SAPIENZA
UNIVERSITÀ DI ROMA

Seconda Facoltà di Medicina e Chirurgia

Dottorato di Ricerca
Metodologie della Ricerca Clinica e Sperimentale in Oncologia
XXIV Ciclo

Tesi di Dottorato

"Clinical relevance of free peritoneal tumor cells detection in gastric and colorectal cancer: a multiple molecular approach"

Dottorando

Dott. Simone Rossi Del Monte

Tutor
Prof. V. Visco

Coordinatore
Prof. V. Ziparo

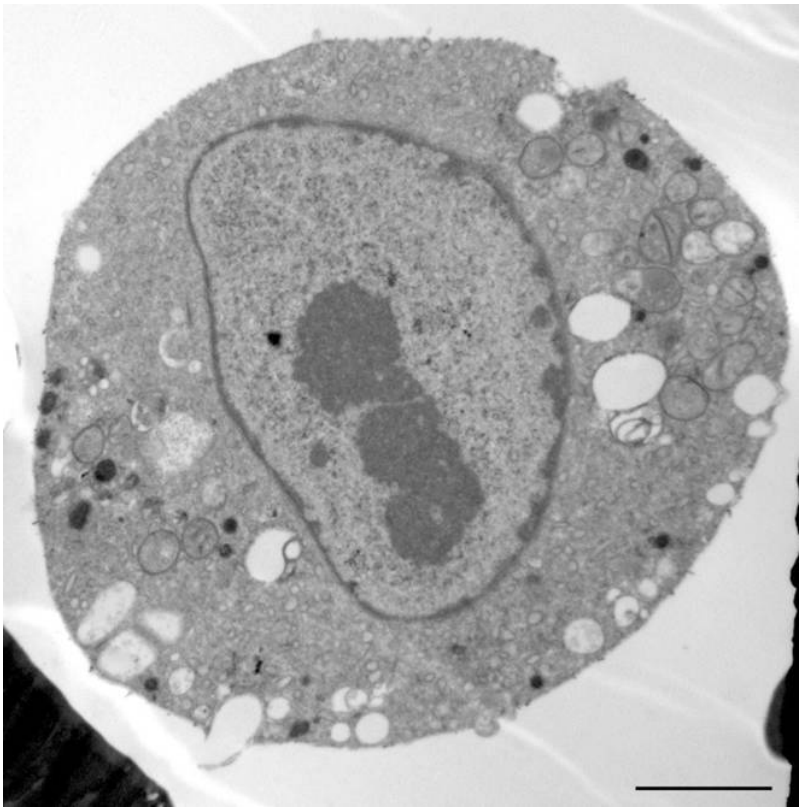
Anno Accademico 2010-2011

Table of Contents

INTRODUCTION

Free peritoneal tumor cells and peritoneal carcinomatosis

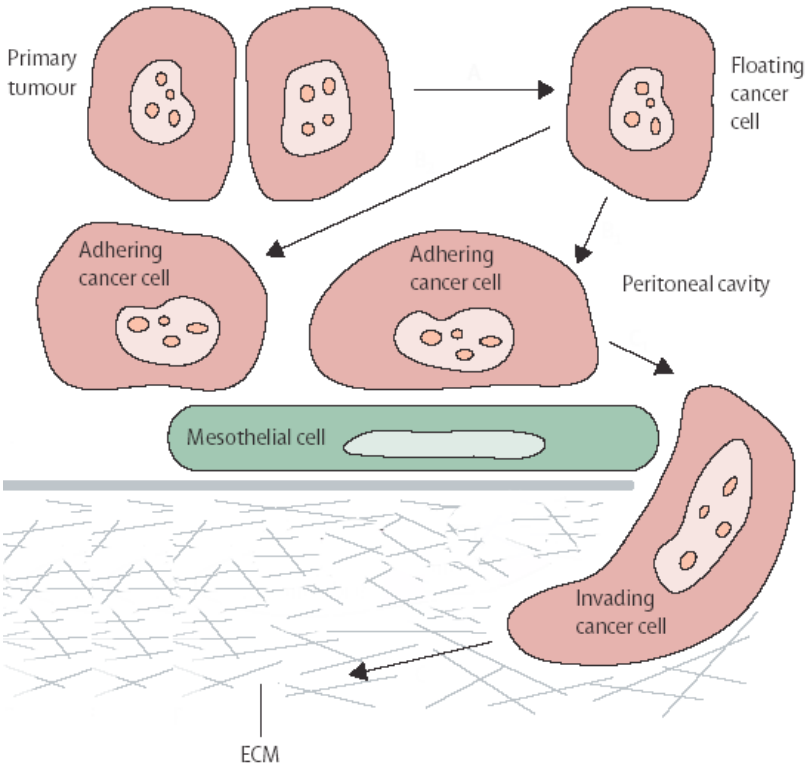
The tumor dissemination starts from the primary tumor and consists of a multistep process. Firstly, individual or clusters of tumor cells must detach from the primary tumor mass and gain access to the peritoneal cavity.



The detachment could occur by several mechanisms and the most frequent one in gastrointestinal cancers is spontaneous exfoliation of tumor cells from cancers that have invaded the serosa. This process

could be mediated by the down-regulation of intercellular adhesion molecules on the tumor cell surface, for example E-cadherin. Cadherins are transmembrane glycoproteins with an extracellular part, a membrane-spanning domain and a cytoplasmatic tail. They form a family with currently about 80 members, but information related to peritoneal carcinomatosis is now restricted to the subfamily of classical (or type I) cadherins. In epithelial tumors the expression or the function of E-cadherin is downregulated, and this has also been confirmed for colorectal, gastric and ovarian cancers with peritoneal carcinomatosis. The presence of viable tumor cells in the peritoneal cavity could also occur by iatrogenic or spontaneous perforation of the primary cancer or from transacted lymphatics and blood vessels during the course of surgical resection. Once the cancer cells are seeded in the peritoneal cavity they spread to different anatomical regions of the abdomen governed by 3 basic forces: gravity, peristaltic movement of the gastrointestinal tract, and negative pressure exerted by diaphragm muscle movements. The successive localization of intraperitoneal dissemination depends on the biology not only of free cancer cells and but also of the tissue that will harbor the metastatic implantation. The process takes place

through 2 routes denominated transmesothelial and translymphatic metastasis. According to the first mechanism the free cancer cells directly attach on distant mesothelium and this process is mediated by adhesion molecules such as CD44, lymphocyte homing molecules, members of integrin superfamily, the selectins and a variety of other leukocyte associated adhesion molecules.



In the successive step, the production of cytokines (interleukins, EGF, HGF, VEGF-C) induces the contraction of mesothelial cells exposing the submesothelial basement membrane. Yonemura et al investigated this phenomena using an animal model and a gastric cell

line, MKN-45-P. Intraperitoneal inoculation of MKN-45-P resulted in mesothelial contraction and eventual exfoliation. However, Jayne et al postulated another mechanism underlying tumor-mesothelial invasion. They used a three dimensional in vitro model of the human peritoneum, and found that colorectal cancer cell lines adhered rapidly to the outer mesothelial monolayer. Closer inspection of points of mesothelial invasion was frequently accompanied by changes in mesothelial cell morphology suggestive of apoptosis, confirmed by DNA fragmentation assays and immunohistochemistry. After attaching to the peritoneum and penetrating the mesothelial barrier, the tumor cells adhere to the submesothelial connective tissue through the interaction of integrins. These molecules are receptors for components of the basement membrane of cancer cells. Kawamura et al studied the expression of various metastasis related genes (integrins subunits, motility factors, proteases, growth factors) between 2 gastric cancer cell lines: MKN-45 and MKN-45-P. The latter was characterized by its high peritoneal metastatic potential. Integrin α2 and α3 subunits were significantly elevated in MKN-45-P compared to MKN-45. These α integrins dimerize with β1-subunits to form adhesion molecules for various basement

membrane proteins, including fibronectin, laminin, and collagen IV, which are secreted by human mesothelium. The invasion of subperitoneal tissue requires the degradation of the peritoneal blood barrier by motility factors and matrix proteinases. The matrix metalloproteinases (MMPs) may play a central role in stromal invasion. Yonemura et al studied the role of MMP-7 in a mouse model of peritoneal carcinomatosis. Specific antisense oligonucleotides inhibited the expression of MMP-7 by the highly metastatic gastric cell line MKN-45P, and suppressed invasion without modifying cell proliferation. Moreover, the survival of MKN-45-P bearing mice, which had been pre-treated with antisense oligonucleotides, was significantly better than control mice. Other potential mediators of stromal degradation are the urokinase plasminogen activating system and the protease inhibitor Bikunin (bik). Subsequently to invasion of the subperitoneal space in the vicinity of capillaries, the cancer cells trigger their proliferation through autocrine and paracrine loops by production of growth factors from cancer cells or stromal cells. Davies et al showed that epidermal growth factor (EGF) enhanced the invasive potential of mammary carcinoma cells when injected into the peritoneal cavities

of rats and that this growth promoting effect was due to the production of EGF by the peritoneal host tissue. The next step in the peritoneal dissemination process is the neoangiogenesis in the subperitoneal space which is mediated by the production of VEGF-A and VEGF-C. Besides the transmesothelial route, peritoneal cancer dissemination could occur by another mechanism denominated the translymphatic process. According to this theory the peritoneal free cancer cells gain access to the subperitoneal lymphatic spaces through lymphatic stomata. Anatomical regions in the peritoneal cavity with a high density of lymphatic stomata are the greater omentum, appendices epiploicae of the colon, inferior surface of the diaphragm, falciform ligament, Douglas pouch and small bowel mesentery. These locations are characterized by the presence of another lymphatic structure which is involved in the translymphatic peritoneal dissemination of free cancer cells, namely the milky spots. Milky spots are very small structures, in contact with the peritoneal membrane, devoid of capsule and consisting of macrophages, lymphocytes and a few plasma cells supported by blood and lymphatic vessels. The exact role of these particular organs is still not clear, but they are similar to lymphatic structures and it is clear that

they play a role in peritoneal cancer dissemination. Lymphatic stomata are found in the milky spots and peritoneal macrophages mobilize into the peritoneal cavity through the lymphatic orifices. The peritoneum layering the Douglas pouch, for example, is rich in subperitoneal lymphatic vessels and milky spots. The intraperitoneal fluid containing free cancer cells, once reaching the pelvic subperitoneal lymphatics, goes toward the rectum and finally flows into the lymph nodes around the iliac artery. On the other hand the peritoneum covering the liver, and the serosal surface of small bowel and spleen are devoid of lymphatic stomata as well as milky spots and thus are involved in peritoneal dissemination of cancer cells only in the late stage of peritoneal carcinomatosis. While the mechanism of peritoneal dissemination in pseudomyxoma peritonei is characterized by the translymphatic process, the dissemination of gastric and colon cancer is characterized by both translymphatic and transmesothelial processes. Pseudomyxoma peritonei is characterized by the accumulation of abundant gelatinous mucin within the peritoneal cavity and diffuse mucinous implants on the peritoneal surface and omentum. The major component of the lesions is mucinous material while neoplastic epithelial cells are

extremely scanty. In the past there was a lack of consensus about the site of origin of this clinical condition, especially in female patients. There were 3 main hypotheses: (1) metastasis from the ovary to the appendix; (2) metastasis from the appendix to the ovary, or (3) an independent origin of the tumor. In exceptionally rare cases other sites have been reported to be the primary sites, such as the colon, common bile duct, pancreas and breast. There is a growing body of evidence, based on morphological, immunohistochemical and genetic studies, suggesting that the primary site of origin is the appendix in majority of the cases. The most popular model explaining tumor progression advocates that an initial neoplastic process (such as a mucinous adenoma) produces mucin continuously inside the appendiceal lumen, leading to obstruction and distension of this structure. The appendix suffers rupture and the mucin material disseminates inside the peritoneal cavity guided by 3 mechanical forces: gravity, hydrostatic pressure exerted by respiratory movements of the diaphragmatic muscle and peristaltic movements of the bowel. The accumulation and deposition of the neoplastic material inside the peritoneal cavity at different locations will be conditioned by the translymphatic model of tumor dissemination, as

