sFAS/sFASL AND sMMP-7: NEW SOLUBLE MARKERS OF PROGNOSIS AND RESPONSE TO THERAPY IN ADVANCED COLORECTAL CANCER

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Memoria de la tesi presentada per obtenir el grau de Doctor en Medicina per la Universitat de Barcelona

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Tutor: Dr. Pere Gascon Vilaplana

A la meva familia,
als meus amics,
als pacients

“Innumerable challenges remain, the understanding of which will be essential to move from generic treatment strategies to principles of rational treatment selection driven by prognostic and predictive human biologic data.”

(Benson AB, 2006)

El Dr Joan Maurel Santasusana, metge oncòleg, i el Dr Pere Gascon Vilaplana, Cap de Servei d´Oncologia Mèdica, ambdós de l´Hospital Clínic de Barcelona,

FAN CONSTAR:

Que el treball clínic, experimental I la redacció de la memoria de la tesi titulada **"sFAS/sFASL AND sMMP-7: NEW SOLUBLE MARKERS OF PROGNOSIS AND RESPONSE TO THERAPY IN ADVANCED COLORECTAL CANCER"** han sigut realitzats per Cristina Nadal Sanmartin i consideren que és apta per el tràmit de la lectura i defensa pública davant d´un tribunal, per optar al grau de Doctor en Medicina per la Universitat de Barcelona.

I per tal que en quedi constància, signen aquest document a Barcelona, 9 de Maig de 2007.

Dr. Joan Maurel Santasusana Dr. Pere Gascon Vilaplana

LIST OF ABREVIATIONS

CRC	Colorectal Cancer
ACRC	Advanced Colorectal Cancer
PS	Performance Status
ALP	Alkalyne Phosphatase
LDH	Lactate Dehydrogenase
WBC	White blood cell
CEA	Carcinoembryonic antigen
OXL	Oxaliplatin
IRI	Irinotecan
5FU	5-Fluoruracil
LM	Levamisole
LV	Leucovorin
UFT	Tegafur/Uracil
CAP	Capecitabine
FOLFOX	Oxaliplatin + 5FU + Leucovorin
FOLFIRI	Irinotecan + 5FU + Leucovorin
FOLFOXIRI	Oxaliplatin + Irinotecan + 5FU + Leucovorin
i.v.	Intravenous Infusion
BSC	Best Supportive Care
US	Ultrasonography
UNL	Upper Limit of Normal
CT	Computarized Tomography
LOH	Loss of heterozygosity
TS	Thymidylate synthase
TP	Thymidine phosphorylase
DPD	Dihydropyrimidine dehydrogenase
sFAS	Soluble FAS
sFASL	Soluble FAS Ligand

MMP-7	Matrix metalloproteinase 7
EGFR	Epidermal growth factor receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
IGFR	Insulin growth factor receptor
IGFBP-3	Insulin growth factor binding protein-3
CTLs	Cytotoxic T lymphocytes
HB-EGF	Heparin binding epidermal growth factor

CONTENTS

1 INTRODUCTION

1.1 ACRC: DEFINITION AND EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with a worldwide incidence of almost a million cases annually[1] (see FIG.1).

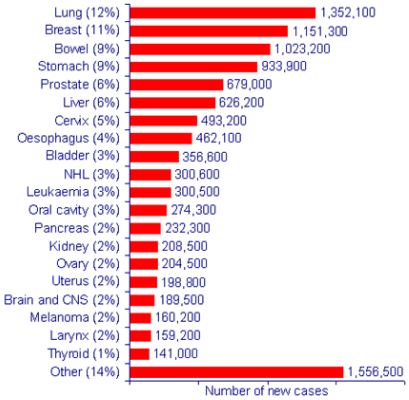


FIG. 1: Cancer incidence worldwide in 2005. www.cancerresearchuk.org

Conceptually, advanced colorectal cancer (ACRC) means that the disease is disseminated or shows metastasis to one or more organs. Despite of advances in screening, 11 to 27% of CRC patients show initially ACRC[2] and up to 25-50% of initially localized CRC patients are destined to metastasize during time[3-6].

Being metastasis the first cause of cancer death, it is understandable that ACRC stands worldwide as the fourth cause of cancer related deaths, just behind lung, stomach and liver. In western countries, ACRC is the third cause of cancer related deaths in both genders (see FIG.2).

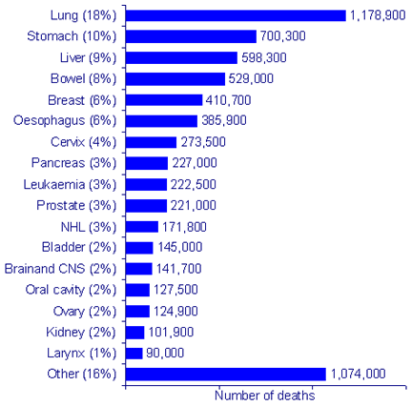


FIG. 2: Mortality related to cancer in 2005. www.cancerresearchuk.org

Over the past decade, CRC incidence and mortality rates have modestly decreased. Incidence decrement could be explained due to early detection, while mortality should be due to improvement in ACRC management, including strategies and new therapies (see FIG.3).

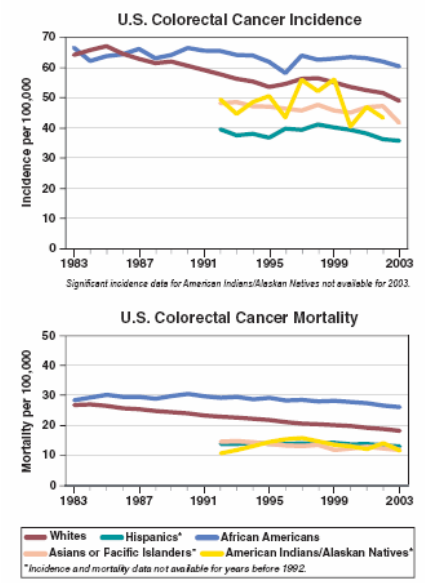


FIG. 3: U.S. CRC incidence and mortality. Source for incidence and mortality data: Surveillance, Epidemiology, and End Results (SEER) Program and the National Center for Health Statistics. Additional statistics and charts are available at http://seer.cancer.gov/.

1.2 ACRC: METASTATIC PATTERNS

Metastatic disease in CRC results from hematogenous, lymphatic and/or intracavitary dissemination of tumor cells. Dissemination can be explained by different ways: (1) spreading of tumor cells to lymph nodes via lymphatic system, (2) seeding through the peritoneal cavity and (3) spreading of tumor cells into venous blood leading to systemic hematogenic tumor cell dissemination[7]. Among patients with ACRC, the most frequent sites of metastases, based on different postmortem series, are: liver (36-81%), lungs (12-54%), pelvis (27-38%), regional nodes (25-59%), peritoneum (17-41%), adrenals (3-27%), small bowel (1-13%), bone (1-18%), thyroid (1-16%), pancreas (1-10%), ovary (1-18%) and brain (1-8%)[8-11] (see FIG. 4)

TABLE 2. *Incidence of Metastases in Colorectal Cancer. Postmortem Findings (by Per Cent)*

	Present Series	Abrams and Lerner[1]	Buirge[6]	Berge et al.[4]	Shindo[28]	Brown[5]	Willis[36]	Cedermark et al.[9]	Mayo and Schlicke[20]	Bacon and Gilbert[2]
Liver	60	65	57	71	80	81	52	48	36	40
Lungs	43	46	15	54	43	54	12	38	16	15
Pelvis	27	NA	NA	NA	38	NA	NA	NA	NA	NA
Regional nodes	55	59	42	"nodes" 77	NA	NA	25	39	NA	31
Peritoneum	32	33	20	41	27	NA	17	NA	20	19
Adrenals	18	17	3	16	NA	27	5	14	7	NA
Small bowel	13	NA	NA	1	NA	NA	NA	NA	12	NA
Bone	10	13	1	14	10	18	2	NA	2	NA
Thyroid	5	1	1	NA	16	6	2	NA	NA	1
Pancreas	10	4	3	2	NA	4	NA	NA	4	4
Ovary	3	12	2	6	NA	3	3	18	7	1
Brain	6	NA	2	7	2	1	2	8	5	0.3

FIG. 4: Incidence and site distribution of metastases in CRC[10]

Metastatic disease can be detected at the same time of primary CRC tumor. In this case we talk about synchronous disease. When metastatic lesions are detected after CRC resection, during the follow-up or surveillance period, we talk about methacronous disease. ACRC includes CRC patients having metastases in one or more organs and patients having minimal metastatic spreading to bulky disease. Disease growth kinetics can vary largely among ACRC. Taking into account all these parameters and combinations, ACRC shows up as a really heterogeneous disease.

ACRC metastatic pattern can be defined as the disease phenotype. It results from tumor-host interaction and reflects tumor biology. Different metastatic

patterns can be presented at ACRC disease onset. Interestingly, metastatic patterns change during disease evolution.

1.3 ACRC: CURRENT DETERMINANTS OF PROGNOSIS AND RESPONSE TO THERAPY

In general, ACRC has a grim prognosis. Median overall survival in all ACRC patients, just receiving best supportive care (BSC), stands about 5 to 6 months[12-14]. Fortunately, especially during the last decade, new therapies and management strategies seem to have raised this media up to 15 to 18 months[15-17].

ACRC disease outcome depends on different variables, as aggressiveness and sensitivity-resistance to administered therapies. Metastatic pattern somehow reflects disease aggressiveness. While long term metachronic ACRC affecting liver, with minimal disease, can even go for curation, synchronous, multi-organ, bulky ACRC has a dismal outcome. Despite of that, patients with similar patterns can respond completely different to administered therapies. ACRC can be instrinsically sensitive or resistant to current therapies. First-line therapy aproaches gives up to 50% of responses, meaning that 50% of tumors show intrinsic resistance. Strickingly, almost all initially sensitive patients become resistant in less than 12 months.

How to recognize which type of ACRC we are facing up? How do we know the way each ACRC is going to behave in terms of aggressiveness and response to therapy?

As defined by the National Cancer Institute (NCI) Dictionary, a prognostic factor is a situation, condition, or a characteristic of a patient, that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring. A predictive factor is a situation or condition that may increase a person's risk of developing a certain disease or disorder. Talking about response

to therapy, it would be a situation or condition that may increase a person's chance of responding to a certain administered therapy.

In the lattest years, clinicians working in the ACRC field have intended to find determinants of prognosis and predictors of response in order to classify ACRC patients. Despite years of research and hundred reports on tumor marker, the number of determinants that have emerged as clinically useful is unexpectably small. There migth be different explanations for this situation. Altman and Riley[18] suggested that there is an evident publication bias, but also inadequate reporting and an excess of retrospective studies. Studies suffer from general methodologic differences, poor design, assays that are not standarized or lack of reproductibility, and inappropiate or misleading statistical analyses that are often based on samples that are too small to draw meaningful conclusions. This condition often causes that, initially reported studies of a marker show great promise but subsequent studies on the same or related markers yield inconsistent conclusions or stand in direct contradiction to the promising results. To improve this situation, Guidelines for **Re**porting of Tumor **Mark**er Studies (REMARK) have been developed by NCI[19]. Strategies such as clear hypothesis and end-points defininition, sample size calculation in accordance to hypothesis, establishment of variable cut-off according to its distribution and mutivariate analysis with other variables are recommended to be implemented. Homogenizing studies will help to achieve robust conclusions. Another explanation is the lack of an evidence-based approach to prognostic and predictive markers. Available studies barely provide acceptable levels of evidence. The majority of studies define prognostic and predictive factors retrospectively. Among them, few have already incorporated REMARK recommendations. A small amount of studies validate prognostic and predictive markers in a prospective manner. There is a surprinsing lack of studies randomizing ACRC patients to selected treatments according to predictive markers.

Until now, widely accepted prognostic factors in ACRC are performance status (PS), number of involved metastatic sites, serum alkaline phosphatase (ALP) levels, white blood counts (WBC) (these fourth conforming Köhne's prognostic indicators), liver involvement or peritoneal carcinomatosis, serum lactate dehydrogenase (LDH) and carcinoembryonic (CEA) levels[15-17, 20,21]. Köhne et al. analyzed retrospectively a serie of 2549 ACRC patients treated with 5FU. Patients could be divided into at least three risk groups, depending on four baseline clinical parameters: PS, WBC count, ALP and number of metastatic sites. LDH was not considered for this classification. Low risk group, with a median survival of 15 months, included patients with PS 0/1 and only one tumour site; Intermediate risk group, with a median survival of 10.7 months, integrated patients with PS 0/1 and more than one tumour site and ALP<300 U/l or patients with PS>1, WBC count <10 x 10^9/l and only one tumour site; High risk group, with a median survival of 6.1 months, accouonted for patients with PS 0/1 and more than one tumour site and ALP≥300 U/l or patients with PS >1 and more than one tumour site or WBC count >10 x 10^9/l. Authors validated the prognostic index in 1276 ACRC patients. The median survival times for the good, intermediate and high risk groups in the validation sample were 14.7, 10.5 and 6.4 months, respectively[20].

The utility of the above mentioned prognostic factors has never been prospectively validated in well-designed and powered clinical trials.

Currently, despite clinicians' intuition, there is quite a bit of controversy related to its value. There is also a general lack of implementation of these prognostic factors in clinical studies. The truth is that we still don't know if these clinical prognostic factors do properly classify ACRC patients.

Besides the mentioned clinical and biochemical variables, a number of biological and molecular characteristics (such as mutations of p53 and p21, K-*ras* mutation, chromosome 18q loss of heterozygosity (LOH), MSI-related germline mismatch repair gene mutations, and high expression of thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine

dehydrogenase (DPD), have been identified that may be of prognostic importance[22-28].

Also, some polymorphisms in thymidylate synthase, methylenetetrahydrofolate reductase, xeroderma pigmentosum group D (XPD), excision repair cross complementing group 1 (ERCC1), x-ray cross complementing group 1, x-ray cross complementing protein 3, uridine diphosphate glucoronosyltransferase (UGT1A1 *28) and glutathione S-transferases (GSTs) genes have been related to differences in progression free survival and thus outcome in ACRC patients[29-32]. Any of those prognostic factors has yet been validated in prospective clinical trials.

Some molecular factors have been shown to be good predictors of response to therapies in ACRC. High expression levels of tumor TS, either measuring proteins or mRNA levels, correlate with poor response to fluoropyrimidines[33-35]. Higher levels of TS were also found in abdominal metastases compared to liver, accounting for different responses to 5FU therapies usually seen[36]. Low levels of tumor TP and DPD, together with TS, have been shown to be independent predictors of response to fluoropyrimidines[28,37].

High Topoisomerase-1 gene expression has been suggested to predict for response to camptothecin (CPT-11) therapy[39,40]. High ERCC-1 gene expression levels, which are independent from TS expression levels, have shown to predict for response to Oxaliplatin. TS and ERCC-1 levels could be predictors of response to Oxaliplatin-5-FU based regimens in ACRC, as has been shown in gastric cancer[41].

Epidermal growth factor receptor (EGFR) staining intensity and percentage of expressing cells, which were initially believed to correlate to Cetuximab response, failed to produce any significative pattern[42].

Some polymorphisms in thymidylate synthase, methylenetetrahydrofolate reductase, xeroderma pigmentosum group D (XPD), excision repair cross complementing group 1 (ERCC1), x-ray cross complementing group 1, x-ray cross complementing protein 3, uridine diphosphate glucoronosyltransferase (UGT1A1 *28) and glutathione S-transferases (GSTs) genes have been linked

to responses to OXL/CPT-11/LV/5-FU in different studies[29-32]. These studies suggest that a pharmacogenetic approach may be an innovative strategy to establish predictive factors which might be of help in selecting ACRC patients for different therapies.

A prospective phase II study, in which the choice of first-line chemotherapy with either 5-FU or a non-5-FU containing regimen was based on TS and DPD expression, has been conducted and has not confirmed higher response rates, as reported in retrospective studies[38].

To summarize, even many of the above mentioned factors have been identified as possible predictors of response in ACRC patients, none of them has succeed in being validated in prospective clinical trials.

1.4 ACRC: MANAGEMENT

Ideally, ACRC management should be different according to the ACRC type of disease, meaning according to prognostic factors and factors predicting response to therapies.

Taking into account that there are no clearly validated prognostic/predictor factors, it is easy to guess that management is not optimized, and thus probably often not adequate. There is an increased need for re-define treatment strategies in patients with ACRC, according to prognostic and predictive factors.

1.4.1 Management Algorithm

According to our guidelines (2001), in our center ACRC management is based on disease metastatic pattern and prognostic factors at the time of disease onset. We had designed a management algorithm according to baseline metastatic patter and commonly used clinical prognostic factors and done estimations of overall survival rates. Roughly 25% of patients have favourable figures (only liver disease, PS 0,1 and LDH < upper limit of normal (ULN). Those patients, who could be classified as Early-Stage, are candidates for local, intended-to-cure treatments. Despite being ARCR, they would show overall survival rates

between 40 to 60% at 3 years. Additionally, 15% of patients have poor PS or are severe disabled due to geriatric syndromes or/and co-morbid diseases that preclude any active treatment strategies different that supportive care. Those could be classified as End-Stage and would have a dismal outcome, with less than 10% overall survival after one year. The rest of patients, classified as Intermediate-Stage, are usually treated with palliative chemotherapy. They would show 2-year overall survival rates around 20 to 40% (see FIG.5). All data comes out from preliminary analyses in our series of ACRC patients.

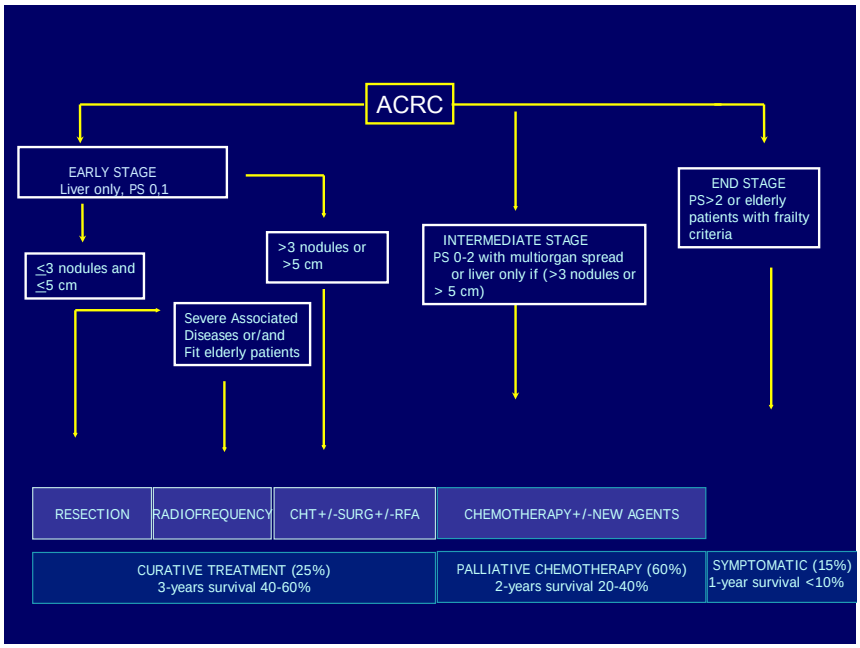


FIG. 5: Management algorithm in ACRC.

1.4.2 Staging Procedures

The indication to have an accurate staging depends on clinical needs. In patients diagnosed at End-stage (fragile patients or PS >2), ultrasonography (US) provide enough information and any other techniques are necessary. Abdominal spiral computarized tomography (CT) and chest x-ray would be

appropriate staging in patients with widely metastatic spread. The value of thoracic CT or/and positron emission tomography (PET) or PET-CT in patients with liver metastases to rule out extra-hepatic disease, is currently being evaluated.

1.4.3 Treatment

Local treatments (surgery and radiofrequency)

Surgery approaches are indicated for Early-stage patients showing good PS and resectability criteria. Approximately 20–30% of patients with metastatic colorectal cancer have disease that is confined to the liver and is potentially resectable[43]. Several recent large series on resection for CRC liver metastases have reported five year survival rates ranging from 25% to 44%, with peroperative mortality of 0–6.6%[44-47]. Ideally, margins of resection should be negative and surgery should include an intraoperative ultrasonograpy of the liver and discard peritoneal carcinomatosis. Despite resectable, elderly patients or patients with severe co-morbid associated diseases may not be good candidates for surgery[48]. Radiofrequency ablation (RFA) has been shown to be a safe and effective treatment for patients unsuitable for liver resection, especially due to bad PS[49,50]. However, its precise role in the management of hepatic colorectal metastases has yet to be defined and no studies have addressed its potential superiority over other treatment modalities in the setting of a randomized controlled trial. A recently published study confirms that patients with three or less nodules and less than 5 cm had better prognosis after RFA treatment[51]. Local recurrences rates vary depending on metastases size and follow-up duration. The RFA-related morbidity is less than 10%[48, 52-54].

Surgery or local treatments for metastatic lesions other than liver have also been performed. There is evidence from cohort studies with historical controls that survival can be improved by lung resection for technically suitable metastatic disease[55]. Long term survival has been reported for patients who undergo resection of pulmonary metastases when these have developed after

apparently curative resection of hepatic colorectal metastases[47]. Recent data suggest that if lung metastases of colorectal origin are resectable, five year survival following thoracotomy is similar to that observed in patients after resection of colorectal liver metastases[56, 57]. Long term survival following peritonectomy (with or without hyperthermia/ hyperthermia+ chemotherapy)[58] and resection of adrenal and splenic metastases have also been recorded[59,60]. As not reported better than systemic chemotherapy, these procedures should not be performed as a standard strategy.

Best supportive care

Palliation can be achieved without active treatment with systemic therapies, such as chemotherapy or biologic therapies. Best supportive care (BSC) consists on applying the best strategies and drugs for palliating sympthoms and improving quality of life. In patients with metastases to other organs than liver and bad PS, BSC should be indicated for symptomatic palliation[61].

Systemic treatments

Systemic therapies account for chemotherapies and biologic therapies, either administered per oral or endovenous. Different chemotherapies have been tried during the past two decades in order to obtain better responses in ACRC. For many years, until the early 1990s, intravenous (i.v.) bolus of 5-Fluoruracil (5-FU) stood as the standard treatment for ACRC, either alone or modulated by Levamisole (LM) or Leucovorin (LV) (high or low doses). During 1990s, continous infusion showed superiority in responses and survival. In the past two decades, the onset of new drugs has changed the scenario. Those drugs have become the new standards in ACRC treatment as, in combination with 5-FU/LV, they achieve high percentages of tumor reduction. Oxaliplatin (OXL) in combination with 5-FU/LV (FOLFOX) or Irinotecan (IRI)/5-FU/LV (FOLFIRI) have increased responses up to 40-50%. Irinotecan and oxaliplatin have also shown to be active in patients refractory to 5-FU/LV[15-17]. Oral fluoropyrimidines such as Capecitabine (CAP) and Tegafur/Uracil (UFT)/LV

seem to have a comparable activity to intravenous bolus 5-FU/LV. Currently, new drugs, specifically what is called biologic therapies are under development. Epidermal growth factor receptor (EGFR) inhibitors and vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) inhibitors, either alone or combined with standard chemotherapies are being tested to keep on improving these results. They hopefully might play a role in the future in the treatment of ACRC[62-66] (see FIG. 6).

1960s:	5-FU
1980s:	Modulated 5-FU
1990s:	Infusional 5-FU
	New cytotoxic agents: raltitrexed, irinotecan, oxaliplatin, oral fluoropyrimidines
2000:	Agents acting on novel targets
	Molecular markers

FIG. 6: Hallmarks in CRC therapy. (Van Cutsem and Verslype, ASCO Educational Book, 2002)

Systemic treatments are used in different settings: neoadjuvant, adjuvant and palliative. The main goal of neoadjuvant approaches is obtaining high percentage of responses. In ACRC, neoadjuvant treatments are used in initially non-resectable liver metastasis to increase resectability and thus curability. It should only be used in liver only disease and in patients with good PS. Even it is clear that this approach improves responses and resectability, we still don't have data about percentages of recurrence after radical surgery and moreover, there is a surprising lack of randomized studies showing disease-free and overall survival advantages[67,68].

In the ACRC subset, adjuvant approaches have their role after an intended-to-cure surgery treatment. Reviewing all randomized studies available in the literature, adjuvant chemotherapy after liver metastasis resection has not showed a significant improvement in disease-free and overall survival[69-71].

The goal of palliative therapies is to delay the onset of disease related symptoms. Increasing the asymptomatic period usually translates into an

increment of quality of life. Often, these therapies also improve overall survival. In patients having extrahepatic metastases and good PS, systemic therapies are the option to improve survival and achieve palliation.

Untreated patients with metastatic colorectal cancer have a median survival of 5 to 6 months. Randomized studies have shown that chemotherapy with bolus of 5FU for ACRC prolongs survival and maintains or improves quality of life[12-14]. Biomodulation of 5-FU with other drugs, including LM, methotrexate, and LV, have been investigated. LV administration, which increases the intracellular pool of reduced folate and stabilizes the FdUMP/TS complex, has been the most successful biomodulatory agent. Studies have shown that the addition of LV to bolus 5-FU improves response rates (23% *v* 11%) compared with single-agent 5-FU[72]. Several studies have shown a higher response rate and a lower toxicity for infusional 5-FU/LV regimens compared with bolus 5-FU/LV regimens[73-79]. Median survival time for infusional regimens has not shown to be longer compared with bolus regimens.

Recently, oxaliplatin (OXL)/5-FU/LV or irinotecan (IRI)/5-FU/LV have increased responses in first-line therapy up to 40-50% with median survival between 15-18 months. Still, 2-year overall survival remains less than 20%[15-17]. First-line combinations of 5-FU in continuous infusion and LV plus OXL (FOLFOX) or IRI (FOLFIRI and IFL) had higher activity in first-line therapy, when compared to 5-FU/LV. FOLFOX has been shown to be superior to IFL in terms of response and toxicity and therefore the later should not be probably considered as standard therapy in randomised trials with new drugs[80]. Combination of FOLFOX plus IRI (FOLFOXIRI) has recently shown better responses, progression-fee survival and overall survival than FOLFIRI alone in first-line therapy, but toxicity was also clearly increased in the experimental arm[81].

Capecitabine (CAP) and tegafur/uracil (UFT), both orally administered fluoropyrimidines, plus LV achieved at least equivalent efficacy compared with bolus FU/LV with the convenience of an oral administration and would be a logical approach for elderly patients[82-85]. New biologic drugs have proved their

activity in ACRC, alone or in combination with standard therapies, such as the antiangiogenic agents bevacizumab[86-92] or Vatalanib (or PTK787)[87,93-98] and the epidermal growth factor monoclonal antibodies inhibitors (Panitumumab[99,100] and Cetuximab[101-104]).

Some of them are currently being evaluated in first and/or second line therapy in combination with standard therapies. Some of the most promising phase III studies ongoing are CRYSTAL study (FOLFIRI+Cetuximab vs FOLFIRI alone in first-line thepapy), PACCE study (FOLFOX4+Bevacizumab+Panitumumab vs FOLFOX4+Bevacizumab in first-line therapy), Study 20050203 (FOLFOX+Panitumumab vs FOLFOX alone in first-line treatment), Study 20050181 (FOLFIRI+Panitumumab vs FOLFIRI alone in second-line treatment).

Until now, despite of increasing cost and toxicity, new agents have just offered improvements in progression-free survival, but not in overall survival. As suggested by Johnson KR last year in Lancet Oncology[213], differences of less than two months in progression-free survival probably do not impact in overall survival. Despite not focusing on overall survival, which is the main criticism, the majority of the available large randomised trials with new agents have important differences between inclusion and exclusion criteria (including confounding factors, as Early Stage patients) and stratification criteria (see FIG) thus being difficult to conclude if benefits are due to drug activity or differences in trial design and patient selection (see FIG.7).

Trial	N	Excluding Criteria	Stratified Criteria	End-point
FUFOX/FOLFOX+/- Bevacizumab	1400	PS 2 and ALP>5	PS and Region	PFS
FOLFOX+/- PTK 787	1168	-	PS and LDH	PFS

FOLFIRI+/- Cetuximab	1200	-	PS and Region	PFS
FOLFOX+/- Panitumumab	900	-	PS and Region	PFS

FIG.7: Exclusion and stratification criteria can lead to misleading results in ongoing clinical trials with biologic compounds in ACRC.

1.5 ACRC: NEED FOR NEW BIOLOGICAL MARKERS OF PROGNOSIS AND RESPONSE TO THERAPY?

As noted above, currently "believed-to-be" prognostic and predictive markers in ACRC are not clearly defined, as they have not been prospectively validated in well-designed and powered clinical trials. This situation perpetuates the lack of implementation of prognostic and predictive factors in clinical studies as well as in daily clinical practice. It is clear that we are loosing the chance of selecting ACRC patients for optimizing treatment.

