in vivo *26*: 39-46 (2012)

Gene Expression Changes Induced by HIPEC in a Murine Model of Gastric Cancer

LUIGINA GRAZIOSI[1], ANDREA MENCARELLI[2], BARBARA RENGA[2], CHIARA SANTORELLI[1], FRANCESCO CANTARELLA[1], WALTER BUGIANTELLA[1], EMANUEL CAVAZZONI[1], ANNIBALE DONINI[1] and STEFANO FIORUCCI[2]

Departments of [1]Surgery and [2]Clinical Medicine, School of Medicine, University of Perugia, Santa Maria della Misericordia Hospital, Perugia, Italy

Abstract. *Background: Peritoneal carcinogenesis (PC) is the most frequent pattern of metastasis in patients with locally advanced gastric cancer. Despite this, there is a consensus on the use of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) for the treatment of PC from gastric cancer. The molecular mechanisms involved in beneficial effects of HIPEC remain unexplored. Materials and Methods: Human gastric cancer MKN45 cells were injected into the peritoneal cavity of immune-deficient NOD-SCID mice. After induction of PC, the animals were randomized into five groups: HIPEC with mitomycin and cisplatin; normothermic intraperitoneal chemotherapy (NIPEC); normothermic intraperitoneal saline; hyperthermic intraperitoneal saline alone; no treatment. After 10 days of treatment, the mice were sacrificed and the extent of PC was assessed. Results: Compared with the other groups of treatment, HIPEC reduced the extent and severity of peritoneal dissemination as measured by assessing the total number of peritoneal and mesenteric nodules (p<0,05) and the HIPEC procedure increased median survival significantly. By gene array analysis, HIPEC was found to effectively modulate the expression of a subset of genes involved in formation of peritoneal metastasis, including adenomatous polyposis coli; beta (3) subunit of the integrin gene; chemokine stromal cell-derived factor-1 receptor; spleen tyrosine kinase; vascular endothelial growth factor receptor 3; collagen, type IV, alpha 2 and Carbossi-terminal binding proteins 1. Conclusion: In the present study we have provided evidence that HIPEC protects against peritoneal dissemination in a mouse model of peritoneal gastric carcinogenesis and brings about specific changes in gene expression wich may be related to this protection.*

Correspondence to: Graziosi Luigina, University of Perugia, Department of General and Emergency Surgery, Hopital Santa Maria della Misericordia, Via G. Dottori, 06132, Perugia. E-mail: luiginagraziosi@yahoo.it

Key Words: Gastric cancer, HIPEC, peritoneal carcinomatosis, MKN45 cells, NOD-SCID mice.

Gastric cancer is one of the leading causes of malignant deaths. Peritoneal dissemination (PC) is the most frequent pattern of metastasis and recurrence in patients with locally advanced gastric cancer. Synchronous peritoneal metastases are found in 10-20% of patients and during follow-up, a further 60% of patients will develop PC. The median survival of patients with PC is about 6 months and no effective curative therapy exists (1). Positive peritoneal cytology (Cy1 according to the Japanese Classification of Gastric Cancer) (2) is a predictive factor for peritoneal recurrence despite curative R0 surgery. In fact, the majority of patients showing lavage cytology-positive intraoperatively develop peritoneal recurrences (3). Despite attempts at curative resection and multimodality therapies, long-term survival of patients with positive cytology is rare and is measured in months, as in patients that show macroscopically evident peritoneal metastases during surgery. Management of patients with positive cytology is debated and no effective treatment strategies have been established (4-6). Waiting for a multi-institutional randomized controlled trial, there is a consensus that cytoreductive surgery (CS) and hyperthermic intraperitoneal chemotherapy (HIPEC) might increase survival in selected patients with gastric cancer with PC. Median survival in those patients treated with HIPEC and CS increased to 15 months, where complete cytoreduction was achieved, compared to 3 months with only basic supportive therapy.

There is no consensus for the treatment of patients with positive cytology and there is no recommendation for neoadjuvant therapy or intraperitoneal treatment in this subset.

We investigated the role of HIPEC in an experimental model of cytology-positive gastric cancer without macroscopic PC and we studied the molecular impact of HIPEC.

Materials and Methods

Cell line. The human gastric cancer cell line, MKN45 and MKN74, were purchased from the Japanese Collection of Research Bioresources, Human Science Research Resources Bank (Osaka, Japan). The gastric cell lines, were maintained in RPMI medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 in air. Cells were regularly passaged to maintain exponential growth.

