY) at 94°C for 1 minute and 72°C for 2 minutes and 30 seconds, with a final extension step at 72°C for 10 minutes. One-microliter aliquots of reaction product were then transferred into second tubes containing the identical reaction mixture except for the primers C and B. Twenty more cycles were run at 94°C for 1 minute and 72°C for 2 minutes and 30 seconds with a final extension step for 10 minutes. For CK20, denaturation was performed for 1 cycle at 94°C for 3 minutes and 38 cycles of 94°C for 1 minute, 60°C for 45 seconds, and 72°C for 45 seconds. For GAPDH, denaturation was performed for 1 cycle at 94°C for 30 seconds and 35 cycles of 94°C for 30 minutes, 58°C for 30 seconds, and 72°C for 45 seconds. Six-microliter quantities of the final products were then run on 1.5 % agarose gels, followed by ethidium bromide staining. The images were collected using a gel imaging system (Bio-Rad, Hercules, CA). Samples with visible 131–base pair (bp), 370-bp, and 496-bp bands were designated as CEA, CK20, and GAPDH positive, respectively. All necessary precautions against contamination of PCRs were rigorously applied. The nucleotide sequences of the PCR products were confirmed by the dideoxy chain termination sequencing method.

Real-Time RT-PCR

The one-step real-time quantitative PCRs were carried out in a 20-μl reaction mixture containing 10 μl 2× SYBR Premix EX Taq II (Takara, Tokyo, Japan), 0.4 μM primers, and 1 μl of template cDNA. The primers were the same with the conventional RT-PCR (Table 1) except for CEA amplification, in which only primer B and primer C were used. Also, each series of real-time RT-PCRs included the same positive and negative control as mentioned above. All real-time RT-PCRs were performed at CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) following the same temperature profile with conventional RT-PCR. Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification.

Serum Tumor Makers

Three milliliters of blood were obtained from each patient 3 days before surgery and 7 days after surgery, respectively. The serum was separated immediately, and then CEA, carbohydrate antigen (CA) 125, and CA19-9 were detected using a full-automatic electrochemistry luminescence immunity analyzer (Roche, Basel, Switzerland). The definitions of CEA, CA125, and CA19-9 positive were ≥5 ng/ml, ≥35 U/ml, and ≥37 U/ml, respectively.

Statistical Analysis

All statistical calculations were done with SPSS 20.0 statistical software (SPSS Inc., Chicago, IL). The figures show the mean ± standard deviation. The chi-square test or Fisher exact probability test and Student's *t* test for paired sample were used to analyze the quantitative data. The real-time quantitative RT-PCR relative quantity analysis used $2^{-\Delta\Delta ct}$ method with GAPDH as control group. The level of statistically significance was set at $P < .05$.

Table 2. Major Clinicopathologic Characteristics of the 50 Patients in This Study

Items	Value, *n* (%)
Gender	
Male	28 (56.0)
Female	22 (44.0)
Age (yr)	
Median age (range)	58 (26-75)
<60	32
≥60	18
Median KPS score (range)	80 (70-90)
PC origin	
Gastric cancer	11 (22.0)
Colorectal cancer	18 (36.0)
Epithelial ovarian cancer	9 (18.0)
Appendix mucinous adenocarcinoma	8 (16.0)
Peritoneum malignant mesothelioma	4 (8.0)
Ascites (ml)	
Scanty ascites	12 (24.0)
<1000 ml	18 (36.0)
≥1000 ml	20 (40.0)
PCI	
Median PCI (range)	19 (0-39)
<20	26 (52.0)
≥20	24 (48.0)
CC scores	
0-1	32 (64.0)
2-3	18 (36.0)

KPS, Karnofsky performance scale.

Results

Major Clinical-Pathological Characteristics

Fifty patients with PC that originated from gastric cancer, colorectal cancer, epithelial ovarian cancer, appendix mucinous adenocarcinoma, and peritoneum malignant mesothelioma, all pathology confirmed, were included in this study. The details of these 50 patients were listed in Table 2.

Result of Conventional Cytology

The IFCC-positive rate before HIPEC versus after HIPEC was 100.0% versus 22.0% ($P = .000$), respectively. Moreover, 14 patients became IFCC negative, and 25 patients were found to have cytolysis after HIPEC (Figure 1).

Result of Conventional RT-PCR

GAPDH shows a clear band at 496 bp for each sample. All the positive controls for CEA, CK20, and GAPDH show clear bands at corresponding sites, and all negative controls do not show any bands (Figure 2). The CEA mRNA positive rate before HIPEC versus after HIPEC was 100.0% versus 86.0% ($P = .012$). The CK20 mRNA positive rate before HIPEC versus after HIPEC was 100.0% versus 96.0% ($P = .495$).