This scenario points to different needs. First of all, currently supposed markers of prognosis and response to therapy should be prospectly validated, in order to do one step ahead in classifying ACRC patients. Secondly, once validated, clinicians should implement them in both clinical trials and routine practice. Third, there is a need of finding markers accurately reflecting ACRC aggressiveness and response to therapy, which means markers with prognostic and predictive value. But, as tumors change over time, aggressiveness and resistance to therapy can also vary. So, those markers should be able to reflect those values anytime during disease evolution. This fact implies that markers should be easy to obtain, through a non-invasive technique, such as venopuncture.

In conclusion, there is a need of prospectively validating markers to determine ACRC behavior, in terms of aggressiveness and sensitivity/resistance to

therapies, and thus to classify ACRC patients, but also there is a need of finding novel markers accurately fulfilling this role anytime during disease history.

1.6 NEW MOLECULAR MARKERS

1.6.1 MMP-7

Matrylisin or matrix-metalloproteinase 7 (MMP-7) (FIG.9) is a proteolytic enzyme belonging to Matrix Metalloproteinase (MMPs) family[105-107] (FIG.8).

MMP subfamily	MMP number	MMP name
Collagenases	1	Interstitial collagenase
	8	Neutrophil collagenase
	13	Collagenase-3
Gelatinases	2	72 kDa Type IV gelatinase
	9	92 kDa Type IV gelatinase
Stromelysins	3	Stromelysin-1
	10	Stromelysin-2
	11	Stromelysin-3
	18	Putative MMP, similar to stromelysins
Membrane-type MMPs	14	MT1-MMP
	15	MT2-MMP
	16	MT3-MMP
	17	MT4-MMP
	24	MT5-MMP
	25	MT6-MMP
Other MMPs	7	Matrilysin (PUMP-1)
	12	Macrophage elastase
	19	Rheumatoid arthritis-associated MMP
	20	Enamelysin
	21	Recently cloned MMP
	22	Recently cloned MMP
	23	Recently cloned MMP

FIG.8: Metalloproteinase family is composed by different subtypes.

It is constitutively expressed in the ductal and glandular epithelium of many tissues[108]. In the lung and intestine it plays a role activating antibacterial peptides such as prodefensins[109].

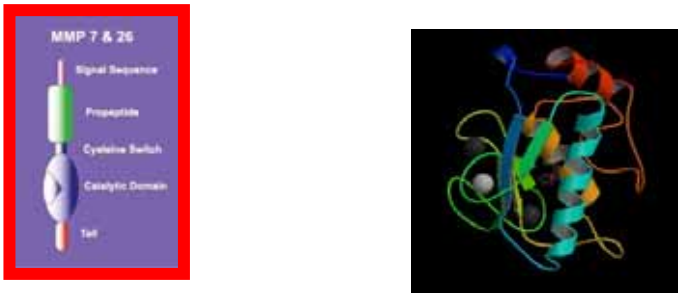


FIG.9: Metalloproteinase-7: domains and tridimensional structure.

MMP-7 is synthesized and secreted by tumor epithelial cells as a 28-KDa proenzyme, that can be activated through proteolytic removal of a 9-Kda prodomain from the N-terminus. Soluble activated form binds to the tumor epithelial cell surface. Both active forms, soluble and membrane-bounded, have proteolytic activity. Its expression is regulated by transcription factors such as AP-1, PEA3 and β-catenin/ tcf4 complex[110-112]. EGFR activation has also been related to MMP-7 expression and activation[113]. By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2, MMP-9), through ectodomain shedding of E-cadherin[114] and receptor activator of nuclear factor-kappa B ligand (RANKL[115] or through cleavage of adhesion molecules, such as integrin β4[116]). MMP-7 is able to induce cell apoptotic impairment. It specifically cleaves critical proteins implicated in the extrinsic apoptotic pathway, such as FAS Ligand (FASL)[117, 118] and Tumor Necrosis Factor-alpha (TNF-α)[119]. FasL shedding is related to the acquisition of an apoptosis resistance phenotype[120].

Additionally, MMP-7 induces cell proliferation through cleavage of Heparin Binding Epidermal Growth Factor (HB-EGF) precursor[121], a Disintegrin and Metalloproteinase family (ADAM) member, ADAM28[122] and degradation of all six Insulin Growth Factor Binding Proteins (IGFBP-1 to -6), increasing the bioavailability of IGF, and thus favoring cancer cell growth and survival[123, 124]. Matrilysin can regulate angiogenesis either inducing a direct proliferative effect on vascular endothelial cells[125] or producing angiogenesis inhibitors

(angiostatin, endostatin, neostatin-7)[126] or enriching the variety of angiogenesis mediators, such as the soluble vascular endothelial growth factor (sVEGF)[127]. Immunoevasion due to MMP-7 would be related to FasL cleavage[117, 118] or to IgG degradation[128].

1.6.2 FAS/FASL

FAS receptor (FAS, CD95) and FAS ligand (FASL, CD95L) are cell surface proteins belonging to tumor necrosis factor (TNF) family. Apoptotic cell death response is triggered upon FASL and FAS binding. FAS/FASL interaction causes FAS receptor homo-oligomerization, recruitment of FAS-associated death domain (FADD) and procaspase-8 proteins, forming what is known as the death-inducing signalling complex (DISC). Procaspase-8 is activated at the DISC and in turn activates the downstream apoptotic extrinsic pathway effector caspase, procaspase-3, leading to the cleavage of structural proteins and causing apoptotic cell death. Caspase-8 can also activate the mitochondrial cell death pathway, in type II cells, through cleaving bcl-2 family members such as Bid[129, 130] (see FIG. 10). A FASL-independent FAS-dependant caspase activation, related to FAS oligomerization domain, has also been described[131].

Soluble forms of FAS and FASL have been found. Soluble FAS (sFAS) comes in various different forms due to alternative splicing phenomena[132, 133]. The majority of these spliced forms maintain the oligodimerization domain which allows them to form homotrimers (between soluble forms) and heterotrimers (when joining transmembrane FAS receptors). A dual either proapoptotic or antiapoptotic role has been attributed to these soluble forms. When forming heterotrimers, they counteract the apoptotic signal[134-136]. While forming homotrimers they are capable of interacting with transmembrane FASL leading to a proapoptotic effect[137]. More convincing data comes from soluble FASL (sFASL) with a dominant antiapoptotic function, resulting from its cleavage by matrix metalloprotease-7 (MMP-7)[138, 139] against a marginal proapoptotic effect[140, 141].

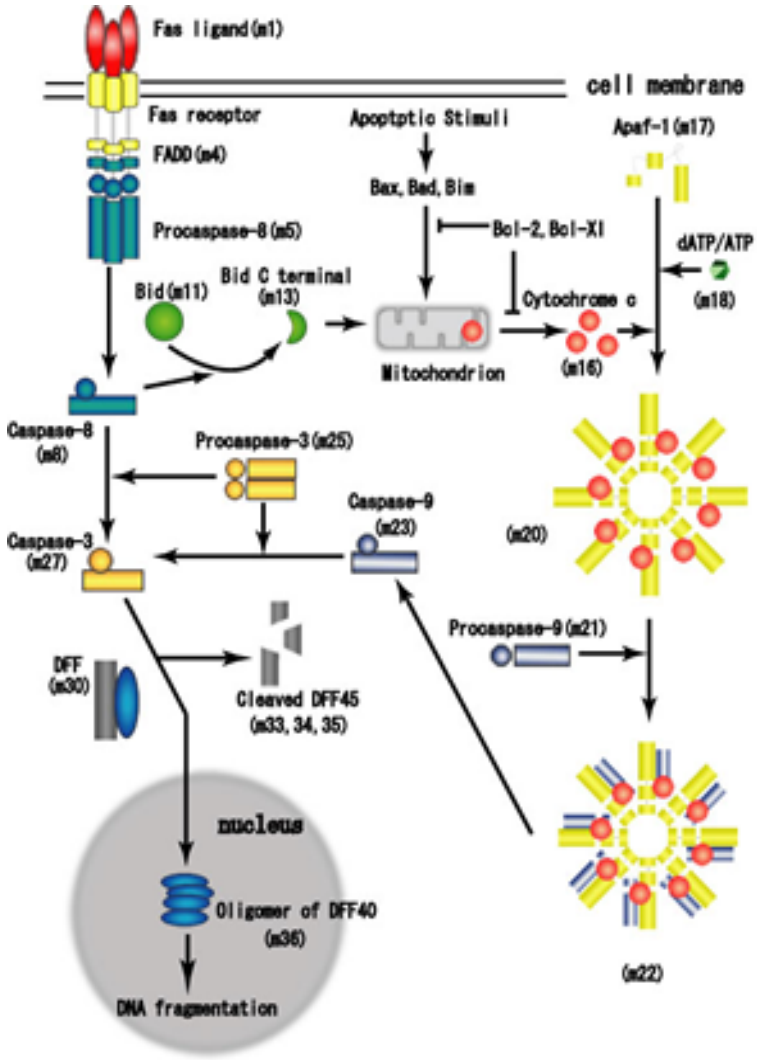


FIG. 10: Diagram describing extrinsic and intrinsic apoptotic pathways.

1.7 ACRC: TUMORIGENIC PROCESS

CRC is a genetically heterogeneous and complex disease. Initially, two major pathways were described as responsible for CRC tumorigenic process: the chromosomal instability pathway and the microsatellite instability pathway. The chromosomal instability or classical pathway accounted for 85% of the tumorigenic processes and was mainly characterized by the sequential allelic losses on chromosomes 5q (APC gene), 17p (TP53) and 18q (DCC/Smad4). The microsatellite instability pathway (MNI), also called the mutator phenotype, only accounted for 15% of the carcinogenic processes. Recently, it has been shown that colorectal carcinogenesis is much more complex, involving new pathways, as the serrated, the TGFβ/Smad and epigenetic pathways, and including infinity of non-pure or mixed pathways[142-144]. General mechanisms of tumorogenesis also include metastasis generation or metastagenesis[145] **(Nadal et al, WJG, 2007, in press)**. Different tumorigenic processes give rise to biologically diverse types of ACRC. Each type has differences in aggressiveness and sensitivity/resistance to treatments.

1.7.1 MMP-7

Matrilysin (MMP-7) has been found overexpressed in a variety of tumors, such as colorectal cancer[146] (see FIG. 11).

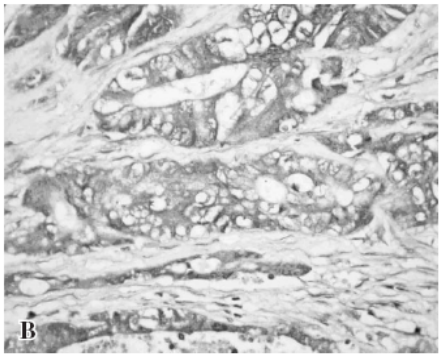


FIG. 11: Immunohistochemical staining for matrilysin in human CRC samples. Staining occurs in the cytoplasm and membrane of tumor cells[147.] (400x)

MMP-7 overexpression is thought to be an early event in the adenoma-carcinoma pathway. MMP-7 is regulated by APC/β-catenin pathway, which is often disrupted, as an early step during CRC tumorigenic process[148-150] (see FIG. 12). It can be also regulated by Ki-Ras oncogenic activation[151].

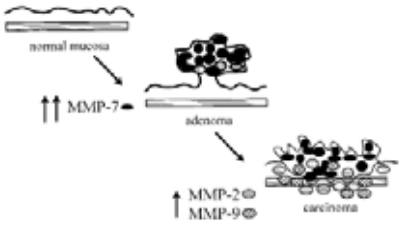


Table 2. MMP EXPRESSION BY TISSUE TYPE

Factor	Normal Mucosa (n = 40)	Adenoma (n = 11)	Carcinoma (n = 34)
MMP-2	3,435 ± 382	6,138 ± 1,310	7,600 ± 1,200*
MMP-7	47 ± 25	2,016 ± 610*	2,400 ± 917*
MMP-9	192 ± 54	265 ± 64	348 ± 50*

MMP, matrix metalloproteinase.
Data are copies per nanogram RNA β-actin.
* $P < .05$, Wilcoxon signed-ranks, vs. normal mucosa.

FIG. 12: Matrilysin overexpression is an early event in CRC carcinogenesis[150].

There is substantial evidence that overexpression of MMP-7 in primary CRC, taking into account the measurement of both activated and pro-forms, is related to a more aggressive phenotype of tumor cells and a poorer prognosis. MMP-7 overexpression has been correlated with invasion and to liver metastasis formation in non-metastatic CRC[152-157]. MMP-7 is also overexpressed in CRC liver metastases compared to normal liver[155]. MMP-7 ability to cleave FASL has been related to chemorresistance in CRC cell lines[138]. Its ability to release EGFligand cleaving HB-EGF increases EGFR pathway activation[158]. MMP-7 progressive increment could be related to acquision of resistance in CRC.

1.7.2 FAS/FASL

The extrinsec apoptotic pathway seems to be physiologically compromised during colorectal cancer progresion. FAS/FASL system can be altered at different levels such as (1) functional blockade of FAS receptor (by FAP-1[159] and FLIP proteins[160]) (2) metalloproteinases activation (by MMP-2 and MMP-7) leading to increment of soluble FAS/FASL fractions and reduction of membrane FAS/FASL[138,139,150,161]), (3) alternative splicing phenomena resulting

in the onset of soluble FAS fractions[132, 133], (4) decoy receptor synthesis[162] and (5) altered expression of FAS/FASL membrane-bound fractions. All these phenomena would be related to some of CRC tumorigenesic typical alterations, such as functional disruption of p53 tumor suppressor protein[163-168], oncogenic activation of KRAS[169-171], NFKβ activation/blockade[148,172,173], activation of the TCF/βcatenin pathway[148-150,173] and methylation of specific promoter regions[174]. Adenoma through carcinoma step has been shown to lead to FASL up-regulation and FAS down-regulation[175]. Zhu et al. reported that in 53 cases of colon carcinomas, 23 cases (43.4%) expressed Fas which was significantly lower as compared to that in normal colonic mucosa (73.3%) (P<0.01), and 45 cases (84.9%) of colon carcinomas expressed FasL, whereas only 2 cases (3.75%) in normal mucosa expressed FasL. Intensity and extent of positive staining varied within individual tumors[176] (see FIG. 13).

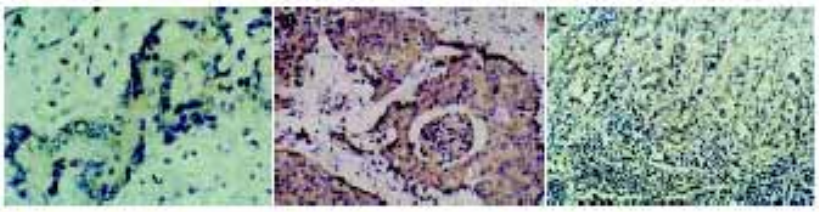


FIG. 13. Immunohistochemical staining of Fas and FasL expression in colon cancer cells. A: Low expression of Fas in colon cancer cells; B: high expression of FasL in colon cancer cells; and C: high expression of FasL in lymph node metastases from colon cancer cells[176].

FasL upregulation in primary CRC is related to lymph node spreading and distant metastasis[177]. FasL expression is progressively increased when comparing normal colonic mucosa to primary CRC to and to liver metastases[178] (see FIG. 14).

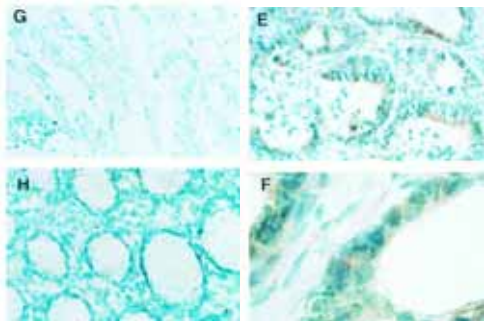


FIG. 14. (G and H) Immunoperoxidase staining of primary tumor of human colonic adenocarcinoma tissue (G), normal human colon mucosa (H) and hepatic metastatic lesion of human colonic adenocarcinoma tissue (E) with anti-FasL N-20 polyclonal antibody (×200). F is a higher magnification (×400) of E[178].

High levels of soluble FAS (sFAS) have been reported in ACRC, compared to those measured in healthy controls and localized disease[179]. Even not shown, it is suggested that mutated p53 would activate alternative splicing phenomena resulting in an augment of soluble FAS fractions[180] (see FIG. 15). Soluble FASL (sFASL) levels have never been measured in CRC patients.

CRC progression is related to the acquisition of a FAS/FASL apoptotic resistant profile, which means chemorresistance to all those drugs that induce apoptosis through the extrinsic pathway. It is also related to immunoescape[176, 181-184].

1.7.3. Tumorigenic Model

Together, MMP-7 and FAS/FASL are altered during CRC tumorigenic process. P53 mutations, RAS activation, NFkβ blockade/activation, TCF-b-catenin pathway activation and methylation of specific promoter regions have been direct or indirectly linked to some of these alterations. FAS expression is decremented and FASL incremented. Moreover, FAS receptor is functionally

blocked, probably by proteins such as FLIP and FAP, but also due to KRAS induced increment of proapototic proteins. MMP-7 expression is progressively enhanced, posssibly due to altered APC/β-catenin pathway and K-RAS activation. MMP-7 activation leads to an increment of soluble FASL fractions and reduction of membrane FASL. Alternative splicing phenomena would be activated, resulting in an augment of soluble FAS fractions.

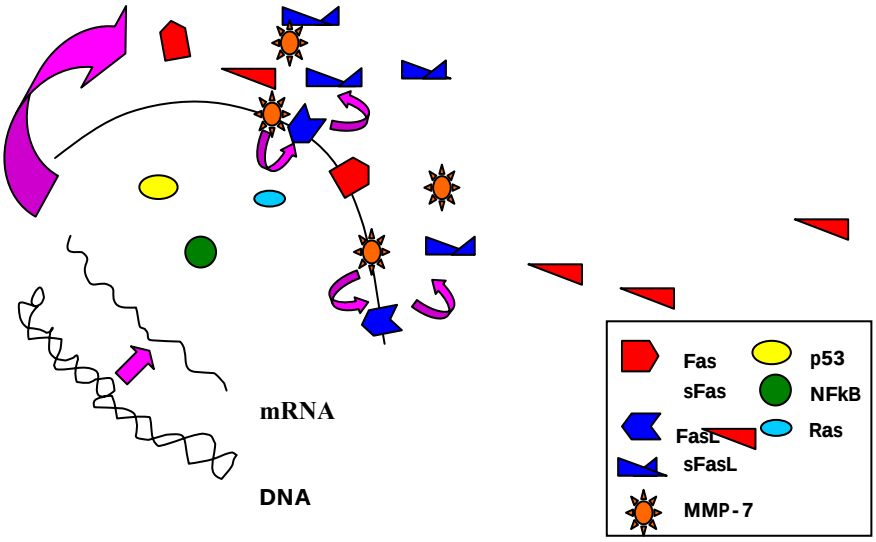


FIG. 15: MMP-7 and FAS/FASL membrane-bounded and soluble forms.

In conclusion, CRC tumorigenic evolution implies an increase of aggressiveness and acquisition of an apoptotic resistance phenotype and ability to immunoevade.

An integrated model showing both alterations during tumorigenic evolution is described in a diagram below (see FIG.16).

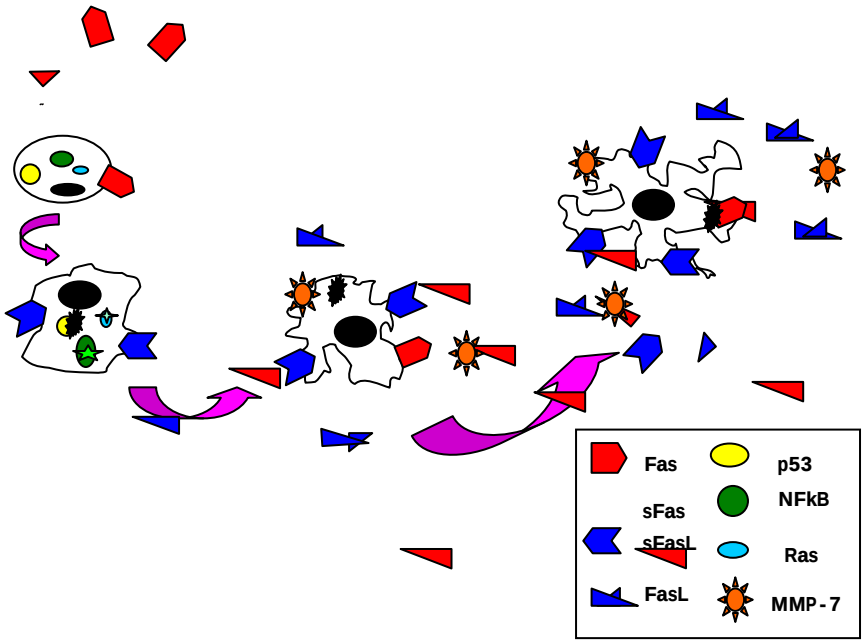
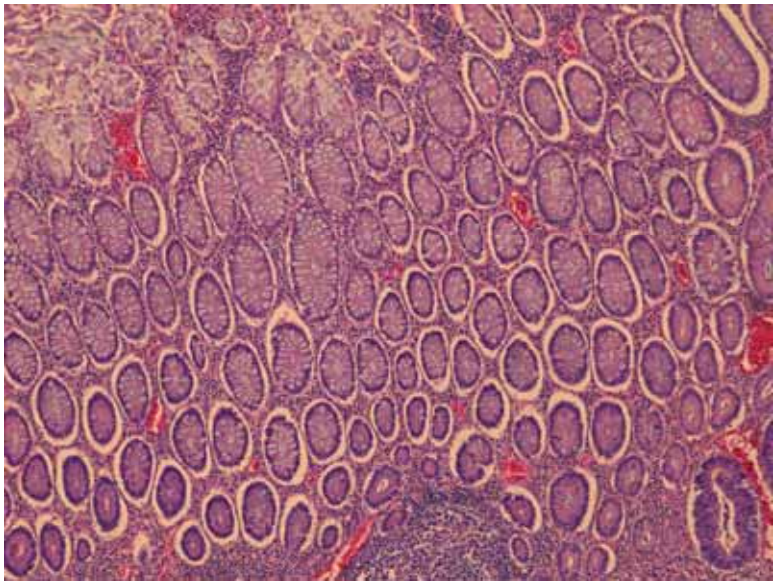
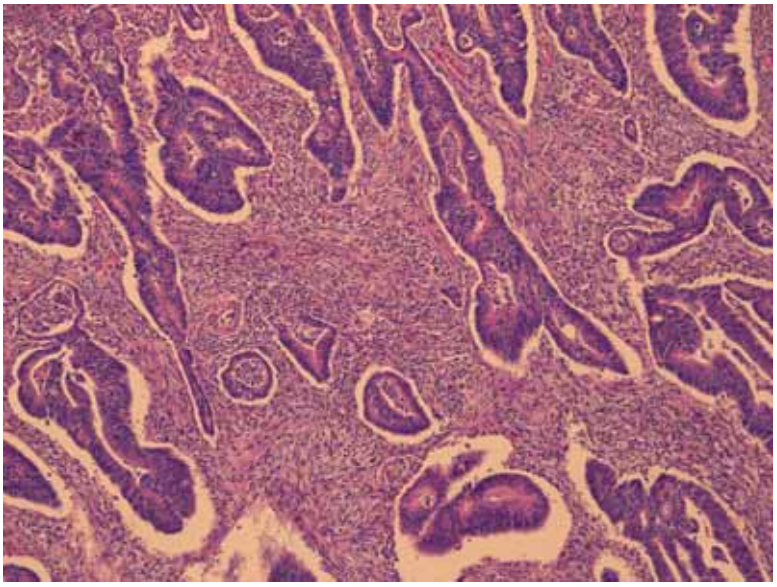


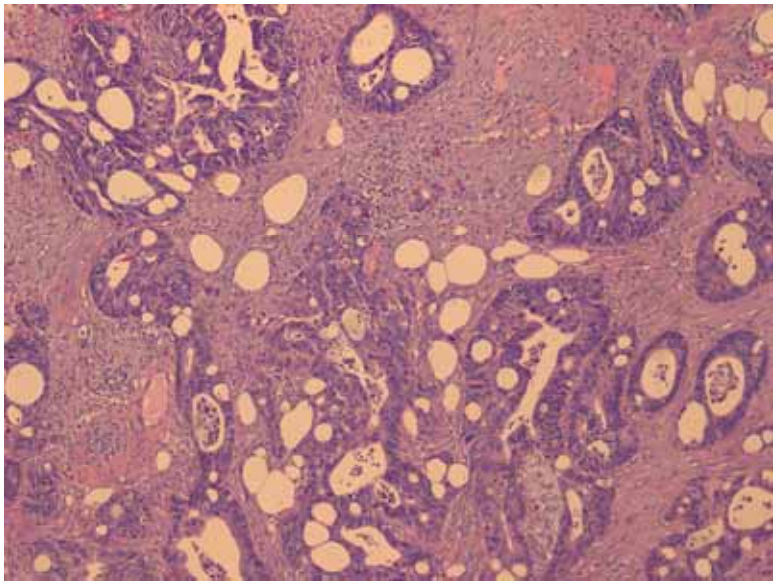
FIG. 16: MMP-7 and Fas/FasL: ACRC tumorigenic model

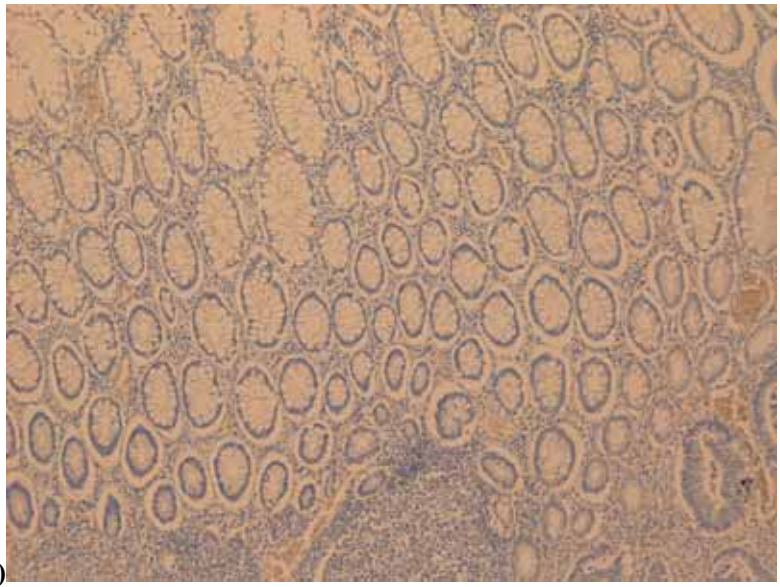
The above mentioned model has been built up with bits and pieces of what has been published in the literature. There are no studies validating it in ACRC series. We are currently evaluating FAS, FASL and p53 expression by immunohistochemistry in series of mucosa-CRC-lymph node metastases-liver metastases in a total of 30 ACRC patients (see FIG. 17 a, b and c). We are also sequencing p53 exons (4 to 9) to establish a correlation between genetic alterations and FAS/FASL expression levels.