mentioned above. The biological course is indolent and progressive and leads the patient to death as a consequence of intestinal obstruction, unless adequately treated. CDX-2 is the product of the caudal-type homeobox gene, which encodes a transcription factor that plays a role as a regulatory protein in proliferation and differentiation of intestinal epithelial cells. CDX-2 expression is uniformly found in almost all cases of colorectal and duodenal adenocarcinomas and appendiceal adenocarcinoma, whereas expression is heterogeneous in adenocarcinomas of gastric, gastro-oesophageal and pancreatobiliary origin. Nonaka et al reported in a series of 42 case of pseudomyxoma peritonei that all cases of peritoneal lesions, showed diffuse and strong immunoreactivity for CDX-2 in a uniform nuclear staining pattern. In a successive evaluation of this marker in the same series of patients, it was shown that immunoexpression was significantly correlated with overall survival by univariate analysis. Mucins are high-molecular-weight glycoproteins, present at the interface between many epithelial and extracellular environments and synthesized by a broad range of epithelial tissues. Genes coding for the protein components of mucin are designated as MUCs. Currently 14 mucin-type glycoproteins have

been assigned to the MUC gene family. Mucins are subdivided into membraneassociated and secreted forms, the former represented by MUC-1 and the latter represented by MUC-2 and MUC- 5AC. MUC-2 is specifically expressed in goblet cells of the small bowel and colon, while MUC-5AC is generally expressed in the stomach and respiratory tracts. The vast majority of mucinous epithelial neoplasms of the appendix coexpress both MUC-2 and MUC-5AC, while mucinous neoplasms of the ovary express only MUC-5AC but not MUC-2. Interestingly, cases of classic pseudomyxoma peritonei show the intestinal⁄appendiceal pattern (MUC-2+ and MUC-5AC+), as do appendiceal mucinous neoplasms, whereas cases of peritoneal implants or pseudomyxoma ovarii associated with primary ovarian mucinous neoplasms show the ovarian pattern (MUC-2- and MUC-5AC+), just as ovarian mucinous neoplasms do. These findings support the notion that pseudomyxoma peritonei is a disease resulting from the accumulation of extracellular secretory-type mucin, particularly related to MUC-2 overexpression by neoplastic cells, thereby rendering MUC-2 expression a potential molecular target to inhibit the progression of the disease.

Peritoneal carcinomatosis from gastric cancer

In the United States 20 to 30% of patients with gastric cancer being explored for potentially curative resection will be found to have peritoneal seeding at the time of surgical exploration. Current standard treatment is systemic chemotherapy which may delay onset of symptoms but is not curative. The median survival of these patients is 5 months with virtually no long-term survivors. Yoo and colleagues reviewed 2328 patients with gastric cancer who underwent curative resection with at least 5-years follow-up. Documented evidence of relapse of the disease was found in 508 patients. Isolated peritoneal recurrence was noted in 34% of patients who relapsed. Hematogenous recurrence occurred in 26% and local-regional persistence of the tumor was seen in 19%. Two or more sites of recurrence were documented in the remaining patients. Serosal invasion and lymph node metastasis were risk factors of relapse in all patterns of recurrence. This high incidence of peritoneal carcinomatosis following curative resections is shared by others, with an average incidence between 20% and 50%. These data show that in an impressive number of patients the recurrence is isolated within the peritoneal cavity. It also suggests that if an effective treatment

could be targeted toward peritoneal dissemination, at least a third of the patients with advanced gastric cancer could experience a better outcome. Systemic chemotherapy for gastric patients presenting with peritoneal seeding at the time of abdominal exploration or as a manifestation of disease recurrence after a curative surgery is uniformly disappointing. Preusser and colleagues published a response rate for advanced gastric cancer of 50%; nevertheless patients with peritoneal dissemination obtained the worst response. Ajani and colleagues, treated patients prior to gastrectomy. At exploration, peritoneal carcinomatosis was the most common cause of failure of intensive neoadjuvant chemotherapeutic treatment. Also, radiation showed limited results in this situation and is expected to cause significant morbidity when applied to such a large field.

Peritoneal carcinomatosis from colorectal cancer

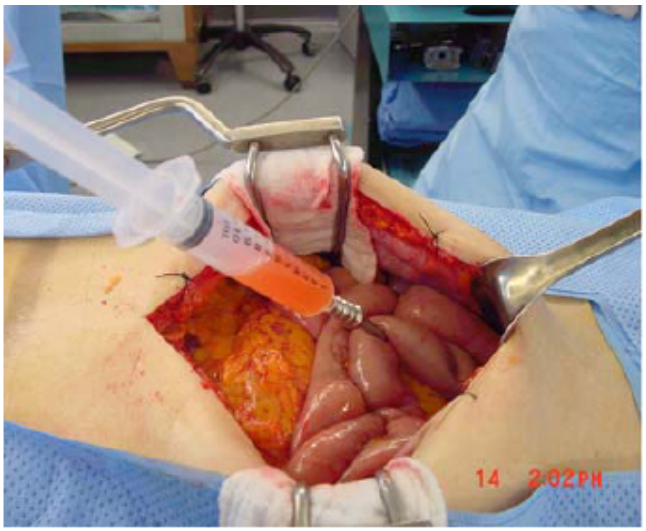
Despite advances for early diagnosis of colorectal cancer, peritoneal carcinomatosis persists as a major problem. Peritoneal implants are present in 10% of patients with colorectal cancer at the time of diagnosis and are the second cause of death after liver metastasis. In

contrast to the other two main sites of colorectal cancer metastasis, liver and lymph nodes, peritoneal seeding is considered a condition uniformly lethal with no perspective of cure. From a database of 3019 colorectal cancer patients, Jayne and colleagues identified 349 patients with peritoneal carcinomatosis. The median survival of this group was 7 months. Unfortunately this recent data showed no improvement in the survival of these patients if compared with the first study of the natural history of peritoneal carcinomatosis published 13 years before by Chu et al. Also, a European multicenter trial (EVOCAPE 1) evaluated prospectively 118 patients with peritoneal carcinomatosis arising from colorectal cancer. The mean survival of those patients was 6.9 months.

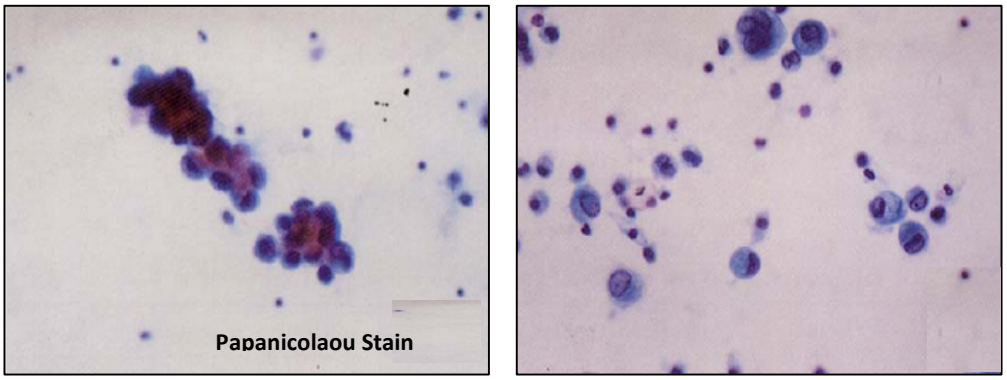
Peritoneal wash cytology

In the majority of reports the peritoneal washing is performed immediately after the laparotomy in the absence of ascites, once achieved a complete hemostasis before the incision into the peritoneum to prevent contamination from leukocytes. One hundred mL of physiologic saline at 37°C is injected into the Douglas cavity, as

recommended by the second edition of the Japanese Classification of Gastric Carcinoma, and after gentle stirring the fluid is aspirated.



All samples are immediately centrifuged (1500 rpm for 10 min) and the precipitates are smeared. The precipitates are promptly fixed with 95% alcohol for Papanicolaou staining, fixed with 100% ethanol for periodic acid-Schiff (PAS) staining, and dried with a drier for May-Giemsa staining. The fixed samples are stained by the staining procedures corresponding to the different fixation methods. The slides are examined by light microscopy by experienced cytopathologists.



The result of the cytological examination include 3 different outcomes: CY0, benign/indeterminate cells on peritoneal cytology; CY1, cancer cells on peritoneal cytology; CYX, peritoneal cytology not performed. The CY0 group in cytological diagnosis comprise “suspicious of malignancy”. Conventional cytological examination is often criticized for its relatively low sensitivity. The overall positive cytology rate depends on the cohort of patients being studied, ranging from 14% to 70%. Bando demonstrated a positive cytology rate of 24%. This series of 1 297 patients included cases with advanced gastric cancer (296 patients with peritoneal metastasis and 77 patients with T4 tumors). Ribeiro reported a positive cytology rate of 41% incidence in a series of 49 patients with both localized and metastatic gastric cancer. Literature reports demonstrated that cytology is strictly related to the extent of the tumor, and with the involvement locoregional lymph-nodes, according with the theory

that cancer cells can directly exfoliate and desquamated from serosa into the peritoneum adjacent to the tumour, or alternatively, seed into the peritoneal space through mesenteric lymphatics through so-called “gaps” in association with milky spots. When only potentially curative resections are included, in fact, the rate of FPTC varies from 4.4-11%, and ranges from 22-30% in gastric carcinoma involving the serosa. The Dutch Gastric Cancer Trial demonstrated positive cytology in only 7.1% of all patients with gastric cancer and in 12% of those with serosal invasion. These results indicate that the conventional cytological examination lacks sensitivity for the detection of residual cancer cells and prediction of peritoneal spread, as evident from the fact that several intraperitoneal recurrences can be observed among patients with negative cytology results and that patients with macroscopically evident peritoneal metastasis can show negative cytology results. Wang et al. showed that only 67% of patients (8/12) with macroscopically evident peritoneal metastasis showed a positive peritoneal cytology. In order to improve sensitivity of conventional cytology Homma et al. performed peritoneal wash cytology in four different cavities: the left subphrenic cavity, right subhepatic cavity, Douglas’ pouch, and inside the omental bursa,

differently form the Japanese Classification of Gastric Cancer recommendation to perform peritoneal wash cytology only in Douglas' pouch. They found that peritoneal wash cytology in multiple cavities allows to increase sensitivity of cytology, showing that eleven (17.7%) patients who had negative peritoneal wash cytology in Douglas' pouch, were positive in the other cavities. Ribeiro, in order to improve sensibility and sensitivity of peritoneal wash cytology, demonstrated also that the number and the arrangement of FPTC are an important parameter of cytological evaluation. In fact, the survival rate appears to change if FPTC are, clustered, isolated, or clustered-plus-isolated type. The reliability of morphologic diagnosis in cytology is limited, and the differential diagnosis between benign reactive mesothelial cells and well-differentiated carcinoma cells is often difficult. Therefore, a more sensitive method for detecting free cancer cells in the peritoneal cavity is needed. In fact, the peritoneal wash cytology has a sensitivity of 90% to 96.7% and nearly 100% specificity in the diagnosis of FPTC. False-positive peritoneal lavage cytology has been recognized by some authors, with a rate of 4.5% to 5%, probably secondary to reactive mesothelial cells.