Study protocol. Several eight-week-old male NOD-SCID mice supplied by the Animal Center of the University of Perugia were housed under pathogen-free conditions. The care and the use of the animals were approved by the Institutional Animal Care and Use Committee of the University of Perugia and were in accordance with European guidelines for the care of experimental animals. Protocols were approved by the Instituto Superiore di Sanità. To examine the potential for peritoneal metastasis of carcinoma cell lines, a single cell suspension of MKN45 and MKN74 cells of 1×10^7 cells in a total volume of 0.2 ml of medium without serum was injected into the peritoneal cavity of each mouse using a 23-gauge needle. The extent of PC was evaluated on the 7th , 14th and 21st day by necroscopy (Figure 1). At given time points, the mice were sacrificed under penthotal (50 mg/kg) anesthesia and the peritoneal and mesenteric nodules counted and removed from each mouse.

At sacrifice, all the tissues were immediately snap frozen in liquid nitrogen and stored at –80˚C until used or fixed in formalin. Tissue sections (5 μm thick) were then stained with hematoxylin and eosin (H&E).

Experimental design. We utilized MKN45 cell lne for this experimental study due to its strong carcinogenesis behaviour compared to that of MKN74 cell line.

At day 1 from MKN45 cells inoculation, the mice were randomly put into five groups of ten animals: HIPEC with mitomycin (8.25 μg/l of perfusate each mouse) and cisplatin (62.5 μg/l of perfusate each mouse); normothermic intraperitoneal chemotherapy with the same chemotherapy solution (NIPEC); normothermic intraperitoneal saline solution (NIPES); hyperthermic intraperitoneal saline solution (HIPES); and no treatment. After 10 days from the intraperitoneal inoculation, the mice were sacrificed under penthotal (50 mg/kg) anesthesia and the peritoneal and mesenteric nodules were counted and removed from each mouse. The primary outcome parameter was the number of peritoneal disseminated nodules. In another set of experiments the animals were again randomly selected and treated as described above and the survival time was assessed.

Intraperitoneal treatments. All animals were anesthetized with an intraperitoneal injection of ketamine (Ketalar; Parke-Davis) 80 mg/kg and xilazine (Rompun; Bayer AG) at 5 mg/kg. Two catheters were introduced into the abdominal cavity through the upper and lower quadrants of the abdomen. The catheters were connected to a closed perfusion system containing 1 l of physiologic solution. The perfusion was performed according to the Coliseum technique at the open abdomen (7). The peritoneal perfusate was warmed in a tube coil using a thermostatically regulated water- bath. The perfusate was intraperitoneally introduced at a temperature of 40˚C in the two groups of animalsfor HIPEC and NIPEC. Perfusion of the peritoneal cavity was performed for 50 min at an infusion speed of 4 ml/min. Mitomycin (8.25 μg/l of perfusate for each mouse) and cisplatin (62.5 μg/l of perfusate for each mouse) were dissolved in 0.9 % sodium chloride and then added to the perfusate in the HIPEC and NIPEC groups. During the intraperitoneal perfusion, the temperature of the abdominal cavity was highly controlled and maintained at the value of 40˚C; the abdomen was massaged gently to achieve a uniform perfusate distribution. After completing the procedure, the abdominal cavity was irrigated with saline for 5 min and then the catheters were removed and the abdominal laparotomy was closed in a double layer using continuous sutures. Immediately after the perfusion, the mice were placed in a warmed cage to limit body heat loss and were given 1 ml of saline subcutaneously to rehydrate them .

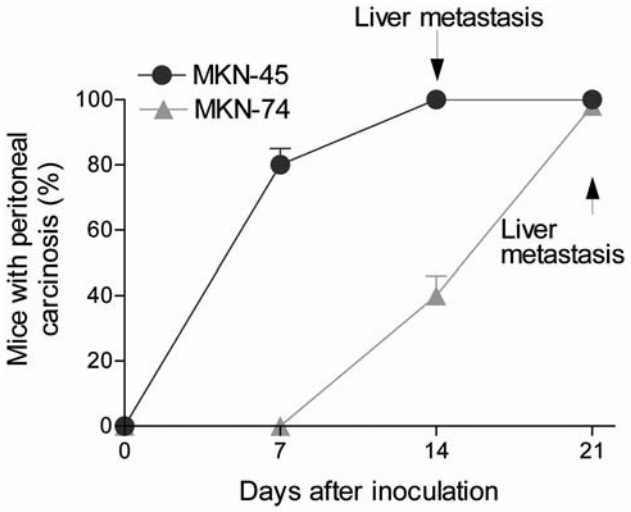

Figure 1. *To induce peritoneal carcinogenesis, a suspension of MKN45 and MKN74 cells were injected into the peritoneal cavity of the mice. The extent of peritoneal carcinogenesis was evaluated on the 7th , 14th and 21st day by necroscopy the peritoneal nodules and visceral metastases were counted and removed from each mouse.*