Result of Real-Time Quantitative RT-PCR

Relative quantity analysis of real-time quantitative RT-PCR shows no significant decrease of the relative expression of CEA mRNA and CK20 mRNA after HIPEC. The relative expressions of CEA mRNA and CK20 mRNA before HIPEC versus after HIPEC were 4.08 ± 1.34 versus 3.22 ± 0.62 ($P = .573$) and 0.47 ± 0.09 versus 0.46 ± 0.06 ($P = .937$), respectively (Figure 3). However, after HIPEC, 18 (36.0%) patients had a decline in CEA mRNA ($P = .000$) and 17 (34.0%) patients had a decline in CK20 mRNA ($P = .000$), including 13 (26.0%) patients who had declines in both CEA mRNA and CK20 mRNA.

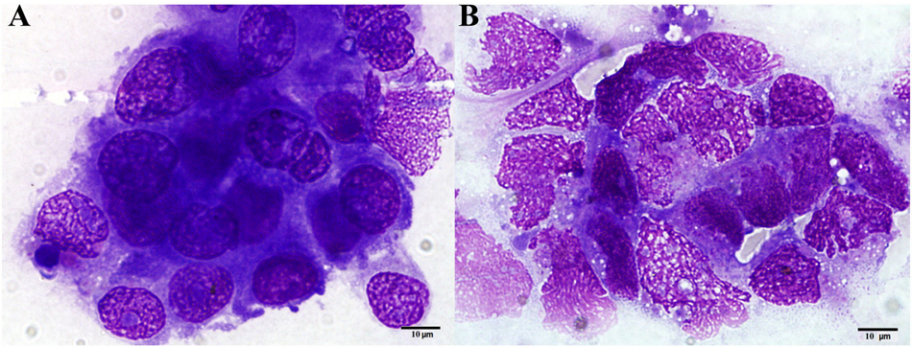

Figure 1. Conventional cytology results: positive IFCCs (A) and cytolysis after HIPEC (B) (Wright's stain, 1000×, scale bar = 10 μm).

Serum Tumor Markers

The serum concentrations of CEA, CA125, and CA19-9 before HIPEC versus after HIPEC were 211.00 ± 156.90 ng/ml versus 41.99 ± 23.67 ng/ml (P = .213), 162.6 ± 46.17 U/ml versus 61.08 ± 10.20 U/ml (P = .015), and 164.00 ± 64.83 U/ml versus 77.53 ± 19.20 U/ml (P = .128), respectively (Figure 4, *A–C*). Moreover, the positive rates of serum CEA, CA125, and CA199 before HIPEC versus after HIPEC were 52.0% versus 28.0% (P = .014), 52.0% versus 44.0% (P = .423), 40.0% versus 28.0% (P = .205), respectively (Figure 4*D*).

Discussion

With the progression of gastrointestinal and gynecological tumors, cancer cells exfoliate from the serosal surface and scatter into the peritoneal cavity as IFCCs, which are a major cause of peritoneal recurrence [14]. Also, division of blood vessels and resection of lymph nodes during the surgery could make cancer cells spill into peritoneal cavity to form IFCCs [15]. There are two different processes that IFCCs could cause PC: the IFCCs attach to peritoneal mesothelial cells, invade into submesothelial tissue, and form metastatic tumors with angiogenesis. Or the IFCCs invade into submesothelial lymphatic space through the milky spots on the peritoneal surface and form metastatic tumors [16].

Patients with IFCCs have a much higher risk for developing peritoneal recurrence than those without IFCCs and have a worse prognosis [17,18]. A number of large-scale clinical trials confirmed IFCCs as one of the most important prognostic factors. Bando et al. [19] reported that in 1297 gastric cancer patients, those with IFCCs had a 5-year survival rate of 2%, and of 30 patients who developed PC, 23 were IFCC positive. Kano et al. [20] reported that in 1039 gastric cancer patients, the IFCC-positive patients had a 5-year survival rate of 15%, and the median overall survival was 11 months. Lee et al. [21] reported 172 IFCC-positive gastric cancer patients, of which 76 patients developed PC. Nashikawa et al. [22] reported that in 410 colorectal cancer patients, the IFCC-positive patients had a 5-year survival of 20.6% and had 10 times higher risk for developing PC than IFCC-negative patients. Zuna et al. [23] reported that in 90 IFCC-positive ovarian cancer patients, the median overall survival was 20 months and the 5-year survival rate was 9.2%.