Other techniques

In order to diminish the false-positive and -negative rates, several authors have used immunocytochemistry and molecular biology techniques, including reverse transcriptase–polymerase chain reaction for carcinoembryonic antigen messenger RNA. Some groups have used immunocytochemical analysis to detect cancer cells in peritoneal lavage. This technique consists in using a panel of different monoclonal antibodies (MoAbs), (B72.3, AR3, BD5, HEA 125, and monoclonal CEA clone 11-7) directed to gastric cancer-associated antigens. MoAbs allow to improve the detection rate of FPTC in peritoneal washing in a percentage comprises between 5% and 15% compared to standard cytology. Nevertheless, the antigens utilized as marker of FPTC at the immunocytology are not cancer specific, and they can be expressed and produced also by non-tumoral cell during inflammatory response (frequently reactive mesothelial cells). Therefore, in order to avoid false positive results of FPTC, immunocytochemical results should be compared to cytological features of malignancy, or utilized as a confirmation of a suspicious feature at the standard cytology. In a recent study we showed the results of PWC in patients underwent gastric resection for gastric

carcinoma, demonstrating that the use of immunocytology was limited to dubious or suspicious glass slides. The dosage of carcinoembryonic antigen (CEA) levels in peritoneal washings was proposed to detect microscopic residual tumor in 1989 by Asao et al. who later found that the 2-year survival rates after curative operation for the patients with and without elevation of CEA levels were 21% (19 patients) and 100% (66 patients), respectively ($P<0.001$). Subsequently other authors investigated other tumor markers: in 1999 Bold et al. showed how elevated peritoneal levels of cancer antigen 125 (CA 125) in the peritoneal washing significantly predicted peritoneal recurrence in patients who underwent curative gastrectomy, but that was not valid for peritoneal levels of CEA . Some other papers followed during the next years most of them showing how peritoneal CEA was more sensitive (positivity rate ~40%) than cytology and that high levels of CEA were related to a greater extension of the disease, to a higher risk of peritoneal carcinomatosis and to a worse prognosis. All these studies used several different methods for assessing the tumor markers dosage in the peritoneal fluid and used even very different cut-off values. The peritoneal dosage of tumor markers was soon abandoned by

researchers in favour of the more reliable and more standardized reverse transcriptase polymerase chain reaction technique. The high sensitivity of reverse transcriptase-polymerase chain reaction (RT-PCR) techniques was already found to allow diagnosis of micrometastases based on tissue-specific mRNA expression in tumor cells in peripheral blood, bone marrow and lymph nodes. In 1997 a Japanese group first proposed the use of RT-PCR for the detection of free peritoneal tumor cells from perioperative peritoneal washings from patients affected with gastric cancer. Briefly in this technique peritoneal lavage samples are centrifuged and the cell pellets are subjected to RNA isolation and amplification. CEA mRNA was firstly used for RT-PCR based molecular detection of peritoneal free tumor cells; later other Authors reported about some gastric cancer cells that do not express CEA mRNA and additional markers were introduced such as cytokeratins, trypsinogen, telomerase, matrix metalloproteinase-7 (MMP-7), dopa decarboxylase, L-3 phophoserine phosphatise. In all studies a greater sensitivity of RT-PCR was reported in comparison with cytology, the global positivity rate ranged between 40% and 70%, being for patients with T1, T2, T3 and T4 stage disease respectively about 10%, 30%, 65% and 90%. Most of

the studies reported how the positive RT-PCR test was significantly and independently related to an increased risk for peritoneal carcinomatosis and to a worse survival. In the largest series reported in literature all patients who presented peritoneal carcinomatosis during the follow up period were positive at time of surgery for RT-PCR on peritoneal washes and omentum while only about 30% of them were positive also for conventional cytology. Some criticism have been moved to this technique, in fact some Authors believe that the expression of some genes used for the identification of tumor cells may be present in inflammatory cells as well, resulting RT-PCR in a high sensitivity and low specificity test. Molecular diagnostic technique has been used also for the prediction of the effects of chemotherapy: in one study the prognosis of patients who showed a change in the result of RT-PCR on the peritoneal washing from positive to negative was significantly improved compared to those who remained positive after chemotherapy. Some problems about the optimization of these techniques still have to be debated for example the possibility of high rate of false positive diagnosis at RT-PCR. This can be due to an illegitimate expression of marker genes in noncancerous cells or to a (too) high sensitivity of the technique that

can even discover markers mRNA from a very small, clinically insignificant, number of cells. Nevertheless in some Japanese Institutions RT-PCR is already used in the clinical practice: patients with negative cytology and positive RT-PCR at preoperative staging laparoscopy are treated with a short-term intraperitoneal chemotherapy. Molecular diagnostic techniques are, however, time-consuming, relatively laborious and more expensive compared with conventional cytodiagnostic methods; there are actually many different methods of molecular diagnosis among institutions, and this causes delay in his routine clinical application. Since 2006, the Japanese Government included the molecular biology assay diagnosis of body fluids in the public health insurance program for patients with solid tumors, facilitating the introduction into clinical practice of genetic diagnostic techniques for peritoneal lavage from patients with gastric cancer, but, at present, only University hospitals and large cancer centers use this techniques.

Aim of the study

In the attempt to further demonstrate the diagnostic/prognostic value of the detection of epithelial-tumor markers in the peritoneal

washes and to rule out the possibility of false positive results using molecular-based techniques alone, in this study we combined the qRT-PCR analysis with an immunomagnetic enrichment followed by immunofluorescence (IF) analysis, for enhancing the specificity of detection of the free peritoneal tumor cells (FPTCs). To this aim, the peritoneal washes were directed to a procedure commonly used for detection of circulating tumor cells CTC from blood samples (20). To detect the disseminated epithelial cells, we used monoclonal antibody against the pan-epithelial marker EpCAM/CD326 and to ascertain their tumor origin we used polyclonal antibodies against the carcinoembryonic antigen (CEA). In this setting, IF microscopy allowed the morphological assessment and unequivocal identification of the FPTCs as well as validation of the molecular analysis. This combined use of immunomagnetic enrichment, IF analysis and real-time qRT-PCR, showing a greater sensibility respect to conventional cytology, was able to permit the detection of free peritoneal tumor cells in both gastric and colorectal cancer and to determine their prognostic value for survival.

MATERIAL AND METHODS

Patients and Surgery

All patients were extensively informed and gave written consent for the investigations. The study was approved by the local ethical commission. Twenty-seven gastric and 48 colorectal patients with cancer who underwent surgery between December 2008 and December 2009 at the A Unit of Surgery of Sant'Andrea Hospital were investigated. Patients with distal extraperitoneal rectum cancer were excluded from the study. Preoperative chemotherapy or radiation therapy was not performed in this series.

Gastric cancer patients (GC) underwent subtotal gastrectomy in 15 cases, total gastrectomy in 8 cases and palliative surgery in 4 cases. Colorectal cancer patients (CRC) underwent right colectomy in 23 cases, left colectomy in 10 cases, anterior resection in 14 cases and palliative surgery in 1 case. All patients underwent open surgery. A control group comprised 6 patients with a variety of non-carcinoma diseases: benign uterus tumor, cholecystolithiasis and colic adenoma. Follow-up data were obtained for a median observation time of 17 months (range 1-27 months).

Samples

Immediately after a midline abdominal incision had been made and before manipulation of the tumor, peritoneal washing was performed. Intraoperatively, 250 mL of saline were instilled into the abdominal cavity over the tumor site and at least 150 mL were reaspirated. Twenty mL were sent for cytological examination which was performed after Papanicolaou and Giemsa stainings. The slides were examined by light microscopy by experienced cytologists unaware of the clinical findings. Patients with suspicious morphological evidence of malignancy by microscopy were included in the positive cytology group.

RNA extraction and cDNA synthesis

Each peritoneal wash sample was centrifuged at 1200 rpm for 10' and total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA) according to the manufacture's procedure. Briefly cells were homogenized in 1 mL TRIzol reagent and RNA was extracted by incubation and centrifugation in 0,2 mL CHCl3. RNA was precipitated from aqueous phase by 0,5 mL of isopropanol. RNA pellet was washed in 75% ethanol and eluted with 0,1%

diethylpyrocarbonate (DEPC)-treated water. Total RNA quantity, purity and absence of ribonuclease digestion were assessed by measuring the optical density ratio 260/280 nm. Total RNA samples were stored at -80°C. After denaturation in DEPC-treated water at 70°C for 10 min, 1 μg of total RNA was used to cDNA synthesis using cDNA synthesis mix (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR primer design

Gene sequences were obtained from the NCBI database. Oligonucleotide primers for CEA and CK20 target genes and GAPDH housekeeping gene were chosen with the assistance of the Beacon Designer 7.0 computer program (Bio-Rad Laboratories). The primers sequences used throughout this study are described in the Table 1. For each primer pair, we performed no-template control and no-reverse-transcriptase control (RT negative) assays, which produced negligible signals (usually >45 in threshold cycle (Ct) value), suggesting that primer dimer formation and genomic DNA contamination effects were negligible. Oligonucleotide primers were purchased from Invitrogen.

Table 1. Primers sequence and amplification efficiency.

Name	Primer Forward	Primer Reverse	Eff.%
GAPDH	5'CATCAGCAATGCCTCCTGCAC3'	5'GTCATGAGTCCTTCCACGATACCAA3'	99.7
CEA	5'AGGACAGAGCAGACAGCAGAG3'	5'GGTTCCAGAAGGTTAGAAGTGAGG3'	94.4
CK20	5'TGCTACTTACCGCCGCCTTC3'	5'CCTTGCCATCCACTACTTCTTGC3'	103

PCR amplification

Real-time PCR was performed using the iCycler Real-Time Detection System (iQ5 Bio-Rad) with optimized PCR conditions. The reaction was carried out in a 96-well plate using iQ SYBER Green Supermix 2X (Bio-Rad) adding each forward and reverse primers and 1 µl of diluted template cDNA to a final reaction volume of 15 µl. All assays included a negative control and were replicated three times. The relative expression of GAPDH was used for standardizing the reaction. The thermal cycling conditions comprised an initial denaturation step at 95°C for 3 minutes, followed by 45 cycles at 95°C for 10 seconds and 60°C for 30 seconds.

Data analyses

Real-time quantitation was performed by using SYBR Green dye as fluorescent signal, with the help of the iCycler IQ optical system software version 3.0a (Bio-Rad), according to the manufacturer's manual. Quantitative values are obtained from the Ct number at which, the increase in signal associated with exponential growth of PCR products, starts to be detected. Target genes (CEA, CK20) amplification was compared with simultaneous amplification of an endogenous reference gene (GAPDH) and each sample was normalized on the basis of its GAPDH content. The target genes CEA and CK20 were tested for expression in tenfold serial dilutions (106-100) of cancer cell lines from colon (HT29, Caco2) and gastric (AGS) carcinoma. Normal human fibroblast cell line from colon (CCD18) and primary culture of human fibroblasts from skin were used as negative controls. For data analysis, receiver-operating characteristic (ROC) curves were used to compare the accuracies of CEA/GAPDH, CK20/GAPDH ratio and determine the cut off value by plotting sensitivity/specificity pairs for the two mRNA ratio. The clinical value of CEA and CK20 detection was assessed based on the diagnostic data from patients with positive cytology made at laparoscopy and from

patients of the control group. The cut off value for CEA and CK20 was defined as 0.66 (gene target/GAPDH ratio). The sensitivity and specificity obtained at the determined cut off were 77% and 100% respectively for the CEA/GAPDH ratio and 100% and 93% for the CK20/GAPDH ratio.