Microarray analysis. Total RNA was prepared from each specimen using Trizol kit (Invitroge) to derive total RNA from cancer peritoneal nodules obtained in the mice inoculated with MKN45 alone or in a combination with HIPEC treatment. The RNA reverse was transcribed with Superscript-II reverse transcriptase (Invitrogen) following the manufacturer's instructions. A total of 100 ng cDNA was pipetted into each well of a 96-well gene array plate (Human Tumor Metastasis RT^2 Profiler™ PCR Array; - http:// www.sabiosciences.com/rt_pcr_product/HTML/PAHS-028A.html - Superarray Bioscence, Frederick, MD, USA) and amplified following the manufacturer's instructions. This gene array is designed to assess 84 genes known to be involved in metastasis (Figure 2). Genes selected for this array encode several classes of protein factors including those for cell adhesion, extracellular matrix components, cell cycle, cell growth and proliferation, apoptosis, transcription factors and regulators and other genes related to tumour metastasis (Figure 3). Array analysis was carried out with the on-line software RT2 Profiler PCR Array Data Analysis (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The signal detected for each gene was normalized to the signal obtained for β-actin or gliceraldehyde 3-phosphate dehydrogenase (GAPDH) on the same gene array to derive gene expression values for each gene.

Up/down regulated genes were whose expression had altered by more than 2.

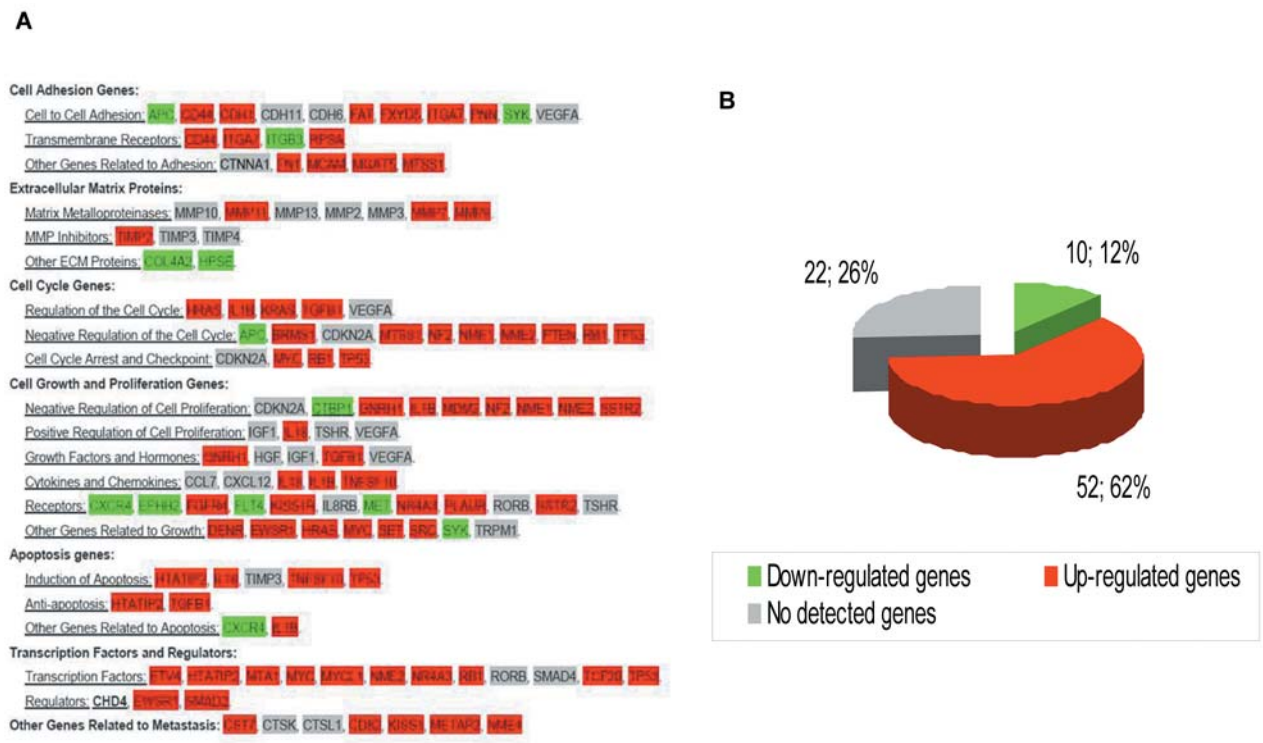

Figure 2. *Results of Human Tumor Metastasis RT² Profiler™ PCR Array (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-028A.html). The gene array was carried out using mRNA isolated from peritoneal gastric cancer nodules obtained from mice treated with and without HIPEC. A: Complete list of analyzed genes divided for pathways. Red: genes up-regulated by HIPEC; green: genes down-regulated by HIPEC; grey: genes not detected or unchanged following HIPEC treatment. B: Pie chart showing the percentage of genes down-regulated (in green), up-regulated (in red) and not detected or unchanged after HIPEC administration.*