So far, the conventional peritoneal lavage cytology, which has a high specificity and a low sensitivity [24], is the most reliable indicator for peritoneal recurrence and the gold standard for detecting IFCCs [25]. New methods like immunohistochemical [26] and flow cytometry [27] method using the monoclonal antibodies to detect tumor-related antigens could partly improve the sensitivity and specificity over routine cytology. In the 1990s, the extremely high sensitivity of RT-PCR technique made it possible to diagnose micrometastasis on the cancer tissue–specific mRNA expression in peripheral vein [28], bone marrow [13], and lymph nodes [29]. In 1997, Nakanishi et al. [30] first reported the successful use of RT-PCR for detecting CEA mRNA of the IFCCs in peritoneal lavage fluid. Kodera et al. [24] reported that using RT-PCR to detect CEA mRNA of peritoneal lavage fluid could effectively predict the prognosis of gastric cancer patients. Recently, more and more

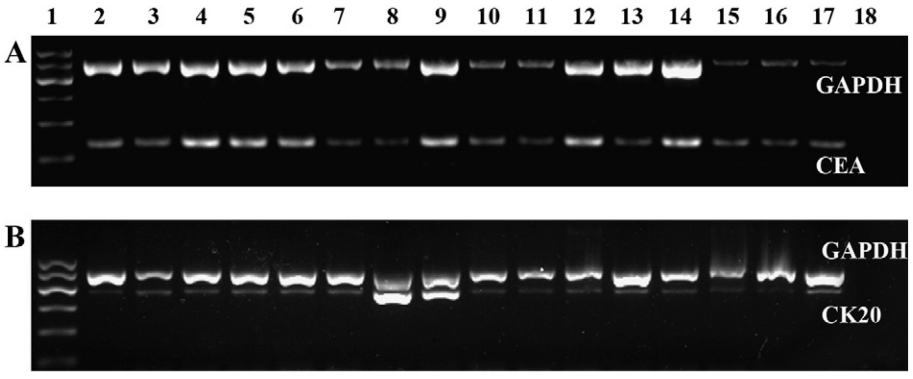

Figure 2. Agarose gel (1.5%) electrophoresis of the RT-PCR product of CEA (A) and CK20 (B). Lane 1 is a DNA maker from 100 bp (bottom) to 600 bp (top); lanes 3 to 17 are the patients' samples; lane 2 is the positive control; lane 18 is the negative control.

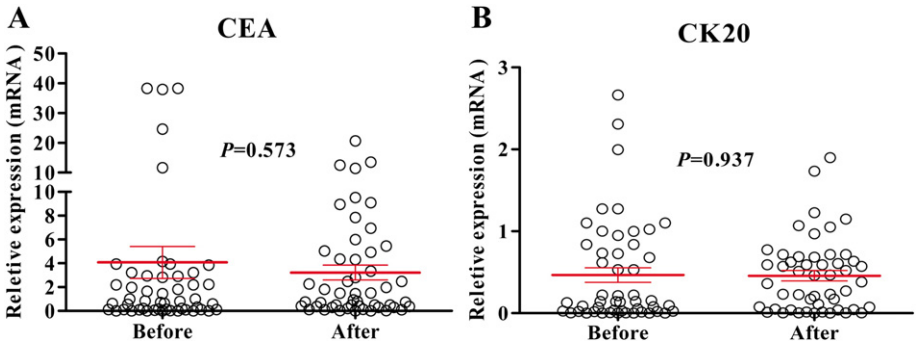

Figure 3. Relative expressions of CEA mRNA (A) and CK20 mRNA (B) before HIPEC and after HIPEC acquired by real-time quantitative RT-PCR. Horizontal lines represent mean ± standard error. Before and after mean before HIPEC and after HIPEC.

researches adopted this RT-PCR–based method for detecting IFCCs because of its higher sensitivity than conventional cytology. Moreover, the use of multiple biomarkers like CEA, CK20 [31], CK19 [32], and MMP [33] for RT-PCR assay could greatly improve both the sensitivity and the specificity of detecting IFCCs and make it a reliable way for clinical use.

There was still no standard treatment for IFCC-positive patients. Conventional radical surgery, which ignores the IFCCs, only focuses on the organ resection and lymph node dissection. Moreover, even strictly following the principle of tumor-free technique during the surgery, some patients who were IFCC negative at laparotomy became IFCC positive after surgery [34]. So now, simple gastrectomy without additional lymphadenectomy was still accepted as the optimal strategy for the treatment. Ito et al. [35] reported that a new oral fluorinated pyrimidine agent (S-1) may delay cancer relapse for gastric cancer patients with free cancer cells detected by real-time RT-PCR but not always eradicate micrometastases. Kuramoto et al. [36] reported that extensive intraoperative peritoneal lavage followed by intraperitoneal chemotherapy could significantly improve the 5-year survival span of gastric cancer patients with IFCCs and recommended this treatment as a standard prophylactic strategy for peritoneal dissemination. HIPEC was also reported to be effective for the prevention of recurrence in patients with IFCCs [37] and be an independent prognostic factor for improving the overall survival of patients with PC that originated from gastric cancer [8], colorectal cancer [38], and ovarian cancer [39].