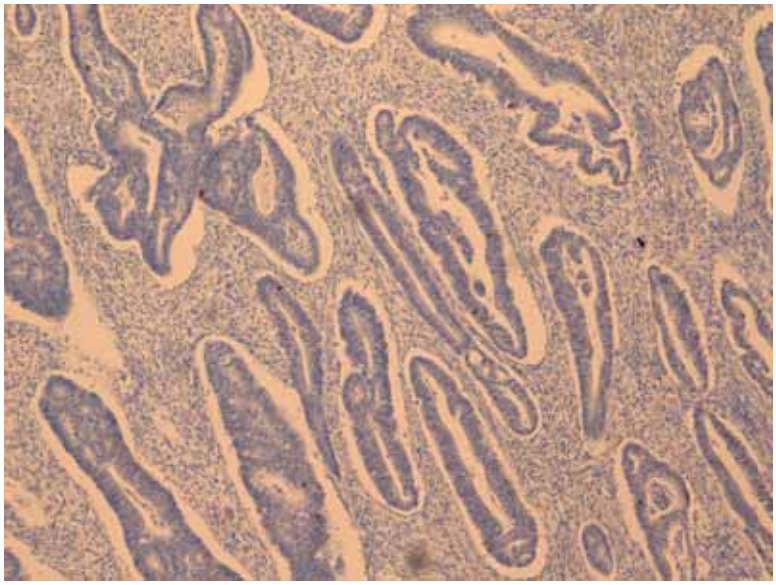
FIG. 17a: Hematoxilin/Eosin staining of **(A)** human colonic mucosae , **(B)** primary CRC, **(C)** peritoneal metastases. (10x) **FIG. 17b:** p53 staining of **(A)** human colonic mucosae , **(B)** primary CRC, **(C)** peritoneal metastases. (10x) **FIG. 17c:** FAS staining of **(A)** human colonic mucosae , **(B)** primary CRC, **(C)** peritoneal metastases. (10x) **FIG. 17d:** FASL staining of **(A)** human colonic mucosae , **(B)** primary CRC, **(C)** peritoneal metastases. (10x)

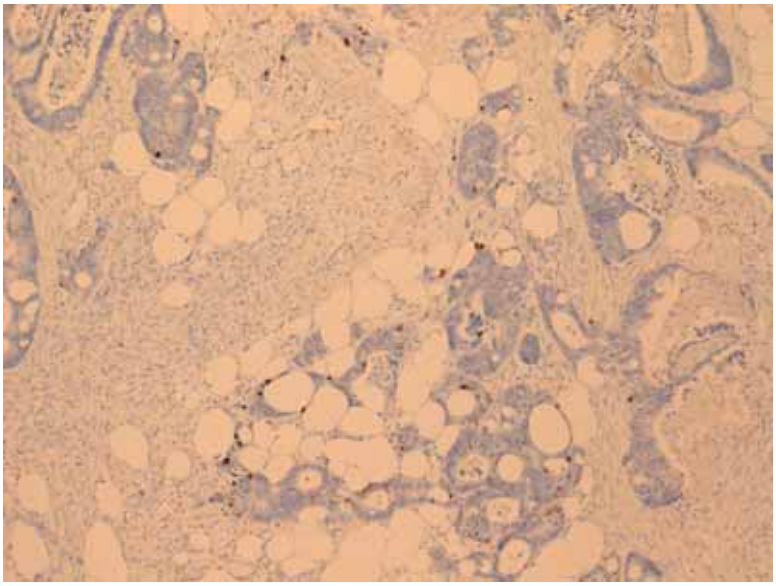
(17aA) 

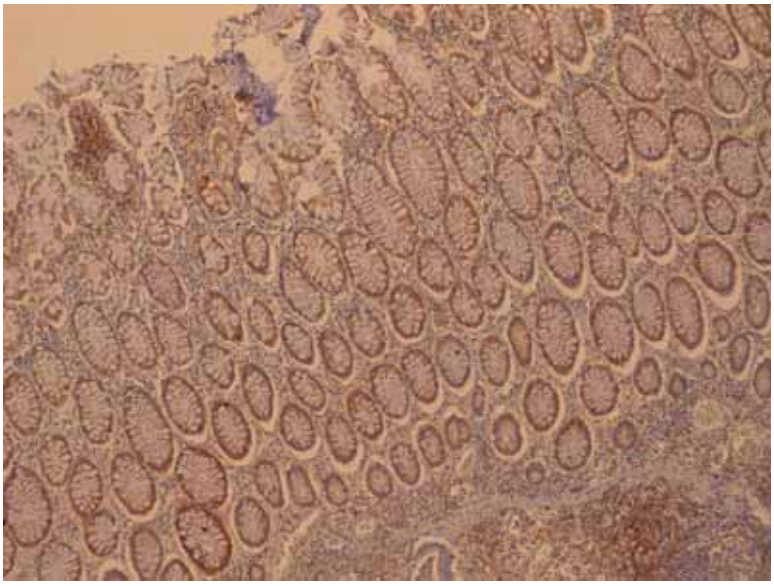
(17aB) 

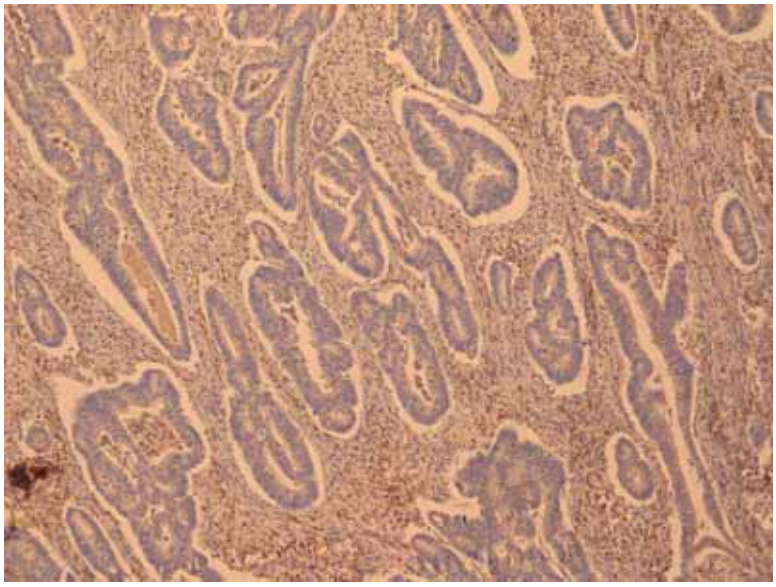
(17aC) 

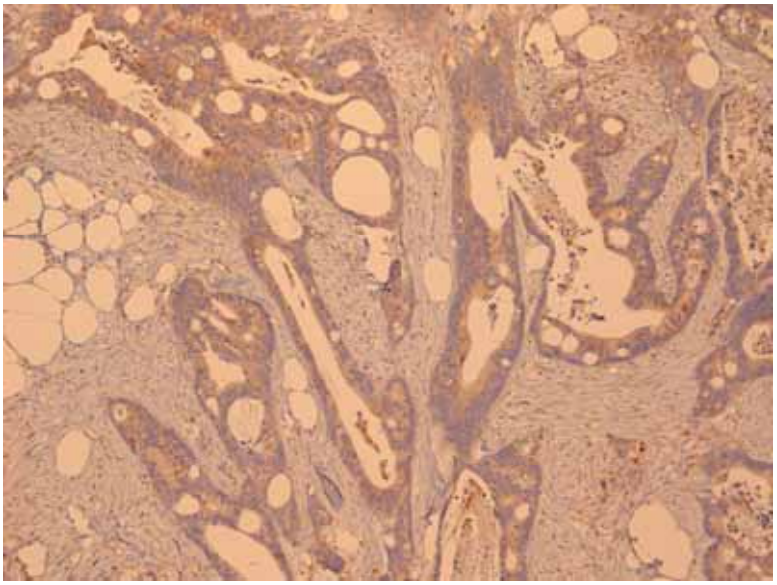
(17bA) 

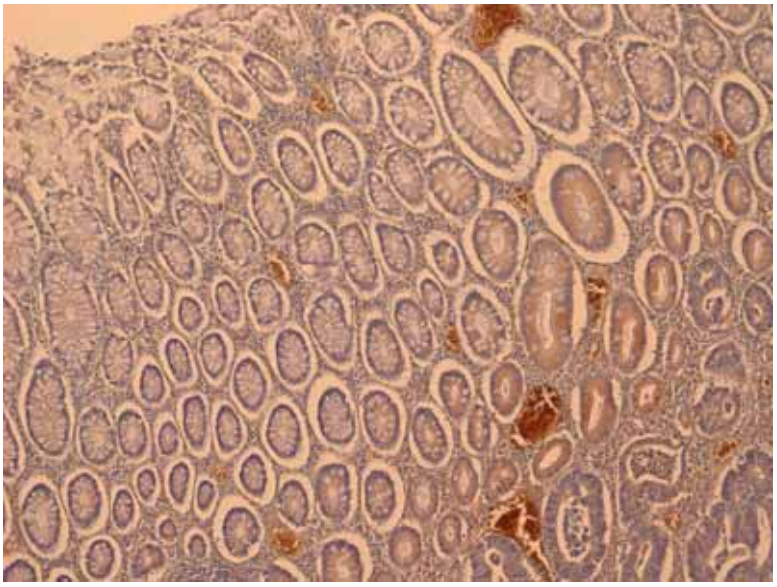
(17bB) 

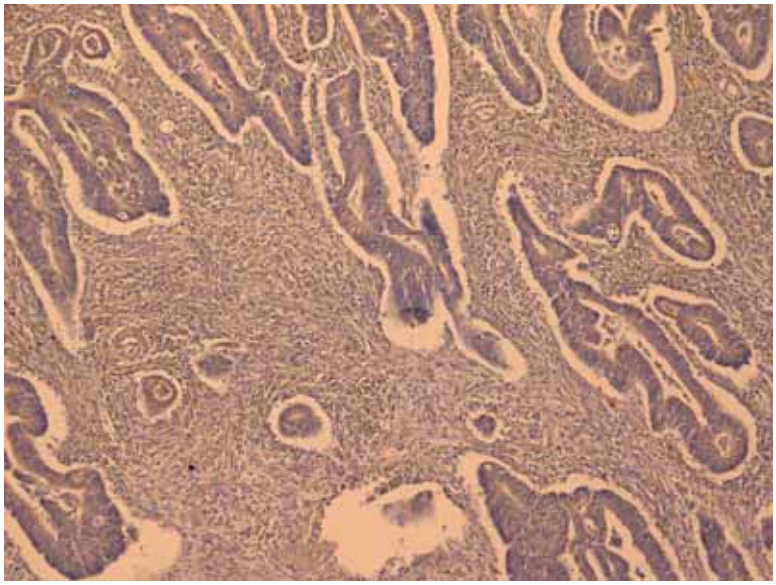
(17bC) 

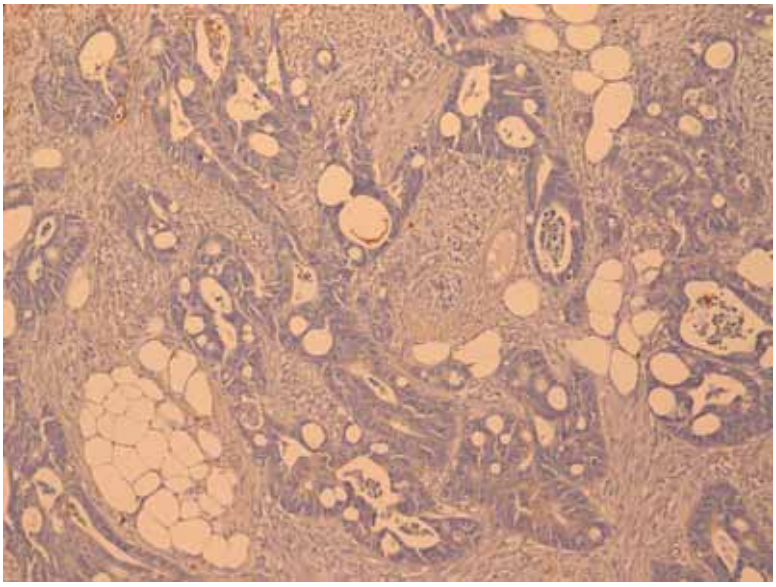
(17cA) 

(17cB) 

(17cC) 

(17dA) 

(17dB) 

(17dC) 

Moreover, we are measuring expression and localizacion of Survivin, a protein related to apoptotic resistance, and MMP-7. Both proteins are regulated by the TCF/beta-catenin pathway (see FIG. 18)[185, 186].

1.8 CHEMOTHERAPY REGULATION OF FAS/FASL AND MMP-7

Cytotoxic drugs can act by inducing apoptosis in sensitive target cells. The precise mechanisms of apoptosis mediated by chemotherapy have not been completely clarified. Membrane-bound forms FAS and FASL have been implicated in chemosensitivity through leading to apoptosis in response to DNA-damaging drugs[187-193]. Some reports have proposed that chemotherapies act through upregulation of FAS and FASL at the tumor cell surface. Upregulation would be secondary to activation of the two cell major sensors of DNA damage: p53 and NFκβ[194-197]. So, chemotherapy drugs could promote apoptosis by increasing FAS/FASL levels and thus favouring interaction and apoptosis.

Commonly used chemotherapeutic drugs in colorectal cancer (OXL, IRI, 5FU) have shown to upregulate Fas and FasL levels “in vitro”. Experiments with HCT115 CRC cell line showed that treatment with different doses of Oxaliplatin (7-25 μM) induces an apoptotic response activating both the intrinsic and extrinsic pathway. Levels of membrane-bound forms of FAS and FASL were upregulated in all cases, after chemotherapy addition[198]. Shao et al. showed in HT-29 CRC cell line, that treatment with CPT-11, a topoisomerase I inhibitor, increased FAS and FASL levels[199]. Backus et al showed that CRC cell lines treated with a thymidilate synthase inhibitor, as 5-FU, upregulated FAS and FASL proteins, especially in p53 wild type cell lines[200]. In all cases, FAS/FASL upregulation appeared to be more intense between 12 and 72h after chemotherapy addition.

Levels of membrane-bound forms of FAS and FASL have also been tested in other CRC cell lines (HT-29 and DLD-1) before and after administration of different chemotherapeutic regimens such as Doxorrubicin, Etoposide and

Cisplatin. The obtained data showed that there is a significant increase of FAS and FASL membrane protein levels [201-203].

Non-CRC cell lines have also been shown to increase FAS and FASL levels after treatment with different chemotherapy drugs.

The accumulated data suggest that upregulation of FAS/FASL membrane-bound forms after chemotherapy treatment is possibly a general mechanism of regulation but depends specifically on the type of chemotherapeutic agent and cell line tested.

Up to date, there is no published evidence of whether chemotherapeutic drugs have some effect in regulating FAS soluble fractions (sFAS and sFASL). Dr. Jordi Codony, working in Experimental Oncology Lab (IDIBAPS), has performed an experiment in HT-29 CRC cell lines to show whether chemotherapy drugs modify FAS/FASL soluble levels.

To establish if chemotherapy regulates the expression of soluble forms "in vivo" we designed a pilot pharmacodynamic study in patients with ACRC receiving chemotherapy. We prospectively selected 20 patients with metastatic colorectal cancer (10 without prior chemotherapy, receiving an OXL-based treatment, and 10 before starting second-line therapy, receiving an IRI-based treatment) (see FIG. 18).

	First line therapy (n=10)	Second line therapy (n=10)
Median Age (range)	67.5 (50-77)	66.5 (44-73)
Sex		
Male	6 (60%)	4 (40%))
Female	4 (40%)	6 (60%)
Median CEA (range)	133.3 (2.1-17121)	69.2 (4.8-295.8)
Median LDH (range)	478 (333-1964)	409 (300-947)
Median ALP (range)	438 (178-1156)	261 (138-1005)
Median basal sFas (range)	15.3 (5.7-31)	24.3 (8.5-73)
Median basal sFasL (range)	0.075 (0.07-0.34)	0.155 (0.07-0.45)
Median basal ratio sFas/sFasL (range)	171.1 (21.1-391.3)	151.8 (40.5-791.4)

FIG. 18: Patient characteristics stratified by therapy line.

Sequential blood extractions were performed before starting treatment and at 2, 24, 48, 72, 96 and 168 hours after treatment. From the obtained serum we measured sFAS and sFASL by ELISA. Clinical data was recorded in a database. Our results were that following chemotherapy treatment, peaks of sFAS increment and sFASL decrement were observed. Statistically significant variations were seen between 24 to 72 hours compared to basal levels. Patients being treated with first-line therapy showed an increase in sFAS levels 48 hours after treatment while sFASL levels did not vary. Patients receiving second-line treatment showed no variation in sFAS levels 48 hours after treatment, while sFASL decreased. In both subsets of patients sFAS/sFASL ratio showed a significant increase after 48 hours (p=0.005) (p=0.009)
(See FIG 19, 20, 21).

	N		sFas	sFasL	Ratio	p
Primera línea	10	Basal	15.3 (5.7-31)	0.08 (0.07-0.3)	171.1 (21.1-391.3)	0.005
		48h	22.9 (12-54)	0.07 (0.07-0.11)	292.9 (168.8-767.1)	
Segunda línea	10	Basal	24.3 (8.5-71)	0.16 (0.07-0.45)	151.8 (40.5-791.4)	0.009
		48h	24.4 (6.4-82)	0.08 (0.07-0.21)	274.9 (30.5-1041.4)	

FIG. 19: sFAS, sFASL ad sFAS/sFASL ratio values according to therapy line.

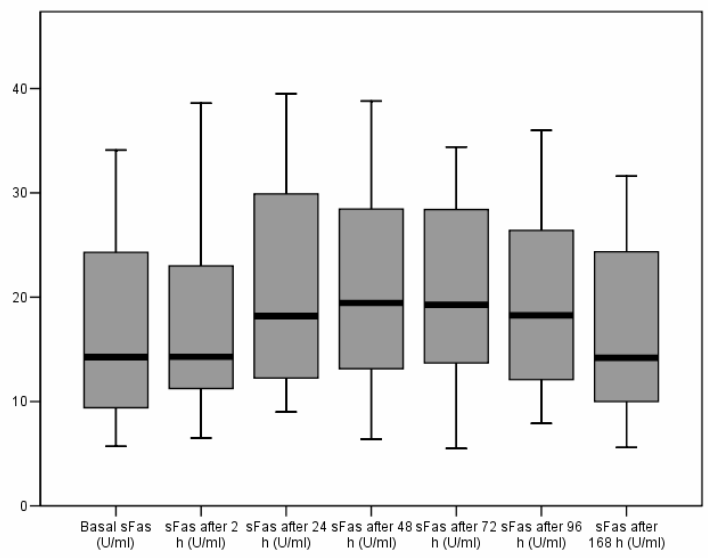


FIG. 20: Median and range of SFAS levels determination (ngr/ml) (basal, 2h, 24h, 48h, 72h, 96h and 168h after chemotherapy)

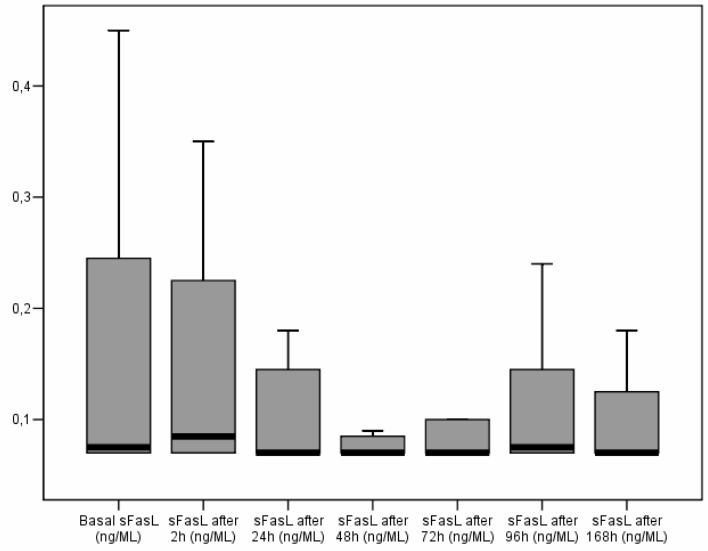


FIG. 21: Median and range of SFASL levels determination (ngr/ml) (basal, 2h, 24h, 48h, 72h, 96h and 168h after chemotherapy)

Our conclusion was that chemotherapy regulates FAS/FASL soluble fractions "in vivo", especially between 24-72hours. We have noticed that sFAS/sFASL ratio increases after chemotherapy addition. This variation is due to sFAS increment and sFASL decrement in chemotherapy-naïve and previously-treated patients respectively **(Nadal et al, publication pendant)**. Our main goal in this study was to perform an accurate pharmacodynamic description of how FAS/FASL soluble fractions vary when chemotherapy was added to patients. This pilot study constitutes the first evidence of FAS/FASL soluble fractions regulation by chemotherapy "in vivo". We believe that our results deserve further investigation in properly adequate-size clinical trials, with chemotherapy or new emergent compounds.

In the current literature there is a complete lack of data regarding MMP-7 regulation by chemotherapy. Dr. Vanessa Almendro, also working in Experimental Oncology Lab has shown a clear increment of MMP-7 expression in OXL resistant HT29 CRC cell lines (ROXI), by ELISA, RT-PCR and immunofluorescence methods (Almendro et al, publication pendant). We are currently collecting data to see variation of MMP-7 levels during time in ACRC patients receiving chemotherapy.

2 HYPOTHESIS AND OBJECTIVES

Current prognostic and predictive markers in ACRC are not clearly defined, as they have not been properly validated in well-designed, prospective and powered clinical trials.

At the present time, we still do not know which type of ACRC we are facing up or the way it is going to behave in terms of aggressiveness and response to therapy. There is no way to determine patients who will not benefit from systemic therapies, or those who will rapidly progress after an initial response.

This scenario points to different needs. First of all, suggested markers of prognosis and response to therapy should be prospectly validated, in order to do one step ahead in classifying ACRC patients. Secondly, once validated, clinicians should implement them in both clinical trials and routine practice. Third, there is a need of finding markers accurately reflecting ACRC aggressiveness and response to therapy, which means markers with prognostic and predictive value. As tumors change over time, aggressiveness and resistance to therapy can also vary. So, those markers should be able to reflect those values anytime during disease evolution. This fact implies that markers should be easy to obtain, through a non-invasive technique, such as venopuncture.

In conclusion, there is a need of prospectively validating markers to determine ACRC behavior, in terms of aggressiveness and sensitivity/resistance to therapies, and thus to classify ACRC patients, but also there is a need of finding novel markers accurately fulfilling this role anytime during disease history.

FAS and FASL are proteins that have been related to chemotherapy apoptotic response. ACRC show a FAS/FASL chemoresistant pattern, but the role of their soluble forms in chemoresistance has never been explored. MMP-7 has been related to an aggressive phenotype in ACRC, but also to chemorresistance

through FASL cleavage. Serum sFAS levels have been shown to be increased in ACRC patients. Serum measurements of sFASL and MMP-7 have never been done in ACRC patients.

Our hypothesis is that serum levels of MMP-7, sFAS and sFASL in ACRC patients can be biologic markers estimating aggressiveness and chemoresistance. As a new concept there is the fact that biologic markers should be obtained not only at the time of diagnosis but anytime during disease history, as tumor biology changes. Also for these reason, they should be obtained in an easy and non-invasive way. MMP-7, sFAS and sFASL serum levels and their variation along time should be predicting ACRC chemorresistance anytime during disease history.

The main objectives of this thesis can be summarized as follows:

1- To determine basal levels of MMP-7 in serum of ACRC patients and establish its prognostic value
2- To determine levels of sFAS and sFASL in serum of ACRC patients before and during chemotherapy treatment and establish its correlation to tumor response and thus its predictive value
3- According to results, to design prospective trials to determine if serum MMP-7 and sFAS/sFASL, as new soluble markers of prognosis and predictors of response to therapy in ACRC, have clinical relevance

3 STUDIES

3.1 Determination of MMP-7 soluble fractions basal levels in serum of ACRC patients and establish its prognostic value

"Serum matrix metalloproteinase 7 (MMP-7) levels identifies poor prognosis advanced colorectal cancer patients"

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Abstract

Purpose

Metalloproteinase 7 (MMP-7) plays an important role in tumor growth, invasion and dissemination, and is secreted to the media. Due to the close implication of MMP-7 in cancer biology, we sought to define the prognostic significance of serum levels of MMP-7 in metastatic colorectal cancer (CRC) and explore its possible impact in the daily clinical practice.

Methods

MMP-7 expression was determined by enzyme-linked immunoabsorbent assay. We assessed serum MMP-7 levels in 87 healthy controls, 96 patients with non-metastatic CRC and 120 patients with advanced CRC. Clinical information was gathered from patient files.

Cox proportional hazards model was used to assess survival. MMP-7 and the variables associated with prognosis were entered and a backward elimination method was employed to adjust the model. Inclusion criteria was $p \leq 0.05$ and exclusion criteria was $p \geq 0.10$.

Results

Advanced CRC patients have a significant higher mean serum MMP-7 levels (13.4 ng/mL) than those in non-metastatic CRC (5.5 ng/mL; $p<0.001$) and healthy controls (4.2 ng/mL; $p<0.001$). In metastatic patients, after adjusting for other prognostic variables, MMP-7 (entered as a continuous variable) is associated with decreased survival (HR 1.016, IC 95% 1.002-1.031).

Conclusions

Serum MMP-7 levels are significantly elevated in patients with advanced CRC. MMP-7 is an independent prognostic factor for survival in advanced CRC. In our sample, the risk of death associated to MMP-7 increase is much higher than the risk of death associated to LDH elevation.

Introduction

Widely accepted prognostic factors in advanced CRC are performance status (PS), serum lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) levels[1-4]**.** Still, controversy related to their value and general lack of implementation in clinical studies points to the need of finding novel markers and useful prognostic indexes to better classify these patients for the clinical practice.

Matrylisin (MMP-7) is a proteolytic enzyme belonging to Matrix Metalloproteinase (MMPs) family[5, 7]. It is constitutively expressed in the ductal and glandular epithelium of many tissues[8]**.** In the lung and intestine it plays a role activating antibacterial peptides such as prodefensins[9]. MMP-7 is synthesized and secreted by tumor epithelial cells as a 28-KDa proenzyme, that can be activated through proteolytic removal of a 9-Kda prodomain from the N-terminus. The soluble activated form binds to the tumor epithelial cell surface.

Both active forms, the soluble and the membrane-bounded, have proteolytic activity. Its expression is regulated by transcription factors such as AP-1, PEA3 and β-catenin/ tcf4 complex[10-12]. By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2, MMP-9), through ectodomain shedding of E-cadherin[13].and receptor activator of nuclear factor-kappa B ligand (RANKL)[14] or through cleavage of adhesion molecules, such as integrin β4[15].

MMP-7 is able to induce cell apoptotic impairment. It specifically cleaves critical proteins implicated in the extrinsic apoptotic pathway, such as FAS Ligand (FASL) [16, 17] and Tumor Necrosis Factor-alpha (TNF-α) [18]. Its shedding is related to the acquisition of an apoptosis resistance phenotype[19]. Additionally, MMP-7 induces cell proliferation through cleavage of Heparin Binding Epidermal Growth Factor (HB-EGF) precursor[20], a Disintegrin and Metalloproteinase family (ADAM) member, ADAM28[21] and degradation of all six Insulin Growth Factor Binding Proteins (IGFBP-1 to -6), increasing the bioavailability of IGF, and thus favoring cancer cell growth and survival[22, 23].

Matrilysin can regulate angiogenesis either inducing a direct proliferative effect on vascular endothelial cells[24] or producing angiogenesis inhibitors (angiostatin, endostatin, neostatin-7)[25] or enriching the variety of angiogenesis mediators, such as the soluble vascular endothelial growth factor (sVEGF) [26].

Immunoevasion due to MMP-7 would be related to FasL cleavage[16, 17] or to IgG degradation[27].

Matrilysin has been found overexpressed in a variety of tumors, such as colorectal cancer[28]. There is substantial evidence that overexpression of MMP-7 in CRC primary tumors, taking into account the measurement of both activated and pro-forms, is related to a more aggressive phenotype of tumor cells and a poorer prognosis. MMP-7 overexpression has been correlated with invasion and to liver metastasis formation in CRC non-metastatic disease[29-34].

As noted above, MMP-7 is secreted to the media. Both soluble MMP-7 forms, active and pro-active, can be measured in serum by a commercial ELISA Kit.

Due to the close implication of MMP-7 in cancer biology and the possibility to measure its soluble forms in serum, this study was performed with the objective to define the prognostic significance of serum levels of MMP-7 in metastatic CRC and explore its possible role in the daily clinical practice.

Serum MMP-7 levels were also measured in healthy volunteers, non-metastatic and advanced colorectal cancer patients, to prove if they can be an indirect estimation of tumor MMP-7 expression and activity.

Patients and Methods

Patients

The study was conducted as a serial collection of serum samples from 120 patients with their first sign of advanced colorectal cancer from July 2001 to December 2004. The patients were in good performance (performance status $\leq$2) and were not initially suitable for liver resection (more than 3 liver nodules and/or $\geq$ 5 cm). All patients had a medical history, clinical examination, full blood count, and a biochemical screen of renal and liver function. Levels of carcinoembryonic antigen (CEA) (Roche ® Germany) were measured using an Elecsys (Roche ®) automated analyzer. Lactate dehydrogenase (LDH) (Roche ®, Germany) and alkaline phosphatase (ALP) (Bayer ®, USA) were measured using and ADVIA 2400 (Bayer ®, USA) automated analyzer. Staging was done with abdominal spiral computed tomography (CT) and chest radiography. Additional techniques such as abdominal ultrasound, chest CT or magnetic resonance imaging (MRI) were done if needed for further staging refinement. Serum samples were obtained before treatment, after written informed consent. Additionally serum MMP-7 was determined in 87 healthy patients without known renal or hepatic dysfunction, and 96 patients with histologically confirmed colorectal adenocarcinoma with non-metastatic disease, before surgical resection. To rule out metastatic disease in non-metastatic CRC patients, staging was done with chest radiography, and abdominal

ultrasonography or spiral CT. The study was approved by Hospital Clinic Ethical Committee.