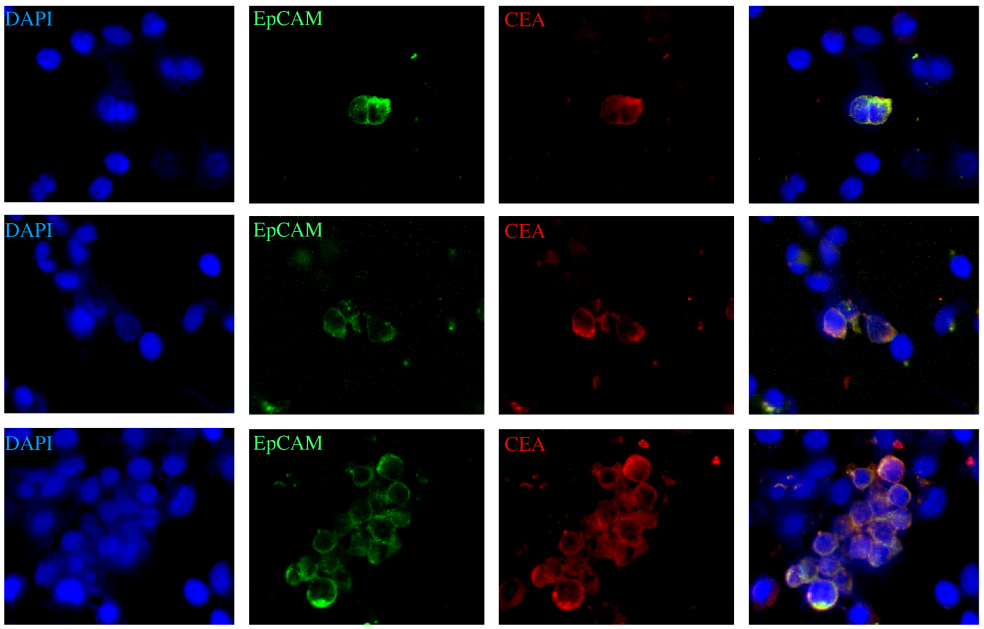
Immunomagnetic enrichment for epithelial cells.

From each patient, 40 mL of peritoneal wash were collected in EDTA (50 µm). Samples were centrifuged at 1300 rpm for 6 min at 25°C and resuspended for magnetic labeling in 80 µL of MACS® separation buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). Immunomagnetic depletion using anti-CD45 microbeads (Miltenyi Biotec) was performed according to the manufacturer's instructions to enrich for FPTCs (Figure 1A). Briefly, MS separation columns (MACS®, Miltenyi Biotec) had been equilibrated with 0,5 mL of MACS® separation buffer and the microbeads labeled cells were subjected to magnetic field trough the column passage. The CD45 negative cells were washed off from the column with 1,5 mL of MACS® separation buffer (Figure 1B) and centrifuged at 1300 rpm for 6 min at 25°C.

Immunofluorescence

CD45 negative cells were incubated with anti-CD326/EpCAM-FITC monoclonal Ab (1:10 in MACS® separation buffer) for 15 min at 4°C (Figure 1C). Cells were then washed, centrifuged at 1300 rpm for 6 min at 25°C and the pellet was resuspended in 10 µL of cell solution and spotted on 8 wells diagnostic slides (Menzel-Glaser, Braunschweig, Germany), left to dry and fixed with acetone for 8 min at -20°C. Cells were then incubated with anti-CEA polyclonal antibodies (Zymed, Invitrogen, Carlsbad, CA, USA) (1:100 in MACS® separation buffer) for 1 h at 25°C. After appropriate washing, the primary antibodies were visualized using goat anti-rabbit IgG-Texas Red (1:400 in MACS® separation buffer) for 30 min at 25°C. Nuclei were stained with DAPI (1 ng/mL, Sigma Chemicals, St Louis, MO, USA). Coverslips were finally mounted with mowiol for observation. Cells were analyzed by conventional fluorescence or by scanning in a series of 0.5 µm sequential optical sections with an ApoTome System (Zeiss, Oberkochen, Germany) connected with an Axiovert 200 inverted microscope (Zeiss). Image analysis was performed by the

Axiovision software (Zeiss). Single optical sections were acquired by a CCD camera and image analysis was performed by the Axiovision software (Zeiss).



Statistics

A cross-tabulation analysis of histopathological findings with qRT-PCR analysis, immunofluorescence evaluation and cytologic examination was performed by the chi-square test for trend or Fisher's exact test. The analysis of cancer specific survival and time to recurrence rates was calculated using the Kaplan-Meier method and compared using the log-rank test. Cox proportional-hazards regression was

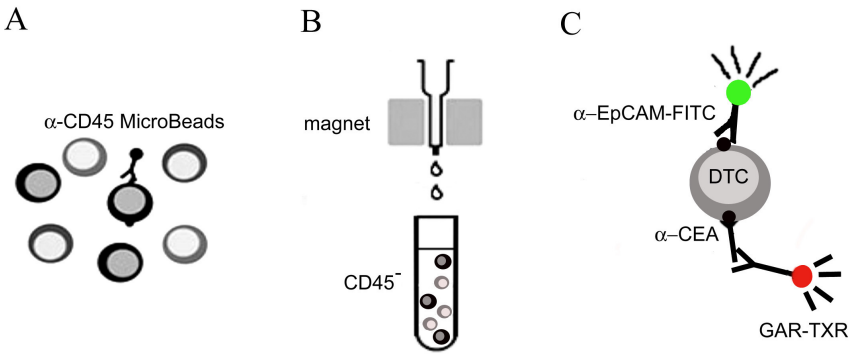
performed to analyze the effect of all variables on survival and recurrence times.

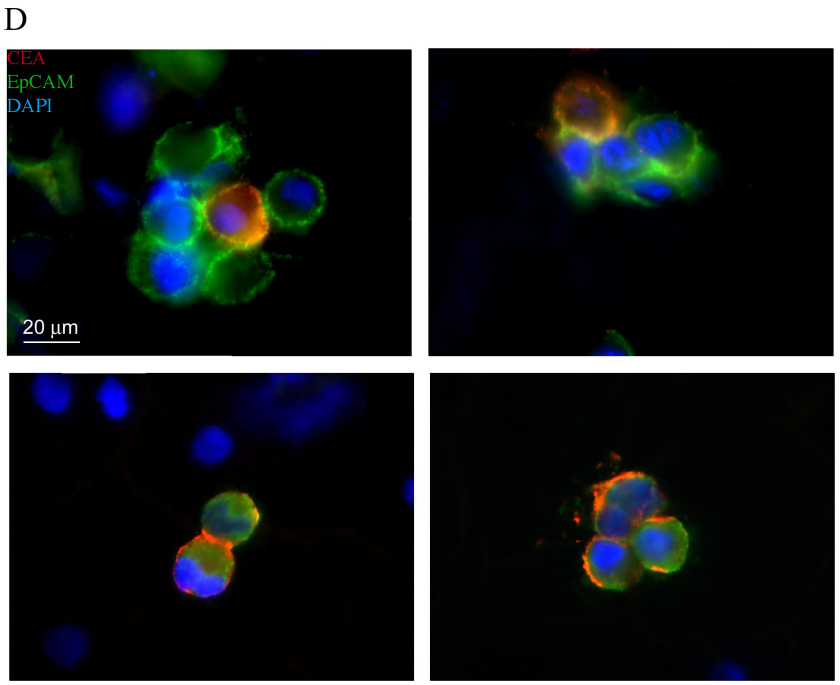
A p value of 0.05 was considered as statistically significant.

RESULTS

The application of immunomagnetic enrichment for epithelial cells and immunofluorescence analysis was performed in peritoneal lavages obtained from patients affected by gastric or colorectal cancers and this results were then associated and compared to the conventional cytology and to the molecular qRT-PCR analysis for the expression of CEA and CK20 mRNA. For the immunomagnetic enrichment we used a consolidated method of immunodepletion of the inflammatory CD45+ cells, which are the major cell population present in the peritoneal washes. After depletion, the CD45-cells washed out from the column were immunolabeled for the epithelial marker CD326/EpCAM and for the tumor marker CEA: cells were then evaluated by immunofluorescence microscopy to search for the FPTCs (Figure 1A-C). In our analysis, only cells double positive for EpCAM and CEA were considered as FPTCs. In addition, careful observation of the cell nuclei stained by DAPI allowed to evaluate the cell viability and to exclude apoptotic or necrotic cells from our analysis (Fig 1D).

Figure 1. A-C. Immunoenrichment and immunofluorescence methods to detect free disseminated peritoneal tumor cells (see text). D. Images of EpCAM/CEA positive FPTCs (yellow) surrounded by epithelial cells positive for EpCAM (green) or IF double negative inflammatory or mesothelial cells.





Gastric Carcinoma

Global positivity rate for cytology, IF and qRT-PCR was 15%, 15% and 78% respectively. Cytology was positive in only 4 patients with T4 tumours, which were also characterized by massive peritoneal carcinomatosis. All these 4 patients were positive qRT-PCR markers and three of them were positive to the IF too. Interestingly, one patient with minor peritoneal carcinomatosis was negative at the cytological examination, but positive at both IF and qRT-PCR analysis. Table 2 shows the results for IF in gastric carcinoma patients.

Table 2. Correlation between immunofluorescence evaluation, cytologic examination and histopathological findings in gastric carcinoma.

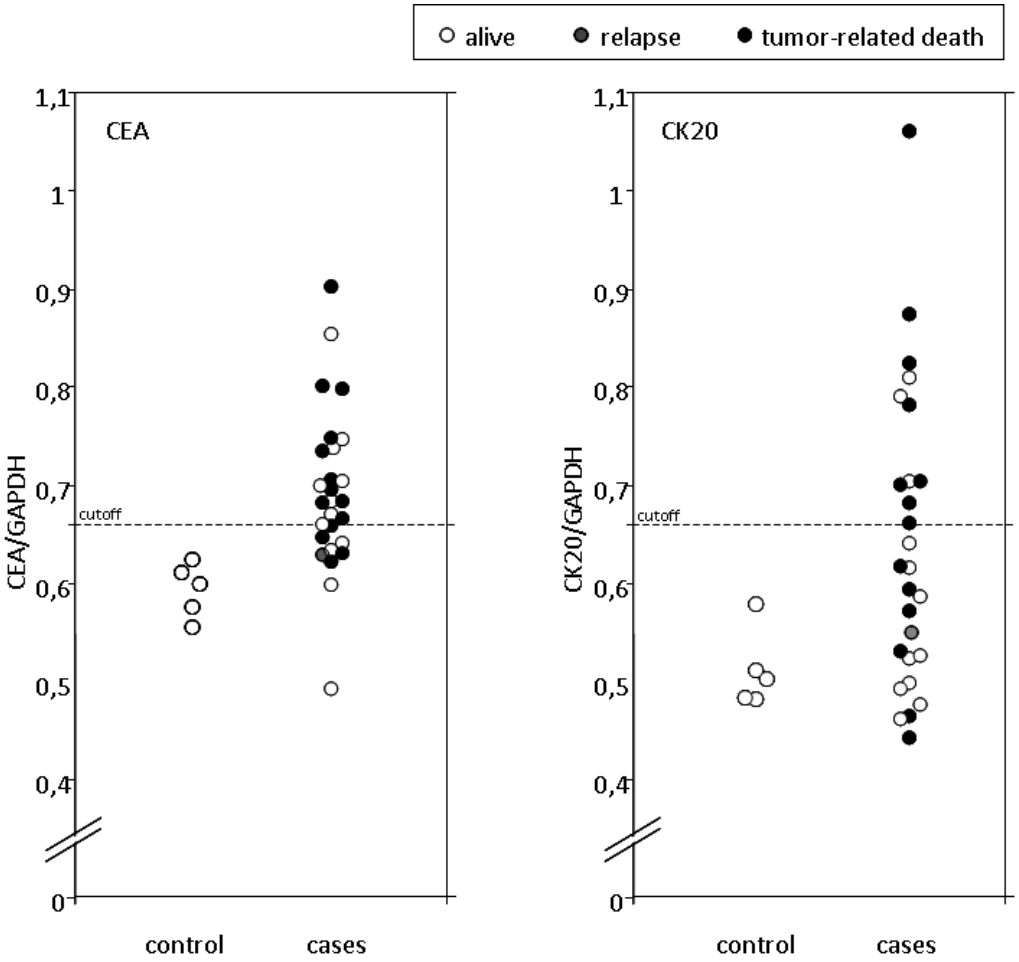
Factor	All patients	IF negative	IF positive	P value
No of patients	27	22 (85.2%)	4 (14.8%)	
Age (years)				
- mean ± SD	69.7 ± 12.8			
Gender				
- Male	14			
- Female	13			
Histology (differentiated/undifferentiated)				
- G1	2	2	0	0.005*
- G2	1	1	0	
- G3	18	18	0	
- G4	6	2	4	
Depth of invasion				
- T1	6	6	0	0.01*
- T2	5	5	0	
- T3	8	8	0	
- T4	8	4	4	

Stage at primary diagnosis				
- I	7	7	0	0.014*
- II	4	4	0	
- III	7	7	0	
- IV	9	5	4	
Cytologic examination				
- negative	23	23	0	0.0014^
- positive	4	1	3	

The chi-square test for trend showed how the worse grading (p=0.005), the deeper invasion of the gastric wall (p=0.01), the advanced stage of disease (p=0.014) and positive cytology (p=0.0014) are all related to the positivity at IF.