Statistical analysis. All values are expressed as the mean±SE of n experiments. The statistical analysis was carried out by GraphPad Prism software The variation between data sets was tested by Student's *t*-test for unpaired samples when we compared two groups. Comparisons of more than two groups were made with a one-way analysis of variance with post hoc Tukey tests. Differences were considered statistically significant if p was <0.05.

Results

HIPEC reduces gastric cancer dissemination and increases the mean survival of MKN45 injected mice. PC was assessed 10 days after MKN45 cell inoculation of 1×10^7/mouse to NOD/SCID mice (Figure 3A B C). While all mice injected with MKN45 cells developed diffuse colonization of the peritoneal cavity, the concomitant treatment with NIPES and HIPES did not change the severity of peritoneal dissemination (Figure 3A B C) HIPEC treatment effectively reduced the extent of peritoneal dissemination as measured by assessing the total number of peritoneal nodules ($p<0.05$) (Figure 3A) and mesenteric nodules ($p<0.05$) (Figure 3B), compared to the other experimental groups. The clinical effectiveness of HIPEC was maintained even in comparison with NIPEC, with the former effectively reducing the number of mesenteric cancer nodules ($p<0.05$) (Figure 3B). In addition, both HIPEC and NIPEC treatments were associated with a slight improvement of general clinical parameters such as the weight loss induced by peritoneal implantation (Figure 3C). The histopathological analysis of peritoneal nodules, stained with H&E, showed no difference in tumor architecture between all experimental groups (Figure 3D). Furthermore, HIPEC treatment compared to other treatment modalities increased the mean survival time from 6 to 9 weeks ($p<0.05$) (Figure 4).

Microarray analysis of cancer peritoneal nodules treated with HIPEC. Because the mentioned above data demonstrates efficacy of the HIPEC treatment in preventing peritoneal dissemination, we then designed a study to investigate the molecular mechanisms involved in this effect. For this purpose, we investigated the effects of HIPEC on the expression of a subset of genes involved in the formation of peritoneal cancer nodules by gene array analysis. Using a specific array designed to investigate the expression of 84 genes known to contribute to the metastatic phenotype, we