Sample Collection

Before the initial treatment, venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 minutes. After that, they were centrifugued at 1500 rpm for 15 minutes. Serum was immediately aliquoted and keeped at -80c, until assayed. The procedure has been performed exactly in the same way in all groups (healthy volunteers, CRC and ACRC).

Serum samples and MMP-7 analysis

MMP-7 (Quantikine®, USA) was determined using a quantitative solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) (RnD Systems Inc®, USA) and tested in duplicate. MMP-7 technique can detect both pro- and active forms of recombinant human MMP-7. High concentrations of MMP-7 were diluted with calibrator, to produce samples with values within the dynamic range of the assay**.**

Statistical Methods

Recorded variables were age, sex, date of birth, date of death or last follow-up, performance status, number and site of metastasis, previous chemotherapy received and sera levels of: CEA, LDH, ALP and MMP-7, as described above.

Assuming a two-sided alpha error of 5% , with our sample size (n=120) and in 18 months of follow-up, the power to detect the differences observed was of 98%. Missing values (<2%) were not included in the multivariate analysis.

Regarding multivariate analysis, overall survival was considered the dependent variable. It was calculated in patients with advanced colorectal cancer as the time from informed consent for biological analysis, to death or censoring data. The prognostic significance of the independent variables regarding survival was assessed using the Cox proportional hazards model as follows. Independent variables were selected sequentially. First, the variables Age, PS, Number of involved organs, LDH, ALP, CEA and MMP-7 were entered and a backward elimination method was employed to adjust the model. Inclusion criteria was $p \leq 0.05$ and exclusion criteria was $p \geq 0.10$[35]. To the best model

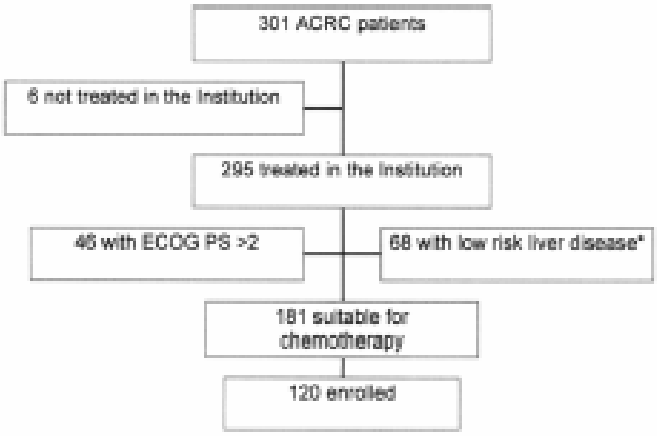
selected with this method, the excluded variables were added, one by one, checking for confusion (>10% variation of the HR of MMP-7) and for any increase of the power of prediction measured by the coefficient of determination[36]. The model without confusion and with the highest power of prediction according to the coefficient of determination was chosen. Quantitative variables were introduced as continuous in the model. Assumed proportionality of the multivariate model was checked plotting the logarithm of the cumulative estimated risk stratified by each independent variable selected. Assumed log-lineal relationship of the multivariate model was checked plotting the *martingale* residuals by each quantitative independent variable selected [37].

Normality of the sample was assessed with the Shapiro-Wilk test. Due to the absence of normality, differences in serum MMP-7 levels between patients with advanced disease, non-metastatic disease and controls was analysed by Mann-Whitney *U* test. The Mann-Whitney *U* test was also used to test for associations between MMP-7 serum levels in patients with advanced disease and clinical characteristics. All *p* values were two-sided, and values <0.05 were considered significant. Survival curves were constructed using the Kaplan-Meier method, assessing significance by the Log-Rank test. Division by quartiles was chosen for plotting. Statistical analyses were performed using SPSS software 12.0.

Results

Patient characteristics

During the study period 301 patients with advanced colorectal cancer were visited in our Oncology Unit. 181 patients were not enrolled owing to: treated outside our Institution (6), poor performance status or elderly with fragility criteria (46), liver resection (61) or radiofrequency ablation (7) as first treatment and patient refusal to participate (61) (see figure 1).



*Low risk liver disease: Fewer than 4 liver nodules of less than 5 cm

FIGURE 1 – Study profile.

Therefore patients enrolled in the study constitute 66% (120/180) of all patients with advanced disease, suitable for chemotherapy treatment diagnosed in the study period. Primary chemotherapy based on oxaliplatin-5-fluorouracil or irinotecan-5-fluorouracil was used to treat 92% (110/120) of patients. Patient characteristics are summarized in Table 1. Median age of the patients in our sample was 66 years, being male patients 62.5 % of them. 80 % of the patients were in good performance status (PS 0-1). 81 % of the sample had metastasis in only one organ and liver was affected in 76.7 % of cases. Most of the patients had not received adjuvant chemotherapy (77.5 %).

TABLE I – CLINICAL CHARACTERISTICS OF THE PATIENTS AT BASELINE

Age, years	
Median	66
Range	33–82
Sex	
Male	75 (62.5)
Female	45 (37.5)
ECOG performance status	
0	45 (37.5)
1	51 (42.5)
2	24 (20)
No. of organs involved	
1	81 (67.5)
>1	39 (32.5)
Type organs involved	
Liver	92 (76.7)
Other than liver	28 (23.3)
Previous adjuvant chemotherapy	
No	93 (77.5)
Yes	27 (22.5)
Serum CEA (ng/ml)	
Median	38
Range	1–24770
Serum LDH (UI/L)	
Median	418
Range	195–15,864
Serum ALP (UI/L)	
Median	341
Range	120–6,561
Serum MMP-7 (ng/ml)	
Median	7.7
Range	2–126.6

Values are *N* (%) unless otherwise indicated.

Basal serum levels of MMP-7 in healthy controls, non-metastatic CRC and advanced CRC

The mean and median serum level of MMP-7 for the advanced CRC patients (n=120) were 13.4ng/ml (SD 17.1) and 7.7ng/mL respectively (range 2 to 126.6 ng/mL) and for the non-metastatic CRC (n=96) group were 5.5ng/ml (SD 3.2) and 4.9ng/mL (range, 1.2 to 19 ng/mL). The difference was statistically significant between these two groups ($p<0.001$). There was also a significant difference between serum MMP-7 levels in patients with

advanced CRC (n=120) and healthy controls (n=87), being the mean and median serum level of MMP-7 4.2ng/m (SD 2.2) and 3.5ng/mL (range 0.5 to 16 ng/ml, p=0.001) and between patients with non-metastatic disease (n=96) and healthy controls (p=0.001). Demographic characteristics between the three groups were stated in Table 2.

TABLE II – DEMOGRAPHIC CHARACTERISTICS OF HEALTHY CONTROLS, LIMITED STAGE AND ADVANCED STAGE PATIENTS

Characteristic	Healthy controls ($n = 87$)	Limited stage ($n = 96$)	Advanced stage ($n = 120$)	p
Sex				
Female (%)	55 (63.2)	38 (39.6)	45 (37.5)	0.0004[1]
Age				
Mean (SD)	56.8 (17.9)	71.1 (12.6)	63.8 (11.1)	<0.0001[2]
MMP-7 (ng/ml)				
Mean (SD)	4.2 (2.2)	5.5 (3.2)	13.5 (17.1)	<0.0001[2]

[1]χ^2-square test.–[2]Kruskal–Wallis test.

Association between basal serum MMP-7 levels in advanced CRC patients and clinical characteristics

Basal serum MMP-7 in advanced CRC patients was significantly associated with ECOG performance status (p<0.001), previous adjuvant treatment (p=0.007), LDH levels (p<0.001), ALP levels (p<0.001), CEA levels (p<0.001) and liver involvement (p=0.01) but not with other covariates such as age, sex or number of involved sites (Table 3).

Association between basal serum MMP-7 levels in advanced CRC patients and overall survival

The median survival for all patients was 17.8 months (range, 15.5 to 20.1). A total of 93 patients (77%) died during follow-up. In the univariate analysis the following variables resulted significant: PS, number of involved organs, LDH, ALP, CEA and MMP-7 (Table 4). Elaborating the multivariate model as described above, the most predictive model was the one containing MMP-

7, PS, number of involved organs and LDH. HR of MMP-7 was 1,016 (IC 95% 1,002 – 1,031; p=0,029). As MMP-7 was entered as a continuous variable, this result is best interpreted as follows: independently of the effect of other variables entered in the multivariate analysis, every increase of 10 units of MMP-7 is associated with a 16% increase of the risk of dying. LDH, ALP and CEA were also treated as continuous variables, being their magnitude of association expressed in Table 3 as percentage of risk increase. Interpreting this in the same way, an increase of 100 units of LDH is associated with a 0, 9 % increase of the risk of dying.

TABLE III – MMP-7 LEVELS ACCORDING TO CLINICAL CHARACTERISTICS

	Mean of MMP-7 (SD)	*P*
Age		
<56	17.2 (16.4)	
56–66	14.9 (18.2)	0.353
67–72	8.6 (6.6)	0.029
>72	12.7 (22.3)	0.100
Sex (*n*)		
Female (45)	13.9 (21.7)	
Male (75)	13.1 (13.8)	0.37
ECOG (*n*)		
PS 0 (45)	9.1 (10.7)	
PS 1 (51)	11.6 (13.3)	0.012
PS 2 (24)	25.8 (16.6)	<0.0001
LDH (n)		
≤450 (66)	8.4 (8.3)	
>450 (52)	20.4 (22.5)	<0.0001
ALP (*n*)		
≤290 (49)	7.7 (8.8)	
>290 (68)	18.1 (20.3)	<0.0001
No. of organs involved (*n*)		
1 (81)	14.1 (19.5)	
>1 (39)	12.2 (10.5)	0.300
Type organ involved (*n*)		
Liver (92)	15.2 (18.9)	
Other than liver (28)	7.9 (6.2)	0.018
Previous adjuvant CHT (*n*)		
Yes (27)	9.0 (9.8)	
No (93)	14.8 (18.5)	0.007
CEA[1]		
≤6	6.5 (5.0)	
6–37.2	8 (5.2)	0.043
37.3–191.7	16.8 (14.7)	<0.0001
>191.7	22.9 (27.3)	<0.0001

[1]CEA was divided by quartils.

TABLE IV – UNIVARIATE AND MULTIVARIATE COX REGRESSION ANALYSES OF POTENTIAL PROGNOSTIC FACTORS FOR OVERALL SURVIVAL.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
Age	1.005	0.987–1.024	0.590			
PS						
0[1]	1			1		
1	1.812	1.126–2.198	0.014	1.736	1.052–2.865	0.083
2	4.894	2.774–8.728	<0.001	3.175	1.618–6.231	0.001
No of involved organs						
1[1]	1			1		
>1	1.580	1.027–2.432	0.038	1.494	0.948–2.353	0.083
LDH[2]	0.012	0.004–0.020	0.002	0.009	0.001–0.018	0.049
ALP[2]	0.060	0.003–0.087	<0.001			
CEA[2]	0.007	0.001–0.013	0.010			
MMP-7	1.028	1.017–1.040	<0.001	1.016	1.002–1.031	0.029

Only PS, number of involved organs, LDH and MMP-7 were selected for the multivariate model (see text for details).
[1]This category was taken as the reference.–[2]Results expressed as percentage of risk increase (see text for details).

Once established the prognostic significance of MMP-7 and the magnitude of this association, quartiles were chosen as a natural division for survival plotting.

Quartiles of MMP-7 were 4.83, 7.50 and 15.92. The Kaplan Meier plot shows statistically significant difference between quartiles (Figure 2).

Although the multivariate model provides a MMP-7 effect adjusted by LDH and other factors, as the latter is one of the most established parameters regarding prognosis, we plotted in Figure 3 survival according to MMP-7 and LDH levels. This figure shows that when MMP-7 is higher than median, irrespectively of LDH levels, survival is worse than when MMP-7 is lower than median. This differences are statistically significant (p=0.003).

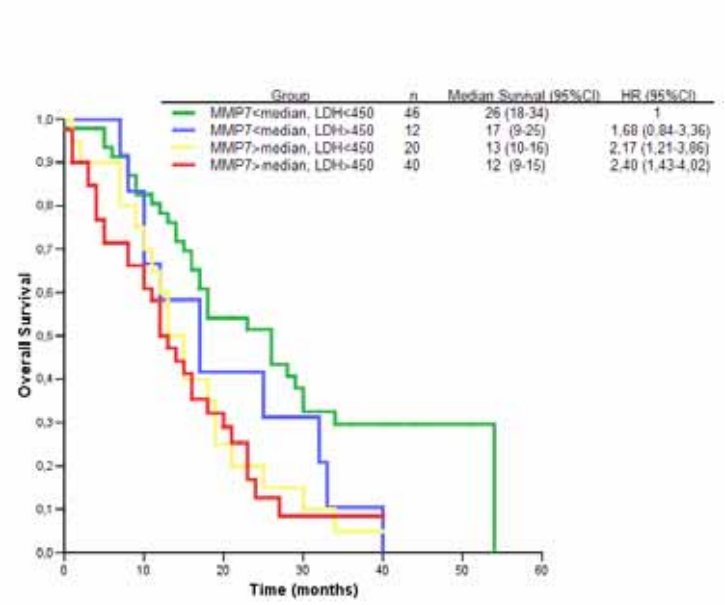


Fig.2. Survival curves showing the association between serum MMP-7 concentration and overall survival.

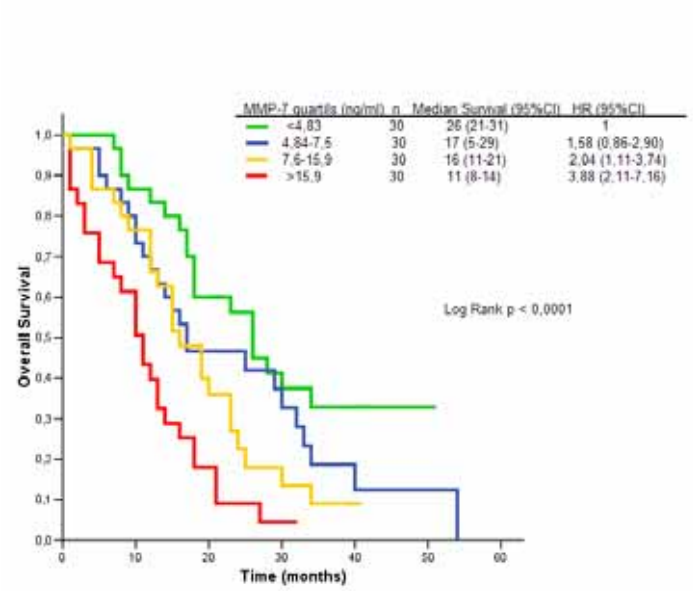


Fig.3. Survival curves according to MMP-7 and LDH levels.

Discussion

The key finding of our study is that MMP-7 is an independent prognostic factor for survival in advanced CRC. Also, in our sample, the risk of death associated to MMP-7 increase is much higher than the one associated to LDH elevation.

Our data confirm that high serum MMP-7 levels tend to correlate with clinical adverse parameters such as high LDH, ALP, CEA levels, liver involvement and poor performance status. Focusing on LDH and MMP-7, in our multivariate survival analysis they came as independent factors, even it is well known that their transcription can be regulated together under hypoxic conditions. Being LDH one of the most accepted clinical parameter to determine prognosis, we tested MMP-7 together with LDH, to determine the prognostic significance of different combinations. The Kaplan Meier curves show statistically significant difference (p=0,003) between curves expressing high and low MMP-7 levels, despite being associated with high or low LDH levels. Patients with high LDH and low MMP-7 levels seem to be associated to a slightly better prognosis, compared with those with both high LDH and MMP-7 levels. Those findings suggest that MMP-7 is possibly even more accurate that LDH in determining prognosis in the group of advanced CRC patients. Interestingly, high serum MMP-7 also correlates with liver involvement during the metastatic spread. Further analyses in the group of liver-only metastases would be required.

Levels of total MMP-7 can be measured in human serum and it is feasible using a simple ELISA technique, as it has been recently shown in few other studies[42]. Serum measurements of total MMP-7 can be considered as an indirect estimation of tumor MMP-7 expression. Other techniques, such as zymography, are useful to distinguish between activated MMP-7 and pro-forms, and might be implemented in the near future for further analysis.

Our work describes how MMP-7 serum level can distinguish between healthy controls, localized and metastatic CRC. Non metastatic CRC patients have significant lower levels of MMP-7 than metastatic CRC, and healthy

controls have also a significant lower level of MMP-7 than non metastatic CRC.

Our results in controls (median MMP-7 level of 3.5, range 0.5 to 16 ng/mL) are consistent with previously published data, where healthy controls (n=28) had a mean MMP-7 serum level of 3, 2 μg/L; st. dv. 1, 5)[43].Although median and mean are different parameters, due to the absence or normality in our sample the former was chosen for description. Both are measurements of central tendency and this conclusion seems to be appropriate.

We have made the observation that serum MMP-7 determinations in advanced CRC are not always homogeneous. Not all patients with advanced colorectal cancer have elevated serum MMP-7 levels compared with healthy controls, suggesting that not all tumours secrete MMP-7 or the protein is secreted at a very low level. Protein levels may therefore reflect differences in the biologic characteristics of cancer cells. We speculate that possibly MMP-7 expression levels are also a qualitative marker in advanced CRC. Advanced CRC expressing high levels of MMP-7 would be related to more invasive, growing and metastatic tumours.

Cancer progression and apoptosis, depends on the interplay between cancer cells, the immune system and the microenvironment. MMP-7 could work as a link between these three major actors. MMP-7 is activated by MMP-2 and MMP-9, both produced by stromal cells, and is also transcriptionally activated by the beta-catenin-tcf-4 complex and oncogenic mutations of *Kras*[44] in tumor cells. Our group and others, have demonstrated that MMP-7 blocks lymphocyte cytotoxicity, by cleaving of the NH_2-terminal ‘’preligand assembly domain’’ of FAS membrane (FASm)[45] and the extracellular surface of FASL membrane (FASLm)[17] leading to a decreased sFAS/sFASL ratio[46], and therefore escaping from the immune system and favoring resistance to chemotherapy. Recently, Wang et al.[47] have demonstrated that MMP-7 increases resistance to Fas-mediated apoptosis and, the authors conclude that high MMP-7 tissue expression is a poor prognostic factor of patients with CRC. Vargo-Gogola T, et al[48] have determined that FasL

cleavage from the cell surface by MMP-7 provides apoptosis resistance and subsequently leads to tumor formation in murine mammary glands. Moreover, in another study, specific cleavage of Fas by MMP-7 resulted in decreased sensitivity of HT-29 colon carcinoma cells to Fas-mediated apoptosis[47]. In the same study, the authors found a markedly increased susceptibility of these cells to Fas-mediated apoptosis when their MMP-7 expression was suppressed by transient transfection of the antisense oligonucleotide for this proteinase. There is then a strong suggestion that increased levels of MMP-7 are associated with the development of refractoriness to chemotherapy agents. Although, this issue requires further clinical validation, in vitro and in vivo studies[47, 48] are highly suggestive of this association which has great clinical implications.

We have observed (unpublished data) that in patients with low MMP-7 but high LDH levels, MMP-7 values can increase during chemotherapy treatment, and would be therefore implicated in early acquired resistance, after initial response. Therefore, we speculate that MMP-7 would be implicated in primary chemo-resistance in the subgroup of patients with well known poor prognosis features, to an even more aggressive phenotype, or both.

This study has some potential limitations. First, only patients suitable for chemotherapy treatment were selected for analysis. We could not rule out that, if patients with favorable characteristics (liver involvement only and, less than 4 nodules and less than 5 cm) or poor prognostic features (performance status >2 or fragility criteria) had been also included, results would have been different. Secondly, although Oxaliplatin-5-fluorouracil or Irinotecan-5-fluorouracil combinations have similar activity in randomized trials, it would be desirable to confirm these results in prospective large series with more homogeneous treatments. Third, we can not rule out MMP-7 being an estimator of tumor burden, but it is clear that MMP-7 is correlated to but not a reflection of LDH, which up to date is the best, even not definitely proven, tumor burden estimator in colorectal cancer. We have

found 12% of patients with high LDH having low MMP-7 levels, which seem to be associated to a slightly better prognosis, compared with those with both high LDH and MMP-7 levels. This finding indicates that in our series, same tumor burden would be differently expressed by LDH and MMP-7. Moreover, in our work, high serum MMP-7 seems to correlate with liver involvement during the metastatic spread, but not with the number of involved organs. Finally, MMP-7 analysis has been done in serum and we have not included studies validating the correlation between MMP-7 expression in primary and metastatic tissues and serum MMP-7.

Detection of serum MMP-7 is feasible and done through a non-invasive technique. It is clearly a good tool not only to detect the subgroup of poor prognosis among de advanced CRC patients, but also it would be worthwhile to use it to evaluate serum MMP-7 levels as a potential marker of liver progression in non-metastatic colorectal cancer.

In summary, the key finding of our study is that having increased MMP-7 levels, in advanced CRC patients, is an independent prognostic factor for survival. This finding might imply a novel strategy for better classification of advanced CRC patients in prospective studies. This is to our knowledge the first time that such an association is reported in advanced CRC. Furthermore, these results represent the clinical confirmation of previous studies performed, either in human tissue samples or in preclinical studies, by other investigators. In our sample, high levels of serum MMP-7 indicate a poor prognosis phenotype, among advanced CRC patients, irrespectively of LDH values. Our results confirm the hypotheses of MMP-7 being a biologic marker selecting for a subset of advanced CRC patients with a qualitative aggressive phenotype. This poor prognosis phenotype can be related to increased ability to growth, invade and disseminate, but also to acquisition of chemoresistance.

3.2 Evaluation of FAS/FASL soluble fractions variation during time and its correlation to tumor response

"FAS/FAS Ligand ratio: A marker of Oxaliplatin-based intrinsic and acquired resistance in advanced colorectal cancer"

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Abstract

Purpose

Oxaliplatin-5-fluorouracil combinations have increased responses in first-line therapy up to 40% in advanced colorectal cancer. Unfortunately, those patients who will respond are unknown and initially sensitive patients become rapidly resistant to current therapies. FAS (CD95) and FAS ligand (FASL; CD95L) have been implicated in chemosensitivity through leading to apoptosis in response to DNA-damaging drugs. Whereas the proapoptotic role of FAS and FASL is well characterized, the function of their soluble forms as predictors of chemosensitivity remains unknown.

Patients and Methods

Blood samples were obtained from68 patients with advanced colorectal cancer who received oxaliplatin-5-fluorouracil combinations in first-line

therapy. Computed tomographic scans were done every 3 months and responses were evaluated by Response Evaluation Criteria in Solid Tumors criteria. ELISA soluble FAS and soluble FASL analysis were done before treatment and every 3 months until disease progression. Ratios between soluble FAS and soluble FASL were established and its values and variations through time were related to treatment responses.

Results

We found a significant increase in soluble FAS levels and a significant decrease in FASL at 3 months compared with baseline (13.2 versus 10.02 ng/mL; P = 0.0001; 0.07 versus 0.14ng /mL; P = 0.007, respectively). A significant increase in the soluble FASL levels up to 9 months (fourth to fifth extractions; 0.26 ng/mL) of therapy compared with first to third extractions (0.11ng/mL; P = 0.003)was also found. A random effect regression statistical model determined that >1.2-fold increase in soluble FAS/soluble FASL ratio was a marker of chemosensitivity (P = 0.001).

Conclusions

These data strongly indicate that an increment of soluble FAS/soluble FASL ratio after treatment could be an excellent marker of chemosensitivity in colorectal cancer. On the other hand, a decreased ratio after treatment can be a predictor of chemoresistance despite an initial response.

Introduction

Colorectal cancer is the most common cancer in western Europe, with f70 new cases a year per 100,000 inhabitants. In spite of advances in screening, 15% to 20% of patients show initially advanced disease, and 30% to 50% are destined to metastasize. Recently, oxaliplatin/5-fluorouracil (5-FU)/leucovorin or irinotecan-5-FU/leucovorin have increased responses in first-line therapy up to 40%, with median survival between 16 and 20 months, but 2-year overall survival still remains <20% (1, 2). Unfortunately, those patients who will benefit from first-line chemotherapy are unknown.

Furthermore, the initially sensitive patients become rapidly resistant to current therapies.

The FAS (CD95) receptor is a cell surface protein that mediates apoptotic cell death on triggering by FAS ligand (FASL). This interaction causes FAS receptor homo-oligomerization and this leads to activation of the caspase cascade (apoptotic extrinsic pathway). A FASL-independent activation of the FAS receptor has also been described (3).

Whereas proapoptotic role of FAS and FASL are well known, more conflicting data come from functionality of soluble forms. Various forms of soluble FAS (sFAS) have been described derived from alternative splicing phenomena (4). The majority of these spliced forms have an oligomerization domain, which allows them to form homotrimers (between soluble forms) and heterotrimers (when joining to transmembrane FAS receptor).

A dual antiapoptotic or proapoptotic function has been advocate for these soluble forms. When they form heterotrimers, they are counteracting the apoptotic signaling (5). While forming homotrimers, they are capable of interacting with transmembrane FASL leading to a proapoptotic effect (6). More convincing data of an antiapoptotic function of soluble FASL (sFASL; resulting from the cleavage of FASL by metalloproteinase-7; ref. 7) or a marginal proapoptotic function (8) has been proposed. The extrinsic apoptotic pathway seems to be physiologically compromised during colorectal cancer progression. It has been shown that adenoma through carcinoma step leads to FASL upregulation and FAS down-regulation (9). sFAS levels have been proven to be elevated in serum of patients with colorectal cancer (10), whereas some colorectal cell lines have turned to be releasing sFASL (11). Together, all these data support the hypothesis of the acquisition of a FAS/FASL apoptotic resistance profile as well as an immunoescape capacity during colorectal cancer progression (12). Cytotoxicity due to 5-FU/leucovorin treatment in p53 wildtype colorectal cell lines can be mediated via FAS (13, 14). It has also been described that this stimulus can produce apoptosis in p53 mutant cells (15). In colorectal

cancer cells, there was an increased level of FASL and apoptosis induction during thymineless death after 5-FU treatment, via activation of nuclear factor-nB and activator protein-1 transcription factors (16), as well as in thymidylate synthase–deficient cells, after treatment with DNA-damaging agents (17). Other drugs, such as camptothecin, seem to induce cell death through recruitment of the FAS-FADD adaptor in a FASLindependent fashion (18, 19). Therefore, it seems that 5-FU (20), capecitabine (21), and antimetabolite therapies can restore the lost of apoptotic capacity of colorectal cancer cells in vitro, either p53 wild-type or mutant, through the extrinsic pathway by regulating FAS and FASL expression and/or function.

Because of the mentioned chemotherapy capacity to modulate FAS/FASL, we hypothesize that these drugs could also modulate the soluble forms and therefore its role in regulating the apoptotic response through the extrinsic pathway as well as the immunologic ‘‘counterattack.’’ Because soluble forms (sFAS and sFASL) can have opposite effects, the ratio between them (sFAS/sFASL) could be a way to measure the final balance of apoptotic and immunoescape effect. This ratio and its variations along chemotherapy treatment could be therefore a useful variable to measure colorectal cancer chemosensitivity and chemoresistance.

Materials and Methods

Patients

Blood samples were obtained from 68 patients treated for advanced colorectal cancer from July 2001 to September 2003. Patients received 85 mg/m2 oxaliplatin on day 1, 200 mg/m2 leucovorin on day 1, and 3 g/m2 5-FU on day 1 in 48-hour continuous infusion every 2 weeks for a maximum of 12 cycles (n = 55) as standard treatment in our institution. Thirteen patients were treated with other oxaliplatin-fluorouracil combinations in multi-institutional clinical trials: 85 mg/m2 oxaliplatin on day 1 and 2.25 g/m2 5-FU on day 1 in 48-hour continuous infusion weekly every 2 weeks (n

= 3), 130 mg/m2 oxaliplatin on day 1, and 1,000 mg/m2 capecitabine on days 1 to 14 every 3 weeks (n = 3) or FOLFOX-4 (n = 7). Eligible criteria were stage IV histologically proven colorectal cancer, measurable metastatic lesions by Response Evaluation Criteria in Solid Tumors criteria, Eastern Cooperative Oncology Group performance status score of 0 to 2, no previous neoplasm in the last 10 years, normal liver and renal function, and no previous chemotherapy for advanced disease. All patients had chest X-ray and a helical computed tomographic (CT) abdominal scan before entry into study and underwent repeated evaluations at least every 3 months. Tumor response was assessed according to Response Evaluation Criteria in Solid Tumors criteria (22) as complete response, partial response, stable disease, and progressive disease. Each tumor measurement by CT scan was compared with previous CT scan. Therefore, patients with initial partial response in first evaluation (second CT versus initial CT) and with stabilization on the second evaluation (third CT versus second CT) were defined as stable disease instead of confirmed partial response. Only those patients with new partial response in second evaluation were defined again as partial response. Patients gave signed informed consent before treatment and the study was approved by the institutional ethics of research committee.