The molecular qRT-PCR method showed a remarkably higher incidence of positivity: in fact, expression of the markers was over the cut-off level in all T2 and T4 patients, in 3 out of 6 of the T1 patients and in 5 out of 8 of T3 patients. Moreover, as shown in Figure 2, there was a clear higher positivity for CEA (70%) respect to CK20 (41%). The combination of positivity for CEA and CK20 was observed in 36% of patients.

Figure 2. Expression levels of CEA and CK20 mRNA in control subjects and gastric cancer patients. The cutoff values of CEA/GAPDH and CK20/GAPDH was 0.66.



The positivity at qRT-PCR was not related to the depth of invasion, stage of disease and to the IF positivity but also associated to the worse grading (p=0.008; table 3).

Table 3. Relationship between qRT-PCR analysis, immunofluorescence evaluation and histopathological findings in gastric carcinoma.

Factor	All patients	qRT-PCR negative	qRT-PCR positive	P value
No of patients	27	6 (22.2%)	21 (77.8%)	
Age (years)				
- mean ± SD	69.7 ± 12.3			
Gender				
- Male	14			
- Female	13			
Histology (differentiated/undifferentiated)				
- G1	2	2	0	0.008*
- G2	1	0	1	
- G3	18	4	14	
- G4	6	0	6	
Depth of invasion				
- T1	6	3	3	0.098*
- T2	5	0	5	
- T3	8	3	5	
- T4	8	0	8	
Stage at primary diagnosis				
- I	7	2	5	0.43*
- II	4	1	3	
- III	7	2	5	
- IV	9	1	8	
Immunofluorescence evaluation				

-	negative	22	5	17	1^
-	positive	5	1	4	

The Kaplan-Meyer survival analysis showed how the positivity of IF and qRT-PCR for FPTCs was a statistically significant negative prognostic factor in both cancer specific overall survival and disease free survival rates (Figures 3-6).

Figure 3. Time to recurrence rates by IF positivity in gastric cancer

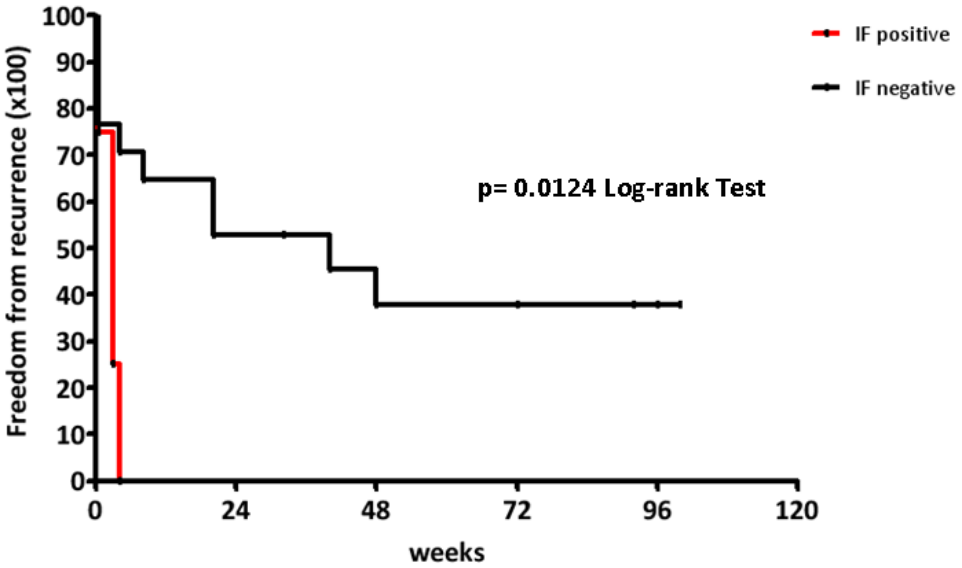


Figure 4. Time to recurrence rates by qRT PCR positivity in gastric cancer

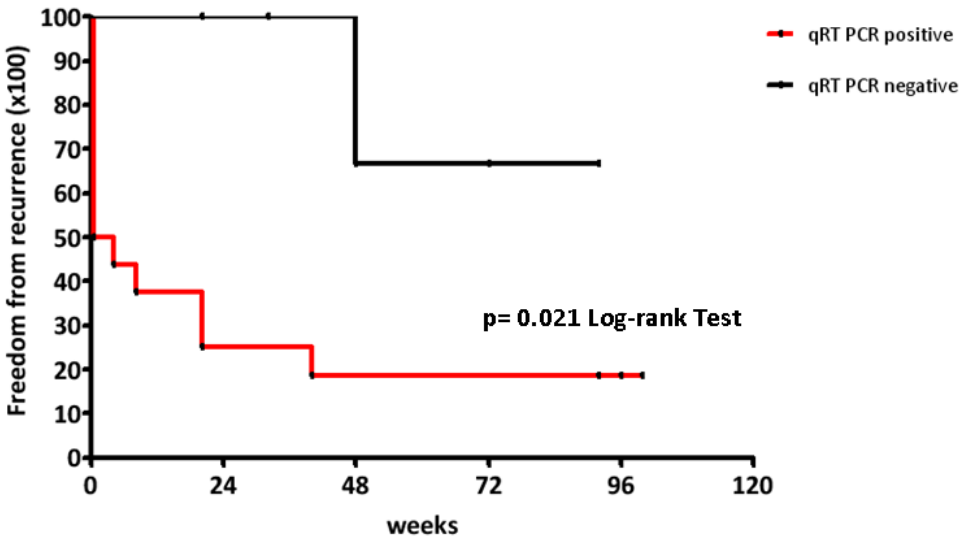


Figure 5. Cancer specific survival rates by IF positivity in gastric cancer

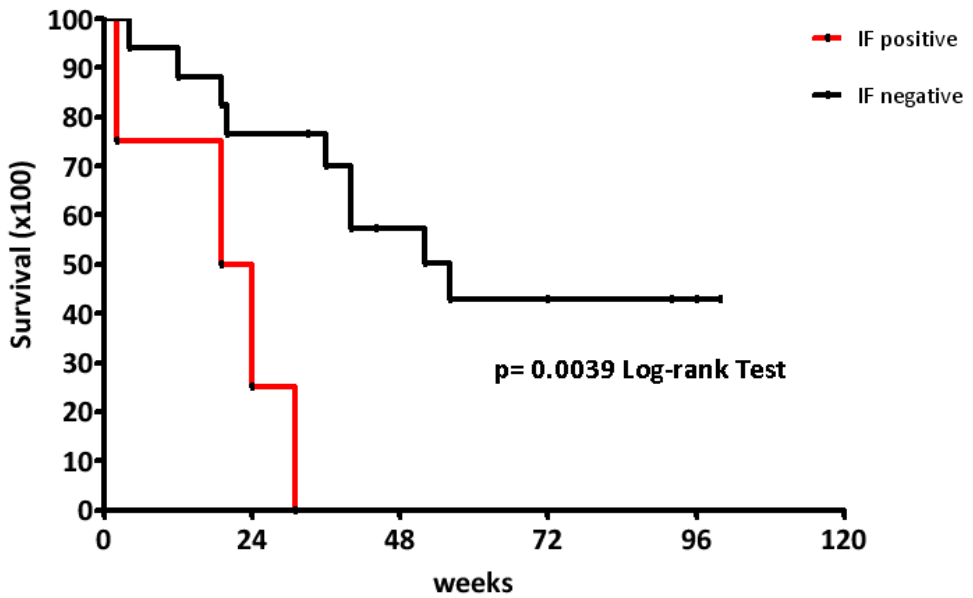
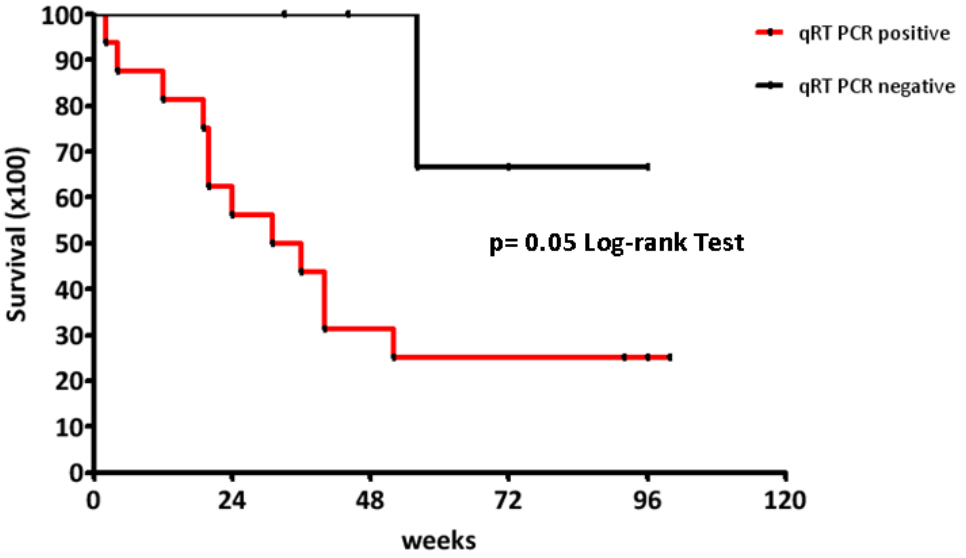


Figure 6. Cancer specific survival rates by qRT PCR positivity in gastric cancer



At the multivariate analysis (Table 4), the stage at primary diagnosis was found to be an independent risk factor in overall survival only, while qRT-PCR resulted to be an independent risk factor in both overall and disease free survival with hazard ratio of 31.3 and 18.5 respectively ($p<0.05$). IF was found to be a statistically significant prognostic factor at univariate analysis (Figures 3 and 5), but it lost its prognostic power at multivariate analysis (Table 4).