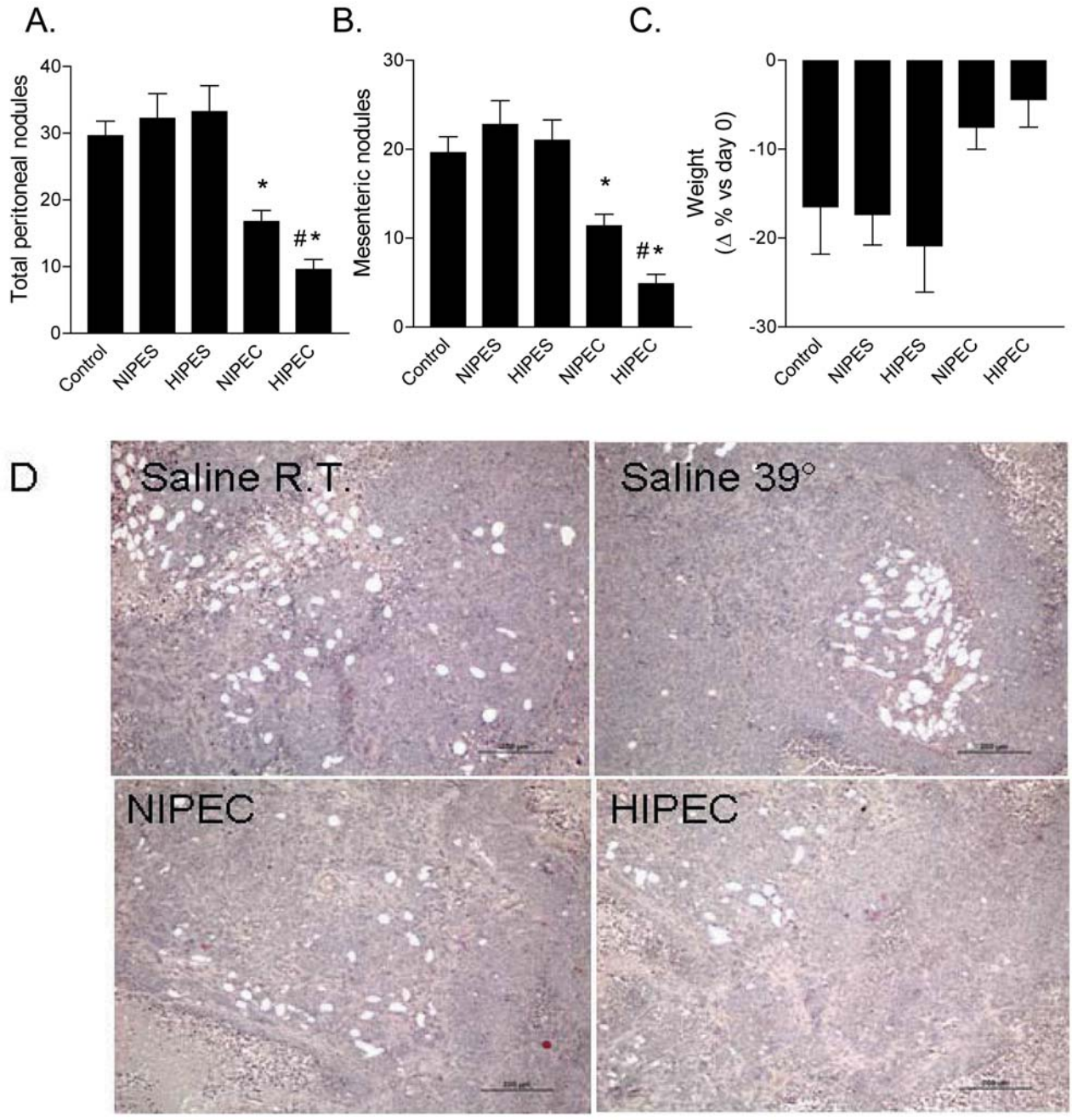

Figure 3. *HIPEC protects against peritoneal dissemination. HIPEC reduces peritoneal dissemination of MKN45 cells as measured by assessing its effects on: A: number of peritoneal nodules; B: number of mesenteric nodules; and C: weight loss calculated as percentage change in weight compared to the basal weight (100%) of mice at day of cells inoculation (day 0). (*p<0.05 versus control group; n=7-10. D: Representative photos of peritoneal nodules stained with H.&E. (Bar 100 μm).*

found that *in vivo* treatment with HIPEC caused a down-regulation of several genes including: the adenomatous polyposis coli (*APC*); beta(3) subunit of the integrin gene (*ITGB3*); chemokine stromal cell-derived factor-1 receptor (*CXCR4*); spleen tyrosine kinase (*SYK*), vascular endothelial growth factor receptor 3/Fms-related tyrosine kinase 4 (*VEGFR3*/*FLT4*); collagen; type IV; alpha 2 (*COL4A2*) and C-terminal binding proteins 1 (*CTBP1*) (Figure 5 and Table I). In contrast somatostatin receptor 2 (*SSTR2*), cystatin-F (*CST7*), SMAD family member 2 (*SMAD2*) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) were up-regulated by HIPEC treatment (Figure 5 and Table II).