Samples and assay

Venous blood samples were drawn into sterile vacuum tubes before the initial treatment and every 3 months until disease progression for a maximum of five extractions (month 12). We have limited the number of extractions to 5 because >90% of the patients have progressed at that time. Blood samples were kept at 4C, centrifuged at 10,000 rpm for 15 minutes, and then immediately frozen at -80C until assayed. FAS and FAS ligand–specific ELISA. A double-antibody sandwich ELISA was constructed to detect sFAS and sFASL in sera using a sFAS and sFASL ELISA kit (Oncogene Research Product, San Diego, CA). This assay uses FAS and FASL antibodies against two epitopes. Standard curves were constructed using serial dilutions of recombinant sFAS and sFASL. The maximum

detectable concentration of sFAS was determined as 100 ng/mL. The maximum and minimum detectable concentrations of sFASL were determined as 1.25 and 0.01 ng/mL, respectively.

Statistical methods

The Mann-Whitney test was used to assess significant associations between continuous variables (FAS and FASL levels) and dichotomous variables [sex, upper limit of normal lactate dehydrogenase (>1 versus <1), number of organs involved (1 versus >1), disease location (liver versus other than liver), adjuvant chemotherapy, previous radiotherapy, and initial Dukes stage (synchronic versus metachronic)]. The Wilcoxon test was also used to ascertain FAS and FASL variations during chemotherapy treatment. The Kruskal-Wallis test was used to assess significant differences in FAS and FASL levels within multiple groups (i.e., Eastern Cooperative Oncology Group performance status). Complete response and partial response were considered as ‘‘sensitive’’ and stable disease and progressive disease were considered as ‘‘refractory.’’ A random effect regression statistical model evaluated the effects of time/therapy on response. A univariate and multivariate analysis for all variables influencing on response was also done. Only variables with a borderline significance ($P < 0.1$) at univariate analysis were included in the multivariate regression model.

Results

Patients and tumor characteristics

Demographic details on the 68 patients included in the study and tumor stage are shown in Table 1.

Table 1. Patient characteristics

	n (%)
No. patients	68
Sex	
Male	42 (61.8)
Female	26 (38.2)
Age, y	
Median	63
Range	33-80
Eastern Cooperative Oncology Group performance status	
0	28 (41.2)
1	27 (39.7)
2	13 (19.1)
No. organs	
1	47 (69.1)
>1	21 (30.9)
Organs involved	
Liver	53 (77.9)
Other than liver	15 (22.1)
Previous adjuvant chemotherapy	15 (22.1)
Previous radiotherapy	7 (10.3)
Response	
Complete response	1 (1.5)
Partial response	30 (44.1)
Stable disease	21 (30.9)
Progressive disease	14 (20.6)
Not evaluable	2 (2.9)

The median of received treatment cycles was 9 (range, 1-12). Twelve of the 68 patients had undergone radical procedures after chemotherapy treatment (11 underwent surgical resection and 1 radiofrequency thermal ablation) but were fully evaluable for response. Two patients were not evaluable for response due to complications after first cycle (1p with pulmonary embolism and 1p with intestinal occlusion).

FAS/FAS ligand levels

Sera were obtained from 68 patients diagnosed with advanced colorectal cancer during the study period with a total of 160 extractions. From 66 patients assessable for response, the average of extractions was 2.4 (range, 1-5). Reasons for extraction discontinuation were per protocol (n = 0.21; median, 3.4; range, 2-5), radical treatment after chemotherapy (n = 0.12; median, 2.2; range, 1-3), patient withdrawal consent (n = 0.1; median, 2), poor medical condition after rapid progression disease (n = 0.4; median, 1), and finished study period (n = 0.28; median, 1.8; range, 1-4). There were no significant associations between sFAS and

sFASL levels and any of the following variables: sex (P = 0.24 and 0.38, respectively), previous chemotherapy treatment (P = 0.32 and 0.35, respectively), lactate dehydrogenase levels (P = 0.43 and 0.77, respectively), previous radiotherapy (P =0.39 and 0.9, respectively), synchronic or metachronic disease (P = 0.37 and 0.21, respectively), number of organs involved (P = 0.45 and 0.31, respectively), and liver involvement (P =0.42 and 0.39, respectively). There were also no significant differences between sFAS and sFASL levels among patients with different performance status grades (P = 0.10 and 0.51, respectively; see Table 2).

Table 2. Baseline characteristics and association with sFAS and sFASL

Characteristic	sFAS (ng/mL)	sFASL (ng/mL)	Ratio
Sex			
Male	11.0 ± 14.3	0.15 ± 0.18	276.5 ± 411.8
Female	8.3 ± 2.5	0.11 ± 0.11	367.9 ± 488.1
Lactase dehydrogenase			
Greater than upper limit of normal	12.2 ± 17.7	0.18 ± 0.24	362.9 ± 553.6
Less than upper limit of normal	8.5 ± 2.8	0.11 ± 0.1	277.6 ± 334.5
No. organs			
1	10.6 ± 13.6	0.15 ± 0.19	275.7 ± 417.5
>1	8.6 ± 3.1	0.1 ± 0.09	391.4 ± 466.4
Location disease			
Liver	10.1 ± 12.9	0.14 ± 0.18	291.5 ± 451
Other	9.3 ± 2.3	0.11 ± 0.11	382.0 ± 367.5
Previous adjuvant			
Chemotherapy			
No	10.2 ± 12.9	0.14 ± 0.18	349.3 ± 466.1
Yes	9.2 ± 2.3	0.14 ± 0.1	177.6 ± 256.4
Previous radiotherapy			
No	10.0 ± 12	0.14 ± 0.17	314.9 ± 443.4
Yes	9.6 ± 2.7	0.12 ± 0.09	281.4 ± 355.4
Eastern Cooperative Oncology			
Group performance status			
0	7.7 ± 2.5	0.11 ± 0.1	280.6 ± 335.1
1	9.6 ± 3.2	0.18 ± 0.24	310 ± 451.4
2	15.7 ± 25.4	0.1 ± 0.08	424.2 ± 577.1
Initial Dukes stage			
Synchronic	10.6 ± 13.1	0.11 ± 0.1	363.6 ± 489.8
Metachronic	8.2 ± 2.1	0.15 ± 0.28	155.0 ± 246.4

NOTE: Mean ± SD (n = 68). All Ps are nonsignificant.

We found a significant increase in sFAS levels and a significant decrease in FASL at 3 months compared with baseline (13.2 versus 10.02 ng/mL; P = 0.0001; 0.07 versus 0.14 ng/mL; P = 0.007, respectively). The median of FAS/FASL ratio increment was 1.2-fold. A significant increase in the sFASL levels up to 9 months (fourth to fifth extractions; 0.26 ng/mL) of therapy

compared with first to third extractions (0.11 ng/mL; P = 0.003) was also found (see Table 3).

Table 3. Circulating sFAS and sFASL and ratio during treatment

Extraction	*n*	sFAS (ng/mL)	sFASL (ng/mL)	Ratio
Basal	68	10.02 (2.9-100)	0.14 (0.01-1.25)	311.5 (5.2-2,000)
3 mo*	46	13.2 (5.7-100)	0.07 (0.01-0.39)	626.6 (27.4-2,170)
6 mo†	26	11.9 (3.5-22.3)	0.11 (0.01-0.46)	313.5 (38.4-1,450)
9/12 mo‡	20	10.3 (6.1-16.7)	0.26(0.01-1.25)	268.6 (13.2-1,670)

NOTE: Mean (range; *n* = 160).
*P = 0.0001 for sFAS basal (Wilcoxon test).
†P = 0.007 for sFASL basal (Wilcoxon test).
‡P = 0.003 for sFASL basal (Wilcoxon test).

Response to chemotherapy

The overall response rate was 45.6%. The levels of the FAS/FASL ratio increment in the group of complete response and partial response (i.e.,‘‘responding’’ tumors; mean, 14.2; range, 0.06-188.4) were significantly different from the levels in the stable disease and progressive disease group (i.e., ‘‘nonresponding’’ tumors; mean, 2.2; range, 0.02-29.2; P = 0.005, Wilcoxon test; Table 4).

Table 4. Association of FAS/FASL changes and response to 5-FU/oxaliplatin

Response	FAS	*P**	FASL	*P*	Ratio	*P*
Complete response/partial response	1.48 (0.56-3.17)	0.05	1.85 (0.01-18)	0.019	14.2 (0.06-188.4)	0.005
Stable disease/progressive disease	1.18 (0.06-2.7)		4.08 (0.04-46)		2.29 (0.02-29.2)	

NOTE: Mean (range).
*Wilcoxon test.

A random effect regression statistical model evaluated the effects of time/therapy on response. We determined that a >1.2-fold increase in sFAS/sFASL ratio was a marker of chemosensitivity (P = 0.001). In addition, we have found a predictor of chemoresistance in a subgroup of patients who, despite presenting a high ratio and an initial CT response, rapidly developed a decreased ratio during treatment, indicating the appearance of chemoresistance. In the univariate analysis of response, only performance status (P = 0.05) and age (P = 0.1), but not lactate

dehydrogenase (P = 0.7), previous adjuvant treatment (P = 0.38), carcinoembryonic antigen (P = 0.33), and number of organs involved (P = 0.93), had a borderline significance. A multivariate regression analysis of response with the relevant clinical variables (age, performance status, and sFAS/sFASL ratio) was done, and only sFAS/sFASL ratio (P = 0.003) and age (P = 0.025) remain as independent factors predicting response.

Discussion

In the present study, the mean of sFAS/sFASL basal levels (sFAS, 10.02 pg/mL; sFASL, 0.14 pg/mL) is similar to that reported previously (23–25). In accordance to some authors, we have not seen any significant relation between sFAS and/or sFASL levels and variables such sex, age, or performance status (23, 24). We have also observed a lower basal level sFAS (8.2 ng/mL), but without reaching significance (P = 0.37), in patients with metachronic compared with synchronic disease (10.6 ng/mL), in accordance with the well-known chemoresistance of this group of patients in randomized advanced colorectal cancer trials (2, 26). We also noted a higher basal levels of sFAS (12.2 ng/mL) in those patients with serum lactate dehydrogenase >1 upper limit of normal compared with 8.5 ng/mL (P = 0.43), also a well-defined, poor prognosis factor of survival in colorectal cancer (1, 2) and described previously in advanced melanoma (27). These data could explain previous reports associating poor prognosis with sFAS levels in gynecologic malignancies and melanoma (24, 25). Also in our knowledge, for the first time in the literature, we have shown a significant increase in sFAS levels after chemotherapy treatment (P = 0.0001). In addition, we have also noted that a ratio increment correlates with tumor response and the subsequent decrease is related to chemoresistance (P = 0.001). Despite these data, it is unclear how chemotherapy regulates, if it does, sFAS and sFASL functions.

We hypothesize that, in advanced colorectal cancer, tumor production of soluble splicing variants (amount and type) leads to a proapoptotic action

(through transmembrane FAS interaction), much more than to an antiapoptotic one. Because in the advanced stages of the disease the matrix metalloproteinases (like matrix metalloproteinase-7) are more active (27–29) and lead to an increase of the sFASL fractions, it is plausible that these events may have a global antiapoptotic and immunoevading action. Supporting this theory, high levels of sFASL have been observed in metastatic pancreatic carcinoma, a notorious resistant neoplasm (30). In mammary tissues from multiparous matrilysin (matrix metalloproteinase-7)–expressing mice, there was decreased FASL expression, suggesting that loss of FASL expression is at least one mechanism of matrilysin-induced resistance to apoptosis (31). Furthermore, CTLs trigger FASmediated apoptosis only after treatment with metalloproteinase inhibitors (matrix metalloproteinase-1). Matrix metalloproteinase-1 induces apoptotosis by increasing the surface expression of FASL and disappearance of sFASL (32).

There have been multiple reports in the literature measuring basal levels of sFAS and sFASL in different neoplasms. However, this is the first study that reflects the dynamics of these soluble fractions during chemotherapy treatment. We conclude that a 1.2-fold increase of FAS/FASL ratio, after receiving chemotherapy, indicates chemosensitivity in colorectal cancer. In addition, a ratio decrease during chemotherapy treatment, despite the initial values, is related to accquired chemoresistance. We suggest that sFAS/sFASL ratio can be useful as a dynamic response predictor in colorectal cancer patients following chemotherapy.

4 DISCUSSION AND PERSPECTIVES

Current prognostic and predictive markers in ACRC are not clearly defined, as they have not been properly validated in well-designed, prospective and powered clinical trials.

At the present time, we still do not know which type of ACRC we are facing up or the way it is going to behave in terms of aggressiveness and response to therapy. There is no way to determine patients who will not benefit from systemic therapies, or those who will rapidly progress after an initial response.

As Benson indicated in 2006 American Society of Clinical Oncology (ASCO) meeting, "innumerable challenges remain, the understanding of which will be essential to move from generic treatment strategies to principles of rational treatment selection driven by prognostic and predictive human biologic data."

This scenario points to different needs. First of all, suggested markers of prognosis and response to therapy should be prospectly validated, in order to do one step ahead in classifying ACRC patients. Secondly, once validated, clinicians should implement them in both clinical trials and routine practice. Third, there is a need of finding markers accurately reflecting ACRC aggressiveness and response to therapy, which means markers with prognostic and predictive value. As tumors change over time, aggressiveness and resistance to therapy can also vary. So, those markers should be able to reflect those values anytime during disease evolution. This fact implies that markers should be easy to obtain, through a non-invasive technique, such as venopuncture.

FAS and FASL are proteins that have been related to chemotherapy apoptotic response. ACRC show a FAS/FASL chemoresistant pattern, but the role of their soluble forms in chemoresistance has never been explored. MMP-7 has been related to an aggressive phenotype in ACRC, but also to

chemorresistance through FASL cleavage. Serum sFAS levels have been shown to be increased in ACRC patients. Serum measurements of sFASL and MMP-7 have never been done in ACRC patients.
Our first approach was based on establishing the role of sFAS/sFASL and MMP-7 serum levels as biologic markers of prognosis and predictors of response to therapy in ACRC.

In the first work, we have shown that serum MMP-7 levels are significantly elevated in patients with ACRC, compared to non-metastatic CRC and to healthy controls. We have made the observation that serum MMP-7 determinations in ACRC are not always homogeneous. Serum MMP-7 levels do not strictly correlate to LDH levels, which is the best estimator of tumor burden up to date. The fact that not all patients with ACRC have elevated serum MMP-7 levels suggests that not all tumours secrete MMP-7 or the protein is secreted at a very low level. According to MMP-7 serum levels, ACRC patients can be divided in two groups. Those with high levels tend to show a dismal outcome while those with low levels are more likely to have a more indolent disease. Protein levels may therefore reflect differences in the biologic characteristics of cancer cells. We speculate that possibly MMP-7 expression levels are also a qualitative marker in ACRC. As it has been shown in CRC primary tumors, high levels of MMP-7 correlate to an aggressive phenotype and poorer prognosis[152-157]. Serum MMP-7 levels seem to be a good estimator of tumor agressiveness within ACRC patients.
Our data confirm that high serum MMP-7 levels tend to correlate with currently used clinical adverse parameters such as high LDH, ALP, CEA levels, liver involvement and poor performance status. Being LDH one of the most accepted clinical parameter to determine prognosis, we tested MMP-7 together with LDH, to determine the prognostic significance of different combinations. The Kaplan Meier curves show statistically significant difference (p=0,003) between curves expressing high and low MMP-7 levels, despite being associated with high or low LDH levels.

Patients with high LDH and low MMP-7 levels seem to be associated to a slightly better prognosis, compared with those with both high LDH and MMP-7 levels. Moreover MMP-7 levels are more accurate in reflecting the risk of death than current used prognostic factors, such as LDH. In our sample, the risk of death associated to MMP-7 increase is significantly much higher than the risk of death associated to LDH elevation. Those findings suggest that MMP-7 is possibly even more accurate that LDH in determining prognosis in the group of ACRC patients.

In conclusion, we have stated that **basal determination of serum MMP-7 in ACRC patients is an independent determinant of prognosis.**

For the first time, we have determined MMP-7 soluble fractions in the serum of ACRC patients. Levels of total MMP-7 can be measured in human serum and it is feasible using a simple ELISA technique, as it has been recently shown in few other studies[204, 205]. As MMP-7 is secreted to the media by tumor cells, serum measurements of total MMP-7 can be considered as an indirect estimation of tumor MMP-7 expression. Other techniques, such as zymography, are useful to distinguish between activated MMP-7 and pro-forms, and might be implemented in the near future for further analysis.

Our work describes how MMP-7 serum levels can distinguish between healthy controls, non-metastatic CRC and ACRC. Non-metastatic CRC patients have significant lower levels of MMP-7 than ACRC, and healthy controls have also a significant lower level of MMP-7 than non-metastatic CRC. Our results in controls (median MMP-7 level of 3.5, range 0.5 to 16 ng/mL) are consistent with previously published data, where healthy controls (n=28) had a mean MMP-7 serum level of 3, 2 μg/L; st. dv. 1, 5)[204, 205]. Although median and mean are different parameters, due to the absence or normality in our sample the former was chosen for description. Both are measurements of central tendency and this conclusion seems to be appropriate.

Detection of serum MMP-7 is feasible and done through a non-invasive technique. It is clearly a good tool not only to detect the subgroup of poor prognosis among ACRC patients, but we were wondering if it also would be worthwhile to use it to evaluate serum MMP-7 levels as a potential marker of liver progression in non-metastatic CRC. Our work evaluating serum MMP-7 in a total of 176 non-metastatic CRC patients points out MMP-7 beig a good marker to predict disease progression ("Serum matrilysin (MMP7) levels are associated with progression, in curatively resected colorectal cancer (CRC) patients" **(2007 ASCO Meeting. Abs: 4124 P:N2; Martínez-Fernández et al.)**

This study has some potential limitations. First, only patients suitable for chemotherapy treatment were selected for analysis (Intermediate Stage). We could not rule out that, if patients with favorable characteristics (liver involvement only and, less than 4 nodules and less than 5 cm)(Early Stage) or poor prognostic features (performance status >2 or fragility criteria)(End Stage) had been also included, results would have been different. Secondly, although OXL-5FU or IRI-5FU combinations have similar activity in randomized trials, it would be desirable to confirm these results in prospective large series with more homogeneous treatments. Third, we can not definitively rule out MMP-7 being an estimator of tumor burden, but it is clear that MMP-7 is correlated to but not a reflection of LDH, which up to date is the best, even not definitely proven, tumor burden estimator in colorectal cancer. We have found 12% of patients with high LDH having low MMP-7 levels, which seem to be associated to a slightly better prognosis, compared with those with both high LDH and MMP-7 levels. This finding indicates that in our series, same tumor burden would be differently expressed by LDH and MMP-7. Moreover, in our work, high serum MMP-7 seems to correlate with liver involvement during the metastatic spread, but not with the number of involved organs. Finally, MMP-7 analysis has been done in serum and we have not included studies validating the correlation between MMP-7 expression in primary CRC tissue

and serum MMP-7. Despite of it, as MMP-7 is secreted to the media by tumor cells, serum measurements of total MMP-7 could be considered as an indirect estimation of tumor MMP-7 expression. Moreover, to our opinion, an accurate correlation between tissue and serum MMP-7 levels would need not only measurements in primary tumor but also in all metastatic lesions, which is not feasible.

In the second work, we present determinations of FAS/FASL soluble fractions in the serum of ACRC patients. In our study, the mean of sFAS/sFASL basal levels (sFAS, 10.02 pg/mL; sFASL, 0.14 pg/mL) is similar to that reported previously in other malignancies[206-208]. In accordance to some authors, we have not seen any significant relation between sFAS and/or sFASL levels and variables such sex, age, or performance status[206, 208]. We have also observed a lower basal sFAS level (8.2 ng/mL), but without reaching significance (P = 0.37), in patients with metachronic compared with synchronic disease (10.6 ng/mL), in accordance with the well-known chemoresistance of this group of patients in randomized ACRC trials[16, 17]. We also noted a higher basal levels of sFAS (12.2 ng/mL) in those patients with serum lactate dehydrogenase >1 upper limit of normal compared with 8.5 ng/mL (P = 0.43)[15, 16]. This data was previously described in advanced melanoma and could explain previous reports associating poor prognosis with sFAS levels in gynecologic malignancies and melanoma[207, 208].

There have been multiple reports in the literature measuring basal levels of sFAS and sFASL in different neoplasms. However, this is the first study that reflects the dynamics of these soluble fractions during chemotherapy treatment. To our knowledge, for the first time in the literature, we have shown a significant increase in sFAS levels after chemotherapy treatment (P = 0.0001). In addition, we have also noted that a sFAS/sFASL ratio increment (>1.2-fold) after receiving chemotherapy, correlates with tumor response, and thus indicates chemosensitivity in ACRC. **Also, sFAS/sFASL**

ratio decrement, despite initial values, is related to chemoresistance (P = 0.001). We suggest that sFAS/sFASL ratio can be useful as a dynamic response predictor in ACRC patients following chemotherapy. In conclusion, we have shown that **variation of soluble FAS and FASL levels in serum of ACRC patients, during chemotherapy treatment, correlates with tumor response to treatment and thus we have established its predictive value.**

But how are sFAS/sFASL and MMP-7 related to each other?

Cancer progression depends on the interplay between cancer cells, the host and the microenvironment. Serum MMP-7 and sFAS/sFASL could work as a link between these three major actors. MMP-7 is activated by MMP-2 and MMP-9, both produced by stromal cells, and is transcriptionally activated by the βcatenin/tcf-4 complex and oncogenic mutations of KRAS[148-151] in CRC cells. Other groups have demonstrated that MMP-7 blocks lymphocyte cytotoxicity, by cleaving the NH_2-terminal ‘’preligand assembly domain’’ of FAS membrane (FASm)[209] and the extracellular surface of FASL membrane (FASLm)[118]. Furthermore, cytotoxic T lymphocytes (CTLs) trigger FAS-mediated apoptosis only after treatment with metalloproteinase inhibitors[210]. All these mechanisms would favour CRC cells escaping from the immune system. Moreover, Vargo-Gogola T, et al [211] showed that in mammary tissues from multiparous matrilysin (MMP-7)–expressing mice, there was decreased FASL expression, suggesting that loss of FASL expression was at least one mechanism of matrilysin-induced resistance to apoptosis. Also, they determined that FasL cleavage from the cell surface by MMP-7 provides resistance to apoptosis and subsequently leads to tumor formation in murine mammary glands. Recently, Wang et al.[139] have demonstrated that MMP-7 increases resistance to Fas-mediated apoptosis in HT-29 CRC cell line. Authors also found a markedly increased susceptibility of these cells to Fas-mediated apoptosis when their MMP-7 expression was suppressed by transient transfection of the antisense oligonucleotide for this proteinase.

There is then a strong suggestion that increased levels of MMP-7 are associated with the development of refractoriness to chemotherapy agents.

MMP-7 is possibly implicated to therapy resistance by other mechanisms. As described in the diagram below (see FIG. 23), MMPs can cleave membrane-bounded proteins other than FAS and FASL, such as epidermal growth factor receptor (EGFR), insulin growth factor receptor (IGFR) or insulin growth factor binding protein-3 (IGFBP-3).

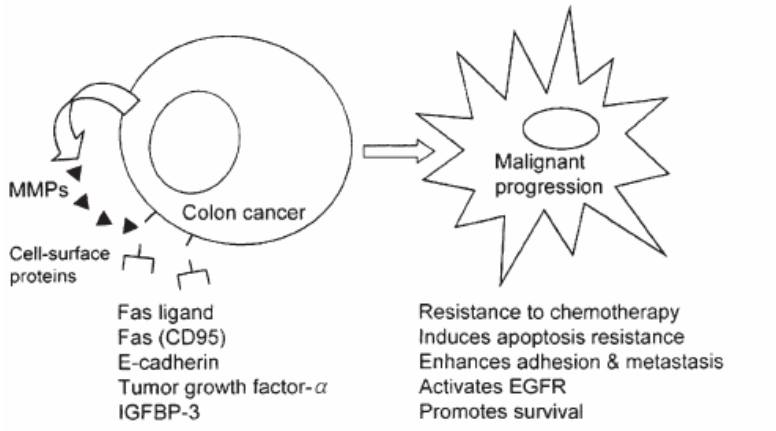

FIG. 23: MMPs can cleave membrane-bounded proteins, such as FAS, FASL IGFBP-3, TGFα, E-Cadherin helping to malignant transformation[148].

Along with this line, our goup has observed that chemotherapy resistant CRC cell lines show a clear increment of MMP-7 expression, either measured by immunoflorescence, ELISA and RT-PCR methods (non published "in vitro" data by Almendro et al.).

Analyzing non-published data from patients treated with OXL-based regimens (n=87), we also have noticed that those with initially high levels of serum MMP-7 (25%) show low response rates to chemotherapy, around 20%, (median overall survival (MOS): 11 months), while those with low basal MMP-7 and LDH levels (55%) show high percentages of response, around 64% (MOS: 25 months). In patients with initial low MMP-7 but high LDH levels (20%), initial response rates are even higher, around 78%, but

MMP-7 values seem to increase quickly during treatment and those initially responders become rapidly resistant (MOS: 13 months) (see FIG. 24 below)

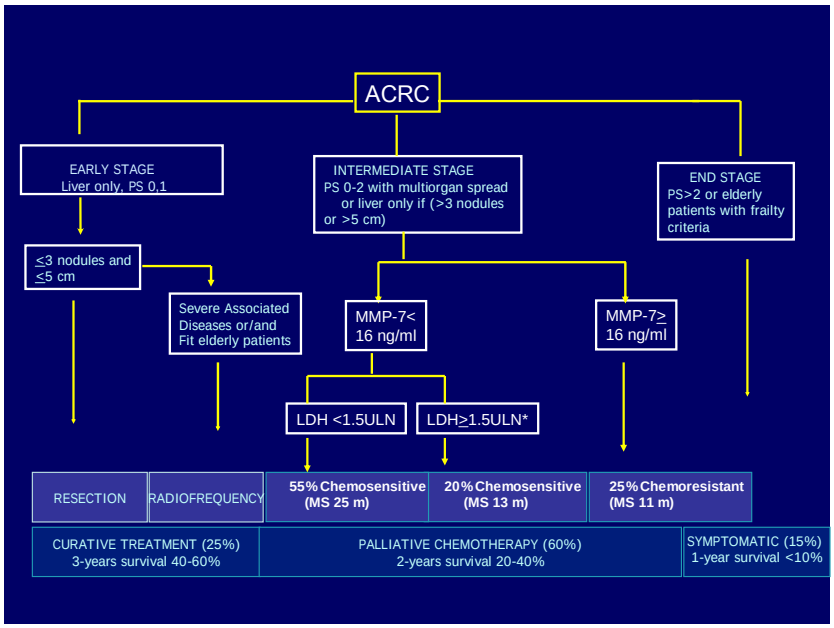

FIG. 24: Data from ACRC patients included in the first study.

Therefore, **we speculate that MMP-7 would be implicated in both primary and acquired chemoresistance**. Although "in vitro" and "in vivo" studies are highly suggestive of this association, this issue requires further clinical validation, as it has great clinical implications.