Table 4. **Correlation between immunofluorescence evaluation, cytologic examination and histopathological findings in colorectal carcinoma.**

Factor	All patients	IF negative	IF positive	P value

No of patients	48	40 (83.3%)	8 (16.7%)	
Age (years)				
- mean ± SD	69.5 ± 12.3			
Gender				
- Male	22			
- Female	26			
Histology (differentiated/undifferentiated)	1	1	0	0.13
- G1	28	21	7	*
- G2	16	15	1	
- G3	3	3	0	
- G4				
Depth of invasion				
- T1	1	1	0	1*
- T2	6	5	1	
- T3	27	22	5	
- T4	14	12	2	
Stage at primary diagnosis				
- I	6	5	1	0.63
- II	24	20	4	*
- III	14	14	3	
- IV	4	4	0	
Cytologic examination				
- negative	48	40	8	-
- positive	0	0	0	

Colorectal Carcinoma

Global positivity rate for cytology, IF and qRT-PCR for FPTCs was respectively 0%, 17% and 42%. Cytology was negative in all patients,

including one patient with peritoneal carcinomatosis; this same patient resulted positive for both CEA and CK20 at the qRT-PCR, but negative at IF. As shown in Table 5, IF was found positive in similar proportions in T2 (1/6 cases, 17%), T3 (5/27 cases, 19%) and T4 patients (2/14 cases, 14%). On the contrary of gastric carcinoma cases, posititive IF was not related to grading, depth of invasion and stage as shown in Table 5.

Table 5. **Correlation between qRT-PCR analysis, immunofluorescence evaluation and histopathological findings in colorectal carcinoma.**

Factor	**All patients**	**qRT-PCR negative**	**qRT-PCR positive**	**P value**
No of patients	48	28 (58.3%)	20 (41.7%)	
Age (years)				
- mean ± SD	69.5 ± 12.3			
Gender				
- Male	22			
- Female	26			
Histology (differentiated/undifferentiated)	1	1	0	0.57*
- G1	28	16	12	
- G2	16	10	6	

- G3	3	1	2	
- G4				
Depth of invasion				
- T1	1	1	0	0.15*
- T2	6	4	2	
- T3	27	17	10	
- T4	14	6	8	
Stage at primary diagnosis				
- I	6	4	2	0.78*
- II	25	14	11	
- III	13	8	5	
- IV	4	2	2	
Immunofluorescence evaluation	40	27	13	0.006
- negative	8	1	7	^
- positive				

In Table 6 are summarized the results for qRT-PCR: as well as the IF, no correlation was found between qRT-PCR and grading, depth of invasion and stage. Of the 8 patients who resulted positive to the IF, 7 of them were positive to qRT-PCR too, indicating a strong correlation between IF and PCR in colorectal carcinoma (p=0.006).

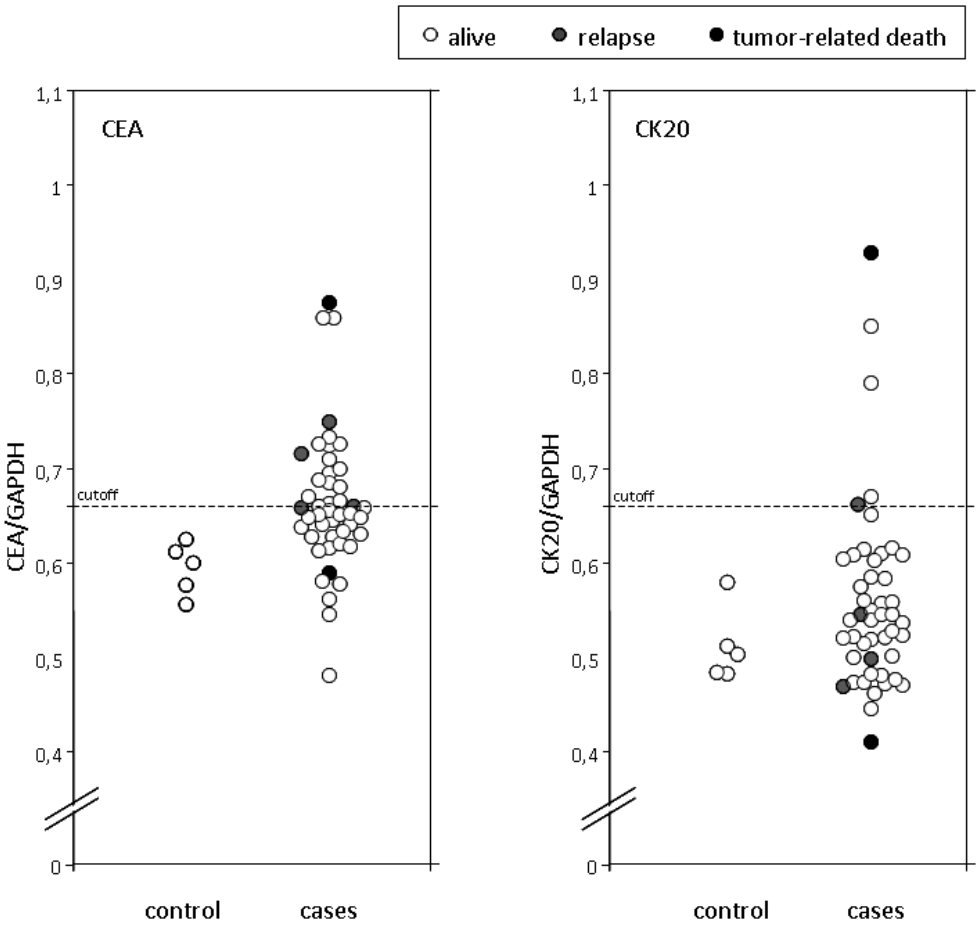
Table 6. **Multivariate Cox population hazards analysis for the gastric cancer patients.**

	Progression-free survival		**Overall survival**	
	P-value	**HR (95% CI)**	**P-value**	**HR (95% CI)**
Depth of invasion	0.13	1	0.65	1
T1-T3		5.81 (0.56-59.7)		1.41 (0.31-6.29)
T4				

Histology				
G1-G2	0.28	1	0.14	1
G3-G4		0.15 (0.01-4.7)		0.04 (0.01-2.87)
Stage at primary diagnosis	0.058	1	0.03	1
I-II		9.08 (0.92-89.5)		11.9 (1.20-118.1)
III-IV				
IF evaluation				
negative				
positive	1	1	0.30	1
		1 (0.20-4.95)		2.49 (0.43-14.2)
qRT-PCR analysis	0.05	1	0.05	1
negative		18.5 (0.70-490.4)		31.3 (0.65-1494.7)
positive				

As shown in Figure 7, there was a higher positivity for CEA (42%) respect to CK20 (10%). In addition, all patients positive for CK20 were also positive for CEA.

Figure 7. Expression levels of CEA and CK20 mRNA in control subjects and colorectal cancer patients. The cutoff values of CEA/GAPDH and CK20/GAPDH was 0.66. The open circles show the alive patients. The gray closed circles show patients who relapse. The black closed circles show patients who died by tumor-relates causes.



The analysis of survival was conducted on disease free survival only, due to the few tumor-related deaths occurred during the follow-up. Figured 8 and 9 shows the Kaplan-Meier survival curves for colorectal carcinoma patients: at Log-rank test worse prognosis was significantly associated to positive qRT-PCR (p=0.018) but not to IF (p=0.88).

Figure 8. Time to recurrence rates by IF positivity in colorectal cancer

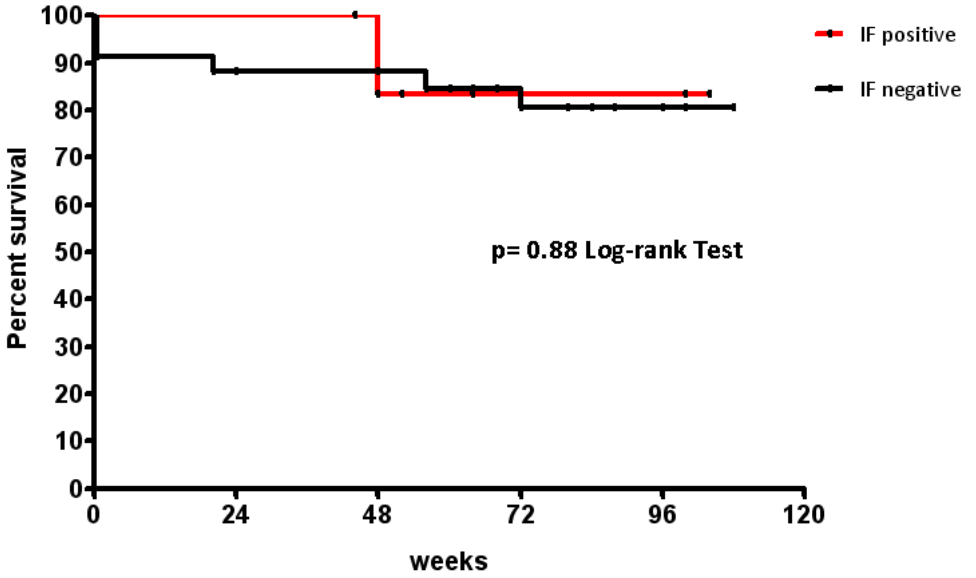
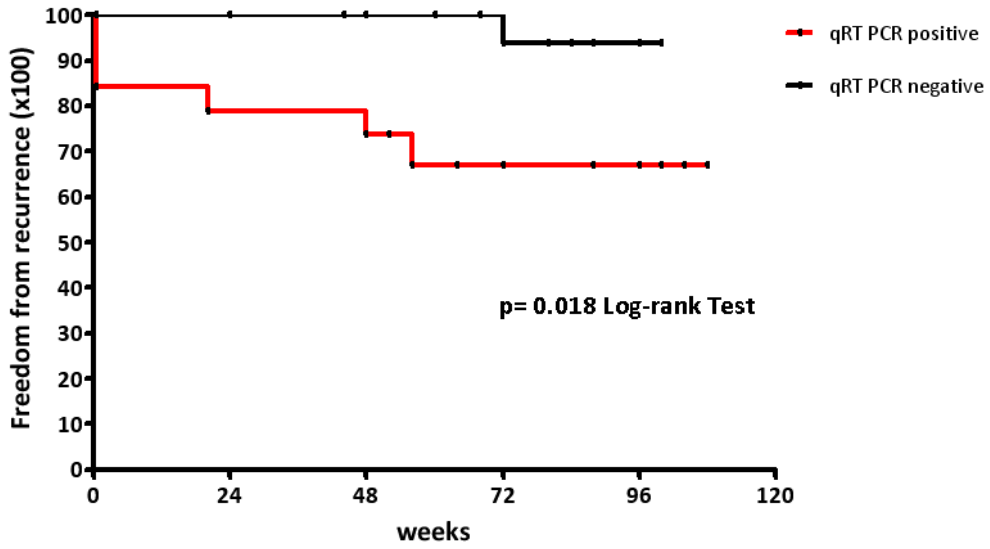


Figure 9. Time to recurrence rates by qRT PCR positivity in colorectal cancer



The multivariate Cox population analysis shows how qRT-PCR was

found to be the only independent risk factor for relapse, with a hazard ratio of 6,95 ($p<0.05$; table 7).

Table 7. **Multivariate Cox population hazards analysis for the colorectal cancer patients.**

	Progression-free survival	
	P-value	**HR (95% CI)**
Depth of invasion		
T1-T3	0.09	1
T4		4.84 (0.76-30.6)
Histology		
G1-G2	0.31	1
G3-G4		0.41 (0.07-2.32)
Stage at primary diagnosis		
I-II	0.17	1
III-IV		3.23 (0.59-17.7)
IF evaluation		
negative		
positive	0.90	1
		1.09 (0.18-6.46)
qRT-PCR analysis		
negative	0.05	1
positive		6.95 (0.78-61.4)

Controls

All samples of peritoneal lavage from the control group resulted negative for cytology, IF and real time qRT-PCR.