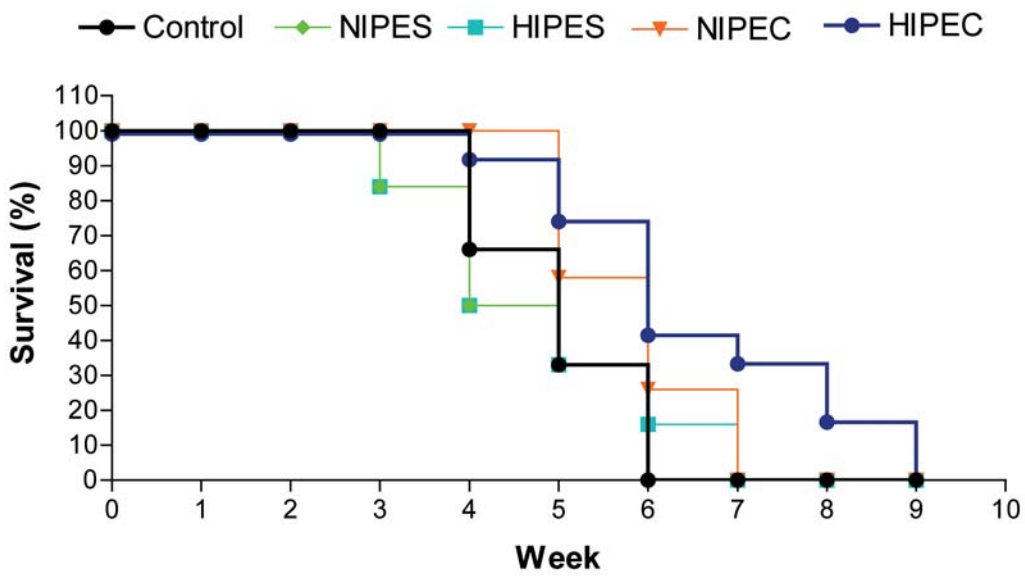

Figure 4. *Effects of HIPEC treatment on survival curve after induction of peritoneal dissemination. After one day from MKN45 cell inoculation, animals were randomized into five groups of 8-10 each and treated by HIPEC, NIPEC, NIPES, HIPES or no treatment, and survival curve was recorded.*

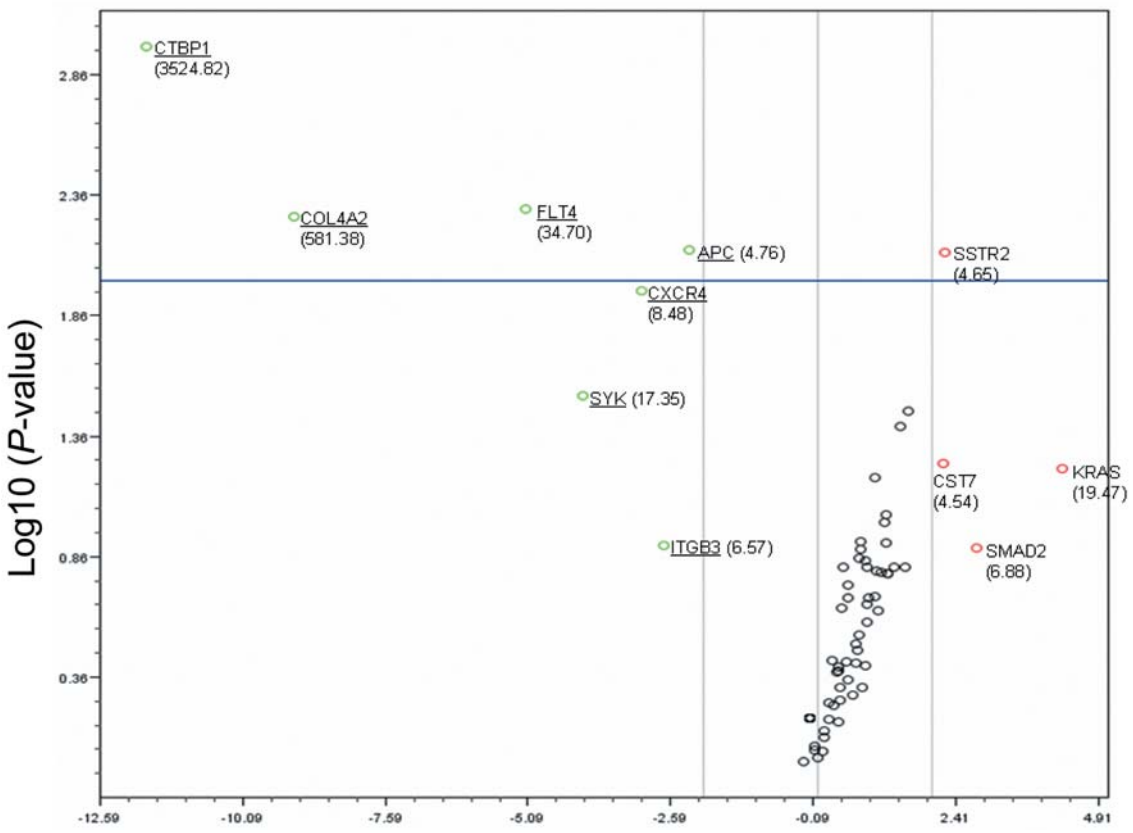

Figure 5. *Microarray analysis of genes involved in peritoneal metastasis. Scatter plot of microarray data showing the genes that are strongly regulated (negatively or positively) by HIPEC. HIPEC treatment day 1 after MKN45 cell inoculation down-regulated the expression gene of APC, ITGB3, CXCR4, SYK, VEGFR3/FLT4, COL4A2 and CTBP1 (*p<0.01 versus control group. In contrast upregulate the expression of these gene: CST7, SSTR2, SMAD2 and KRAS (p<0.01 versus the untreated control group). Microarray was performed using 3 peritoneal nodules from different mice for each experimental group.*