Putting together all the data we have now risen up a novel theory. ACRC patients express different levels of MMP-7 **(Maurel et al, Int J Cancer, 2007)**[212]. Our results confirm the hypotheses of MMP-7 being a biologic marker selecting for a subset of ACRC patients with worse prognosis. This poor prognosis can be related to increased ability to growth, invade and disseminate, but also to immunoevasion and therapy resistance, as expression of MMP-7 is related to FASL cleavage. Serum detection of high

soluble MMP-7 would be linked to increased levels of soluble FASL and a decrement of soluble FAS/FASL ratio, which is related to chemoresistance. As previously stated, an increment of sFAS/sFASL ratio would be related to chemosensitivity **(Nadal et al, Clin Cancer Res, 2005)**[145]. **A serum pattern with high MMP-7 and high sFASL would be then related to bad prognosis as well as to chemoresistance. ACRC patients with high MMP-7 and high sFASL, whether basal or during therapy, would show chemoresistance and a dismal outcome.** ACRC patients showing high basal serum levels of MMP-7 and sFASL would show intrinsic or primary resistance to therapies. Those ACRC patients presenting this pattern during treatment would show accquired resistance (see an example below in FIG. 25). **Also patients showing an increment of sFAS/sFAS ratio after treatment, would show chemosensitivity.**

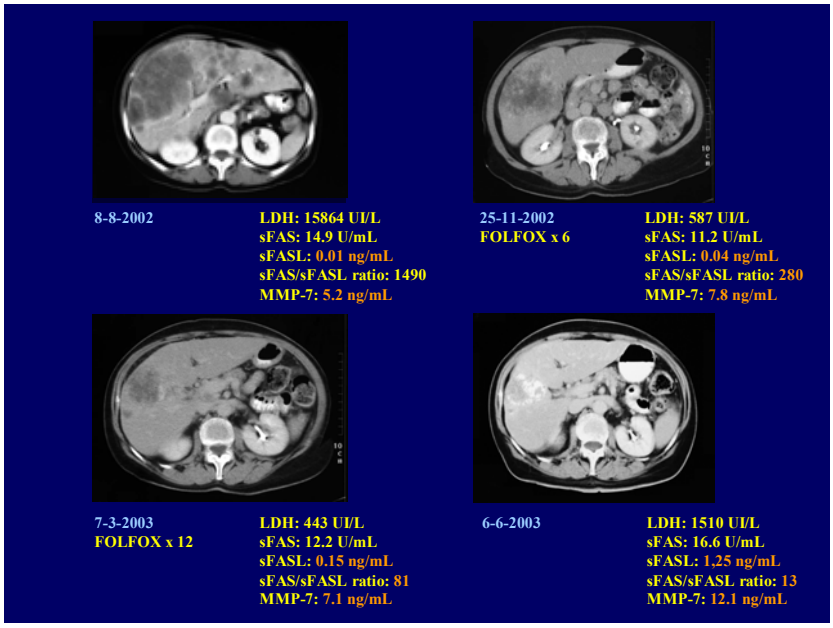

FIG. 25: An example of a patient with initial low serum MMP-7 levels and high LDH. She was treated with chemotherapy for 6 month with a decrease of LDH levels and clinical response. sFAS/sFASL ratio decreased

immediately after treatment. Three months after finishing treatment patient was asymptomatic and CT scan did not reveal a progression. But serum MMP-7 and sFASL were already increased. Two months after, disease progression was confirmed and patiet died within a month.

As these considerations might imply a novel strategy for better prediction of response and outcome in ACRC patients, we have designed a prospective multicentric trial in order to clarify the role of serum MMP7 and sFAS/sFASL in intrinsic and accquired chemoresistance **(HCB-05-1 Trial**: *"Estudio farmacodinámico evaluando el papel de MMP-7 y las formas solubles de FAS y FASL en pacientes tratados con Irinotecan y Cetuximab, en cáncer de colon metastático refractario a Irinotecan y Oxaliplatino"*.
In summary, we are determining MMP-7 and sFAS/sFASL serum levels in ACRC patients prior to receive a third line treatment with IRI+Cetuximab. (after failure to OXL and IRI). Serum is obtained before treatment, 48 hours after treatment **(Nadal et al, publication pendant)** at 2, 4, 6, 9 and 12 month, or until disease progression. Response assessment is done by RECIST criteria. The study has been design to see that ACRC patient sensitive to IRI+cetuximab will present an increment of the ratio at 48h compared with the resistant phenotype. Up to date, the study has recruited all initially planned patients (74). Analysis will be performed shortly. We will also analyze if high serum MMP-7 and sFASL is related to intrinsic and/or acquired chemoresistance.
We had also designed another prospective study to evaluate the role of basal serum MMP-7 and sFAS/sFASL as markers of therapy resistance in chemotherapy-naïve ACRC patients. It is a prospective, randomized and multicentric study by GEMCAD. Patients are randomized to receive up to six cicles of XELOX+Bevacizumab versus same schedule followed by Bevacizumab until disease progression. Primary end-point will be overall survival and secondary objetives will be progression-free survival and

quality of life. MMP-7 and sFAS/sFASL serum levels will be recorded initially and latter correlated to response and outcome.

As we pointed out before, there are no well-designed and powered studies prospectively validating prognostic and predictive markers in ACRC. Our goal is to analyze a complete ACRC database (GEMCAD database) and according to results, design and perform a prospective trial in order to validate prognostic and predictive markers.

Our current work in basic research is based on the study of MMP-7 mediated mechanisms of resistance. We are mainly focusing on EGFR, RAS and IGFR/IGFBP-3 systems (DrCodony, Dra Almendro).

5 CONCLUSIONS

In summary, we conclude that:

-Basal levels of MMP-7 can be measured in serum and are an independent determinant of prognosis in ACRC patients.

-Variation of serum sFAS/FASL levels in ACRC patients receiving chemotherapy correlates with tumor response

-Determination of a serum sFAS/sFASL ratio decrement, usually due to sFASL increment, is related to chemoresistance

-Serum sFAS/sFASL ratio could be used as a dynamic predictor of response to therapy in ACRC patients and its value shoul be validated in prospective trials

-Our observations, in both clinic and basic fields, point to MMP-7 as being implicated in both primary and acquired chemoresistance in ACRC

-A newly generated hypothesis is that a serum pattern with high MMP-7 and high sFASL, whether basal or during therapy, would imply chemoresistance and a dismal outcome.

-MMP-7 and sFAS/sFASL, as new proposed soluble markers, can be easily detected through a non-invasive technique.

-MMP-7 and sFAS/sFASL, as new proposed soluble markers, can be detected anytime during disease history, and dynamically reflect tumor biology, which changes over time.

-According to exposed results and hypothesis, we have designed prospective trials to determine the clinical relevance of serum MMP-7 and sFAS/sFASL, as new soluble markers of chemoresistance in ACRC.

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7 ANNEX

7.1 Nadal et al. World Journal Gastroenterology, 2007, in press

7.2 Maurel et al. International Journal of Cancer, 2007

7.3 Nadal et al. Clinical Cancer Research, 2005

7.4 HCB-05-1 Trial

Ms: wjg/2006/005440 **TOPIC HIGHLIGHTS (REVIEW)**

Is there a genetic signature for liver metastasis in colorectal cancer?

Running title: Liver metastases gene signature in colorectal cancer

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Abstract

Even though liver metastasis account for the vast majority of all cancer deaths in patients with colorectal cancer (CRC), fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered. Determination of gene expression profiles by microarray technology has improved our knowledge in CRC molecular pathways. However, defined gene signatures are highly variable among studies. Expression profiles and molecular markers have been specifically linked to liver metastases mechanistic paths in CRC. However, to date, any of the identified signatures or molecular markers has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice. To obtain a genetic signature for liver metastases in CRC, measures to improve reproducibility, to increase consistency, and to validate results need to be implemented. Alternatives to expression profiling by microarray technology have kept on being used. In the recent past years, many genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastases in CRC.

Key words: Colorectal cancer; Liver metastasis; Genetic signature; Expression profile; Arrays

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with a worldwide incidence of almost a million cases annually, both in males and females[1]. Despite of advances in screening, approximately 25% of patients have initially detectable liver metastases (synchronous metastases), and further 25% of patients will develop liver metastases during the course of their disease (metachronous disease) [2]. From all patients who will die of advanced colorectal cancer (ACRC), 60% to 70% show liver metastasis[3]. Metastatic spread to the liver is the major contributor to mortality in patients with CRC.

CRC is a genetically heterogeneous and complex disease. Initially, two major pathways were described as responsible for CRC tumorigenic process: the chromosomal instability pathway and the microsatellite instability pathway. The chromosomal instability or classical pathway accounted for 85% of the tumorigenic processes and was mainly characterized by the sequential allelic losses on chromosomes 5q (APC gene), 17p (TP53) and 18q (DCC/Smad4). The microsatellite instability pathway (MNI), also called the mutator phenotype, only accounted for 15% of the carcinogenic processes. Recently, it has been shown that colorectal carcinogenesis is much more complex, involving new pathways, as the serrated, the TGFβ/Smad and epigenetic pathways, and including infinity of non-pure or mixed pathways[4-6].

General mechanisms of tumorogenesis also include metastasis generation. But, is the knowledge referred to CRC tumorigenic pathways extensible to metastasis generation? What do we really know about the molecular determinants of liver metastases formation in CRC?

MECHANISMS OF LIVER METASTASIS

Colorectal liver metastasis, or dissemination and colonization by colorectal tumor cells coming from the primary CRC to the liver, is a complex process and has many different steps. In order to metastasize, tumour cells detach from the primary tumor, invade and migrate through the stroma and intravasate into the lymphatic and/or venous vessels. Whichever is the vasculature entrance, cells will mainly end up travelling through the portal vein system. During transportation they manage to survive to mechanical stresses and escape from the immune system. Same stresses keep on acting once cells arrest in the liver capillaries. Some of those arrested cells manage to adhere to endothelial cells, contact the extracellular matrix and extravasate to the surrounding tissues. Kupffer cells, belonging to the monocyte-macrophage system, are a perfect barrier to unwanted hosts. Being in the liver parenchyma, tumor cells establish a crosstalk with the stroma and create a microenvironment. Only if this microenvironment is favourable to tumor cells, signals of proliferation and neoangiogenesis will lead to macroscopic liver metastasis formation[7-9]. Even though liver metastasis account for the vast majority of all cancer deaths in patients with colorectal cancer, fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered.

Genetic signatures: The breakthrough

The availability of DNA arrays technology, allowing genome-wide analyses of gene expression, has been providing new insights in the determination of gene expression or transcriptional profiles. Expression profiling studies in CRC have mainly been focusing on comparisons of normal mucosa, adenoma and primary carcinomas. Few studies have thrown light to differences between primary tumors and metastases. For this reason and, in contrast to the many molecular alteration involved in the CRC adenoma to carcinoma step characterized to date, comparatively little information is available on the possible mechanisms of metastases, even less for liver specific metastases[10].

Talking about metastases means referring to two different aspects: The metastatic ability and the tropism or organ-specificity. Metastatic ability accounts for the potential to establish a distant secondary tumor. Organ-specificity or tropism means the capacity of doing it in a specific tissue. The ability to metastasize together with the specificity to do it in an organ and not to another can be genetically marked by what is called a metastatic signature.Studies checking for mRNA or protein levels take into account facts as expression regulation, splicing mechanisms, epigenetic phenomena, and complexity of post-translational changes or modifications. Then, a metastatic signature is not a gene list but a translation of a functional status of gene expression. Metastatic signatures are gene expression patterns conditioned by both an intrinsic gene composition and phenomena regulating its expression.

In order to determine metastatic signatures by microarray technology in CRC, three different strategies have been followed. (Table 1)

First approach consists on comparing transcriptional profiles of primary CRC from metastases-free patients to those affected by metastatic spread during a 5-year follow-up period. The main goal is finding gene expression profiles as prognostic markers of metastatic spread. Identification of a gene set capable of classifying CRC patients according to prognosis or 5-year survival rate was done by Bertucci *et al*[11]. A total of 219 and 25 genes were found to be respectively down- and up-regulated in metastatic samples when compared to non-metastatic. Moreover, a 46 gene set signature was isolated discrimining between CRC with and without lymph node metastases. Arango *et al*[12] checked the expression profile of Dukes C CRC and reported two different signatures according to survival. Barrier *et al*[13] built an accurate 30-gene tumor-based prognosis predictor for stage II and III colon cancer patients, based on gene expression measures. The group of Komuro *et al* [14] analyzed gene expression profiles in a total of 89 CRC. After stratifying by right and left locations, they were able to extract gene expression profiles characteristic of the presence versus absence of lymph node metastasis, with an accuracy of more than 90%. Kwon *et al*[15] analyzed the gene-expression profiles of colorectal cancer cells from 12 tumors. Sixty genes possibly associated with lymph node metastasis in CRC were selected on the basis of clinicopathological data. Wang *et al*[16] analyzed RNA samples from 74 patients with Dukes' B CRC. Gene expression profiling identified a 23-gene signature that predicted recurrence. This signature was validated in 36 independent patients. The overall performance accuracy was 78%. D' Arrico *et al* compared the transcriptional profiles of 10 radically resected primary CRC from patients who did not develop distant metastases within a 5-year follow-up period with those of 10 primary/metastatic tumor pairs from patients with synchronous liver metastases. The study was conducted on laser-microdissected bioptic tissues. Arrays of 7864 human cDNAs were utilized. Non-metastasizing primary tumors were clearly distinct from the primary/metastatic tumor pairs. Of 37 gene expression differences found between the 2 groups of primary tumors, 29 also distinguished nonmetastasizing tumors from metastases. Gene encoding for mannosyl (alpha-1, 3-)-glycoprotein beta-1,4-N-acetyl-glucosaminyl-transferase (GnT-IV) became significantly upregulated in primary/metastatic tumor pairs ($P < 0.001$), supporting the existence of a specific transcriptional signature distinguishing primary CRC with different metastatic potential[17].

Second approach consists on comparing gene expression of primary tumors with their matched metastases. Studies comparing gene expression between primaries and corresponding metastases indicate that they show a high transcriptional resemblance. The above mentioned study found a striking transcriptional similarity between the primary tumors and their distant metastases[17]. Another study by Koehler *et al*[18] determined expression profiles from 25 CRC and 14 corresponding liver metastases using cDNA arrays containing 1176 cancer-related genes. Most primary tumours and matched liver metastases clustered together. A specific expression

signature in matching metastases was not found, but a set of 23 classifier genes with statistically significant expression patterns in high- and low-stage tumours was identified. Gene expression studies in breast cancer also support the notion that primary tumors genetically ressemble much more to their matched metastasis than to their primary counterparts[19]. Agrawal *et al*[20] found a signature of 11 markers of tumor progression when comparing gene expression among different stages, including liver metastases in a total of 60 samples.

Utilization of CRC cell lines, with different metastatic potential, for expression profiling is another approach. Studies with cDNA microarrays have identified genes differentially expressed in primary versus metastatic CRC cell lines. Differential expression of 11 genes has been found between SW480 and SW620 CRC cell lines[21]. Unfortunately, metastatic signatures resulting from the above mentioned studies do not show too much in common. Gene expression patterns do not overlap enough to show consistency. Leaving patterns aside, few genes reported in at least two independent studies, have been linked to metastatic ability. (see Table 1 ***)

It is interesting to remark that any expression profile has been specifically linked to liver metastases in CRC. Leaving aside gene expression profiling, other techniques such as genomic profiling has also been used to determine metastatic ability in CRC. Genomic analyses of primaries and their matched metastases[23] showed that CRC primary tumors resemble to their corresponding metastases. Array-based comparative genomic hybridization (CGH) was used to detect genetic alterations in CRC that predicted survival after liver resection[24]. Genome wide copy number analysis revealed the involvement of Cycline D3 in liver metastases formation in CRC[25].

Genetic signatures: Handicaps and pitfalls

When determining metastatic expression profiles or signatures by array technology, several confounders have to be taken into account. Studies have important methodological differences. Those can be due to the use of different array platforms (Affymetrix, cDNA nylon membranes) or experimental conditions. Tissue sampling is almost always an issue. Availability of frozen tissues is not the norm in many institutions. Formalin-fixed or paraffin-embedded tissues usually yield low quality RNA and/or DNA. Creation of frozen-tissue tumor banks is rapidly increasing. Also methodology for RNA isolation can lead to different results. The number of samples used varies enormously in different studies. Relatively small cohorts of tumors have been analyzed in the referred studies, especially if they include the analysis of matched metastases. Selection of homogeneous samples among tumor heterogeneity can often be a problem. Anatomical localization (right *vs* left sided, colon *vs* rectum) and genetic instability status (MSI/classical) may justify the variability of the CRC gene expression profile characterized to date. Macrodissection techniques include tumor tissue with both tumor cells but also tumor stroma and valid tissue samples should include at least 50% of tumor cells. One of the major criticisms to "metastatic signature"-seeking works is the fact that tumors are analyzed as a whole, mixing tumor cells with microenvironment and stroma components. Certainly, data coming from these experiments is a mixture representing gene expression of tumor cells, stroma cells as well as their interactions. Moreover, expression data can be highly conditioned by host genetic background. Resulting data can be highly interesting in terms of defining prognosis, but not to understanding the mechanisms of metastasis generation. Microdissection techniques help to avoid this problem. Laser capture microdissection (LCM) allows isolation of only tumor cells and is considered the gold standard in microdissection procedures[26]. It is a time-consuming technique and it is not available in all institutions. Other strategies include subtracting non-tumor cells signatures from gene expression data [27]. It is still unclear whether the analysis of pure tumor cell populations will lead to an appropriate result in terms of indicating prognostic.

Description of metastatic signatures has been done in the basis of the transcription analysis of tumors. Data coming from DNA microarray analysis is often overwhelming and mixed. After collection, this amount of data needs to be analyzed. Analysis of differentially expressed genes is often altered by different criteria to define low-quality spots, distinct normalization procedures, different baseline references for ratio calculations, and arbitrary criteria for cut-off values applied to fold-change and significance level. Commonly, quantitative levels of expression are the basis to filter the raw data. During filtering, information coming from qualitative data can be lost [10]. Moreover, final data has to be interpreted and integrated to have sense, in biological

terms. This step is highly subjective, and probably is often leading to unreal conclusions. Near all studies lack of internal and external validation tests for the generated lists of genes. Different selection algorithms should be tested in order to improve the accuracy of the classifier sets [10].

In conclusion, to obtain a genetic signature for liver metastases in CRC, measures to improve reproducibility, to increase consistency, and to validate results need to be implemented.

Genes involved in liver metastases formation in CRC

Alternatives to expression profiling by microarray technology have also been used in the recent past years. Many genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastases in CRC[28]. (Table 2)

Adhesion: **Different proteins involved in adhesion/deadhesion processes have been linked to liver metastasis development in CRC. Deadhesion is a necessary step for tumor cells to detach from tumor and disseminate. Adhesion is needed for circulating cells to contact helping counterparts in the dissemination process. It is also needed to attach to the vascular endothelium, induce endothelial retraction, and subsequently bind to glycoproteins of the basement membrane to extravasate.**

E-cadherin/α-catenin is a cell to cell adhesion complex that keeps tumor cells together. Cells detaching from the primary CRC undergo an epithelial to mesenchymal transition, during which E-cadherin downregulatates in favour of other cadherins, such as N-cadherin. This process is known as the "cadherin switch" and leads to acquisition of a mesenchymal phenotype that favours invasion and migration through the stroma and thus dissemination of tumor cells[29]. Downregulation of E-cadherin/α-catenin expression has been related to tumor aggressiveness[30,31] and metastatic potential[32,33] in gastrointestinal cancers. Low expression of α-catenin and E-cadherin in CRC patients has been associated to an increment of β-catenin[34-36], advanced stages[33,37,38] and acquisition of metastatic potential[39,40]. Immunohistochemical studies show that CRC metastasizing to liver have a significant (P = 0.014) reduction or complete absence of E-cadherin expression when compared to non liver metastases[34].

Epithelial cell adhesion marker (EpCAM) is a widely expressed adhesion molecule. It has been found to present a more diffuse pattern and higher expression in CRC compared to non malignant tissues[41]. EpCAM has a role in modulating cadherin mediated cell-cell interactions[42] and its expression has been linked to downregulation of cadherin levels[43], suggesting that possibly this protein plays a role in ETM processes, facilitating migration and dissemination of tumor cells. Supporting this notion, isolation of EpCAM positive cells in blood samples of advanced CRC patients[44] has recently been achieved. All these preliminary data suggests that possibly EpCAM has a role in CRC cells dissemination. Whether there is or not liver specificity remains unknown.

Sialyl Lewis X (sLex or CD15s) and A (sLea) are oligosaccharides commonly found in surface glycoproteins of metastatic tumor cells[45]. sLex and sLea are natural ligands for E-selectin, a receptor which has been found to be expressed by activated endothelial cells. Interaction between sLex and sLea would induce endothelial adhesion of tumor cells and thus favour stasis, extravasation and metastases formation. sLex and sLea expression in primary CRC have been related to poor prognosis[46] and metastatic potential[46-48] in CRC patients. sLex and sLea stain significantly positive in vessel invasion CRC cells developing metastases compared to those that do not (71.4% *vs* 31%)[49]. sLex and sLea have been found to be present in the surface of tumor cells[50] in CRC patients developing liver metastases. In the same line, CRC liver metastases express sLex and sLea in a large proportion of tumor cells than in primary tumors[48,51]. E-selectin is overexpressed by endothelial cells from tumor and non-tumor vessels in CRC patients developing liver metastasis in contrast to those that do not[52,53]. In general, as it has been demonstrated in "in vivo" models, glycosilated ad syalilated mucins correlate to liver metastases formation[54]. Some proteins allow the adhesion of CRC cells to blood components, such as platelets and leukocytes. Among those proteins, P-Selectin and L-Selectin can be cited.

This interaction facilitates to tumor emboli formation favouring protection of tumor cells from immune attack and also enhancing their ability to contact blood vessels by mechanical factors. This interaction between tumor cells and blood cells also increases the contact with endothelial surface, facilitating stasis and thus enhancing chances of extravasation[55].

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein containing significant amounts of sLex and sLea. Expression of (CEA) has been clearly correlated to generation of liver metastases in experiments transfecting CEA to CRC cell lines or administering CEA to animal models previous to CRC cell injection[56]. Initially it was speculated that CEA would act as an adhesion molecule, facilitating tumor cell aggregation and interaction to endothelial surface. However, studies with immunosuppressed mice show that administration of intravenous CEA results in an increase of hepatic colonization and retention of CRC cells, but not an increase of adhesion[57]. Kupffer cells, expressing a CEA receptor, bind to and degrade it, activating a signaling cascade that ends up releasing IL-1, 6 and TNF-α which, in turn, facilitate CRC cells stasis and growth[58,59]. The ability to secrete CEA offers to CRC cells a selective advantage to form metastases in the liver.

Integrins are molecules that can bind to many ECM components such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing those integrins are more likely to adhere to ECM components surrounding microvasculature. High expression of α6β4 and α5β3 integrins has been related to a more aggressive CRC phenotype[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastases formation by CRC cells in animal models[62] and results have shown that αvβ5 integrin is useful as an adhesion molecule and its inhibition diminished liver metastases formation.

Osteopontin (OPN) is a secreted phosphoglycoprotein capable of binding and inducing integrin-mediated cell survival, motility and anti-apoptotic intracellular pathways. OPN has been isolated in gene expression profiling studies as a candidate marker for CRC progression[20]. CRC liver metastases express OPN at higher ratios than primary CRC or normal mucosa[63]. OPN upregulation can occur due to TCF4/LEF transcription factor activation[64]. Mechanisms by which OPN promotes liver metastases formation in CRC is unknown, but can be related to up-regulation of Upa[65], c-Met receptor and integrins[66].

Other adhesion molecules such as the intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) have been measured in ACRC patients showing higher serum levels when compared to non-advanced CRC or healthy controls[67,68]. Despite of that, neither clinical nor physiological relation has been established with specific development of liver metastases.

CD44 glycoprotein, more specifically v6 and v8-10 splicing variants, have been related to metastases and disease recurrence in CRC[69,70]. There is quite a bit of controversy in the real value of CD44 in liver metastases formation as plasma levels have not been linked to advanced stages of the disease[71] and immunohistochemical studies measuring CD44v6 staining have not found significant differences when comparing CRC metastasizing to liver or not[34].

Invasion: **Invasion processes are crucial to explain liver metastases formation in CRC. Invasion occurs mainly due to basal membrane and extracellular matrix (ECM) degradation in both intravasation and extravasation steps. Some of the enzymes responsible for degradation are proteases. Among proteases, matrix metalloproteases (MMPs), cathepsines and plasminogen activators are the most relevant.**

Matrylisin (MMP-7) is a proteolytic enzyme belonging to MMPs family[72,73]. It is synthesized and secreted by tumor epithelial cells as a 28-KDa proenzyme, that can be activated through proteolytic removal of a 9-KDa prodomain from the N-terminus. The soluble activated form binds to the tumor epithelial cell surface. Both active forms, the soluble and the membrane-bounded, have proteolytic activity. Its expression can be regulated by epidermal growth factor through transcription factors such as PEA3[74] or AP-1 and β-catenin/ tcf4 complex. By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2, MMP-9), through ectodomain shedding of E-cadherin[75] and

receptor activator of nuclear factor-kappa B ligand (RANKL) [76] or through cleavage of adhesion molecules, such as integrin β4 [77].

Matrilysin has been found overexpressed in CRC [78]. MMP-7 overexpression in localized CRC disease has been correlated with invasion and to liver metastasis formation[79,80]. Colorectal liver metastases show intense expression of MMP-7 compared to normal liver, and differences are more evident when comparing the MMP-7 activated form, measured by zymography, emphasizing the role of MMP-7 in CRC liver metastases formation[81]. Testing liver metastasis formation in "in vivo" models, it has been shown that treating colorectal cancer cells with MMP-7 specific antisense oligonucleotide leads to a decrease[82], while adding active MMP-7 results to an increase of liver metastasis generation[83].

MMP-9 and MMP-2 seem also to have a role in liver metastases formation in CRC. High MMP-9 and MMP-2 levels have been detected by immunohistochemistry in the tumor-stroma interface in both primary CRC and liver metastases[84,85]. Moreover, MMP-2 and -9 activities seem to be higher in metastasis than in the originating primary tumor[86]. A close correlation between high MMP-9 RNA levels and worse survival and higher risk of liver relapse after surgery has also been established[81].

Cathepsines have equally been implicated in liver metastases formation in CRC. They are a family of proteolytic enzymes with a wide variety of physiological functions. They act as serin-proteases, cystein-proteases or aspartate-proteases. They are stored as proforms in cell lysosomes and secreted to the ECM secondarily to inflammatory and oncogenic stimuli[87].

Cathepsins B, L and D are especially involved in ECM degradation in CRC. Their levels and activity[87-88] have been found to be elevated in the invasion edge of CRC. Still, Cathepsin B is the most valuable in determining invasion in CRC[89]. Cathepsin B degrades ECM directly or indirectly, by stimuling other proteases or blocking their inhibitors[87]. It can be detected in early stages of CRC but it is a good marker to determine metastatic disease[90,91]. High plasma and urine levels of Cathepsin B have been found in CRC patients[92]. "In vivo" experiments show that inhibition of Cathepsine B, by selective compounds, results in reduction of liver metastases formation up to a 60% and reduction of liver metastases burden up to an 80%[93]. A proteolytic profile, taking into account MMP and cathepsin expression, has been defined for CRC by some authors[94].

Urokinase plasminogen activator receptor (uPAR) is a factor involved in metastases development in several cancers[95,96]. Its binding to urokinase plasminogen activator (uPA) enhances plasmin production which, in turn, degrades ECM and activates pro-MMPs. Inhibition of uPAR expression is associated to decreased motility and invasiveness in the human CRC cell line HCT116[97]. High uPAR expression in CRC has been related to low 5-year survival[98]. Use of antisense uPAR mRNA in a nude mice model inhibited CRC liver metastases development[99].

During invasion, apart from basal membrane and ECM degradation processes, cancer cells have to migrate through the stroma. Acquisition of a mesenchymal phenotype during ETM and ability to survive independently of the tumor cell population are the clues for succeeding. To gain the ability to disseminate, tumor cells have to detach from the tumor population overcoming anoikis and transiting from an epithelial to a mesenchymal phenotype. As a principle, cells need to be in contact with other cells in order to survive. If they loss contact or penetrate to ECM they undergo through anoikis. Overcoming anoikis, an apoptotic program related to tumor cell population detachment, is a necessary requirement to disseminate. Integrins are responsible for epithelial cancer cell cross-talk with the ECM in order to overcome anoikis, survive and migrate.

"In vitro" experiments have shown that activation of Src and Akt pathways are linked to decreased sensitivity of detached CRC cells to anoikis[100]. Downregulation of αvβ3 integrin has also been linked to resistance to anoikis in CRC cells[101,102]. Integrins can bind to many ECM components such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing those integrins are more likely to invade and migrate through the ECM[103,104]. High expression of α6β4 and α5β3 integrins has been related to more aggressive CRC phenotypes[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastases formation by CRC cells in animal models[62] showing that αvβ-integrin inhibition did not affect migration within the liver parenchyma. The role of

integrins in the migration and invasion through the ECM in order to generate liver metastases has not been extensively explored.