In this study, conventional peritoneal lavage cytology, conventional RT-PCR, and real-time quantitative RT-PCR methods were used for detecting IFCCs. Before HIPEC, the IFCC, CEA mRNA, and CK20 mRNA positive rates achieved by these methods were all 100%, far more than the other studies had reported [2,26,30,33], because the patients included in this study were all with visible peritoneal recurrence (median PCI = 22, range = 2-39). However, IFCC, CEA mRNA and CK20 mRNA positive rate became 22.0%

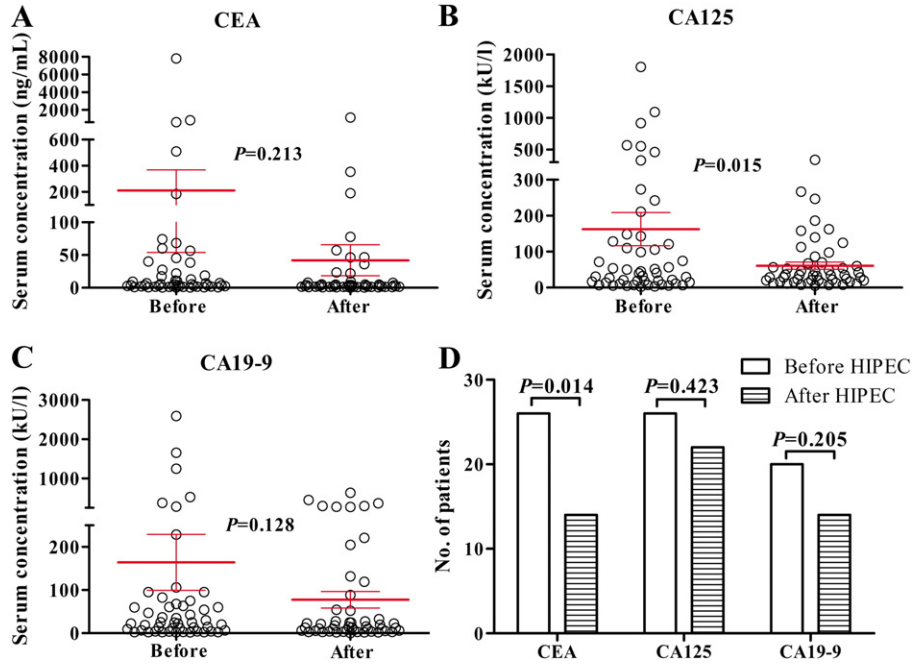

Figure 4. Serum CEA (A), CA125 (B), and CA19-9 (C) concentrations before HIPEC and after HIPEC, and the number of patients with positive serum CEA, CA125, and CA19-9 (D). The definitions of positive serum CEA, CA125, and CA19-9 were ≥5 ng/ml, ≥35 U/ml, and ≥ 37 U/ml, respectively. Horizontal lines represent mean ± standard error. Before and after means before HIPEC and after HIPEC.

(P = .000), 86.0% (P = .012), and 96.0% (P = .495) after HIPEC, respectively. Even though HIPEC could not realize significant decline of CEA mRNA and CK20 mRNA positive rates, the relative quantity analysis shows that the relative expression of CEA mRNA decreased in 18 (36.0%) patients (P = .000) and the relative expression of CK20 mRNA decreased in 17 (34.0%) (P = .000). Moreover, the positive rate of serum CEA also significantly decreased from 52.0% to 28.0% after HIPEC (P = .014).

In China, most of the patients with malignancy were diagnosed at an advanced stage; traditional radical surgery–based therapy cannot eradicate the IFCCs, which are the major cause for the high peritoneal recurrence rate and the poor prognosis. HIPEC combines the chemotherapeutic effect and the hyperthermic effect and can eradicate IFCCs effectively. Also, extensive peritoneal lavage of the heat fluid with chemotherapy drugs can reduce IFCCs. Our results suggest that HIPEC could achieve partial cytological cure for PC. More studies and long follow-up are needed to verify the impact of this approach on patient outcomes.

Conclusion

HIPEC could effectively eradicate IFCCs and partially achieve cytological cure for PC.

Acknowledgement

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