Table I. *Molecular effects of hyperthermic intraperitoneal chemotherapy (HIPEC): downregulated genes. HIPEC treatment one day after MKN45 cell inoculation down-regulated the expression of APC, ITGB3, CXCR4, SYK, VEGFR3/FLT4, COL4A2 and CTBP1 genes (p< 0.01 versus the untreated control group). Microarray analysis was performed using 3 peritoneal nodules from different mice for each experimental group.*

Gene	Fold change
Adenomatous polyposis coli (*APC*)	4.76
Beta(3) subunit of the integrin gene (*ITGB3*)	6.57
Chemokine stromal cell-derived factor-1 receptor (*CXCR4*)	8.48
Spleen tyrosine kinase (SYK)	17.35
Vascular endothelial growth factor receptor 3/Fms-related tyrosine kinase 4 (*VEGFR3/FLT4*)	34.7
Collagen, type IV, alpha 2 (*COL4A2*)	581
C-Terminal binding proteins 1 (*CTBP1*)	3524

These patterns of expression indicate a specific regulatory effect of HIPEC that could be exploited to design experimental clinical protocols.

Discussion

In the present study we have provided evidence that HIPEC protects against peritoneal dissemination in a murine model of peritoneal gastric cancer carcinogenesis when PC is not macroscopically evident. This is, therefore, the first experimental study demonstrating that HIPEC might have utility as a prophylatic approach for patients with advanced gastric cancer at high risk of developing PC when the peritoneal cytology is positive. Several studies have demonstrated that the prognosis of patients at this stage of the disease, following surgery alone, is very poor and similar to that of patients with peritoneal macroscopically evident carcinomatosis. The lack of efficient systemic chemotherapy combined with the fact that the peritoneum is the preferential site of gastric cancer dissemination, represents the reason to investigate the role of intraperitoneal chemotherapy in both prophylatic and therapeutic settings. In this therapeutic setting, phase II-III trials have revealed a relative benefit of HIPEC and cytoreductive surgery. Infact, there is no scientific evidence that supports a real benefit of preventive intraperitoneal chemotherapy for these patients, and therefore our experimental study should represent the basis for a clinical study.

From the molecular standpoint, using a specific array designed to investigate the expression of 84 genes known to contribute to the metastatic phenotype, we found that *in vivo* treatment with HIPEC caused a significant shift in cell phenotype. Thus, while the expression of 22 genes (26%) was undetected/unchanged, the expression of 52 genes (62%) was increased and the expression of 12 genes (10%) was reduced in response to treatment. Among these genes, we observed that HIPEC treatment caused a strong down-regulation of the expression of *CTBP1*, encoding a transcriptional co-repressor involved in tumorigenesis and tumor progression; *COLA42*, encoding a protein related to epithelial–mesenchymal transformation (8-10), and *CXCR4* and *VEGFR3*, (Figure 4 and Table I) wich are notably involved in gastric cancer cell growth and metastatic dissemination (11-16). *CTBP1* and *CTBP2* are closely related and evolutionarily conserved transcriptional corepressors (8). CTBP-mediated repression of adhesion molecules such as E-cadherin suggests that CTBP is important in promoting the epithelial-to-mesenchymal transition EMT, a step that contributes to the malignant property of tumor cells due to the loss of intercellular adhesion in tumors, acquisition of motile and invasive phenotypes, and resistance to apoptosis (8). Certain tumor suppressors also target CTBPs to restrain their tumor promoting activity. Down-regulation of CTBPs mediated by some tumor suppressors resulted in p53-independent apoptosis and reduced tumor cell migration and invasion (9).

Table II. *Molecular effects of hyperthermic intraperitoneal chemotherapy (HIPEC): up-regulated genes. HIPEC treatment one days after MKN45 cell inoculation upregulated the expression of CST7, SSTR2, SMAD2 and KRAS genes (p< 0.01 versus the untreated control group). Microarray analysis was performed using 3 peritoneal nodules from different mice for each experimental group.*

Gene	Fold change
Cystatin-F (*CST7*)	4.54
Somatostatin receptor 2 (*SSTR2*)	4.65
SMAD family member 2 (*SMAD2*)	6.88
v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*)	19.47

Expression of COL4A2 chain in cancer peritoneal nodules was also strongly down-regulated by *in vivo* treatment with HIPEC. Collagen IV in normal epithelial basement membrane is mainly derived from fibroblasts from the connective stromal tissue. Nevertheless, neoplastic cells are able to synthesize and deposit collagen IV in the absence of fibroblasts indicating that it might be a feature acquired through malignant progression related to epithelial–mesenchymal transformation (10). Further

on, high levels of type IV collagen in peritoneal fluids in patients with peritoneal disseminated gastric and colorectal cancers correlate directly with shorter survival rates (10).