Angiogenesis: **Different angiogenic factors have been related to metastasis formation, as they can promote primary tumor growth and increase tumor cell chances to contact blood and thus disseminate. But probably angiogenesis has a major role in metastasis generation regulating micrometastases outgrowth. Balance between angiogenic/antiangiogenic factors in the microenvironment of the metastatic tissue can promote metastases formation by directly stimulating tumor cell growth or by increasing blood vessels formation and supply. Even in quiescent tumor cells, alteration of angiogenic balance can induce metastasis formation. This phenomenon is known as "angiogenic switch" [105] and causal factors are still under investigation.**

Expression levels of vascular endothelial growth factor (VEGF) in the primary CRC have been related to a poor prognosis[106]. VEGF isoforms patterns have been defined using reverse transcription polymerase chain reaction (RT-PCR) analysis in 61 primary CRC. Patients developing liver metastases showed expression of VEGF121 + VEGF165 + VEGF189 at a significantly higher incidence (12 of 16, 75%) than those without liver metastasis (20 of 45, 44%) ($P = 0.036$)[107]. VEGF expression in primary CRC seems clearly associated to increased chances of dissemination. However, other studies support exactly the contrary[108]. When VEGF mRNA levels were measured in 31 pairs of primary CRC and corresponding liver metastases, no significant differences were detected (median value 3.79 *vs* 3.97: $P = 0.989$). On an individual basis, there was a significant correlation in VEGF mRNA expression between primary CRC and matched liver metastases ($r = 0.6627$, $P < 0.0001$). VEGF mRNA levels of patients having two or more liver metastatic tumors were significantly higher than those of the patient who had solitary liver metastatic tumor in both primary cancer (5.02 *vs* 3.34: $P = 0.0483$) and liver metastases (4.38 *vs* 3.25: $P = 0.0358$)[109]. Together these results indicate that VEGF is probably not more active in metastases than in primary tumors. Despite of that, increased blood supply and tumor vessel formation, as estimators of angiogenic activity, have been found to be higher in liver metastases that in primary CRC. Some molecular mediators have been though to fulfill this role, as angiopoietin-2 (Ang-2) [110].

Other distinctive molecules related to angiogenesis and liver metastatic progression are plateled-derived endothelial cell growth factor or thymidine phosphorylase (PD ECGF or dThdPase). Inhibitors of angiogenesis, such as angiostatin, endostatin and thrombospondin-1 (TSP-1), either secreted by the primary or the metastatic CRC cells, can also regulate liver metastasis growth. Frequency of hepatic recurrence was significantly higher in patients with TSP-1-negative primary CRC[111]. Angiostatin transfected cells developed liver metastases in lower proportion than controls, in experiments performed in animal models[112]. Removal of primary CRC resulted in an increase in metabolic activity in its liver metastasis, while a decrease in plasma levels of angiostatin and endostatin was seen. This finding indicates that primary tumor suppressed angiogenesis in its distant metastasis, and that removal of the primary lesion caused a flare-up in vessel neoformation and, thus, enhanced metabolic activity in its liver metastasis[113].

Other molecules mentioned above, also contribute to liver metastasis formation through angiogenesis regulation. MMP-7 induces a direct proliferative effect on vascular endothelial cells[114], produces angiogenesis inhibitors (angiostatin, endostatin, neostatin-7) [115] and activators (sVEGF) [116]. MMP-2 and MMP-9 stimulate degradation of ECM increasing availability of angiogenic activators. E-selectin acts facilitating endothelial cell migration. α and β integrins play an important role sending survival signals for endothelial cells mantainance[117].

Cell growth: **Once established in the liver tissue microenvironment and, in order to grow, micrometastases need growth factor stimuli. Degradation of ECM results in an increased availability of growth and inhibitory factors. The resulting balance will then determine micrometastasic growth. Extrapolation to a non-physiological situation can be highly illustrative. Liver tissue thermal ablation was performed in mice models bearing CRC liver metastases. After ablation, increased expression of FGF-2 and VEGF was detected in**

the surrounding tissue. Latter on a greater amount of metastases occupied the regenerated thermal-ablated lobe compared with controls (55%±4% *vs* 29%±3%; *P* < 0.04) [118].

Tumor cells growth factor receptors also seem to determine success in metastatic liver growth. Her-2/neu has been detected by immunohistochemistry in 5-50% of primary CRC[119]. The mechanism of overexpression seems not to be linked to gene amplification. Her-2/neu positive CRC were associated to higher postoperative non-liver specific recurrence rates (39.3% *vs* 14.6%, *P* = 0.013) and worse prognosis at 5 years (55.1% *vs* 78.3%)[120]. Other studies show that primary CRC with high c-erbB-2 expression (27%), determined by immunohistochemical techniques, develop liver metastases more often than CRC with low c-erb-2 expression (3%) [121].

Epidermal growth factor receptor (EGFR) has been reported to be highly expressed and/or gene amplificated in metastatic CRC tissue samples in 72% up to 82%[122-124]. Some studies have reported that expression of EGF receptor in CRC is associated to aggressiveness and metastatic ability. EGFR status has been shown to express similarly when measured in primary CRC and corresponding liver metastases[125]. However, some authors have seen that its status in the corresponding metastatic site is not always the same[126,127]. Conventional immunohistochemistry technique has not been able to reveal any association between EGFR expression and outcome predicted by the biologic role of EGFR in tumor behavior[128].

C-Src gene, codifying for pp60 tyrosine kinase, has been reported to be mutated and thus highly activated in CRC, implying an increase in the proliferative potential. High activation is present especially in those CRC that metastasize to liver[129,130]. Prostaglandin E_2 (PGE_2)-induced transactivation of the EGF receptor (EGFR) in colorectal carcinoma cells has been recently found to be mediated by β-arrestin 1, which acts as an important mediator in G protein-coupled receptor-induced activation of c-Src. Interaction of beta-arrestin 1 and c-Src seems to be critical for the regulation of CRC metastatic spread of disease to the liver "in vivo" [131].

Cell survival: **CRC cells need molecular factors, specifically growth factors, in order to survive in the liver parenchyma. However, there is also the need to survive to the immune sytem action (immunoescape) and to overcome anoikis.**

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is known to be expressed in human hepatic NK cells[132]. CRC cells expressing TRAIL-receptor would undergo apoptosis upon triggering the ligand. Same would happen in CRC cells expressing tumor necrosis factor receptor FAS (Apo-1; CD95) when contacting its corresponding ligand FASL (Apo-1L; CD95L) expressing cells, as activated lymphocytes.

During CRC tumorigenic process, cells tend to downregulate FAS receptor expression and upregulate FASL[133]. Fas expression is significantly down-regulated in liver metastasis compared with corresponding primary colorectal carcinoma[134]. The link between functional Fas status and malignant phenotype was investigated using a matched pair of naturally occurring primary (Fas-sensitive) and metastatic (Fas-resistant) human colon carcinoma cell lines in both in vitro and in vivo (xenograft) settings. Results showed that loss of Fas function was linked to, but alone was insufficient for, acquisition of a detectable metastatic phenotype. Also, showed that metastatic subpopulations pre-existed within the heterogeneous primary tumor, and that anti-Fas interactions served as a selective pressure for their outgrowth. Thus, Fas-based interactions may represent a novel mechanism for the biologic or immunologic selection of certain types of Fas-resistant neoplastic clones with enhanced metastatic ability[135]. Moreover, univariate and multivariate analyses revealed that Fas/CD95 expression in CRC resected liver metastases is a significant prognostic indicator of survival[136]. Increase in TRAIL sensitivity, due to changes in the balance between TRAIL receptors TRAIL-R1 and -R2 and "decoy" receptors TRAIL-R3 and -R4, has also been described during malignant progression in CRC. Still, studies measuring receptors by flow cytometry have not been conclusive[137].

Experimental metastases studies with a CRC cell line allowed the characterization of metastatic derivatives, showing that they were less susceptible for killing by syngeneic NK cells, due to a decreased sensitivity towards TRAIL- and CD95L[138]. Data suggest that CRC cells forming metastases acquire the ability to surpass immune surveillance through desensitization to FAS/TRAIL killing. As detailed before, integrins and Src activation may

contribute to CRC progression and liver metastasis in part by activating survival pathways that decrease sensitivity of detached cells to anoikis[100].

Other molecules related to liver metastatic spreading: k-ras (12p) activation, present in 40%-50% of sporadic CRC[4], has been related to a decrease in overall survival and disease free survival in CRC[6,139,140]. p53 (17p) abolition, occurring in 70%-80% of CRC[4] and resulting in accumulation of abnormal protein detectable by immunohistochemistry, has been linked to a bad prognosis[6,141-143]. Deletion or mutation of DCC (deleted in colorectal cancer) gene has also been related bad prognosis tumours[144-147]. Even p53, Ras and/or DCC alterations have been linked to metastatic spreading in CRC, still there is no evidence specifically relating them to liver metastases formation. The human nm23 gene, consisting in two genes, nm23-H1 and nm23-H2, is a candidate metastasis suppressor gene. Its role in CRC is still confusing. Some authors claim that a reduced protein expression, secondary to gene alterations, is associated to metastases development[148,149]. Genetic alterations were detected in four among eight CRC associated with metastases in lymph nodes, lung, or liver, while no alteration was observed in 12 additional CRC specimens without metastasis[150]. Others have found that gene overexpression is linked to higher recurrences, liver metastases and decreased overall survival[151,152]. This contradiction could be explained being overexpression of nm23 a reflection of a deletion in the nm23 gene, leading to accumulation of an altered protein product. However, more recent works have not been able to relate nm23 expression to prognosis[153-155]. PRL-3 protein tyrosine phosphatase gene gained importance in 2001 when an article was published in Science showing that it was expressed at high levels in each of 18 cancer metastases studied but at lower levels in nonmetastatic tumors and normal colorectal epithelium[156]. Latter on, new data came up establishing an unexpected and unprecedented specificity in metastatic gene expression profiles: PRL-3 was apparently expressed in CRC metastases to any organ but was not expressed in metastases of other cancers to the same organs or in nonmetastatic CRC[157]. At that time PRL-3 was determined as a potential marker for liver metastasis of CRC with a negative impact in prognosis[158]. CRC specificity was objected in further studies. Some authors claim that PRL-3 would act enhancing cell motility and thus facilitating extravasation into the liver tissue[159]. The mechanism of action is still under investigation but it has already been related to integrin α1[160] and the Rho family of small GTPases[161].

CONCLUSION

Huge amount of experimental data points to the statement of tumor cells having a metastatic signature. This signature would codify not only for the ability but also for organ-specificity in forming metastases. DNA microarray technology has significantly improved efficiency allowing wide-range analysis of gene expression. Until now, many authors have provided gene expression profiles that have been related to CRC liver metastases. Despite of that, in order to obtain the real genetic signature for liver metastases in CRC by transcription profiling, measures to improve reproducibility, to increase consistency, and to validate results need to be implemented. Seeking for metastatic signatures through expression profiling is one more tool to fight cancer, but its indiscriminate use can be misleading. Advances in molecular assays on isolated cells and in the study of cell-cell and cell-stroma interactions will probably enable the dissection of the metastatic cascade. Genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have already been linked to mechanisms of liver metastases in CRC. The improvement in knowledge around the molecular pathways involved in development of colorectal liver metastasis will lead us to a better approach to prevention and treatment of this disease.

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Serum matrix metalloproteinase 7 levels identifies poor prognosis advanced colorectal cancer patients

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Metalloproteinase 7 (MMP-7) plays an important role in tumor growth, invasion and dissemination, and is secreted to the media. Because of the close implication of MMP-7 in cancer biology, we sought to define the prognostic significance of serum levels of MMP-7 in nonmetastatic colorectal cancer (CRC) and explore its possible impact in the daily clinical practice. MMP-7 expression was determined by enzyme-linked immunosorbent assay. We assessed serum MMP-7 levels in 87 healthy controls, 96 patients with nonmetastatic CRC and 120 patients with advanced CRC. Clinical information was gathered from patient files. Cox proportional hazards model was used to assess survival. MMP-7 and the variables associated with prognosis were entered and a backward elimination method was employed to adjust the model. Inclusion criteria was $p \leq 0.05$ and exclusion criteria was $p \geq 0.10$. Advanced CRC patients have a significant higher serum serum MMP-7 levels (15.4 ng/ml) than those in nonmetastatic CRC (5.5 ng/ml; $p < 0.001$) and healthy controls (4.2 ng/ml; $p < 0.001$). In metastatic patients, after adjusting for other prognostic variables, MMP-7 (entered as a continuous variable) is associated with decreased survival (HR 1.016, IC 95% 1.002–1.031). Serum MMP-7 levels are significantly elevated in patients with advanced CRC. In conclusion, MMP-7 is an independent prognostic factor for survival in advanced CRC. In our sample, the risk of death associated to MMP-7 increase is much higher than the risk of death associated to lactate dehydrogenase elevation.
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Key words: colorectal cancer; prognosis; matrix metalloproteinases

Widely accepted prognostic factors in advanced colorectal cancer (CRC) are performance status (PS), serum lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) levels.[1–4] Despite of it, novel markers and useful prognostic indexes are needed to better classify these patients for clinical practice.

Matrylisin (MMP-7) is a proteolytic enzyme belonging to Matrix Metalloproteinase (MMPs) family.[5,6] It is constitutively expressed in the ductal and glandular epithelium of many tissues.[7] In the lung and intestine it plays a role activating antibacterial peptides such as prodefensins.[8] MMP-7 is synthesized and secreted by tumor epithelial cells as a 28-KDa proenzyme that can be activated through proteolytic removal of a 9-KDa prodomain from the N-terminus. The soluble activated form binds to the tumor epithelial cell surface. Both active forms, the soluble and the membrane-bounded, have proteolytic activity. Its expression is regulated by transcription factors such as AP-1, PEA3 and β-catenin/ tcf4 complex.[9–11] By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2 and MMP-9), through ectodomain shedding of E-cadherin,[12] and receptor activator of nuclear factor-kappa B ligand (RANKL),[13] or through cleavage of adhesion molecules, such as integrin β4.[14]

MMP-7 is able to induce cell apoptotic impairment. It specifically cleaves critical proteins implicated in the extrinsic apoptotic pathway, such as FAS ligand (FASL)[15,16] and tumor necrosis factor-alpha (TNF-α).[17] Its shedding is related to the acquisition of an apoptosis resistance phenotype.[18] Additionally, MMP-7 induces cell proliferation through cleavage of heparin binding epidermal growth factor (HB-EGF) precursor,[19] a disintegrin and metalloproteinase family (ADAM) member, ADAM28[20] and degradation of all 6 insulin growth factor binding proteins (IGFBP-1 to -6), increasing the bioavailability of IGF and thus favoring cancer cell growth and survival.[21–23] Matrilysin can regulate angiogenesis either by inducing a direct proliferative effect on vascular endothelial cells[24] or producing angiogenesis inhibitors (angiostatin, endostatin and neostatin-7),[24] or enriching the variety of angiogenesis mediators, such as the soluble vascular endothelial growth factor (sVEGF).[25] Immunoevasion due to MMP-7 would be related to FASL cleavage,[15,16] or to IgG degradation.[26]

Matrilysin has been found overexpressed in a variety of tumors such as CRC.[27] There is substantial evidence that overexpression of MMP-7 in CRC primary tumors, taking into account the measurement of both activated and pro forms, is related to a more aggressive phenotype of tumor cells and a poorer prognosis. MMP-7 overexpression has been correlated with invasion and to liver metastasis formation in CRC nonmetastatic disease.[28–32]

As noted earlier, MMP-7 is secreted to the media. Both soluble MMP-7 forms, active and proactive, can be measured in serum by a commercial ELISA Kit.

Because of the close implication of MMP-7 in cancer biology and the possibility to measure its soluble forms in serum, this study was performed with the objective to define the prognostic significance of serum levels of MMP-7 in metastatic CRC and explore its possible role in the daily clinical practice.

Serum MMP-7 levels were also measured in healthy volunteers, nonmetastatic and advanced CRC patients, to prove if they can be an indirect estimation of tumor MMP-7 expression and activity.

Patients and methods

Patients

The study was conducted as a serial collection of serum samples from 120 patients with their first sign of advanced CRC from July 2001 to December 2004. The patients were in good performance (PS ≤ 2) and were not initially suitable for liver resection (more

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than 3 liver nodules and or ≥ 5 cm). All patients had a medical history, clinical examination, full blood count and a biochemical screen of renal and liver function. Levels of carcinoembryonic antigen (CEA) (Roche® Germany) were measured using an Elecsys (Roche®) autoanalyzer. Lactate dehydrogenase (LDH) (Roche®, Germany) and alkaline phosphatase (ALP) (Bayer®, USA) were measured using an ADVIA 2400 (Bayer®, USA) autoanalyzer. Staging was done with abdominal spiral computed tomography (CT) and chest radiography. Additional techniques such as abdominal ultrasound, chest CT or magnetic resonance imaging (MRI) were done if needed for further staging refinement. Serum samples were obtained before treatment, after written informed consent. Additionally serum MMP-7 was determined in 87 healthy patients without known renal or hepatic dysfunction, and 96 patients with histologically confirmed colorectal adenocarcinoma with nonmetastatic disease, before surgical resection. To rule out metastatic disease in nonmetastatic CRC patients, staging was done with chest radiography and abdominal ultrasonography or spiral CT. The study was approved by Hospital Clinic Ethical Committee.

Sample collection

Before the initial treatment, venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 min. After that, they were centrifuged at 1,500 rpm for 15 min. Serum was immediately aliquoted and kept at −80°C, until assayed. The procedure has been performed exactly in the same way in all groups (healthy volunteers, CRC and ACRC).

Serum samples and MMP-7 analysis

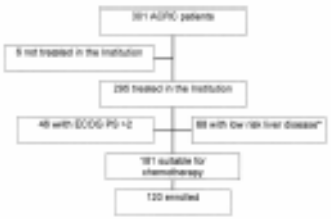
MMP-7 was determined using a quantitative solid phase sandwich enzyme linked immuno sorbent assay (ELISA) (RnD Systems, USA) and tested in duplicate. MMP-7 technique can detect both pro- and active forms of recombinant human MMP-7. High concentrations of MMP-7 were diluted with calibrator, to produce samples with values within the dynamic range of the assay.

Statistical methods

Recorded variables were age, sex, date of birth, date of death or last follow-up, PS, number and site of metastasis, previous chemotherapy received and sera levels of: CEA, LDH, ALP and MMP-7, as described above. Missing values (<2%) were not included in the multivariate analysis.

Assuming a 2-sided alpha error of 5%, with our sample size ($n = 120$) and in 18 months of follow-up, the power to detect the differences observed was of 90%.

Regarding multivariate analysis, overall survival was considered the dependent variable. It was calculated in patients with advanced CRC as the time from informed consent for biological analysis, to death or censoring date. The prognostic significance of the independent variables regarding survival was assessed using the Cox proportional hazards model as follows. Independent variables were selected sequentially. First, the variables Age, PS, number of involved organ, LDH, ALP, CEA and MMP-7 were entered and a backward elimination method was employed to adjust the model. Inclusion criteria was $p \leq 0.05$ and exclusion criteria was $p \geq 0.10$.[31] To the best model selected with this method, the excluded variables were added, one by one, checking for confusion (>10% variation of the HR of MMP-7) and for any increase of the power of prediction measured by the coefficient of determination.[32] The model without confusion and with the highest power of prediction according to the coefficient of determination was chosen. Quantitative variables were introduced as continuous in the model. Assumed proportionality of the multivariate model was checked plotting the logarithm of the cumulative estimated risk stratified by each independent variable selected. Assumed log-linear relationship of the multivariate model was checked plotting the martingale residuals by each quantitative independent variable selected.[33]



*Low risk liver disease: Fewer than 4 liver nodules of less than 5 cm

Figure 1 – Study profile.

Normality of the sample was assessed with the Shapiro-Wilk test. Because of the absence of normality, differences in serum MMP-7 levels between patients with advanced disease, nonmetastatic disease and controls was analyzed by Mann–Whitney *U* test. The Mann–Whitney *U* test was also used to test for associations between MMP-7 serum levels in patients with advanced disease and clinical characteristics. All p values were 2-sided, and values <0.05 were considered significant. Survival curves were constructed using the Kaplan–Meier method, assessing significance by the Log-Rank test. Division by quartiles was chosen for plotting. Statistical analyses were performed using SPSS software 12.0.

Results

Patient characteristics

During the study period, 301 patients with advanced CRC were visited in our Oncology Unit. One hundred eight-one patients were not enrolled owing to: treated outside our institution (6), poor PS or elderly with fragility criteria (46), liver resection (61) or radiofrequency ablation (7) as first treatment and patient refusal to participate (61) (Fig. 1). Therefore patients enrolled in the study constitute 66% (120/180) of all patients with advanced disease, suitable for chemotherapy treatment diagnosed in the study period. Primary chemotherapy based on oxaliplatin-5-fluorouracil or irinotecan-5-fluorouracil was used to treat 92% (110/120) of patients. Patient characteristics are summarized in Table I. Median age of the patients in our sample was 66 years, being male patients 62.5% of them. Eighty percent of the patients were in good PS (0–1). Eighty-one percent of the sample had metastasis in only 1 organ and liver was affected in 76.7% of cases. Most of the patients had not received adjuvant chemotherapy (77.5%).

Basal serum levels of MMP-7 in healthy controls, nonmetastatic CRC and advanced CRC

The mean and median serum level of MMP-7 for the advanced CRC patients ($n = 120$) were 13.4 ng/ml (SD 17.1) and 7.7 ng/ml respectively (range: 2–126.6 ng/ml) and for the nonmetastatic CRC ($n = 96$) group were 5.5 ng/ml (SD 3.2) and 4.9 ng/ml (range, 1.2–19 ng/ml). The difference was statistically significant between these 2 groups ($p < 0.001$). There was also a significant difference between serum MMP-7 levels in patients with advanced CRC ($n = 120$) and healthy controls ($n = 87$), being the mean and median serum level of MMP-7 4.2 ng/ml (SD 2.2) and 3.5 ng/ml (range 0.5–16 ng/ml, $p = 0.001$) and between patients with nonmetastatic disease ($n = 96$) and healthy controls ($p = 0.001$). Demographic characteristics between the 3 groups were stated in Table II.

Association between basal serum MMP-7 levels in advanced CRC patients and clinical characteristics

Basal serum MMP-7 in advanced CRC patients was significantly associated with ECOG PS ($p < 0.001$), previous adjuvant

TABLE I – CLINICAL CHARACTERISTICS OF THE PATIENTS AT BASELINE

Age, years	
Median	66
Range	33–82
Sex	
Male	75 (62.5)
Female	45 (37.5)
ECOG performance status	
0	45 (37.5)
1	51 (42.5)
2	24 (20)
No. of organs involved	
1	81 (67.5)
>1	39 (32.5)
Type organs involved	
Liver	92 (76.7)
Other than liver	28 (23.3)
Previous adjuvant chemotherapy	
No	93 (77.5)
Yes	27 (22.5)
Serum CEA (ng/ml)	
Median	38
Range	1–24770
Serum LDH (U/L)	
Median	418
Range	195–45,864
Serum ALP (U/L)	
Median	341
Range	120–6,561
Serum MMP-7 (ng/ml)	
Median	7.7
Range	2–126.6

Values are *N* (%) unless otherwise indicated.

treatment ($p = 0.007$), LDH levels ($p < 0.001$), ALP levels ($p < 0.001$), CEA levels ($p < 0.001$) and liver involvement ($p = 0.01$) but not with other covariates such as age, sex or number of involved sites (Table III).

Association between basal serum MMP-7 levels in advanced CRC patients and overall survival

The median survival for all patients was 17.8 months (range, 15.5–20.1). A total of 95 patients (77%) died during follow-up. In the univariate analysis the following variables resulted significant: PS, number of involved organs, LDH, ALP, CEA and MMP-7 (Table IV). Elaborating the multivariate model as described earlier, the most predictive model was the one containing MMP-7, PS, number of involved organs and LDH. HR of MMP-7 was 1.016 (IC 95% 1.002–1.031; $p = 0.029$). As MMP-7 was entered as a continuous variable, this result is best interpreted as follows: independently of the effect of other variables entered in the multivariate analysis, every increase of 10 units of MMP-7 is associated with a 16% increase of the risk of dying. LDH, ALP and CEA were also treated as continuous variables, being their magnitude of association expressed in Table III as percentage of risk increase. Interpreting this in the same way, an increase of 100 units of LDH is associated with a 0.9% increase of the risk of dying.

Once established the prognostic significance of MMP-7 and the magnitude of this association, quartiles were chosen as a natural division for survival plotting.

Quartiles of MMP-7 were 4.83, 7.50 and 15.92. The Kaplan–Meier plot shows statistically significant difference between quartiles (Fig. 2).

Although the multivariate model provides a MMP-7 effect adjusted by LDH and other factors, as the latter is one of the most established parameters regarding prognosis, we plotted in Figure 3 survival according to MMP-7 and LDH levels. This figure shows that when MMP-7 is higher than median, irrespectively of LDH levels, survival is worse than when MMP-7 is lower than median. This differences are statistically significant ($p = 0.003$).

TABLE II – DEMOGRAPHIC CHARACTERISTICS OF HEALTHY CONTROLS, LIMITED STAGE AND ADVANCED STAGE PATIENTS

Characteristic	Healthy controls ($n = 87$)	Limited stage ($n = 56$)	Advanced stage ($n = 120$)	*P*
Sex				
Female (%)	55 (63.2)	38 (39.6)	45 (37.5)	0.004[1]
Age				
Mean (SD)	56.8 (17.9)	71.1 (12.6)	63.8 (11.1)	<0.001[2]
MMP-7 (ng/ml)				
Mean (SD)	4.2 (2.2)	5.5 (3.2)	13.5 (17.1)	<0.001[2]

[1]χ^2-square test.–[2]Kruskal–Wallis test.

TABLE III – MMP-7 LEVELS ACCORDING TO CLINICAL CHARACTERISTICS

	Mean of MMP-7 (SE)	*P*
Age		
<56	17.2 (16.4)	
56–66	14.9 (18.2)	0.353
67–72	8.6 (6.4)	0.029
>72	12.7 (22.3)	0.800
Sex (*n*)		
Female (45)	13.9 (21.7)	
Male (75)	13.1 (13.8)	0.37
ECOG (*n*)		
PS0 (45)	9.1 (10.7)	
PS1 (51)	11.6 (13.3)	0.012
PS2 (24)	25.8 (16.6)	<0.0001
LDH (*n*)		
≤450 (69)	8.4 (8.3)	
>450 (52)	20.4 (22.5)	<0.0001
ALP (*n*)		
≤280 (49)	7.7 (8.8)	
>280 (68)	18.1 (20.3)	<0.0001
No. of organs involved (*n*)		
1 (81)	14.1 (19.5)	
>1 (39)	12.2 (10.5)	0.300
Type organ involved (*n*)		
Liver (92)	15.2 (18.9)	
Other than liver (28)	7.9 (6.2)	0.018
Previous adjuvant CHT (*n*)		
Yes (27)	9.0 (9.8)	
No (93)	14.8 (18.5)	0.107
CEA[1]		
≤6	6.5 (5.0)	
6–37.2	8 (5.2)	0.083
37.3–191.7	16.8 (14.7)	<0.0001
>191.7	22.9 (27.3)	<0.0001

[1]CEA was divided by quartils.

Discussion

The key finding of our study is that MMP-7 is an independent prognostic factor for survival in advanced CRC. Also, in our sample, the risk of death associated to MMP-7 increase is much higher than the one associated to LDH elevation.

Our data confirm that high serum MMP-7 levels tend to correlate with clinical adverse parameters such as high LDH, ALP, CEA levels, liver involvement and poor PS. Focusing on LDH and MMP-7, in our multivariate survival analysis they came as independent factors, even it is well known that their transcription can be regulated together under hypoxic conditions. Being LDH one of the most accepted clinical parameter to determine prognosis, we tested MMP-7 together with LDH to determine the prognostic significance of different combinations. The Kaplan–Meier curves show statistically significant difference ($p = 0.003$) between curves expressing high and low MMP-7 levels, despite being associated with high or low LDH levels. Patients with high LDH and low MMP-7 levels seem to be associated to a slightly better prognosis, when compared those with both high LDH and MMP-7 levels. Those findings suggest that MMP-7 is possibly even more accurate that LDH in determining prognosis in the group of

TABLE IV – UNIVARIATE AND MULTIVARIATE COX REGRESSION ANALYSIS OF POTENTIAL PROGNOSTIC FACTORS FOR OVERALL SURVIVAL

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
Age	1.005	0.987–1.024	0.590			
PS						
0[1]	1			1		
1	1.812	1.126–2.198	0.014	1.736	1.052–2.865	0.083
2	4.894	2.774–8.728	<0.001	3.175	1.618–6.231	0.001
No of involved organs						
1[1]	1			1		
>1	1.580	1.087–2.432	0.038	1.494	0.948–2.353	0.083
LDH[2]	0.012	0.004–0.020	0.002	0.009	0.001–0.018	0.049
ALP[2]	0.060	0.033–0.087	<0.001			
CEA[2]	0.007	0.001–0.013	0.010			
MMP-7	1.028	1.017–1.040	<0.001	1.016	1.003–1.031	0.029

Only PS, number of involved organs, LDH and MMP-7 were selected for the multivariate model (see text for details).
[1]This category was taken as the reference.–[2]Results expressed as percentage of risk increase (see text for details).