DISCUSSION

Peritoneal cytology has been introduced by many institutions as prognostic marker in both gastric and colorectal cancer. In gastric cancer its importance has been increasing during the last years and it has been proposed to use percutaneous or laparoscopic peritoneal lavage in the preoperative staging of patients (21). Actually in some cases positive peritoneal cytology from patients with gastric cancer is being used as indication for neoadjuvant chemotherapy or as absolute contraindication to surgery. It has been clearly assessed from many studies its value as negative prognostic marker: although positivity for peritoneal cytology increases with the stage of the disease, it has been found from different studies how its prognostic significance is independent. In fact, analyzing patients from the same stage of disease, those with positive peritoneal cytology had worse prognosis. The 7th TNM edition (Sobin LH, Gospodarowicz MK, Wittekind C. International Union Against Cancer (UICC) TNM classification of malignant tumours, 7th edition. New York: Wiley-Liss; 2010) has given great importance to peritoneal cytology,

including in the M1 group those patients with positive washings even in absence of visible peritoneal implants.

In colorectal cancer the use of peritoneal cytology is less used and standardized than in gastric cancer, probably for the minor incidence of peritoneal carcinomatosis in this type of neoplasm. Most studies on patients affected with colorectal cancer show that the detection of single cancer cells in peritoneal cavity has prognostic relevance (22-23), but in other cases results were different (24).

The primary problems with conventional peritoneal cytology are the lack of sensitivity (positivity of 14-21% in gastric cancer and 0-11% in colorectal cancer) and the high operator-dependent feature of this test. In fact most of patients with positive peritoneal lavage develop peritoneal carcinomatosis, but it is even developed by many of the patients with negative peritoneal washing. Since the knowledge about the presence of isolated tumor cells in the peritoneal cavity has been growing in importance for the treatment strategy in both gastric and colorectal cancer, clinicians need new and more sensitive and specific techniques to retrieve these new prognostic factors. The simplest technique that gives little advantage on the results of traditional cytopathology is to integrate it with immunocytochemical

methods, using monoclonal antibodies directed to gastric cancer-associated antigens (10).

Kodera et al. (16) proposed the use of real time qRT-PCR for the detection of free peritoneal tumor cells from patients affected with gastric cancer: a greater sensitivity of real time qRT-PCR was reported in comparison with cytology: all patients who presented peritoneal carcinomatosis during the follow up period were positive at time of surgery for real time qRT-PCR on peritoneal washes and omentum while only about 30% of them were positive even for conventional cytology.

After 1998 some more Authors, mostly Japanese, reported about the use of real time qRT-PCR for the detection of isolated peritoneal tumor cells from gastric cancer patients and all of them concluded confirming how real time qRT-PCR is a more specific and sensitive technique than cytopathology and that it was found to be as independent prognostic marker. Similar studies about colorectal cancer are also present in the literature, but less frequently. In their study Guller et al. (25) report that, on a total of 39 colorectal cancer patients, 10 of them resulted positive for the RT-PCR (CEA and CK20) at the peritoneal lavage. During the follow up period 8 of them had

recurrence and positive peritoneal real time qRT-PCR was found to be an independent prognostic factor.

Hara et al. (26) published the first and only study comparing the results of RT-PCR on peritoneal lavage in gastric and colorectal cancer patients. They found that prognosis in positive RT-PCR patients was worse in both colorectal and gastric cancer; they also found that, among real time qRT-PCR positive cases, peritoneal carcinomatosis was significantly more frequent in gastric cancer patients but not in colorectal patients. They concluded stating that colorectal carcinoma cells must have some biological characteristics that make them with a low-peritoneal metastatic potential.

Some criticism have been moved to this molecular technique, since some Authors believe that the expression of some genes used for the identification of tumor cells may be present in inflammatory cells as well, resulting real time qRT-PCR in a high sensitivity and low specificity test (19).

Some problems about the optimization of the molecular techniques still have to be debated: for example, the possibility of high rate of false positive diagnosis at RT-PCR. This can be due to an illegitimate expression of marker genes in noncancerous cells (27) or to a too

high sensitivity of the technique that can even detect mRNA markers from a very small, clinically insignificant, number of cells. Nevertheless in some Japanese Institutions real time qRT-PCR is already used in the clinical practice: patients with negative cytology and positive real time qRT-PCR at preoperative staging laparoscopy are treated with a short-term intraperitoneal chemotherapy (28).

To our knowledge nothing is reported about the use of IF for the detection of free peritoneal cancer cells in enriched samples of peritoneal lavages. Our study combined for the first time the use of real time qRT-PCR with IF and immunomagnetic enrichment of epithelial cells to detect free peritoneal tumor cells in gastric and colorectal cancer. For each technique we used two different markers: CEA and CK20 for the qRT-PCR and CEA and EpCAM for IF. Our results confirmed the low sensitivity of the traditional cytology: in fact, it was positive only in four cases of gastric cancer with associated massive peritoneal carcinomatosis and in none of colorectal cancers. All cytological positive samples resulted positive also for IF and real time qRT-PCR. On the contrary, no false positive were found at the qRT-PCR or IF examination in the group of patients with non-

malignant diseases, further demonstrating the validity of our procedure.

In comparison with cytology, both IF and real time qRT-PCR showed higher positivity rates, being 15% and 78% for gastric cancer patients and 17% and 42% for colorectal cancer patients respectively. Among the gastric cancer patients, IF was positive not only in the 3 of them with massive carcinomatosis, but also in 1 case with minor extent of peritoneal dissemination. Interestingly, in colorectal cancer patients we found positivity even in early stages of disease.

The positivity rate for qRT-PCR in gastric cancer patients was impressive, comprising more than 3/4 of the patients, distributed in all T1-T4 stages of disease. In contrast, in colorectal cancer patients the qRT-PCR positivity was found in less than half of patients, most of them with T3-T4 disease. All patients positive at IF were also positive at qRT-PCR, except for one colon cancer and one gastric cancer patients.

Our data showed how positive IF resulted to be significantly associated to grading, depth of invasion, stage of disease and cytology in gastric cancer. On the opposite for colorectal cancer IF was note related to any of the examined clinicopathological factors.

In the survival study positive IF was associated to worse overall and disease free survival in gastric patients at the univariate analysis; at the multivariate analysis IF was not found to be an independent prognostic factor in gastric cancer patients. In colorectal cancer cases IF was not a statistically significant prognostic factor in both univariate and multivariate analysis.

RT-PCR positivity was associated to higher grading in gastric cancer and only to positive IF in colorectal cancer. In both gastric and colorectal cancer RT-PCR was found to be one of the strongest independent prognostic factors.

From these data we can notice that IF seems to be associated to the most common clinicopathological factors in GC, but it has no prognostic value in both gastric and colorectal cancer patients. On the other hand RT-PCR is not frequently associated to other clinicopathological factors but resulted to be independently relevant for the prognosis in both gastric and colorectal cancer.

In conclusion, we believe that the combination of conventional real time qRT-PCR with immunoenrichment and IF, which permit morphological assessment and unequivocal identification of the FPTCs as well as validation of the molecular analysis, could be an

useful and more powerful procedure for the detection of free peritoneal tumor cells. More studies on these cells are requested to understand their prognostic power and any other possible clinical application. Since the treatment of cancer is going toward the personalized therapy, as well as for the circulating tumor cells, in the future the characterization of peritoneal tumor cells may be interrogated to guide molecularly targeted therapies, assess treatment effect and detect development of drug resistance.

REFERENCES

- *A. Gervasoni, R. M. Monasterio Munoz, G.S. Wengler, A. Rizzi, A. Zaniboni, O. Parolini. Molecular signature detection of circulating tumor cells using a panel of selected genes. Cancer letters 2008; 263:267-279.*
- *Abe N, Watanabe T, Toda H, Machida H, Suzuki K, Masaki T et al. Prognostic significance of carcinoembryonic antigen levels in peritoneal washes in patients with gastric cancer. Am J Surg 2001;181:356-61.*
- *Ajani JA, Ota DM, Jessup JM, Ames FC, McBride C, Boddie A, Levin B, Jackson DE, Roh M and Hohn D (1991) Resectable gastric carcinoma. An evaluation of preoperative and postoperative chemotherapy. Cancer 68, 1501-1506.*
- *Asao T, Fukuda T, Yazawa S, Nagamachi Y. CEA levels in peritoneal washings from gastric cancer patients as a prognostic guide. Cancer Lett 1989;47:79-81.*
- *Baba h, Korenaga D, Okamura T., Saito A and Sugimachi K. Prognostic factors in gastric cancer with serosal invasion. Arch Surg 1989; 124:1061-1064.*
- *Bando E, Yonemura Y, Takeshita Y, et al. Intraoperative lavage for cytological examination in 1297 patients with gastric carcinoma. Am J Surg 1999; 178: 256-262.*
- *Benevolo M, Mottolese M, Cosimelli M, et al. Diagnostic and prognostic value of peritoneal immunocytology in gastric cancer. J Clin Oncol 1998; 16: 3406-3411.*
- *Boku T, Nakane Y, Minoura T, Takada H, Yamamura M, Hioki K et al. Prognostic significance of serosal invasion and free intraperitoneal cancer cells in gastric cancer. Br J Surg 1990;77:436-9.*
- *Bold RJ, Ota DM, Ajani JA, et al. Peritoneal and serum tumor markers predict recurrence and survival of patients with resectable gastric cancer. Gastric Cancer 1999;2:1-7.*

- *Bonenkamp JJ, Songun I, Hermans J, van de Velde CJ. Prognostic value of positive cytology findings from abdominal washings in patients with gastric cancer. Br J Surg 1996;83:672-4.*
- *Burchill SA, Bradbury MF, Pittman K, Southgate J, Smith B, Selby P. Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. Br J Cancer 1995;71:278-81.*
- *Cetin B, Atalay C, Aslan S, Babacan B, Hatipoglu C, Akinci M et al. Peritoneal carcinoembryonic antigen level for predicting locoregional and distant spread of gastric cancer. Surg Today 2005;35:919-24.*
- *Chu DZ, Lang NP, Thompson C, Osteen PK and Westbrook KC (1989) Peritoneal carcinomatosis in nongynecologic malignancy. A prospective study of prognostic factor. Cancer 63, 364-367.*
- *Fujimura T, Ohta T, Kitagawa H, Mori T, Ishii T, Niwa H et al. Trypsinogen expression and early detection for peritoneal dissemination in gastric cancer. J Surg Oncol 1998;69:71-5.*
- *Fujiwara Y, Doki Y, Taniguchi H, Sohma I, Takiguchi S, Miyata H et al. Genetic detection of free cancer cells in the peritoneal cavity of the patient with gastric cancer: present status and future perspectives. Gastric Cancer 2007;10:197-204.*
- *Goeminne JC, Guillaume T, Salmon M, Machiels JP, D'Hondt V, Symann M. Unreliability of carcinoembryonic antigen (CEA) reverse transcriptase-polymerase chain reaction (RT-PCR) in detecting contaminating breast cancer cells in peripheral blood stem cells due to induction of CEA by growth factors. Bone Marrow Transplant 1999;24:769-75.*
- *Guller U, Zajac P, Schnider A, et al. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. Ann Surg. 2002; 236:768-75.*
- *Gunderson LL and Sosin H (1982) Adenocarcinoma of the stomach: Areas of failure in a reoperation series (second or symptomatic look), clinico-*

pathologic correlation and implications for adjuvant therapy. Int J Radiat Biol Phys 8, 1-11.