CXCR4 expression in primary gastric carcinomas correlates with the development of PC and malignant gastric ascites contains high concentrations of CXCL12 also called stromal-derived-factor-1α (SDF-1α) (11). Supporting a mechanistic role of this chemokine receptor in peritoneal dissemination, in fact CXCR4 antagonism protects against experimental peritoneal carcinomatosis (11). In addition, the expression of CXCR4 ligand, CXCL12, is significantly increased in gastric carcinoma cells compared with non-neoplastic mucosa and is markedly upregulated in nodal positive gastric carcinoma compared to nodal negative cases (12) strongly advocating the case for a mechanistic role of the CXCR-4/CXCL12 axis in causing gastric carcinoma cell proliferation and migration (11). In addition to CXCR4, *in vivo* treatment with HIPEC caused a 34-fold reduction in the expression of VEGFR3 in peritoneal nodules. Full-length vascular endothelial growth factor (VEGF)-C and VEGF-D isoforms are high affinity ligands for VEGFR-3 and are intimately involved in the regulation of lymphangiogenis (13-16). In addition to its expression on lymphatic endothelial cells, VEGFR-3 is also expressed in a variety of human malignancies, including gastric cancers. Similarly to expression of VEGF-C and VEGF-D, the relative abundance of these factors is inversely correlated with patients survival (15, 16).

In summary, we have provided evidence that HIPEC might have utility in preventing peritoneal dissemination of gastric cancer in a rodent model of peritoneal cancerogenesis with positive cytology. In addition to these clinical benefits we have shown that HIPEC has profound molecular effects and modulates the expression of genes involved in the attachment and dissemination of neoplastic cells. The present study highlights the urgent need of clinical studies for identify clinical benefit and molecular mechanisms of this treatment.

Competing Interests

The Authors declare that they have no competing interests.

Authors Contributions

LG designed the study, contributed to the experimental work and wrote the manuscript. BR contributed to the microarray analysis. AM contributed to the experimental work and wrote the manuscript. CS and FC contributed to animal data and histological analysis. EC and WB contributed to animal data. AD and SF designed the study, contributed to data interpretation and wrote the manuscript. All Authors have read and approved the final manuscript.

References

1 Jemal A: Cancer statistics. CA Cancer J Clin *59*: 225-249, 2009.
2 Japanese Gastric Cancer Association. *In*: Japanese Classification of Gastric Cancer Carcinoma. second English edition *1*: 5-30, 1998.
3 Bando E: Intraoperative lavage for cytological examination in 1.297 patients with gastric carcinoma. Am J Surg *178*: 256-262, 1999.
4 Bentrem D: The value of peritoneal cytology as a preoperative predictor in patients with gastric carcinoma undergoing a curative resection. Ann Surg Oncol *12*: 347-253, 2005.
5 Bonenkamp JJ: Prognostic value of positive cytology findings from abdominal washings in patients with gastric cancer. Br J Surg *83*: 672-674, 1996.
6 Burke EC: Peritoneal lavage cytology in gastric cancer: an independent predictor of outcome. Ann Surg Oncol *5*: 411-415, 1998.
7 Kodera Y: Peritoneal washing cytology: prognostic value of positive findings in patients with gastric carcinoma undergoing a potentially curative resection. J Surg Oncol *72*: 60-64, 1999.
8 Sugarbaker PH: Peritonectomy procedures. Ann Surg *221*: 29-42, 1995.
9 Thiery JP: Epithelial mesenchymal transitions in tumour progression. Nat Rev Cancer *2*: 442-454, 2002.
10 Chinnadurai G: The transcriptional corepressor CtBP: a foe of multiple tumor suppressors. Cancer Res *69*: 731-734, 2009.
11 Korenaga D: Peritoneal collagen type IV concentration in adenocarcinoma of the gastrointestinal tract and its relationship to histological differentiation, metastasis, and survival. Surg Today *28*: 780-786, 1998.
12 Yasumoto K: Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer. Cancer Res *66*: 2181-2187, 2006.
13 Ingold B: Vascular CXCR4 expression – A novel antiangiogenic target in gastric cancer? PLoS One *5(4)*: e10087, 2010.
14 Karkkainen MJ: Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene *19*: 5598-5605, 2000.
15 Stacker SA: Lymphangiogenesis and cancer metastasis. Nat Rev Cancer *2*: 573-583, 2002.
16 Ni XF: Serum VEGFR-3 and survival of advanced gastric cancer patients treated with FOLFOX. World J Gastroenterol *16*: 63-69, 2010.
17 Jüttner S: Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. J Clin Oncol *24*: 228-240, 2006.

Received August 9, 2011
Revised November 25, 2011
Accepted November 28, 2011