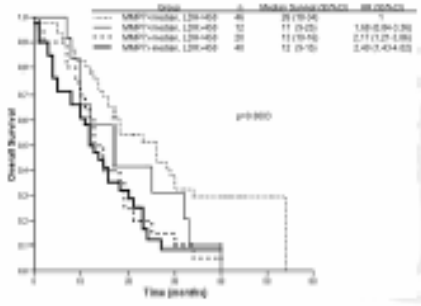


FIGURE 2 – +++

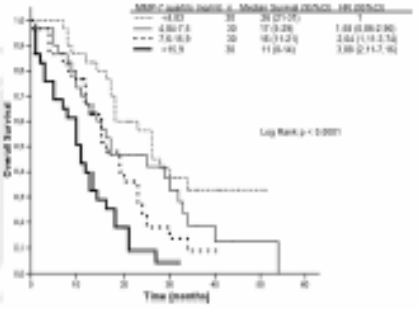


FIGURE 3 – +++

advanced CRC patients. Interestingly, high serum MMP-7 also correlates with liver involvement during the metastatic spread. Further analyses in the group of liver-only metastasis would be required.

Levels of total MMP-7 can be measured in human serum and it is feasible using a simple ELISA technique, as it has been recently shown in few other studies.[38] Serum measurements of total MMP-7 can be considered as an indirect estimation of tumor MMP-7 expression. Other techniques, such as zymography, are useful to distinguish between activated MMP-7 and pro-forms and might be implemented in the near future for further analysis.

Our work describes how MMP-7 serum level can distinguish between healthy controls, localized and metastatic CRC. Nonmetastatic CRC patients have significant lower levels of MMP-7 than metastatic CRC, and healthy controls have also a significant lower level of MMP-7 than nonmetastatic CRC.

Our results in controls (median MMP-7 level of 3.5, range 0.5–16 ng/ml) are consistent with previously published data, where healthy controls ($n = 20$) had a mean MMP-7 serum level of 3, 2 μg/L; st. dv. 1, 5.[39] Although median and mean are different parameters, because of the absence or normality in our sample the former was chosen for description. Both are measurements of central tendency and this conclusion seems to be appropriate.

We have made the observation that serum MMP-7 determinations in advanced CRC are not always homogeneous. Not all patients with advanced CRC have elevated serum MMP-7 levels when compared with healthy controls, suggesting that not all tumors secrete MMP-7 or the protein is secreted at a very low level. Protein levels may therefore reflect differences in the biologic characteristics of cancer cells. We speculate that possibly MMP-7 expression levels are also a qualitative marker in advanced CRC. Advanced CRC expressing high levels of MMP-7 would be related to more invasive, growing and metastatic tumours.

Cancer progression and apoptosis, depends on the interplay between cancer cells, the immune system and the microenvironment. MMP-7 could work as a link between these 3 major actors. MMP-7 is activated by MMP-2 and MMP-9, both produced by stromal cells, and is also transcriptionally activated by the beta-catenin-tcf-4 complex and oncogenic mutations of *Kras*[40] in tumor cells. Our group and others, have demonstrated that MMP-7 blocks lymphocyte cytotoxicity, by cleaving of the NH_2-terminal "proligand assembly domain" of FAS membrane (FASpg)[41] and the extracellular surface of FASL membrane (FASLm)[42] leading to a decreased sFAS/sFASL ratio,[42] and therefore escaping from the immune system and favoring resistance to chemotherapy. Recently, Wang *et al.*[43] have demonstrated that MMP-7 increases resistance to Fas-mediated apoptosis and, the authors conclude that high MMP-7 tumor expression is a poor prognostic factor of patients with CRC. Vargo-Gogola *et al.*[44] have determined that FASL cleavage from the cell surface by MMP-7 provides apoptosis resistance and subsequently leads to tumor formation in murine

mammary glands. Moreover, in another study, specific cleavage of Fas by MMP-7 resulted in decreased sensitivity of HT-29 colon carcinoma cells to Fas-mediated apoptosis.[43] In the same study, the authors found a markedly increased susceptibility of these cells to Fas-mediated apoptosis when their MMP-7 expression was suppressed by transient transfection of the antisense oligonucleotide for this proteinase. There is then a strong suggestion that increased levels of MMP-7 are associated with the development of refractoriness to chemotherapy agents. Although, this issue requires further clinical validation, *in vitro* and *in vivo* studies[43,44] are highly suggestive of this association which has great clinical implications.

We have observed (unpublished data) that in patients with low MMP-7 but high LDH levels, MMP-7 values can increase during chemotherapy treatment, and would be therefore implicated in early acquired resistance, after initial response. Therefore, we speculate that MMP-7 would be implicated in primary chemoresistance in the subgroup of patients with well known poor prognosis features, to an even more aggressive phenotype, or both.

This study has some potential limitations. First, only patients suitable for chemotherapy treatment were selected for analysis. We could not rule out that, if patients with favorable characteristics (liver involvement only and, less than 4 nodules and less than 5 cm) or poor prognostic features (PS >2 or fragility criteria) had been also included, results would have been different. Second, although Oxaliplatin-5-fluorouracil or Irinotecan-5-fluorouracil combinations have similar activity in randomized trials, it would be desirable to confirm these results in prospective large series with more homogeneous treatments. Third, we cannot rule out MMP-7 being an estimator of tumor burden, but it is clear that MMP-7 is correlated to but not a reflection of LDH, which up to date is the best, even not definitely proven, tumor burden estimator in CRC. We have found 12% of patients with high LDH having low MMP-7 levels, which seem to be associated to a slightly better prognosis, when compared those with both high LDH and MMP-7 levels. This finding indicates that in our series, tumor burden would be differently expressed by LDH and MMP-7. Moreover, in our work, high serum MMP-7 seems to correlate with liver involvement during the metastatic spread, but not with the number of involved organs. Finally, MMP-7 analysis has been done in serum and we have not included studies validating the correlation between MMP-7 expression in primary and metastatic tissues and serum MMP-7.

Detection of serum MMP-7 is feasible and done through a noninvasive technique. It is clearly a good tool not only to detect the subgroup of poor prognosis among de advanced CRC patients, but also it would be worthwhile to use it to evaluate serum MMP-7 levels as a potential marker of liver progression in nonmetastatic CRC.

In summary, the key finding of our study is that having increased MMP-7 levels, in advanced CRC patients, is an independent prognostic factor for survival. This finding might imply a novel strategy for better classification of advanced CRC patients in prospective studies. This is to our knowledge the first time that such an association is reported in advanced CRC. Furthermore, these results represent the clinical confirmation of previous studies performed, either in human tissue samples or in preclinical studies, by other investigators. In our sample, high levels of serum MMP-7 indicate a poor prognosis phenotype, among advanced CRC patients, irrespectively of LDH values. Our results confirm the hypothesis of MMP-7 being a biologic marker selecting for a subset of advanced CRC patients with a qualitative aggressive phenotype. This poor prognosis phenotype can be related to increased ability to growth, invade and disseminate, but also to acquisition of chemoresistance.

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FAS/FAS Ligand Ratio: A Marker of Oxaliplatin-Based Intrinsic and Acquired Resistance in Advanced Colorectal Cancer

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Abstract

Purpose: Oxaliplatin-5-fluorouracil combinations have increased responses in first-line therapy up to 40% in advanced colorectal cancer. Unfortunately, those patients who will respond are unknown and initially sensitive patients become rapidly resistant to current therapies. FAS (CD95) and FAS ligand (FASL; CD95L) have been implicated in chemosensitivity through leading to apoptosis in response to DNA-damaging drugs. Whereas the proapoptotic role of FAS and FASL is well characterized, the function of their soluble forms as predictors of chemosensitivity remains unknown.

Patients and Methods: Blood samples were obtained from 68 patients with advanced colorectal cancer who received oxaliplatin-5-fluorouracil combinations in first-line therapy. Computed tomographic scans were done every 3 months and responses were evaluated by Response Evaluation Criteria in Solid Tumors criteria. ELISA soluble FAS and soluble FASL analysis were done before treatment and every 3 months until disease progression. Ratios between soluble FAS and soluble FASL were established and its values and variations through time were related to treatment responses.

Results: We found a significant increase in soluble FAS levels and a significant decrease in FASL at 3 months compared with baseline (13.2 versus 10.02 ng/mL; $P = 0.0001$; 0.07 versus 0.14 ng/mL; $P = 0.007$, respectively). A significant increase in the soluble FASL levels up to 9 months (fourth to fifth extractions; 0.26 ng/mL) of therapy compared with first to third extractions (0.11 ng/mL; $P = 0.003$) was also found. A random effect regression statistical model determined that >1.2-fold increase in soluble FAS/soluble FASL ratio was a marker of chemosensitivity ($P = 0.001$).

Conclusions: These data strongly indicate that an increment of soluble FAS/soluble FASL ratio after treatment could be an excellent marker of chemosensitivity in colorectal cancer. On the other hand, a decreased ratio after treatment can be a predictor of chemoresistance despite an initial response.

Colorectal cancer is the most common cancer in western Europe, with ~70 new cases a year per 100,000 inhabitants. In spite of advances in screening, 15% to 20% of patients show initially advanced disease, and 30% to 50% are destined to metastasize. Recently, oxaliplatin/5-fluorouracil (5-FU)/leucovorin or irinotecan-5-FU/leucovorin have increased responses in first-line therapy up to 40%, with median survival between 16 and 20 months, but 2-year overall survival still remains <20% (1, 2). Unfortunately, those patients who will benefit from first-line chemotherapy are unknown. Furthermore, the initially sensitive patients become rapidly resistant to current therapies.

The FAS (CD95) receptor is a cell surface protein that mediates apoptotic cell death on triggering by FAS ligand (FASL). This interaction causes FAS receptor homo-oligomerization and this leads to activation of the caspase cascade (apoptotic extrinsic pathway). A FASL-independent activation of the FAS receptor has also been described (3).

Whereas proapoptotic role of FAS and FASL are well known, more conflicting data come from functionality of soluble forms. Various forms of soluble FAS (sFAS) have been described derived from alternative splicing phenomena (4). The majority of these spliced forms have an oligomerization domain, which allows them to form homotrimers (between soluble forms) and heterotrimers (when joining to transmembrane FAS receptor). A dual antiapoptotic or proapoptotic function has been advocate for these soluble forms. When they form heterotrimers, they are counteracting the apoptotic signaling (5). While forming homotrimers, they are capable of interacting

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with transmembrane FASL leading to a proapoptotic effect (6). More convincing data of an antiapoptotic function of soluble FASL (sFASL; resulting from the cleavage of FASL by metalloproteinase-7; ref. 7) or a marginal proapoptotic function (8) has been proposed.

The extrinsic apoptotic pathway seems to be physiologically compromised during colorectal cancer progression. It has been shown that adenoma through carcinoma step leads to FASL up-regulation and FAS down-regulation (9). sFAS levels have been proven to be elevated in serum of patients with colorectal cancer (10), whereas some colorectal cell lines have turned to be releasing sFASL (11). Together, all these data support the hypothesis of the acquisition of a FAS/FASL apoptotic resistance profile as well as an immunoescape capacity during colorectal cancer progression (12).

Cytotoxicity due to 5-FU/leucovorin treatment in p53 wild-type colorectal cell lines can be mediated via FAS (13, 14). It has also been described that this stimulus can produce apoptosis in p53 mutant cells (15). In colorectal cancer cells, there was an increased level of FASL and apoptosis induction during thymineless death after 5-FU treatment, via activation of nuclear factor-κB and activator protein-1 transcription factors (16), as well as in thymidylate synthase–deficient cells, after treatment with DNA-damaging agents (17). Other drugs, such as camptothecin, seem to induce cell death through recruitment of the FAS-FADD adaptor in a FASL-independent fashion (18, 19). Therefore, it seems that 5-FU (20), capecitabine (21), and antimetabolite therapies can restore the lost of apoptotic capacity of colorectal cancer cells *in vitro*, either p53 wild-type or mutant, through the extrinsic pathway by regulating FAS and FASL expression and/or function.

Because of the mentioned chemotherapy capacity to modulate FAS/FASL, we hypothesize that these drugs could also modulate the soluble forms and therefore its role in regulating the apoptotic response through the extrinsic pathway as well as the immunologic "counterattack." Because soluble forms (sFAS and sFASL) can have opposite effects, the ratio between them (sFAS/sFASL) could be a way to measure the final balance of apoptotic and immunoescape effect. This ratio and its variations along chemotherapy treatment could be therefore a useful variable to measure colorectal cancer chemosensitivity and chemoresistance.

Patients and Methods

Patients. Blood samples were obtained from 68 patients treated for advanced colorectal cancer from July 2001 to September 2003. Patients received 85 mg/m^2 oxaliplatin on day 1, 200 mg/m^2 leucovorin on day 1, and 3 g/m^2 5-FU on day 1 in 46-hour continuous infusion every 2 weeks for a maximum of 12 cycles (*n* = 55) as standard treatment in our institution. Thirteen patients were treated with other oxaliplatin-fluorouracil combinations in multi-institutional clinical trials: 85 mg/m^2 oxaliplatin on day 1 and 2.25 g/m^2 5-FU on day 1 in 48-hour continuous infusion weekly every 2 weeks (*n* = 3), 130 mg/m^2 oxaliplatin on day 1, and 1,000 mg/m^2 capecitabine on days 1 to 14 every 3 weeks (*n* = 3) or FOLFOX4 (*n* = 7). Eligible criteria were stage IV histologically proven colorectal cancer, measurable metastatic lesions by Response Evaluation Criteria in Solid Tumors criteria, Eastern Cooperative Oncology Group performance status score of 0 to 2, no previous neoplasm in the last 10 years, normal liver and renal function, and no previous chemotherapy for advanced disease.

Table 1. Patient characteristics

	n (%)
No. patients	68
Sex	
Male	42 (61.8)
Female	26 (38.2)
Age, y	
Median	63
Range	33–80
Eastern Cooperative Oncology Group performance status	
0	28 (41.2)
1	27 (39.7)
2	13 (19.1)
No. organs	
1	47 (69.1)
>1	21 (30.9)
Organs involved	
Liver	53 (77.9)
Other than liver	15 (22.1)
Previous adjuvant chemotherapy	15 (22.1)
Previous radiotherapy	7 (10.3)
Response	
Complete response	1 (1.5)
Partial response	30 (44.1)
Stable disease	21 (30.9)
Progressive disease	14 (20.6)
Not evaluable	2 (2.9)

All patients had chest X-ray and a helical computed tomographic (CT) abdominal scan before entry into study and underwent repeated evaluations at least every 3 months. Tumor response was assessed according to Response Evaluation Criteria in Solid Tumors criteria (22) as complete response, partial response, stable disease, and progressive disease. Each tumor measurement by CT scan was compared with previous CT scan. Therefore, patients with initial partial response in first evaluation (second CT versus initial CT) and with stabilization on the second evaluation (third CT versus second CT) were defined as stable disease instead of confirmed partial response. Only those patients with new partial response in second evaluation were defined again as partial response. Patients gave signed informed consent before treatment and the study was approved by the institutional ethics of research committee.

Samples and assay. Venous blood samples were drawn into sterile vacuum tubes before the initial treatment and every 3 months until disease progression for a maximum of five extractions (month 12). We have limited the number of extractions to 5 because >90% of the patients have progressed at that time. Blood samples were kept at 4 °C, centrifuged at 10,000 rpm for 15 minutes, and then immediately frozen at −80 ° until assayed.

FAS and FAS ligand–specific ELISA. A double-antibody sandwich ELISA was constructed to detect sFAS and sFASL in sera using a FAS and sFASL ELISA kit (Oncogene Research Product, San Diego, CA). This assay uses FAS and FASL antibodies against two epitopes. Standard curves were constructed using serial dilutions of recombinant sFAS and sFASL. The maximum detectable concentration of sFAS was determined as 100 ng/mL. The maximum and minimum detectable concentrations of sFASL were determined as 1.25 and 0.01 ng/mL, respectively.

Statistical methods. The Mann-Whitney test was used to assess significant associations between continuous variables (FAS and FASL levels) and dichotomous variables (sex; upper limit of normal lactate dehydrogenase (>1versus<1), number of organs involved(1 versus >1).

disease location (liver versus other than liver), adjuvant chemotherapy, previous radiotherapy, and initial Dukes stage (synchronic versus metachronic)]. The Wilcoxon test was also used to ascertain FAS and FASL variations during chemotherapy treatment. The Kruskal-Wallis test was used to assess significant differences in FAS and FASL levels within multiple groups (i.e., Eastern Cooperative Oncology Group performance status). Complete response and partial response were considered as "sensitive" and stable disease and progressive disease were considered as "refractory." A random effect regression statistical model evaluated the effects of time/therapy on response. A univariate and multivariate analysis for all variables influencing on response was also done. Only variables with a borderline significance ($P < 0.1$) at univariate analysis were include in the multivariate regression model.

Results

Patients and tumor characteristics. Demographic details on the 68 patients included in the study and tumor stage are shown in Table 1. The median of received treatment cycles was 9 (range, 1-12). Twelve of the 68 patients had undergone radical procedures after chemotherapy treatment (11 underwent surgical resection and 1 radiofrequency thermal ablation) but were fully evaluable for response. Two patients were not evaluable for response due to complications after first cycle (1p with pulmonary embolism and 1p with intestinal occlusion).

FAS/FAS ligand levels. Sera were obtained from 68 patients diagnosed with advanced colorectal cancer during the study period with a total of 160 extractions. From 66 patients assessable for response, the average of extractions was 2.4 (range, 1-5). Reasons for extraction discontinuation were per protocol ($n = 0.21$; median, 3.4; range, 2-5), radical treatment after chemotherapy ($n = 0.12$; median, 2.2; range, 1-3), patient withdrawal consent ($n = 0.1$; median, 2), poor medical condition after rapid progression disease ($n = 0.4$; median, 1), and finished study period ($n = 0.28$; median, 1.8; range, 1-4).

There were no significant associations between sFAS and sFASL levels and any of the following variables: sex ($P = 0.24$ and 0.38, respectively), previous chemotherapy treatment ($P = 0.32$ and 0.35, respectively), lactate dehydrogenase levels ($P = 0.43$ and 0.77, respectively), previous radiotherapy ($P = 0.39$ and 0.9, respectively), synchronic or metachronic disease ($P = 0.37$ and 0.21, respectively), number of organs involved ($P = 0.45$ and 0.31, respectively), and liver involvement ($P = 0.42$ and 0.39, respectively). There were also no significant differences between sFAS and sFASL levels among patients with different performance status grades ($P = 0.10$ and 0.51, respectively; see Table 2).

We found a significant increase in sFAS levels and a significant decrease in FASL at 3 months compared with baseline (13.2 versus 10.02 ng/mL; $P = 0.0001$; 0.07 versus 0.14

Table 2. Baseline characteristics and association with sFAS and sFASL

Characteristic	sFAS (ng/mL)	sFASL (ng/mL)	Ratio
Sex			
Male	11.0 ± 14.3	0.15 ± 0.18	276.5 ± 411.8
Female	8.3 ± 2.5	0.11 ± 0.11	387.9 ± 488.1
Lactate dehydrogenase			
Greater than upper limit of normal	12.2 ± 17.7	0.16 ± 0.24	362.9 ± 553.6
Less than upper limit of normal	8.5 ± 2.8	0.11 ± 0.1	277.6 ± 394.5
No. organs			
1	10.6 ± 13.6	0.15 ± 0.19	276.7 ± 417.5
>1	8.6 ± 3.1	0.1 ± 0.09	391.4 ± 466.4
Location disease			
Liver	10.1 ± 12.9	0.14 ± 0.18	291.5 ± 451
Other	9.3 ± 2.3	0.11 ± 0.11	382.0 ± 367.5
Previous adjuvant			
Chemotherapy			
No	10.2 ± 12.9	0.14 ± 0.18	349.3 ± 466.1
Yes	9.2 ± 2.3	0.14 ± 0.1	177.6 ± 256.4
Previous radiotherapy			
No	10.0 ± 12	0.14 ± 0.17	314.9 ± 443.4
Yes	9.6 ± 2.7	0.12 ± 0.09	281.4 ± 355.4
Eastern Cooperative Oncology			
Group performance status			
0	7.7 ± 2.5	0.11 ± 0.1	260.6 ± 336.1
1	9.6 ± 3.2	0.18 ± 0.24	310 ± 451.4
2	15.7 ± 25.4	0.1 ± 0.08	424.2 ± 577.1
Initial Dukes stage			
Synchronic	10.6 ± 13.1	0.11 ± 0.1	383.6 ± 469.8
Metachronic	8.2 ± 2.1	0.16 ± 0.28	155.0 ± 246.4

NOTE: Mean ± SD ($n = 68$). All P were nonsignificant.

Table 3. Circulating sFAS and sFASL and ratio during treatment

Extraction	n	sFAS (ng/mL)	sFASL (ng/mL)	Ratio
Basal	68	10.02 (2.9-100)	0.14 (0.01-1.25)	311.5 (5.2-2,000)
3 mo*	46	13.2 (5.7-100)	0.07 (0.01-0.39)	626.8 (27.4-2,170)
6 mo†	28	11.9 (3.5-22.3)	0.11 (0.01-0.46)	313.5 (38.4-1,450)
9/12 mo‡	20	10.3 (6.1-16.7)	0.26 (0.01-1.25)	268.6 (13.2-1,670)

NOTE: Mean (range; n = 160).
*P = 0.0001 for sFAS basal (Wilcoxon test).
†P = 0.007 for sFASL basal (Wilcoxon test).
‡P = 0.003 for sFASL basal (Wilcoxon test).

ng/mL; $P = 0.007$, respectively). The median of FAS/FASL ratio increment was 1.2-fold. A significant increase in the sFASL levels up to 9 months (fourth to fifth extractions; 0.26 ng/mL) of therapy compared with first to third extractions (0.11 ng/mL; $P = 0.003$) was also found (see Table 3).

Response to chemotherapy. The overall response rate was 45.6%. The levels of the FAS/FASL ratio increment in the group of complete response and partial response (i.e., "responding" tumors; mean, 14.2; range, 0.06-188.4) were significantly different from the levels in the stable disease and progressive disease group (i.e., "nonresponding" tumors; mean, 2.2; range, 0.02-29.2; $P = 0.005$, Wilcoxon test; Table 4). A random effect regression statistical model evaluated the effects of time/therapy on response. We determined that a >1.2-fold increase in sFAS/sFASL ratio was a marker of chemosensitivity ($P = 0.001$). In addition, we have found a predictor of chemoresistance in a subgroup of patients who, despite presenting a high ratio and an initial CT response, rapidly developed a decreased ratio during treatment, indicating the appearance of chemoresistance. In the univariate analysis of response, only performance status ($P = 0.05$) and age ($P = 0.1$), but not lactate dehydrogenase ($P = 0.7$), previous adjuvant treatment ($P = 0.38$), carcinoembryonic antigen ($P = 0.33$), and number of organs involved ($P = 0.93$), had a borderline significance. A multivariate regression analysis of response with the relevant clinical variables (age, performance status, and sFAS/sFASL ratio) was done, and only sFAS/sFASL ratio ($P = 0.003$) and age ($P = 0.025$) remain as independent factors predicting response.

Discussion

In the present study, the mean of sFAS/sFASL basal levels (*sFAS*, 10.02 pg/mL; *sFASL*, 0.14 pg/mL) is similar to that reported previously (23–25). In accordance to some authors, we have not seen any significant relation between *sFAS* and/or sFASL levels and variables such sex, age, or performance status (23, 24). We have also observed a lower basal level *sFAS* (8.2 ng/ml), but without reaching significance ($P = 0.37$), in patients with metachronic compared with synchronic disease (10.6 ng/mL), in accordance with the well-known chemoresistance of this group of patients in randomized advanced colorectal cancer trials (2, 26). We also noted a higher basal levels of *sFAS* (12.2 ng/mL) in those patients with serum lactate dehydrogenase >1 upper limit of normal compared with 8.5 ng/mL ($P = 0.43$), also a well-defined, poor prognosis factor of survival in colorectal cancer (1, 2) and described previously in advanced melanoma (27). These data could explain previous reports associating poor prognosis with sFAS levels in gynecologic malignancies and melanoma (24, 25).

Also in our knowledge, for the first time in the literature, we have shown a significant increase in sFAS levels after chemotherapy treatment ($P = 0.0001$). In addition, we have also noted that a ratio increment correlates with tumor response and the subsequent decrease is related to chemoresistance ($P = 0.001$). Despite these data, it is unclear how chemotherapy regulates, if it does, *sFAS* and *sFASL* functions. We hypothesize that, in advanced colorectal cancer, tumor production of soluble espliicing variants (amount and type) leads to a proapoptotic action (through transmembrane FAS interaction), much more than to an antiapoptotic one. Because in the advanced stages of the disease the matrix metalloproteinases (like matrix metalloproteinase-7) are more active (27–29) and lead to an increase of the sFASL fractions, it is plausible that these events may have a global antiapoptotic and immunoevading action. Supporting this theory, high levels of sFASL have been observed in metastatic pancreatic carcinoma, a notorious resistant neoplasm (30). In mammary tissues from multiparous matrilysin (matrix metalloproteinase-7)–expressing mice, there was decreased FASL expression, suggesting that loss of FASL

Table 4. Association of FAS/FASL changes and response to 5-FU/oxaliplatin

Response	FAS	P*	FASL	P	Ratio	P
Complete response/partial response	1.48 (0.56-3.17)	0.05	1.85 (0.01-18)	0.019	14.2 (0.06-188.4)	0.005
Stable disease/progressive disease	1.18 (0.06-2.7)		4.08 (0.04-46)		2.29 (0.02-29.2)	

NOTE: Mean (range).
*Wilcoxon test.

expression is at least one mechanism of matrilysin-induced resistance to apoptosis (31). Furthermore, CTLs trigger FAS-mediated apoptosis only after treatment with metalloproteinase inhibitors (matrix metalloproteinase-1). Matrix metalloproteinase-1 induces apoptosis by increasing the surface expression of FASL and disappearance of sFASL (32).

There have been multiple reports in the literature measuring basal levels of sFAS and sFASL in different neoplasms. However, this is the first study that reflects the dynamics of these soluble fractions during chemotherapy treatment. We conclude that a 1.2-fold increase of FAS/FASL ratio, after receiving chemotherapy, indicates chemosensitivity in colorectal cancer. In addition, a ratio decrease during chemotherapy treatment, despite the initial values, is related to acquired chemoresistance. We suggest that sFAS/sFASL ratio can be useful as a dynamic response predictor in colorectal cancer patients following chemotherapy.

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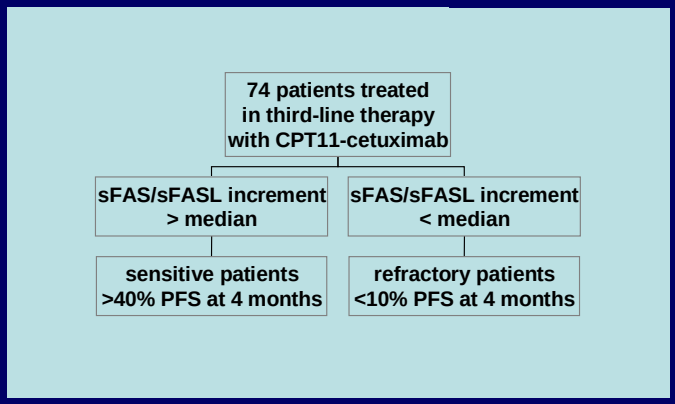
HCB-05-1

- ACRC patients treated in third-line therapy with irinotecan 180 g/m2 and cetuximab after failure to OXL and irinotecan
- Serum were obtained before treatment at 48h and until PD (2,4,6,9,12 months)
- Evaluation of MMP-7, sFAS and sFASL.
- Study design (patient sensitive to cetuximab will present an increment of the ratio at 48h compared with the resistant phenotype)

HCB-05-01 Trial



Inclusión Abril 2007

N. Centro	Centro	Inclusión
1000	Hospital Clínic Barcelona	24
1001	Hospital Miguel Servet	17
1006	Hospital Parc Taulí	16
1004	Hospital Clínico Zaragoza	12
1005	IVO	6
1007	Hospital General Terrasa	1
1002	Hospital La Fe	0
1003	Hospital Peset Valencia	0
1008	ICO Girona	0
Total		76

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