- *Hagiwara A, Takahashi T, Kojima O, et al. Prophylaxis with carbon adsorbed mitomycin against peritoneal recurrence of gastric cancer. Lancet 1992;339:629-631.*
- *Hara M, Nakanishi H, Jun Q, et al. Comparative analysis of intraperitoneal minimal free cancer cells between colorectal and gastric cancer patients using quantitative RT-PCR: possible reason for rare peritoneal recurrence in colorectal cancer. Clin. Exp. Metastasis. 2007; 24:179-89.*
- *Hayes N, Wayman J, Wadehra V, Schott DJ, Raimes SA, Griffin SM,. Peritoneal cytology in the surgical evaluation of gastric carcinoma. Br. J Cancer 1999;79:520-4*
- *Homma Y, Ushida S, Yamada M, Kobayashi H, Suzuki K. Positive peritoneal washing cytology in multiple cavities can predict poor prognosis of advanced gastric cancer patients. Ann Surg Oncol 2010;17:455-60.*
- *Japanese Research Society for Gastric Cancer. Japanese classification of gastric carcinoma. 2nd English ed. Gastric Cancer 1998;1:11-24.*
- *Jayne DG, Fook S, Loi C and Seow-Choen F (2002) Peritoneal carcinomatosis from colorectal cancer. Br J Surg 89, 1545-1550.*
- *Juhl H, Stritzel M, Wroblewski A, Henne-Bruns D, et al. Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow of gastric, colorectal and pancreatic cancer patients. Int J Cancer 1994; 57: 330-335.*
- *Katsuragi K, Yashiro M, Sawada T, Osaka H, Ohira M, Hirakawa K. Prognostic impact of PCR-based identification of isolated tumour cells in the peritoneal lavage fluid of gastric cancer patients who underwent a curative R0 resection. Br J Cancer. 2007; 97:550-6.*
- *Khair G., Monson. J.R., Greenman J. Epithelial molecular markers in the peripheral blood of patients with colorectal cancer. Dis. Colon Rectum 50 2007;1188-1203.*

- *Kodera Y, Nakanishi H, Ito S, et al. Quantitative detection of disseminated free cancer cells in peritoneal washes with real-time reverse transcriptase-polymerase chain reaction: a sensitive predictor of outcome for patients with gastric carcinoma. Ann Surg 2002; 235: 499-506.*
- *Kodera Y, Nakanishi H, Ito S, Mochizuki Y, Ohashi N, Yamamura Y et al. Prognostic significance of intraperitoneal cancer cells in gastric carcinoma: analysis of real time reverse transcriptase-polymerase chain reaction after 5 years of followup. J Am Coll Surg 2006;202:231-6.*
- *Kodera Y, Nakanishi H, Yamamura Y, et al. Prognostic value and clinical implications of disseminated cancer cells in the peritoneal cavity detected by reverse transcriptase-polymerase chain reaction and cytology. Int J Cancer. 1998;79:429-33.*
- *Kodera Y, Yamamura Y, Shimizu Y, et al. Peritoneal washing cytology: prognostic value of positive findings in patients with gastric carcinoma undergoing a potentially curative resection. J Surg Oncol 1999; 72: 60-64; discussion 64-65.*
- *Koga S, Hamazoe R, Maeta M, Shimizu N, Murakami A and Wakatsuki T (1988) Prophylactic therapy for peritoneal recurrence of gastric cancer by continuous hyperthermic peritoneal perfusion with mitomycin C. Cancer 61, 232-237.*
- *Koga S, Kaibara N, Iitsuka Y, Kudo H, Kimura A, Hiraoka H. Prognostic significance of intraperitoneal free cancer cells in gastric cancer patients. J Cancer Res Clin Oncol 1984;108:236-8.*
- *Kowalewska M, Chechlicska M, Markowicz S, Kober P, Nowak R. The relevance of RT-PCR detection of disseminated tumour cells is hampered by the expression of markers regarded as tumour-specific in activated lymphocytes. Eur J Cancer 2006;42:2671-4.*
- *Kowalewska M, Chechlinska M, Nowak R. Carcinoembryonic antigen and cytokeratin 20 in peritoneal cells of cancer patients: are we aware of what we are detecting by mRNA examination?. Br J Cancer. 2008;98:512-3.*

- *La Torre M, Ferri M, Giovagnoli MR, Sforza N, Cosenza G, Giarnieri E, Ziparo V. Peritoneal wash cytology in gastric carcinoma. Prognostic significance and therapeutic consequences. Eur J Surg Oncol. 2010; 36:982-6.*
- *Landry J, Tepper JE, Wood WC, Moulton EO, Koerner F and Sullinger J (1990) Patterns of failure following curative resection of gastric carcinoma. Int J Radiat Biol Phys 19, 1357-1362.*
- *Li JK, Zheng M, Miao CW Zhang JH, Ding GH, Wu WS. Peritoneal lavage cytology and carcinoembryonic antigen determination in predicting peritoneal metastasis and prognosis of gastric cancer. World J Gastroenterol 2005;11:7374-7.*
- *Moertel CG, Rleming TR, MacDonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 1990;322:352-358.*
- *Mori N, Oka M, Hazama S, Iizuka N, Yamamoto K, Yoshino S et al. Detection of telomerase activity in peritoneal lavage fluid from patients with gastric cancer using immunomagnetic beads. Br J Cancer 2000;83:1026-32.*
- *Mori T, Fujiwara Y, Sugita Y, Azama T, Ishii T, Taniguchi K et al. Application of molecular diagnosis for detection of peritoneal micrometastasis and evaluation of preoperative chemotherapy in advanced gastric carcinoma. Ann Surg Oncol 2004;11:14-20.*
- *Nakanishi H, Kodera Y, Torii A, Hirai T, Yamamura Y, Kato T et al. Detection of carcinoembryonic antigen-expressing free tumor cells in peritoneal washes from patients with gastric carcinoma by polymerase chain reaction. Jpn J Cancer Res 1997;88:687-92.*
- *Nekarda H, Gess C, Stark M, Mueller JD, Fink U, Schenck U et al. Immunocytochemically detected free peritoneal tumour cells (FPTC) are a strong prognostic factor in gastric carcinoma. Br J Cancer 1999;79:611-9.*

- *Noura S, Ohue M, Seki Y, Yano M, Ishikawa O, Kameyama M. Long-term prognostic value of conventional peritoneal lavage cytology in patients undergoing curative colorectal cancer resection. Dis Colon Rectum. 2009;52:1312-20.*
- *Oyama K, Terashima M, Takagane A, Maesawa C. Prognostic significance of peritoneal minimal residual disease in gastric cancer detected by reverse transcription-polymerase chain reaction. Br J Surg 2004; 19: 435-43.*
- *Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer. 2008 May;8(5):329-40.*
- *Preusser P, Wilke H, Achterrath W, Fink U, Lenaz L, Heinicke A, Meyer J, Meyer HJ and Buente H (1989) Phase II study with the combination of etoposide, doxorubicin and cisplatin in advanced measurable gastric cancer. J Clin Oncol 7, 1310-1317.*
- *Ribeiro U Jr, Gama-Rodrigues JJ, Bitelman B, Ibrahim RE, Safatle-Ribeiro AV, Laudanna AA et al. Value of peritoneal lavage cytology during laparoscopic staging of patients with gastric carcinoma. Surg Laparosc Endosc 1998;8:132-5.*
- *Sadeghi B, Arvieux C, Glehen O, Beaujard AC, Rivoire M, Baulieux J, Fontaumard E, Brachet A, Caillot JL, Faure JL, Porcheron J, Peix JL, Francois Y, Vignal J and Gilly FN (2000) Peritoneal carcinomatosis from non-gynecologic malignancies: results of the EVOCAPE 1 multicentric prospective study. Cancer 88, 358-363.*
- *Sakakura C, Takemura M, Hagiwara A, Shimomura K, Miyagawa K, Nakashima S et al. Overexpression of dopa decarboxylase in peritoneal dissemination of gastric cancer and its potential as a novel marker for the detection of peritoneal micrometastases with real-time RT-PCR. Br J Cancer 2004 9;90:665-71.*

- *Schoenfeld A, Luqmani Y, Smith D, O'Reilly S, Shousha S, Sinnett HD et al. Detection of breast cancer micrometastases in axillary lymph nodes by using polymerase chain reaction. Cancer Res 1994 1;54:2986-90.*
- *Schott A, Vogel I, Krueger U, et al. Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker. Ann. Surg. 1998;. 227:372-9.*
- *Shimomura K, Sakakura C, Takemura M, Takagi T, Fukuda K, Kin S et al. Combination of L-3-phosphoserine phosphatase and CEA using real-time RT-PCR improves accuracy in detection of peritoneal micrometastasis of gastric cancer. Anticancer Res 2004;24(2C):1113-20.*
- *Sugarbaker PH (2004) Carcinomatosis from gastrointestinal cancer. Ann Med 36, 9-22.*
- *Sugarbaker PH, Graves T, DeBruijn EA, Cunliffe WJ, Mullins RE, Hull WE, Oliff L and Schlag P (1990) Early postoperative intraperitoneal chemotherapy as an adjuvant therapy to surgery for peritoneal carcinomatosis from gastrointestinal cancer: pharmacological studies. Cancer Res 50, 5790-5794.*
- *Sugita Y, Fujiwara Y, Taniguchi H, et al. Quantitative molecular diagnosis of peritoneal lavage fluid for prediction of peritoneal recurrence in gastric cancer. Int J Oncol 2003;23:1419-23.*
- *Suzuki T, Ochiai T, Hayashi H, Hori S, Shimada H, Isono K. Peritoneal lavage cytology findings as prognostic factor for gastric cancer. Semin Surg Oncol 1999;17:103-7.*
- *Vogel P, Rüschoff J, Kümmel S, Zirngibl H, Hofstädter F, Hohenberger W et al. Immunocytology improves prognostic impact of peritoneal tumour cell detection compared to conventional cytology in gastric cancer. Eur J Surg Oncol 1999;25:515-9.*
- *Wang JY, Lin SR, Lu CY, Chen CC, Wu DC, Chai CY et al. Gastric cancer cell detection in peritoneal lavage: RT-PCR for carcinoembryonic antigen*

transcripts versus the combined cytology with peritoneal carcinoembryonic antigen levels. Cancer Lett 2005;223:129-35

- *Wang Z, Zhang X, Xu H, Zhou X, Jiang L, Lu C. Detection of peritoneal micrometastasis by reverse transcriptase-polymerase chain reaction for heparanase mRNA and cytology in peritoneal wash samples. J Surg Oncol 2005;90:59-65.*
- *Wind P, Norklinger B, Roger V, Kahlil A, Guin E, Parc R. Long-term prognostic value of positive peritoneal washing in colon cancer. Scand. J. Gastroenterol. 1999; 34:606-10.*
- *Wisbeck WM, Beecher EM and Russell AH (1986) Adenocarcinoma of the stomach: Autopsy observations with therapeutic implications for the radiation oncologist. Radiother Oncol 7, 13-18.*
- *Wu CC, Chen JT, Chang MC, et al. Optimal surgical strategy for potentially curable serosa-involved gastric carcinoma with intraperitoneal free cancer cells. J Am Coll Surg 1997; 184: 611-617.*
- *Yamamoto M, Baba H, Kakeji Y, Endo K, Ikeda Y, Toh Y et al. Prognostic significance of tumor markers in peritoneal lavage in advanced gastric cancer. Oncology 2004;67:19-26.*
- *Yonemura Y, Fujimura T, Ninomiya I, Kim BS, Bandou E, Sawa T et al. Prediction of peritoneal micrometastasis by peritoneal lavaged cytology and reverse transcriptase-polymerase chain reaction for matrix metalloproteinase-7 mRNA. Clin Cancer Res 2001;7:1647-53.*
- *Yoo CH, Noh SH, Shin DW, Choi SH and Min JS (2000) Recurrence following curative resection for gastric carcinoma. Br J Surg 87, 236-242.*